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THE POTENTIAL OF POLYMERIC NANOPARTICLES FOR ORAL DELIVERY OF AMPHOTERICIN B: PREPARATION, SCALE-UP, *IN VITRO* AND *IN VIVO* EVALUATION

BY

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination, which has led to the award of a degree.

Signed: *Date:*

Dedicated To

My Loving Wife and

Parents

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PUBLICATIONS

1. **Italia, J.L**. Singh, D., Kumar, M.N.V.R, 2009. High-performance liquid chromatographic analysis of amphotericin B in rat plasma using α-naphthol as an internal standard. **Analytica Chimica Acta**, 634, 110-114.
2. **Italia, J.L.,** Sharp, A., Carter, K.C., Warn, P., Kumar, M.N.V.R, 2010. Peroral delivery of amphotericin B aided by polyester nanoparticles lead to better or comparable *in vivo* anti-fungal activity to that of intravenous Ambisome® or Fungizone™ (Manuscript communicated).
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ABSTRACT

Amphotericin B (AMB) is the drug of choice for treatment of systemic fungal infections and visceral leishmaniasis (VL). However, due to poor biopharmaceutical characteristics including low solubility and intestinal permeability, AMB is negligibly absorbed upon oral administration. Hence, AMB is conventionally administered as intravenous infusion, which is associated with severe infusion-related adverse effects and nephrotoxicity necessitating prolonged hospitalisation and monitoring of the patient. These factors severely limit the clinical use of AMB. Lipid-based formulations of AMB have been developed but these formulations are substantially more expensive and still require parenteral administration. The aim of the project was to develop an oral nanoparticulate formulation of AMB (AMB-NPs), which would allow non-invasive treatment and enhance its accessibility to patients. High-performance liquid chromatographic (HPLC) methods were developed for *in vitro* and *in vivo* analysis of AMB. AMB-NPs were prepared using nano-precipitation method. The formulation was optimised thoroughly based on particle size, entrapment efficiency and AMB loading by varying the process parameters. The lead formulation was ~115 nm in size with ~70% entrapment efficiency at 30% (w/w of polymer) AMB loading. A scale-up method that allowed production of batches up to 500 and 1000 mg of AMB-NPs was developed, which was mild, fast and reproducible. Ultrafiltration process was assessed for the purification of AMB-NPs, which was found to be superior to centrifugation in terms of particle recovery, removal of solvent and unentrapped drug. AMB-NPs were freeze-dried using 10% w/v of sucrose as cryo/lyo-protectant. The freeze-dried formulation was stable at 4°C for the study period of 3 months. AMB-NPs had significant ($p<0.0001$ and $p=0.0001$) *in vivo* anti-fungal activity in murine

models of disseminated and invasive pulmonary aspergillosis (IPA) models, where AMB-NPs were as effective as intaperitoneally administered Fungizone® and intravenous AmBisome® in reducing the kidney fungal burdens. AMB-NPs were significantly ($p<0.05$) more effective in reducing parasite burden than AMB solution in the bone marrow macrophages (BMMs) infected with *L. donovani*. In murine model of VL, oral treatment with AMB-NPs caused significant ($p<0.005$) reduction in liver parasite burden with no detectable nephrotoxicity. Together, the results indicate that AMB-NPs could be used as an oral therapy for systemic fungal infection and VL.

ABBREVIATIONS

AMB	Amphotericin B
AMB-NPs	Amphotericin B nanoparticles
AR	Analytical reagent
BCS	Biopharmaceutics classification system
BMMs	Bone marrow macrophages
BUN	Blood urea nitrogen
CFU	Colony forming units
CME	Clathrin-mediated endocytosis
CV	Coefficient of variation
CvME	Caveolae-mediated endocytosis
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EPA	Electron pair acceptor
EPD	Electron pair donor
FAE	Follicle-associated epithelia
FcR	Fc receptors
Hb	Haemoglobin
HI-FCS	Heat-inactivated fetal calf serum
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
INF- γ	Interferon-gamma
IPA	Invasive pulmonary aspergillosis
IS	Internal standard
DL	Detection limit
QL	Quantitation limit

LR	Laboratory reagent
M-cells	Membranous microfold cells
ME	Matrix effect
ND	Non-detectable
NPs	Nanoparticles
NS	Non-significant
PC	Plasma creatinine
PDA	Photo-diode array
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactide-co-glycolide)
QC	Quality control
RES	Reticulo-endothelial system
RME	Receptor-mediated endocytosis
SD	Standard deviation
SE	Standard error of mean
TCA	Tricarboxylic acid cycle
TNF-α	Tumor necrosis factor-alpha
UV	Ultraviolet
VE-TPGS	Vitamin E-TPGS
VL	Visceral leishmaniasis
WBC	White blood cells
WHO	World Health Organization

SYMBOLS

λ	Wavelength
°C	Degree Celsius
μg	Microgram
μl	Microlitre
h	Hour
kg	Kilogram
mBar	Millibar
ml	Millilitre
mM	Millimolar
N/m	Newton per meter
ng	Nanogram
nm	Nanometer
o/w	Oil-in-water
s	Second
s/o	Solid-in-oil
s/o/o	Solid-in-oil-in-oil
s/o/w	Solid-in-oil-in-water
Sf	Final size
Sf/Si	Ratio of final to initial size
Si	Initial size
Tg	Glass transition temperature
Tg′	Glass transition temperature of maximally cryo-concentrated solution
v/v	volume by volume
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
w/v	Weight by volume
w/w	Weight by weight

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CHAPTER 1

GENERAL INTRODUCTION

1. Introduction

Infectious diseases are one of the leading causes of mortality worldwide, which account for around 15 million deaths annually (Morens *et al*., 2008). These numbers can further increase considerably as chronic diseases like cervical cancer, Kaposi's sarcoma and *Helicobacter pylori* ulcers are being re-classified as infectious diseases. A proportion of these diseases, which pose a health treat are systemic fungal infections and leishmaniasis.

The increasing incidence of systemic fungal infections is a growing concern in immunocompromised patients (Richardson, 2005). The advances in the healthcare sector have witnessed an increase in the population of immunocompromised patients those are at the risk of developing systemic fungal infections. (Pfaller, 1992; Fridkin and Jarvis, 1996; Groll *et al*., 1996; Upton and Marr, 2006). Common opportunistic fungal infections include aspergillosis, candidiasis and mucormycosis. Pulmonary infection is the most common form of aspergillosis; however, systemic dissemination may occur in 25-50% of the patients. High mortality rates have been reported in invasive pulmonary aspergillosis (IPA) patients and the outcome of the therapy is dependent on several factors, including early start-up of anti-fungal therapy, severity of underlying disease as well as granulocyte recovery (Chong *et al*., 2006).

Leishmaniasis is a group of parasitic diseases caused by different species of parasites belonging to the genus *Leishmania* that ranges from cutaneous to visceral leishmaniasis (VL) depending on the parasite species involved and the host immune status (Barral *et al.,* 1991; Grimaldi *et al.,* 1991; Liew and O'Donnel, 1993). Amongst these, VL is the most devastating disease and is responsible for high levels of mortality and morbidity in infected patients.

Overall leishmaniasis affects ~12 million people worldwide and it is endemic in 88 countries, mainly in tropical and sub-tropical countries. There are approximately 2 million new cases reported every year and around 350 million people are at risk in endemic areas (Desjeux, 1996; Desjeux, 2004). Leishmaniasis is one of the most neglected diseases in developing countries; however, the incidence of leishmaniasis has been increasing worldwide and it has a wider geographical distribution than before, which could be a potential threat for developed countries as well (Desjeux, 2001).

Amphotericin B (AMB) is currently considered as a first-line treatment for systemic fungal infections and VL. The conventional formulation of AMB (Fungizone®, a colloidal dispersion with sodium deoxycholate) is given by slow intravenous infusion over 2-6 h. Its use is associated with severe infusion and drug-related adverse effects. About 70-90% of the patients treated with AMB are likely to be affected by infusion-related adverse effects such as nausea, vomiting, fever, chills, gastrointestinal disturbances, thrombophlebitis, bronchospasm, hypoxia, hypotension/hypertension and cardiac arrhythmia (Gallis *et al.,* 1990; Goodwin *et al.,* 1995; Levy *et al.,* 1995). These effects are caused due to production of pro-inflammatory cytokines by immune cells as AMB is a microbial product (Sau *et al.,* 2003). Pre-treatment with other medicaments like acetaminophen, diphenhydramine, chlorpheniramine, meperidine or nefopam reduce the incidence and severity of these effects (Goodwin *et al.,* 1995; Rosa *et al.,* 1997). Use of corticosteroids is also beneficial, but they can have detrimental effect on the course of the infection due to immunosuppressive effect (Zygmunt and Tavormina, 1966).

Renal impairment or nephrotoxicity is the major problem in patients treated with AMB, which is characterised by increase in blood urea nitrogen (BUN) and plasma creatinine (PC) levels. Nephrotoxicity is cumulative and can lead

to hypokalemia, hypomagnesemia and acidosis (Gallis *et al.,* 1990). Nephrotoxicity occurs in 24-80% of patients treated with AMB (Gallis *et al.,* 1990; Clements and Peacock, 1990) and necessitates careful monitoring of the toxicity parameters during and after AMB therapy. Vasoconstriction provoked by AMB has been reported as a possible cause of nephrotoxicity, which leads to reduction in the blood flow to the kidneys and reduced glomerular filtration rate (Gerkens and Branch, 1980). Hemolysis is another important toxicity associated with AMB therapy, which can leads to serious consequences such as hemolytic anaemia and jaundice (due to accumulation of lysed products of haemoglobin (Hb) in blood circulation).

Recent advances in drug delivery technology have resulted in the development of lipid-based formulations of AMB. After many attempts to decrease the toxicity of AMB by using liposomes, emulsions and other systems, three lipid-based formulations of AMB (AmBisome®, Amphocil® and Abelcet®) have been developed. Various lipid-based formulations of AMB, dosage regimens and cost of therapy for systemic fungal infections and VL are summarised in Table 1.1. The lipid-based formulations are less toxic compared to Fungizone® and allow administration of higher dosage of AMB thus enhancing effectiveness of the therapy. However, these formulations are much more expensive and still require parenteral administration. Therefore, development of an effective, safe and less expensive oral formulation of AMB would have significant applications for the treatment of disseminated fungal infections and would dramatically increase access to treatment of VL.

Table 1.1 Dosage regimens and cost estimates of AMB formulations for treatment of systemic fungal infections and VL

Formulation	Unit cost (50-mg vial)	Systemic fungal infection		VL	
		Regimen	Cost per Week of therapy (£)	Regimen	Cost per therapy (£)
Fungizone®	3.44	0.7 mg/kg/day	24.08	1 mg/kg/day, 15 infusions daily or on alternate days	72. 24
		1.5 mg/kg/day	77.70		
AmBisome®	96.69	3 mg/kg/day	3384.15	2 mg/kg/day for 5 days	210.00†
		5 mg/kg/day	4737.81	7.5 mg/kg single infusion	165.00†
Abelcet®	50.00	5 mg/kg/day	2074.73	2 mg/kg/day for 5 days	700.00
				7.5 mg/kg single infusion	550.00
Amphotec®/	104.10	3 mg/kg/day	3389.40		
Amphocil®		4 mg/kg/day	3991.05		

† Costs are calculated based on availability of AmBisome® at $20 per 50-mg vial through the Gilead Sciences AmBisome Access Program in developing countries.

All drug cost values are for a 70 kg patient.

1.1. Mechanism of action of AMB

Early research has shown that the anti-fungal activity of AMB is related to its ability to interact with ergosterol, a critical component of the fungal cell membrane. AMB binds to ergosterol or cholesterol of the cell membranes, which leads to formation of pores (Fig. 1.1) and resultant leakage of essential small molecules such as K^+, Na^+ and Ca^{++} followed by cell death (Bolard, 1986). Ergosterol is essential membrane component of most of the fungal species and some protozoan (e.g., *Leishmania*) species. AMB, by virtue of its affinity to ergosterol to that of cholesterol, exhibits relative selectivity for fungi and *Leishmania* parasites. However, AMB has tendency to accumulate in organs, particularly the kidneys, where its affinity for cholesterol becomes deleterious due host cells destruction, resulting in toxicity (Ng *et al*., 2003).

1.2. A brief overview on systemic fungal infections

Over the recent decade, advancements in the healthcare system have led to significant rise in the number of life-threatening pathogenic and opportunistic fungal infections (Richardson, 2003). This rise is largely due to an increase in the number of immunocompromised patients, which include transplant recipients, cancer patients, HIV-patients and those receiving extensive immunosuppressive therapy. In addition, an increase in the number of invasive medical procedures such as surgical procedures, indwelling catheters or skin damage through radiation therapy have increased the opportunity for fungal cells to breach barriers, which normally protect against infection. Thus, invasive mycoses pose a major challenge in oncology, hematology and intensive care patients (Richardson, 2005).

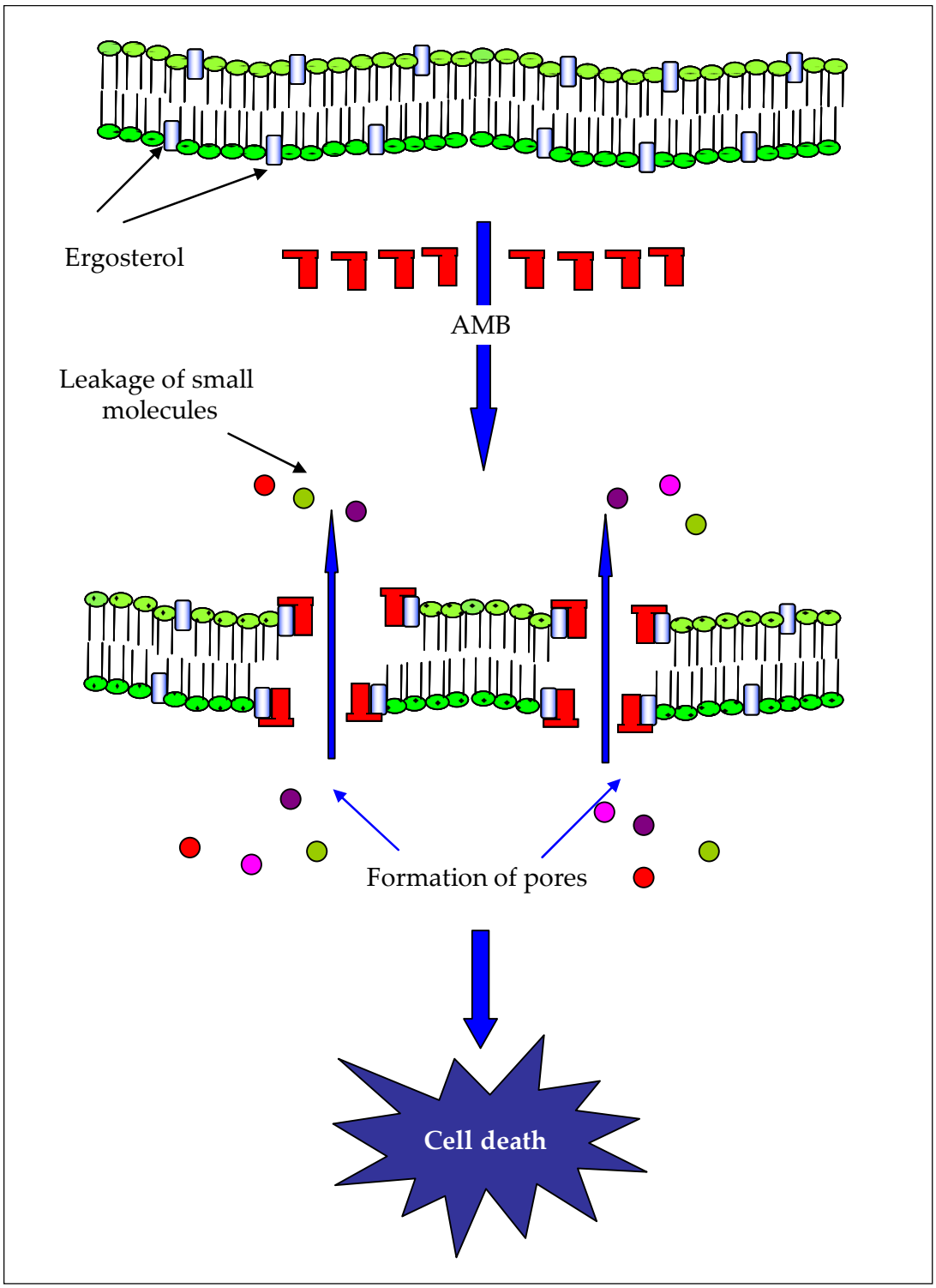


Fig. 1.1 Mechanism of action of AMB

In immunocompromised patients, systemic fungal infections may account for as many as 30% of deaths (Cornely, 2007). Fungal cells are eukaryotic and have many similar biochemical characteristics compared to their mammalian host. This similarity makes it difficult to develop drugs, which selectively act on fungal pathogens and do not induce host cell damage.

Majority of the systemic fungal infections are caused by *Aspergillus* and *Candida* species. Among the pathogenic species of *Aspergillus*, *A. fumigatus* is the primary causative agent of invasive/disseminated fugal infections, followed by *A. flavus, A. terreus, A. niger* and *A. nidulans* (Denning, 1998; Morgan *et al.*, 2005).

1.2.1. Invasive pulmonary aspergillosis (IPA)

Aspergillus can cause a number of ailments depending upon the immune status of the host (Denning, 1998; Latge, 1999). In asthma and cystic fibrosis patients, *Aspergillus* can cause allergic bronchopulmonary aspergillosis, which is basically a hypersensitive response to fungal components. In tuberculosis patients, repeated exposure to conidia can cause non-invasive aspergillomas due to pre-existing lung cavities such as healed lesions. The most devastating disease caused by *Aspergillus* is IPA, which occurs in the severely immunocompromised patients.

The incidences of IPA have increased considerably during last two decades and despite improved diagnosis methods and therapeutic intervention, the fatality rate is very high. The mortality rate of IPA patients is more than 50% in patients with neutropenia and even higher (~90%) in patients receiving hematopoietic stem-cell transplantation (Yeghen *et al.*, 2000).

1.2.1.1. Infectious life cycle of *A. fumigatus*

Aspergillus species are ubiquitous in nature and have a saprophytic lifestyle. The fungus grows on dead or decaying organic matters in the environment. *Aspergillus* undergoes asexual reproduction that leads to formation of airborne spores called conidia (Morris *et al.,* 2000; Falvey and Streifel, 2007). The infectious cycle begins when the conidia are inhaled by humans (Fig. 1.2). Upon inhalation, the conidia are deposited in the bronchioles or alveolar spaces. The conidia are normally removed by mucociliary clearance but only those remain are encountered by epithelial cells or alveolar macrophages. In immunocompetent people, alveolar macrophages are the primary defense against the pathogenic organisms entering the lungs and are responsible for the phagocytosis and killing of *Aspergillus* conidia as well recruitment of neutrophils to the infection site. Conidia, which escape macrophagic destruction and germinate, are targeted by infiltrating neutrophils, which are capable of destroying hyphae. When these primary host defenses become dysfunctional (e.g., in immunocompromised people), *Aspergillus* conidia may survive and grow in the pulmonary system leading to development of IPA (Schaffner *et al.,* 1982).

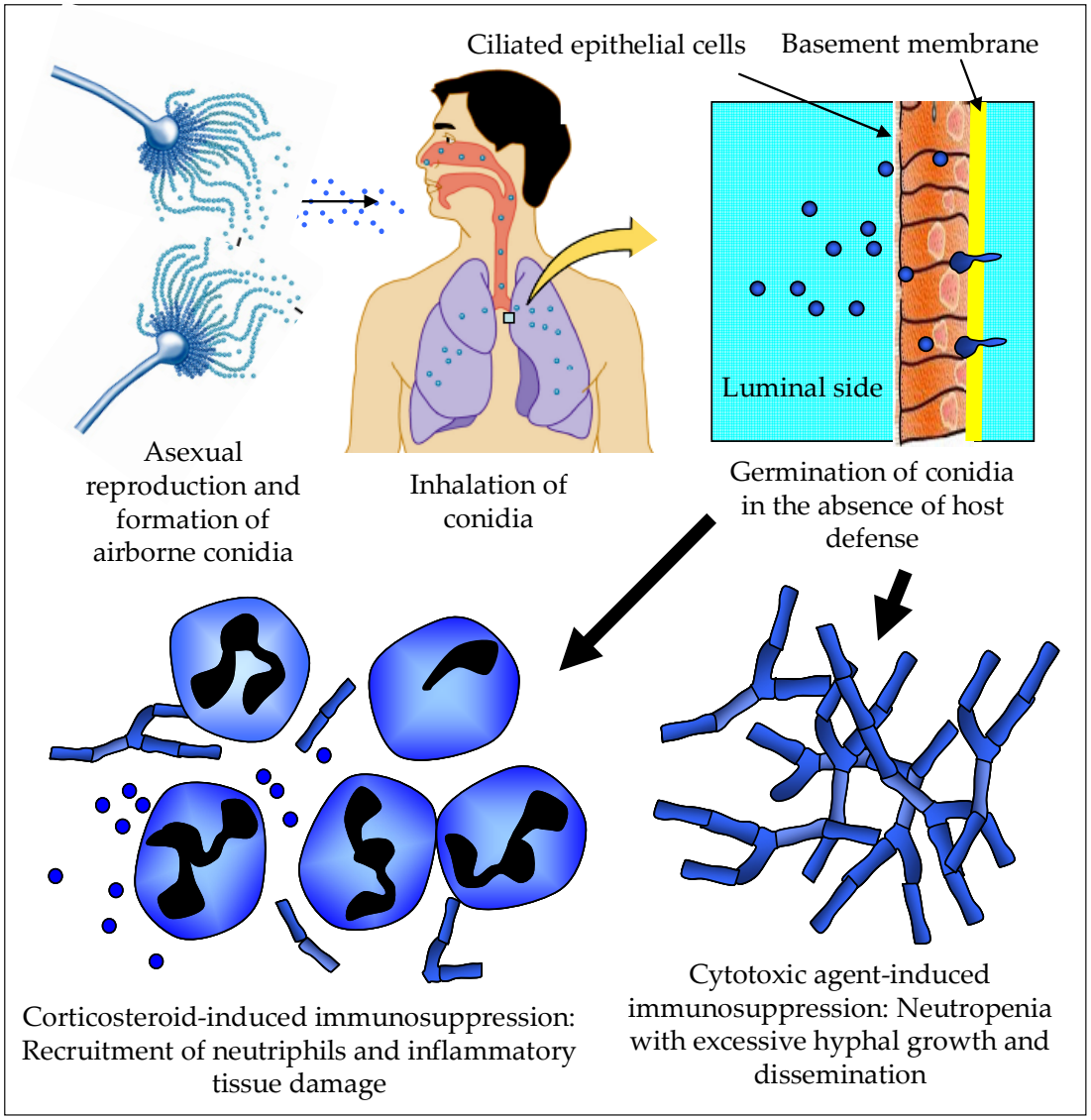


Fig. 1.2 Infectious life cycle of *A. fumigatus*. *Aspergillus* reproduces asexually and undergoes sporulation that leads to formation of airborne conidia. Inhalation of conidia by immunocompromised patient leads to establishment and germination of conidia into lungs. In the patient with corticosteroid-induced immunosuppression, neutrophils are recruited in the lungs as a host defense leading to fungal control with considerable inflammatory response while in case of neutropenic patients (cancer chemotherapy), uncontrolled hyphal growth and in severe cases dissemination occurs (adapted from Dagenais and Keller, 2009).

1.2.1.2. Risk factors and clinical symptoms of IPA

The majority of the incidences of IPA have been observed in immunocompromised patients. The important risk factors for IPA are shown in Table 1.2 (Gerson *et al*., 1984; Schaffner, 1985; Denning *et al*., 1991; Minamoto *et al*., 1992; Hibberd and Rubin, 1994). Prolonged neutropenia is the prime risk factor, which is often associated with the cytotoxic therapy like cyclophosphamide following transplantation or hematological disorders. Cyclophosphamide depletes circulating white blood cells including those of neutrophils. In neutropenic patients, IPA is characterised by thrombosis and hemorrhage due to rapid and extensive hyphal growth (Stergiopoulou *et al*., 2007; Chiang *et al*., 2008). The lack of inflammatory infiltrates (neutrophils) results in low levels of inflammation. In absence of neutrophil recovery, angioinvasion and dissemination to other organs can occur. Non-neutropenic patients, those receiving corticosteroid therapy are also susceptible to IPA, although the pathology of the disease is quite different from that of neutropenic patients. In non-neutropenic patients, no angioinvasion occurs and symptoms are limited to fungal development with pyogranulomatous infiltrates, tissue necrosis and excessive inflammation. Though steroids interfere with immune cell function, neutrophils are recruited to the lung, which prevent hyphal invasion but the severe inflammation induced causes tissue pathology. The usual symptoms are bronchopneumonia, cough, increased sputum production, dyspnoea and fever. Other symptoms include isolated tracheobronchitis, which is associated with severe inflammation of the airways due to ulceration and plaque formation. This may results in obstruction of the airway and secondary atelectasis, which is common in the HIV-patients and in recipients of lung transplant (Judson and Sahn, 1994; Nathan *et al*., 2000).

Table 1.2 Major risk factors for IPA

No.	Risk factors
1	Prolonged neutropenia (>3 wk) or neutrophil dysfunction (chronic granulomatous disease)
2	Immunosuppressive therapy with corticosteroid (especially prolonged, high-dose therapy)
3	Transplantation (highest risk is with lung and bone marrow)
4	Hematological malignancy (risk is higher with leukemia)
5	Cytotoxic (cancer) chemotherapy
6	AIDS (risk increases with lower CD4 count)

1.2.2. Disseminated aspergillosis

Untreated IPA often leads to disseminated infection by hematogenous or contiguous spread. The dissemination may occur to organs including the brain causing symptoms, which can include seizures, ring-enhancing lesions, cerebral infarctions, intracranial hemorrhage, meningitis and epidural abscess. Other dissemination sites include the skin, kidneys, pleura, heart, esophagus or liver (Denning, 1998). Multifocal disease within one organ (i.e., IPA and tracheobronchial aspergillosis) is distinctly different from that of disseminated infection. The mortality rates are as high as 90% or more with disseminated aspergillosis even with appropriate anti-fungal therapy (Patterson *et al*., 2000; Lin *et al*., 2001).

1.2.3. Other systemic fungal infections

Apart from *A. fumigatus* and *Candida albicans, Cryptococcus neoformans* are the other common causative organisms of systemic fungal infections. However, in recent years, other pathogenic fungi have emerged as competent

pathogens including yeast like *Candida glabrata*, *Candida krusei, Candida tropicalis* and *Trichosporon,* filamentous fungi such as *Fusarium*, *Rhizopus* and *Rhizomucor* and agents of *Phaeohyphomycosis* (Richardson, 2003).

1.2.4. Treatment of systemic fungal infections

Currently available treatment options for systemic fungal infections are summarised in the Table 1.3. Apart from AMB, Flucytosine (Ancotil®, Valeant) has been used for the treatment of systemic fungal infections but it has a limited spectrum of activity that includes *Candida* species, *Cryptococcus* species and some filamentous fungi. Flucytosine has to be used in the combination with other anti-fungal agents because of the emergence of resistance (Eilard *et al.,* 1976).

Azoles, a new class of anti-fungal agents, were introduced in the markets in 1980's. They inhibit the enzyme lanosterol demethylase (14α-sterol demethylase or P450DM), which is a critical enzyme necessary for the biosynthesis of ergosterol. Exposure of fungus cells to azoles leads to depletion of ergosterol and thus disruption of the cell membrane. Though lanosterol demethylase is also present in the mammals, azoles have a higher affinity for fungal lanosterol demethylase at therapeutic concentrations. Ketoconazole (Nizoral®; Pharmaceutica) is the only member of the imidazole class of systemic anti-fungal agents that is currently used. The development of newer triazole class of compounds such as fluconazole (Diflucan®; Pfizer Pharmaceuticals), which are less toxic and more effective, has now largely replaced ketoconazole. It can be administered by both, oral and intravenous routes. However, fluconazole has limited activity against *Aspergillus* species or most other mould fungi (Bodey, 1992; Denning *et al*., 1992).

Table 1.3 Drugs used in treatment of systemic fungal infections

Class	Drug	Trade name	Route	Adverse effects	Reference
Polyene antibiotic	AmphotericinB	Fungizone®	Intravenous	Infusion related adverse effects, hemolysis, nephrotoxicity	Gallis *et al.*, 1990;
Pyrimidinone	Flucytosine	Ancotil® Ancobon®	Oral	Gastro-intestinal side effects, hepatotoxicity and bone marrow depression	Vermes *et al.*, 2000
Imidazole	Ketoconazole	Nizoral®	Oral or intravenous	Nausea, vomiting, hepatotoxicity and mild cardiovascular effects including prolongation of the QT interval, atrial fibrillation, a decreased ejection fraction and torsades de pointes	Urcuyo and Zaias, 1982;
Triazole	Fluconazole Itraconazole Posaconazole Voriconazole	Diflucan® Sporanox® Noxafil® Vfend®	Oral or intravenous		Cornely *et al.*, 2007; Eiden *et al.*, 2007; Fung *et al.*, 2008
Echinocandins	Caspofungin Micafungin Anidulafungin	Cancidas® Mycamine® Eraxis®	Intravenous	Mild hepatotoxicity	Menichetti, 2009; Sucher *et al.*, 2009

Itraconazole, another compound from the same triazole class, is also available as oral and intravenous formulations. It overcomes the limitation of fluconazole and exhibits activity against most of the *Aspergillus* isolates and a subset of fluconazole resistant *Candida* strains (Groll and Walsh, 2002). Recently, two second-generation triazole molecules (voriconazole and posaconazole) have been approved for the treatment of systemic fungal infections. Both of these molecules have good therapeutic efficacy against acute invasive aspergillosis and candidiasis (Boucher *et al*., 2004; Mathew and Nath, 2009). However, drug-interaction is the significant problem with these molecules as they interact with other drugs, which are substrates of cytochrome P450 3A4 (e.g., immunosuppressants like cyclosporine, tacrolimus, sirolimus, cyclophosphamide and most of the anti-ritroviral drugs including nevirapine, efavirenz, indinavir, aprenavir and ritonavir etc.). Since the majority of patients with systemic fungal infections are receiving multiple medicines, complications may occur with co-administration. Moreover, azoles also cause adverse effects as shown in Table 1.3.

The echinocandins (caspofungin, micafungin and anidulafungin) are a newer class of semi-synthetic lipopeptide, which have anti-fungal activity against a variety of yeast and moulds species (Sucher *et al.,* 2009). Echinocandins acts as the non-competitive inhibitors of β-(1,3)-Dglucan synthase, an essential component of the cell wall of many fungi that is not present in mammalian cells (Odds *et al.,* 2003; Wiederhold and Lewis, 2003). This offers unique mechanism of action as glucan synthesis enzymes are absent in the mammals, which makes it to act selectively upon the fungal cells with minimal damage to the host and hence lower toxicity (Boucher *et al*., 2004). This mechanism is different from that of other anti-fungal agents, which

usually involves interaction with ergosterol function or biosynthesis and thus caspofungin present a unique opportunity for the combination therapies.

1.3. A brief overview on Leishmaniasis

Leishmania are obligatory intracellular parasites, which causes wide spectrum of diseases including localised/diffused cutaneous leishmaniasis, muco-cutaneous leishmaniasis, leishmaniasis recidivans, VL (also known as kala-azar) and post-kala-azar dermal leishmaniasis. The causative species and the geographical distribution are summarised in Table 1.4. In cutaneous leishmaniasis, parasites are restricted to macrophages of the skin, whereas in VL, parasites are concentrated in deeper sites including the liver, spleen and bone marrow (Sundar and Chatterjee, 2006). VL is much more severe than cutaneous leishmaniasis and is fatal if left untreated (Chappuis *et al*., 2007). Approximately 500,000 new cases and more than 50,000 deaths from VL occur annually (Desjeux, 1996). More than 90% of cases of VL occur in just six countries including Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Fig 1.3).

Table 1.4 Different clinical forms of leishmaniasis, causative parasite species and its geographic location (Grevelink and Lemer, 1996)

Disease	New World parasite	Old World parasite	Geographical location
Localised cutaneous leishmaniasis	*L. b. braziliensis* *L. b. guyanensis* *L. b. panamensis* *L. m. mexicana* *L. m. amazonensis*	*L. major* *L. tropica* *L. aethiopica* *L. infantum*	North Africa, India, Middle East, China, South Russia, Pakistan, Mediterranean, Central and South America, Texas, Caribbean
Diffuse cutaneous leishmaniasis	*L. m. amazonensis* *L. mexicana* *L. m. pifanoi*	*L. aethiopica*	Venezuela, Bolivia, Mexico, Dominican Republic, Brazil, Ethiopia
Leishmaniasis recidivans	*L. braziliensis*	*L. tropica*	Central and South America, Middle East
Muco-cutaneous leishmaniasis	*L. b. braziliensis* *L. b. panamensis*		Brazil, Venezuela, Peru, Ecuador, Colombia
VL	*L. donovani/chagasi*	*L. donovani* *L. infantum* *L. tropica*	China, India, Bangladesh, Asia, Sudan, Africa, East Russia, Mediterranean, South America
Post kala-azar dermal leishmaniasis	*L. donovani/chagasi*	*L. donovani* *L. tropica*	India, Bangladesh, East Africa, Sudan, South America

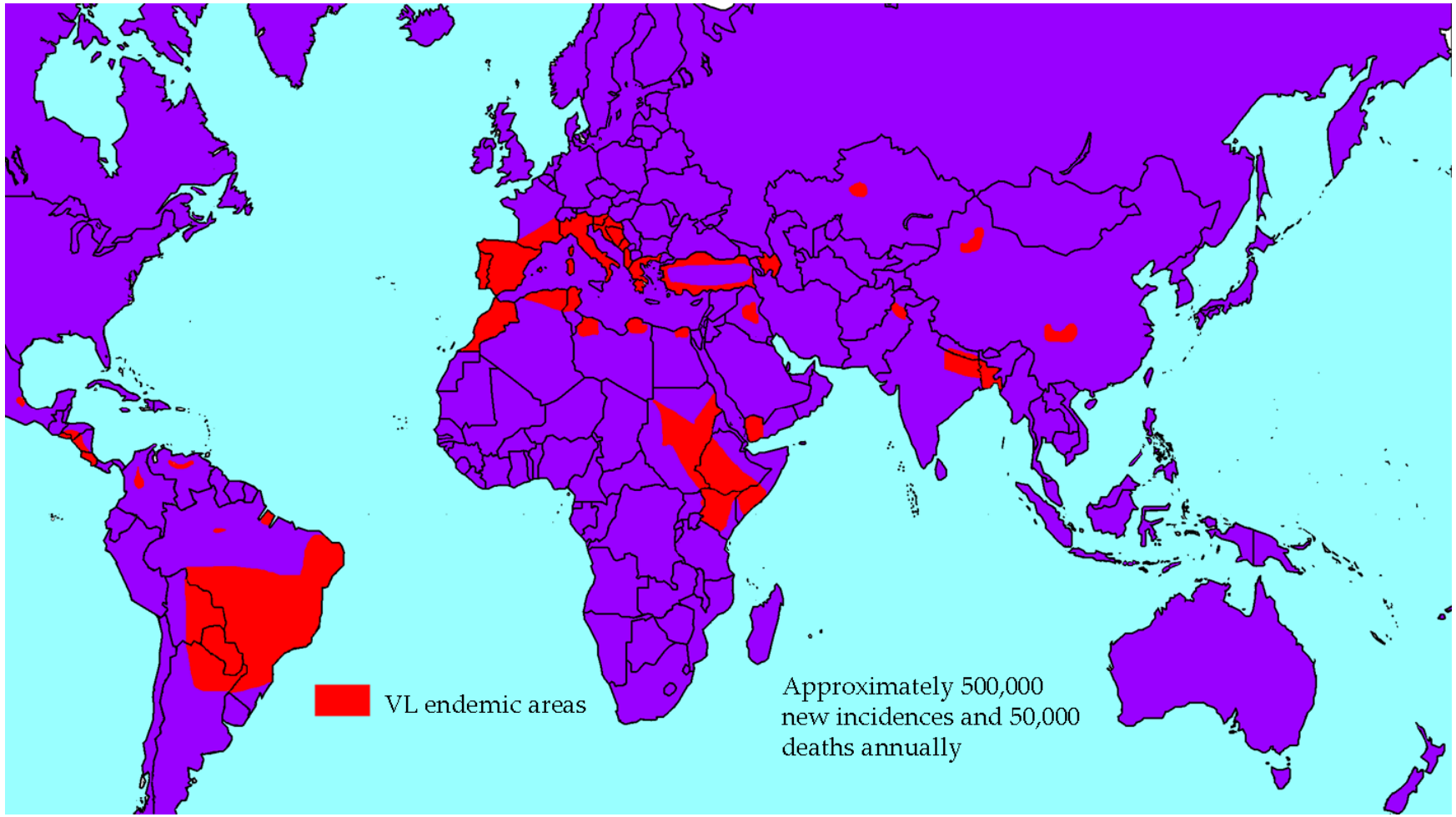


Fig. 1.3 Geographical distribution of VL. More than 90% of the cases occur in six countries – Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan (adapted from Desjeux, 2004).

1.3.1. Life cycle of *Leishmania* species

Transmission of leishmaniasis occurs via the bite of a female phlebotomine hematophagus sandfly. The parasite's life cycle involves two different stages, as extracellular metacyclic promastigotes found in the gut of the sandfly and as intracellular amastigotes found in macrophages (Fig. 1.4). The life cycle starts when the sandfly bites and deposits flagellated promastigotes into the host during a blood meal. The promastigotes are then phagocytosed by dendritic cells or macrophages in the dermis. The parasites transform into non-motile amastigotes (2-4 μm in diameter) and lose their flagella within the phagolysosomes/parasitophorous vacuoles of the macrophages. The amastigotes multiply by binary fission, an increase in parasite number causes rupture of the macrophage, and the released parasites are taken up by other macrophages. The released amastigotes may disseminate and infect the vital organs, principally the reticulo-endothelial system (RES) including spleen, liver and bone marrow (Sacks and Noben-Trauth, 2002). The life cycle is completed when a sandfly feeds on the infected host and ingests macrophages infected with amastigotes or parasites present in the blood. In the gut of the sandfly, the amastigotes differentiate into extracellular promastigotes (20 μm in size) and multiply. The promastigotes undergo several stages of maturation to form the infectious metacyclic form, which migrate to the alimentary tract of sandfly and concentrate in the mouthparts (Kamhawi, 2006).

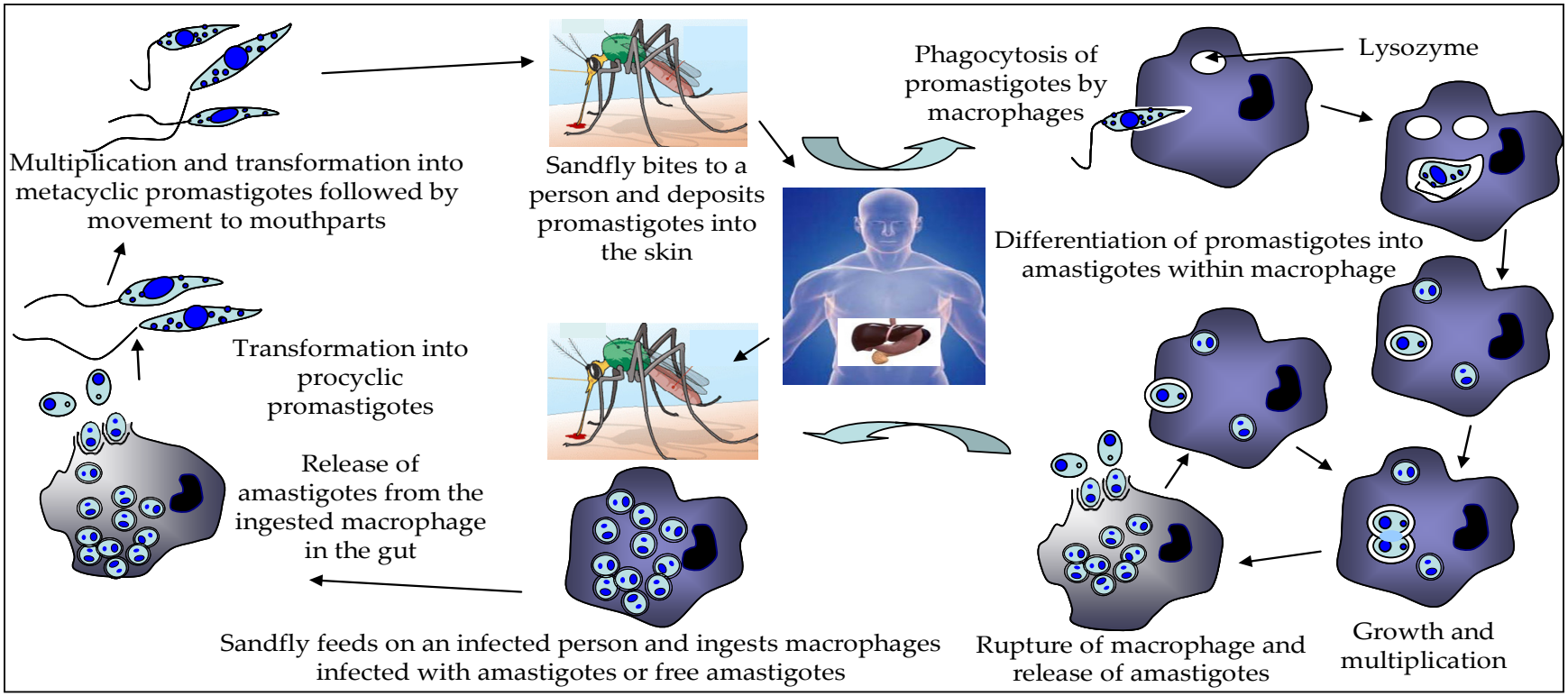


Fig 1.4 Life cycle of the *Leishmania* parasites. The human host is infected with the parasites via the bite of a female sandfly. The promastigotes entering the skin are phagocytosed by macrophages and transform into intracellular amastigotes. The parasites replicate causing rupture of the macrophages. The released parasites enter other macrophages as well as the RES. When a sandfly feeds on an infected host, it ingests infected macrophages or free amastigotes. The parasites quickly transform into to rapidly dividing promastigotes in the gut of the sandfly. The parasites multiply and undergo a series of maturation stages to form infective metacyclic promastigotes. The metacyclic promastigotes migrate to salivary glands, ready for transmission to the next host. Adapted from Handman, 2001.

1.3.2. Clinical symptoms of leishmaniasis

Leishmania infection can be unapparent or sub-clinical or it can display a spectrum of manifestations that ranges from cutaneous involvement, through destruction of mucous membranes or fatal systemic visceral disease.

Localised cutaneous leishmaniasis usually affects unclothed parts of the body, which are easily bitten by the sandfly vector, i.e., face, neck and arms. Primary symptoms include erythemateous papules on exposed areas of the body where infected sandfly vectors have fed (Balanã-Fouce *et al.,* 1998). The incubation period can range from 1 week to 3 months. The primary lesion often develops into an ulcer, which can be pruritic but is not painful. The ulcer may remain relatively dry with a central crust or it may exude seropurulent material. Subcutaneous nodules or cords may develop around the ulcer due to local lymphangitic spread of the parasites. Draining lymph nodes may become enlarged and parasites are found in biopsy specimens (Al-Gindan *et al.,* 1989). Inflammatory satellite papules, along with subcutaneous indurations, may develop around the primary lesion due to a reaction to local dissemination of the parasites or its antigenic products (Kubba *et al.,* 1987; Kubba *et al.,* 1988). Frequently, cutaneous leishmaniasis produces self-healing lesions, but lesion may persist for a long time (1 year or more) in absence of treatment. Diffuse cutaneous leishmaniasis is a rare complication of cutaneous disease in which lesions are disseminated, resembling lepromatous leprosy. It usually starts as an initial regular ulcerative cutaneous lesion but then disseminates to affect multiple sites of the skin. The lesions are non-ulcerative nodules, often scattered over the cooler areas of the body including limbs, buttocks and face (Suzanne *et al.,* 1996).

Leishmaniasis recidivans or recurrent leishmaniasis is another rare form of cutaneous disease, which is usually associated with *L. tropica* and less commonly *L. braziliensis* (Salman *et al.,* 1999). The disease involves development of new papular and non-ulcerating lesion beyond the border of the original scar as satellite lesions or in the center of a healed lesion.

Muco-cutaneous leishmaniasis is caused by metastasis of parasites to mucosal sites from a primary cutaneous lesion by lymphatic or haematogenous dissemination. The disease often starts with nasal inflammation and stuffiness, followed by ulceration of the nasal mucosa and perforation of the septum. In some cases, the lips, cheeks, soft palate, pharynx or larynx are also involved. Invasion can extend to the neighbouring skin, oropharynx and even trachea. Dissemination to the mucous membranes of the eye and genitals occurs rarely. Mucosal leishmaniasis never heals spontaneously, is difficult to treat and secondary bacterial infections are common (Marsden, 1986). It is potentially fatal.

VL is the most severe form of leishmaniasis caused by the dissemination of parasites throughout the reticulo-endothelial system (RES) (Herwaldt, 1999). The incubation time for VL ranges from weeks to months and in immunocompromised individuals, it can occur years after they have left an endemic region. The symptoms at the onset of disease include irregular fever accompanied by sweating, weakness and weight loss. Splenomegaly, hepatomegaly, lymphoadenopathy, anemia, emaciation, pancytopenia and hyperglobulinemia may develop with the progression of disease (Guerin *et al*., 2002). A small erythematous papule usually appears on the leg as the primary lesion of VL. A patchy blackening of the skin can occur due to increased melanoblastic activity and enhancement of the natural skin color because of xerosis. The skin generally becomes dry and rough, and the hair

becomes very brittle, which sometime leads to complete alopecia (Suzanne *et al.,* 1996). Post-kala-azar dermal leishmaniasis can occur as a complication of VL. It appears as rash, due to discrete erythematous papules on the cheeks, chin, ears and extensor aspects of forearms, buttocks and lower legs. Lesions heal spontaneously over a few months due to acquired cellular immunity.

1.3.3. Treatment of leishmaniasis

The treatment options available for VL are summarised in Table 1.5. Pentavalent antimonials were introduced in 1945 and were used as the first-line treatment against different types of leishmaniasis (Berman 1988; Olliaro and Bryceson, 1993). Two formulations were available; sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®) (Papadopoulou *et al.,* 1998). The recommended dosage regimen for cutaneous or VL consists of once-daily injections of 20 mg/kg of body weight of sodium stibogluconate or meglumine antimoniate for 28-30 days. Antimonials were considered as the gold standard for treatment of leishmaniasis but their clinical use is now lmited because of widespread antimony resistance in India (Ashutosh *et al.,* 2007). Antimonial drug use was associated with adverse effects such as acute pancreatitis and cardiac arrhythmia (Chappuis *et al*., 2007).

Pentamidine, an aromatic diamidine, was initially proven to be useful in animonial-resistant VL cases in India; however, its higher cost, unacceptable toxicity, including irreversible insulin dependent diabetes mellitus, limited its use (Jha, 1983; Jha *et al.,* 1991; Das *et al.,* 2001; Olliaro *et al.,* 2005).

Table 1.5 Drugs used in treatment of VL.

Class	Name of drug	Trade name	Route	Adverse effects	References
Pentavalent antimonials	Sodium stibogluconate	Pentostame®	Intravenous or Intramuscular	Abdominal pain, nausea, hepatotoxicity and pancreatitis, myalgias and arthralgias, thrombocytopenia/leucopenia, cardiotoxicity	Lawn *et al.*, 2005; Chappuis *et al*., 2007; Shahian and Alborzi, 2009
	Meglumine antimoniate	Glucantime®			
Diamidine	Pentamidine isethionate	Pentamidine®	Intramuscular	Nausea, vomiting or diarrhea, hyperglycaemia, pancreatitis, azotemia	Andersen *et al.*, 1986; Balslev and Nielsen, 1992
Aminoglycoside antibiotic	Paromomycin sulfate	Humatin®	Intramuscular	Ototoxicity, nephrotoxicity	Sundar *et al.*, 2007
Polyene antibiotic	Amphotericin B	Fungizone®	Intravenous	Infusion related adverse effects, hemolysis, nephrotoxicity	Gallis *et al.*, 1990;
Hexadecyl-phosphocholine	Miltefosine	Impavido® Miltex®	Oral	Nausea, vomiting and/or diarrhea, teratogenic	Berman, 2008
8-aminoquinoline	Sitamaquine		Oral	Vomiting, methemoglobinemia and transient nephrotoxicity	Jha *et al.*, 2005

AMB has been proven to be highly effective in the treatment of VL (Thakur *et al.,* 1999) and antimonial-resistant cases of muco-cutaneous leishmaniasis. Initially AMB was used as second-line treatment for VL but it is now considered as first-line therapy due to high efficacy and no proven clinical resistance. However, the risk of severe adverse effects restricts administration of AMB to hospitals capable of monitoring and intervention of serious drug-induced toxicity.

Paromomycin, an aminoglycoside class of antibiotic, was identified as an anti-leishmanial drug in the 1960's. Intramuscular injections of paromomycin have shown potential in treating the antimony-resistant VL in India. Several Phase 2 clinical trials in India and Kenya have shown promising results with 90% cure rate occurring in normal and antimony-resistant cases of VL following treatment with 15 mg/kg daily for 20 days (Thakur *et al.,* 2000). The efficacy was confirmed in a phase III clinical trial comparing intramuscular injections of paromomycin (21 mg/kg for 21 days) to intravenous AMB (Fungizone® 1 mg/kg every other day for 30 days). However, more adverse effects were observed as compared to AMB (Sundar *et al*., 2007). Intramuscular administration of paromomycin (14 mg/kg daily for 20 days) has been found to be beneficial for the treatment of cutaneous leishmaniasis with cure rates of ~59%; however, toxicity was the limiting factor (Berman, 1997; Croft and Yardley, 2002). A topical cream containing paromomycin (twice-daily application for 10-30 days) was used to treat cutaneous leishmaniasis without systemic adverse effects (El-On *et al.,* 1992; Asilian *et al.,* 1995; Ben Salah *et al.,* 1995).

Introduction of miltefosine, an alkylphosphocholine ester, originally developed as chemotherapeutic agent for breast cancer, is perhaps the most significant advance as it is the first oral therapy for VL. The anti-leishmanial

activity of miltefosine against VL was discovered in the mid-1980's and its use was demonstrated in several experimental animal models (Croft *et al.*, 1987; Croft *et al.*, 1996; Le Fichoux *et al.*, 1998; Croft *et al.*, 2003). The successful cure of 94% of the VL patients in a Phase 3 trial led to miltefosine being approved for oral therapy of VL in India, Columbia and Germany. The recommended dosage of miltefosine is 2.5 mg/kg/day orally for 28 day. Oral miltefosine is also found to be effective in the treatment of cutaneous leishmaniasis (Soto *et al.*, 2004; Soto and Toledo, 2007). Miltefosine exhibits only transient gastro-intestinal adverse effects and is reasonably well tolerated. However, it has a major drawback of teratogenicity, which precludes its use in the pregnant women (Berman, 2008). Serious care should be taken during the treatment with miltefosine and female patients of childbearing age should be protected against pregnancy during treatment. A phase IV trial with miltefosine gave an ultimate cure rate of 81%, suggesting that a relapse will occur in some patients (Bhattacharya *et al.*, 2007). In addition, drug resistance may develop as it has a very long half-life of 150-200 h and resistance is easily induced *in vitro* (Pérez-Victoria *et al*., 2006; Seifert *et al*., 2003). Combination treatment with other drugs and direct observation of the treatment are the possible ways to prevent the development of miltefosine-resistant strains of *Leishmania* (Sundar and Olliaro, 2007). Miltefosine is also effective in the treatment of canine leishmaniasis. This would be beneficial in reducing the animal reservoir of the parasites that could be transmitted to humans via sandflies. However, the dogs are never cured parasitologically; therefore, the policy of using the same drug for treating humans and dogs could favour the emergence of miltefosine-resistant parasites (Dujardin *et al*., 2008).

Another drug that could be useful in oral treatment of VL is sitamaquine (8-aminoquinoline derivative), which is currently in Phase IIb clinical trials. Its anti-leishmanial activity was first identified in the 1970's by studies carried out at the Walter Reed Army Institute of Research, USA. Subsequent pre-clinical and clinical investigations have demonstrated oral efficacy against *L. donovani* (Jha *et al.,* 2005). Sitamaquine induces only mild adverse effects, which include methemoglobinemia and transient renal-toxicity. Similar to miltefosine, a sitamaquine-resistance could be induced *in vitro* in the *Leishmania* strains by stepwise exposure to higher concentrations indicating that *in vivo* resistance could occur (Bories *et al.,* 2008).

1.4. Biopharmaceutics and physicochemical aspects of AMB

AMB has a polyene structure consisting of a macrolactone ring, a rigid non-polar heptene unit on one side and more flexible polar polyol region on the other side (Fig. 1.5). This arrangement gives amphipathic characteristics to the AMB molecule. A carboxyl and a mycosamine group, which are located at one end of the molecule, are charged at neutral pH and responsible for the amphoteric nature of AMB. Consequently, AMB has very low aqueous solubility, which is one of the key factors in limiting its oral bioavailability and therapeutic applications. AMB is unstable at acidic pH of stomach. The high molecular weight (924.08 Da) and large cyclic structure of AMB account for the low intestinal permeability of the molecule.

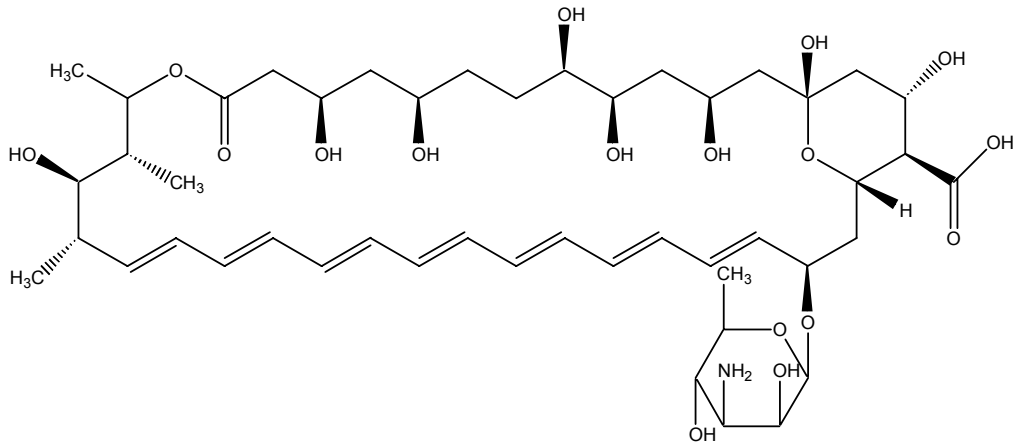


Fig. 1.5 Chemical structure of AMB.

In 1995, Lipinski has retrospectively analysed a large database of drug compounds, selecting those, which are likely to have physical chemical characteristics consistent with good absorption and derived a mnemonic called the "Rule of Five" (Lipinski *et al*., 2001). The 'Rule of Five' states that poor absorption is more likely when a compound possesses:

- more than five H-bond donors
- more than ten H-bond acceptors
- ClogP greater than five (or MlogP greater than 4.15)
- molecular mass over 500 Da

According to the "Rule of Five", the structural characteristics confer low oral bioavailability to AMB. Together, these characteristics also place AMB in class IV under Biopharmaceutics classification system (BCS).

The BCS is a scientific framework for classifying a drug substance based on its aqueous solubility and intestinal permeability (Fig. 1.6) (Amidon *et al.*, 1995). BCS has opened up various avenues and strategies for formulation development based on solubility and permeability issues. The United States Food and Drug Administration (USFDA) issued the biowaiver guidelines based on the BCS in 2000, which offers biowaivers to class I (highly soluble and highly permeable) drugs. Many reports have demonstrated the increase in solubility of poorly soluble small molecules by modifying the shape, size, and functional groups present on the molecule, as well as the increase in permeability by the incorporation of lipid components into the drug.

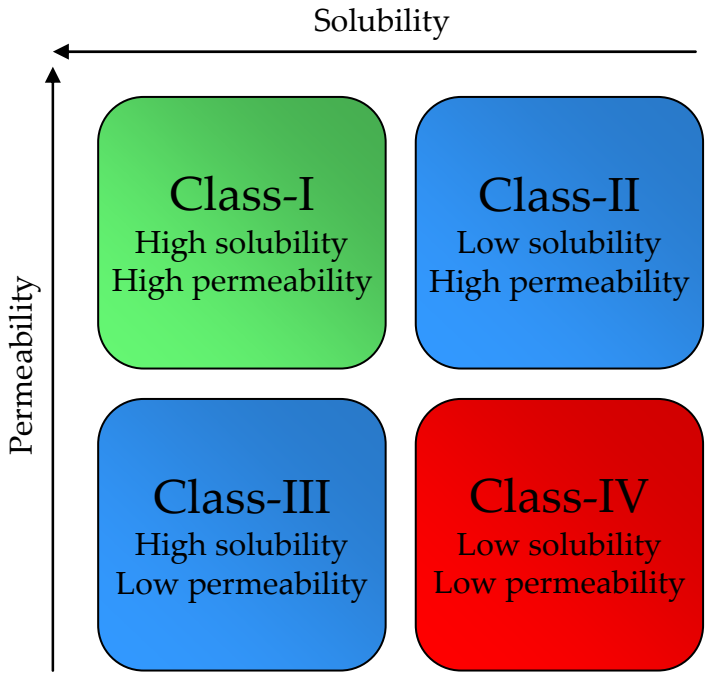


Fig. 1.6 The Biopharmaceutics Classification System (BCS) of drugs based up on the aqueous solubility and intestinal permeability.

Since the NPs could provide a carrier-driven cellular entry mechanism irrespective of the solubility or permeability of the entrapped drug, this approach offers opportunities for the modulation of both, solubility and permeability at the same time. The unique properties of these carrier systems could be used for the drugs that belong to class II (low solubility-high permeability), class III (high solubility-low permeability), or class IV (low solubility-low permeability) drugs. With an increase in the use of computational and combinatorial processes in drug design, often based on receptor morphology, many of the new drugs approved fall into the category of class II or IV, with significant solubility problems, and nanoparticulate delivery approaches could play a major role in delivery of such drugs. Nifedipine, a BCS class II drug, when encapsulated in poly (ε-caprolactone) (PCL) and Eudragit®, shows significantly increased bioavailability (Kim *et al.,* 1997). Similarly, acyclovir (a BCS class III drug), when formulated as poly(d,l-lactic acid) (PLA) nanospheres, resulted in improved ocular pharmacokinetics compared with the free drug (Giannavola *et al.,* 2003). In a recent study, it was shown that paclitaxel (a BCS class IV drug), when loaded in PEG–poly(lactide-co-glycolide) NPs showed greater tumor growth inhibition compared to free paclitaxel (Danhier *et al*., 2009).

The oral route remains to be the most convenient and widely used means of systemic drug delivery. Physico-chemical properties of the drug molecule and gastro-intestinal physiology play vital roles in the design of an oral drug delivery system. Low aqueous solubility and permeability are the prime factors responsible for the poor oral bioavailability of AMB and thus its encapsulation in a suitable drug carrier, which is capable of shielding unfavorable biopharmaceutical properties of the drug and enhances the intestinal uptake, presents a possible solution. The formulation must also

improve the stability of the drug in the harsh gastric pH as well as protect it from enzymatic degradation in the gastro-intestinal tract. Effective release of AMB from the carriers in its active monomeric form after entering into the body is also the pre-requisite to achieve desired therapeutic effect. All these factors must be balanced to develop a successful oral formulation of AMB.

1.5. Oral AMB delivery systems

The initial *in vivo* studies were conducted in 1995 using an oral AMB suspension (Jambor *et al*., 1955-1956). AMB was formulated as colloidal suspension by first solubilising in N,N-dimethylacetamide and hydrochloric acid, followed by mixing with water. Treatment of *Candida albicans* infected mice with oral dosages of 20-40 mg/mouse twice a day for two days resulted in 90-100% survival of the animals. A second study was conducted using an oral solution of AMB prepared with 0.5% lecithin. The study resulted in the similar dose-dependent efficacy at the dose of 4, 8, 16 or 32 mg/mouse twice a day for two days (Jambor *et al*., 1955-1956). In another study, AMB formulations were prepared by suspending amorphous AMB in saline, which was supplemented with penicillin and streptomycin at a concentration of 1000 units/ml each. The prepared suspension was administered orally to the mice infected with *Coccidioides immitis* at the dose of 12 mg/mouse/day for 23 days. Though treatment resulted in 100% survival of the mice, post-mortem cultures for spleen and omentum were positive for 90% of the animals indicating incomplete eradication of the fungi (Oura *et al*., 1955-1956).

Unlike the pre-clinical studies, the clinical studies required much higher doses to elicit any significant therapeutic response. Moreover, significant

gastrointestinal adverse effects were observed in the human studies. In human clinical trials conducted in patients with systemic fungal infections resulted in poor absorption and efficacy of orally administered AMB formulations (Utz *et al*., 1957-1958). Other studies in humans also supported the finding that AMB was negligibly absorbed from the orally administered formulations in treated individuals and the serum levels were not even quantifiable. (Littman *et al*., 1958; Louria, 1958; Kravetz *et al*., 1961). Subsequently, other route of administration were investigated which included intravenous, intra-articular, intra-thecal, intra-pulmonary and intra-thoracical. Intravenous therapy was found to be most effective for the treatment of fungal infections and leishmaniasis (Littman *et al*., 1958; Ghosh and Ghosh, 1967; Robbie *et al*., 1999).

Various other formulations strategies have been attempted in order to develop an oral formulation of AMB. Santangelo *et al*., (2000) reported a cochleate formulation of AMB for oral administration. Cochleates are multi-layered structures consisting of a continuous spiral sheet of lipid bilayer without internal aqueous compartment. They are formed by the interaction of negatively charged phosphatidylserine and calcium cations and were initially used as carriers for vaccine and gene delivery (Zarif and Mannino, 2000). Since AMB is amphipathic molecule, it can be efficiently incorporated into membrane bilayers. Cochleates are thought to facilitate the interaction of AMB with biological membranes by acting as fusion intermediates. This formulation was evaluated in the rodent models of both disseminated candidiasis and disseminated aspergillosis. Oral treatment with cochleate-AMB at the doses of 0.5, 1, 2.5, 5, 10 and 20 mg/kg/day for 15 consecutive days improved the survival of *Candida*-infected mice and suppressed the fungal burdens (in kidney, lung and spleen) in the dose-dependent manner

but complete eradication of fungus could only be achieved in the lung (Santangelo *et al*., 2000). In the disseminated aspergillosis model, oral treatment with the cochleate-AMB at the doses of 20 and 40 mg/kg/day for 14 days significantly decreased fungal tissue burden (Delmas *et al*., 2002). However, only 70% survival of the animals could be achieved.

Nanosuspensions of AMB were developed for improving the intestinal uptake and bioavailability of AMB (Kayser *et al*., 2003). These formulations have been shown to improve the adherence of the drug to the gastro-intestinal mucosa with improved surface area, solubility and drug contact time and thus improving the intestinal uptake. The nanosuspension was prepared by suspending AMB at a concentration of 0.4% in an aqueous solution of Tween 80, Pluronic F68 and sodium cholate, followed by homogenisation. The therapeutic efficacy of the formulation was evaluated in *L. donovani* infected BALB/c mice. Oral treatment of infected mice with nanosuspension at the dose of 5 mg/kg/day for 5 days resulted in 28.6% reduction of liver parasite burdens as compared to controls. Oral administration of micronised AMB, AmBisome® or Fungizone® did not show any significant reduction in the liver parasite burden (Kayser *et al*., 2003).

Lipid nanospheres containing AMB were prepared following a melt-dispersion technique, using one of three lipidic mixtures composed of glyceryl tristearate and/or glyceryl monstearate (main constituents) and 20% w/w egg phosphatidyl choline (stabiliser) (Amarji *et al*., 2007). The lipid nanosphere formulation improved the oral absorption of AMB in rats compared to aqueous AMB suspension, with the highest absorption been observed with formulation with smallest particle size (Amarji *et al*., 2007). The exact mechanism of nanoparticles (NPs) uptake was not well

understood; however, enhanced lymphatic uptake was thought to be responsible for improved bioavailability.

Risovic *et al.,* (2003) reported Pecol®-based AMB formulation with improved oral bioavailability. This formulation suppressed the fungal burdens 95% at the dose of 50 mg/kg for 4 days in rats infected intravenously with *A. fumigatus* (Sachs-Barrable *et al.,* 2008). Recently, a lipid-based oral formulation of AMB with significant anti-fungal and leishmanicidal activity has been reported (Thornton and Wasan, 2009). The formulation was made as a self-emulsifying drug delivery system using proprietary mixture of mono- and diglycerides with phospholipids. This system improved the aqueous solubility of AMB by 50-fold and significantly protected AMB from gastrointestinal degradation (Wasan *et al.,* 2009a). The formulation exhibited improved bioavailability of AMB upon oral administration (Gershkovich *et al.,* 2009). This formulation suppressed liver parasite burdens by 99.8±0.2% at the dose of 40 mg/kg/day for 5 days in mice infected with *L. donovani* (Wasan *et al.,* 2009b). It also exhibited significant anti-fungal activity upon oral administration and reduced the fungal burdens by ~75 and 95% in *A. fumigatus*-infected rats at the dose of 10 and 20 mg/kg/day, respectively, for 2 days (Wasan *et al.,* 2009a). This was a significant improvement considering non-availability of oral formulation for AMB; however, the formulation has not been evaluated in the IPA model, which is relatively difficult to treat and now considered as generally accepted standard.

1.6. Polymeric NPs

Polymeric NPs have been promisingly investigated as the drug delivery carriers due to their ability to improve oral bioavailability of the poorly

soluble/permeable drugs, better durg entrapment, controlled drug release and ability to circumvent the adverse effects (Barratt, 2003; Bala *et al.,* 2004; Kumari *et al*., 2010). NPs have a wide array of applicability, as they are suitable for both oral and parenteral administration.

1.6.1. Gastrointestinal uptake of NPs

Intestinal tissues contains highly specialied enterocytes, called the membranous microfold (M) cells, which collect antigens and macromolecules from the gastrointestinal lumen. The M-cells are derived from crypt cells (Gebert *et al.,* 1996) and exhibit different morphological characteristics compared to absorptive enterocytes such as underdeveloped microvillous and glycocalyx structures, the presence of apical microfolds, increased intracellular vacuolisation and absence of mucus. Both, absorptive enterocytes and M-cells togather constitute the follicle-associated epithelia (FAE), which is responsible for shuttling colloidal antigens/particles, small bacteria and viruses, via a transcytotic mechanism to lymphoid microcompartments those in close contact with their covering epithelia (Sanderson and Walker, 1993). The solitary lymphoid nodules are widespread along the entire intestine and tend to increase in number in the ileum as oval aggregates in the antimesometrial gut wall, the so-called Peyer's patches (Jani *et al.,* 1989). The physiological transport/uptake mechanism associated with M-cells is thought to be of particular relevance for colloidal carriers, such as NPs. Two types of pathways can be considered for the uptake of NPs through the gastrointestinal epithelium, the paracellular transport and endocytosis.

1.6.1.1. The paracellular route of NPs uptake

The paracellular spaces are sealed by tight junctions and contribute less than 1% of the mucosal surface area. Since the pore diameter of these junctions was reported to be less than 10 Å, signicant paracellular transport of macromolecules and NPs is unlikely to occur. However, paracellular permeability for peptides has been repored to be improved by polymers such as chitosan (Schipper *et al.,* 1997), poly(acrylate) (Lehr *et al.,* 1990; Kriwet and Kissel, 1996) or starch (Bjork *et al.,* 1995). The enhancement of paracellular transport of peptides is thought to occur by charge mediated polymer binding to epithelia, leading to structural reorganisation of tight junction-associated proteins (in case of chitosans) or by a reduction of free extracellular Ca^{+2} concentration (in case of polyacrylates). Moreover, the inactivation/inhibition of proteolytic enzymes, located in the gut lumen (e.g. trypsin) or at the brush border (aminopeptidases) contributes to higher absorption of peptides.

Since the hypothetical mechanisms for increasing the paracellular permeability have been derived mostly from cell culture models, their relevance for the *in vivo* situation is unclear. Paracellular uptake of NPs>50 nm cannot be easily explained by a temporary widening of tight junctions.

1.6.1.2. The endocytotic pathway of NPs uptake

Adsorptive endocytosis, by way of phagocytosis, clathrin coated pits and vesicles, and fluid phase endocytosis, has been described as the major mechanism of NPs uptake by M-cells (Buda *et al.,* 2005).

1.6.1.2.1. Phagocytosis

Phagocytosis has a critical physiological role in the defence of the organism against nonself elements, infectious agents (most bacteria and some viruses) as well as exogenous inert particles—including nanoparticulate carriers. Phagocytosis primarily occurs in professional phagocytes such as macrophages, monocytes, neutrophils and dendritic cell (Aderem and Underhill, 1999) although other types of cells (such as epithelial cells, endothelial cells and fibroblasts), which are referred to as nonprofessional phagocytes, also exhibit phagocytic activity (Rabinovitch, 1995). The phagocytic pathway of entry into cells can be explained using three different steps: recognition of the particles by opsonisation; adhesion of the opsonised particles to the macrophage; ingestion of the particle.

Opsonisation is a process, which occurs before the actual phagocytosis. This process consists in tagging the foreign particle by proteins called opsonins, making the particle noticeable to macrophages. This process typically takes place in the bloodstream rapidly after introduction of the particles. Major opsonins include immunoglubulins (Ig) G (and M) and complement components (C3, C4, C5) (Vonarbourg *et al.*, 2006), in addition to other serum proteins (including laminin, fibronectin, C-reactive protein, type-I collagen) (Owens and Peppas, 2006). The opsonised particles then attach to the macrophage surface through specific receptor-ligand interactions. The main and best-studied receptors for this purpose include the Fc receptors (FcR) and the complement receptors. Other receptors, including the mannose/fructose and scavenger receptors, can be involved in the phagocytosis (Aderem and Underhill, 1999), while new opsono-receptors like CD44 are still being discovered (Vachon *et al.,* 2006). Receptor ligation leads to the starting of a signalling cascade mediated by Rho-family GTPases

(Caron and Hall, 1998), which triggers actin assembly, forming cell surface extensions called pseudopodia that zipper up around the particle and engulf it.

1.6.1.2.2. Clathrin-mediated endocytosis

Endocytosis via clathrin-coated pits, or clathrin-mediated endocytosis (CME), occurs constitutively in all mammalian cells, which fulfils crucial physiological roles of nutrient uptake and intracellular communication. The CME process serves as the main mechanism of internalisation for macromolecules and plasma membrane constituents. CME via specific receptor-ligand interaction is the best described mechanism, and was previously referred to as ''receptor-mediated endocytosis'' (RME). However, it is now clear that alternative non-specific endocytosis via clathrin-coated pits also exists (as well as receptor-mediated but clathrin-independent endocytosis).

Receptor-dependent CME is a shared pathway for the internalisation of a variety of ligand-receptor complexes (Mukherjee *et al.*, 1998). This mode of endocytosis is of paramount importance not only for ligands, but also for many viruses (e.g., influenza) (Marsh and Helenius, 2006) and for drug-loaded nanocarriers. The Receptor-dependent CME typically occurs in a membrane region enriched in clathrin, which is the main cytosolic coat protein. Formation of the endocytic vacuole is driven by assembly of a basket-like structure formed by polymerisation of clathrin units (Kanaseki and Kadota, 1969). Clathrin has a three-leg structure called triskelion. These triskelia assemble in polyhedral lattice on the cytosolic surface of the cell membrane helping the membrane to deform into a coated pit of ~150 nm. As

the process continues, the pit becomes deeply invaginated until fission of the vesicle occurs. This step requires presence of GTPase dynamin, which finally end up to formation of clathrin-coated vesicles. The resulting endocytic vesicle have an average size of 100-120 nm (Conner and Schmid, 2003; Bareford and Swaan, 2007). This vesicle transports its cargo to ''early'' endosomes, which are acidified to pH ~6 by ATP-dependent proton pumps. At this stage, some receptors (e.g., LDL receptor, transferrin and its receptor) and ligands dissociate and are recycles for another round of delivery. The early endosomes then mature into late endosomes (having pH ~5), which, after fusion with prelysosomal vesicles containing acid hydrolases, generate a harsh environment prone to degradation of the internalised cargo (Mukherjee et *al.,* 1998; Bareford and Swaan, 2007). In the case of polarised cells, the recycled molecules either return to the membrane from which they were internalised, or cross the cell and get delivered to the opposite membrane in a process called transcytosis (Matter and Mellman, 1994).

Receptor-independent CME, another CME mechanism, which involves non-specific adsorptive pinocytosis, has been simply referred to as fluid-phase endocytosis (Bareford and Swaan, 2007). Compounds absorbed by this pathway circumvent direct binding with membrane constituents, but often display non-specific charges and hydrophobic interactions with the cell membrane. Fluid entry occurs via clathrin-coated vesicles as described above, also internalising receptor ligands located in these pits, together with extracellular fluid and its content (Bareford and Swaan, 2007). Apart from the different mode of interaction with the membrane, the major characteristic of this pathway is a slower internalisation rate compared to the receptor-dependent CME (Strømhaug *et al.,* 1997).

1.6.1.2.3. Caveolae-mediated endocytosis (CvME)

Although CME is the predominant endocytosis mechanism in most cells, the alternative pathways of endocytosis have been recently identified, of which, CvME being the major one. Caveolae are characteristic flask-shaped membrane invaginations, with a size 50–100 nm range (Mukherjee *et al.*, 1997; Conner and Schmid, 2003; Bareford and Swaan, 2007; Mayor and Pagano, 2007). They are lined by a dimeric protein called caveolin, and enriched with cholesterol and sphingolipids. Caveolae are particularly abundant in endothelial cells, constituting about 10–20% of the cell surface (Conner and Schmid, 2003). They are also observed in smooth muscle cells and fibroblasts. CvMEs are involved in endocytosis and trancytosis of various proteins, viruses (typically the SV40 virus) (Marsh and Helenius, 2006) and received increasing attention for drug delivery applications using nanocarriers.

Overall, the endocytotic processes are characterised by pinching of membrane vesicles from the plasma membrane, followed by an internalisation of the engulfed extracellular materials. The endocytic pathway is controlled by a series of highly complicated molecular sorting events, which result in the transport of membrane vesicles to subcellular compartments. Of particular interest are transcytotic processes at mucosal surfaces by which macromolecules or particles, internalised at the apical plasma membrane of the epithelial cells, are transported to the contralateral plasma membrane and released to the basolateral compartment. During endocytotic processes various types of membrane vesicles have been observed, ranging from the actin-dependent formation of phagosomes, involved in particle uptake, to smaller clathrin-coated vesicles. While RME is an active transport mechanism, requiring receptors at the apical cell

membrane, the adsorptive endocytosis does not require specific ligand-receptor interactions at the cells surface. This process is initiated by an unspecific physical adsorption of particulate material to the cell surface by electrostatic forces, H-bonding or hydrophobic interaction and is followed by an invagination of the local plasma membrane, forming intracellular vesicles. Similar to RME this process occurs predominantly at the base of microvilli. Adsorptive endocytosis is energy-dependent, saturable and can lead to either intracellular processing or a transcytotic transport of the engulfed macromolecules or particles. Since specific receptors are not required, adsorptive endocytosis depends primarily on size and surface properties of the adsorbed material. Therefore, this mechanism may to have potential for oral delivery of nanoparticulate carriers.

1.6.2. Polymer matrix

Appropriate selection of the polymer matrix is necessary in order to develop a successful drug delivery system. The polymer could be nondegradable or degradable. A major disadvantage with non-biodegradable polymers is that surgery is required to eliminate these polymers from the body once they are depleted of the drug; however, these non-degradable polymers can be successfully used where the removal of the implants is easy. Degradable polymers, on the other hand, do not require surgical removal and hence are preferred to non-biodegradable polymers in many applications. However, the biodegradable polymer degrades to smaller absorbable molecules; hence, it is important to make sure that the resulting monomers are nontoxic. The most commonly used biodegradable polymers are PLA, PGA, or their copolymer PLGA. These polymers are known to be biocompatible and

nontoxic and have been used for biomedical applications for more than two decades.

1.6.2.1. Physicochemical properties of PLGA

Chemical structures of PLA, PGA and PLGA are shown in Fig. 1.7. PLGA synthesized from L-PLA and poly glycolide (PGA) exhibits crystalline behavior while those prepared from D,L-PLA and PGA exhibits amorphous characteristics. PLGA containing more than 70% glycolide is amorphous in nature. The degree of crystallinity and the melting point of the polymers depend on the molecular weight of the polymers (Li and McCarthy, 1999). The molecular weight affects the mechanical strength of the polymer and thus its ability to be formulated as a drug delivery carrier. In addition, it also affects the rate of hydrolysis and biodegradation.

The PLGA copolymers, those available commercially, are usually characterised in terms of intrinsic viscosity, which is directly related to the molecular weight of the polymers. The crystallinity of the polymer directly affects its mechanical strength, swelling behavior, capacity to undergo hydrolysis and the biodegradation rate. The crystallinity of PLGA is dependent on the type and molar ratio of the lactide and glycolide monomers in the copolymer chain. Lactic acid, being more hydrophobic than glycolic acid, makes lactide-rich PLGA copolymers less hydrophilic and subsequently slows down the degradation process.

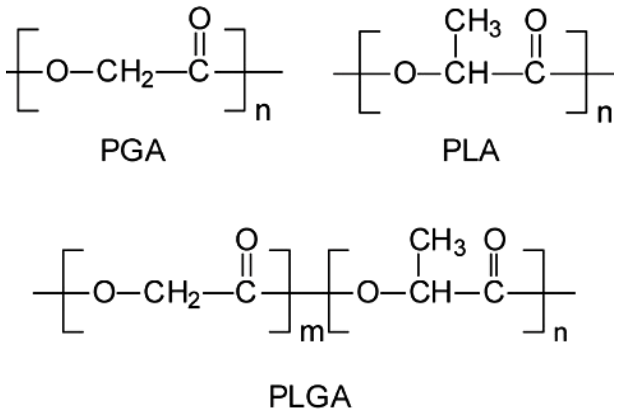


Fig. 1.7 Chemical structures of PLA, PGA and PLGA

Glass transition temperature (Tg) is another important parameter determining the physical strength of the delivery system. The Tg of PLGA copolymer decreases with decrease in molecular weight and in lactide content (Gilding and Reed, 1979; Athanasiou *et al.,* 1996; Li and McCarthy, 1999; Ravivarapu *et al.,* 2000). The Tg of PLGA is 45–55 °C, which is above physiological temperature of 37°C, providing it sufficient strength to be formulated as a drug delivery system.

1.6.2.2. Biodegradation and elimination of PLGA

In both conditions, *in vitro* and *in vivo*, PLGA copolymers undergoes degradation in aqueous environment (hydrolytic or biodegradation) through cleavage of backbone ester linkage. Bulk erosion is the main degradation pathway for PLGA copolymer. This occurs by random scission of ester bonds in the polymer backbone proceeding homogenously throughout the matrix (Vandervoort and Ludwig, 2002). Furthermore, hydrolysis is enhanced by the accumulation of acidic products and the reduction of pH facilitated by the carboxylic acid end groups, which is an autocatalytic degradation process (Li *et al.,* 1990a; Li *et al.,* 1990b; Pistner *et al.,* 1993). The degradation of these polymers differs *in vivo* and *in vitro*, mainly because, although *in vivo* there is no major influence of enzymes during the glassy state of the polymer, these enzymes can play a significant role when the polymer becomes rubbery (Li *et al.,* 1990b). Cöferich proposed a three-phase mechanism for PLGA biodegradation. Initially, random chain scission occurs with a significant decrease in molecular weight of polymer without appreciable weight loss or formation of soluble monomer products. This phase is followed by a decrease in molecular weight with rapid loss of mass and formation of

soluble mono and oligomeric products. Finally, soluble monomer products are formed from soluble oligomeric fragments, resulting in complete polymer degradation (Cöferich, 1996).

Several factors affects the polymer degradation process including method of preparation; presence of low molecular weight compounds (monomers, oligomers, catalysts); size, shape and morphology; polymer properties including molecular weight, chemical structure, hydrophobicity, crystallinity, and Tg); physicochemical parameters (pH, temperature and ionic strength of the environment); site of implantation; and the mechanism of hydrolysis (Jain, 2000).

The degradation behavior of polymer is also dependent on hydrophobicity of the polymer; the more hydrophobic the polymer, the more slower its degradation. The hydrophobicity of the polymer is influenced by the ratio of crystalline to amorphous regions, which in turn is determined by copolymer composition and monomer stereochemistry. Lactic acid, being more hydrophobic than glycolic acid, makes lactide-rich PLGA copolymers more hydrophobic and subsequently slows down the degradation process.

Overall, the degradation time will be shorter for low molecular weight, more hydrophilic, more amorphous polymers and copolymers with higher content of glycolide. Normally, PLGA 50:50 copolymers have the fastest half-life of degradation, around 50 to 60 days, whereas PLGA 65:35, 75:25, and 85:15 have progressively longer degradation half-lives *in vivo*.

It is known that PLGA biodegrades to form lactic acid and glycolic acid. Lactic acid enters tricarboxylic acid cycle (TCA) and subsequently meabolised to CO_2 and H_2O and eliminated from the body. Glycolic acid may

excrete unchanged in urine or it enters TCA cycle and eliminated as CO_2 and H_2O.

1.6.2.3. Immune reactions/biocompatibility

Development of nanoparticulate formulation requires knowledge of cellular and tissue responses, which determine the biocompatibility of the polymer. The biocompatibility of a material is determined by two main factors, the host reactions induced by the material itself and by its degradation products in the body. Consequently, determination of the degradation rate of polymer and local tissue clearance is important for predicting the polymer concentration in the tissue and the resultant immune response (Sinha *et al.*, 2004).

Understanding of the inflammatory and healing responses to materials is required for the evaluation of the biocompatibility of drug delivery systems because inflammation, wound healing, and foreign body responses are considered components of the tissue or cellular host responses to injury. PLGA polymers have the advantage of being well characterised and are being already used for commercially available US FDA approved microparticulate formulations (Table 1.6) (Jain, 2000; Mundargi *et al*., 2008).

Since PLGA copolymer biodegradation products are formed at a very slow rate, they do not affect normal cell function. PLGA polymers have been evaluated for toxicity and safety in extensive animal studies and are currently used in humans for resorbable sutures, bone implants, screws, and contraceptive implants (Hanafusa *et al.*, 1995; Katz, 2001). These polymers are also used as graft materials for artificial organs development, and recently as supporting scaffolds in tissue engineering research (Langer, 1997; Mooney *et*

al., 1997; Eiselt *et al.,* 1998). Long-term biocompatibility was demonstrated by the absence of any untoward effects on intravascular administration of PLGA NPs to arterial tissue in pig and rat models of chronic restenosis (Guzman *et al.,* 1996; Panyam and Labhasetwar, 2003).

Table 1.6 PLGA-based products on market

Drug	Trade name	Company
Leuprolide acetate	Lupron Depot®	TAP
Growth hormone	Nutropin Depot®	Genetech
Buserelin acetate	Suprecur® MP	Aventis
Triptorelin pamoate	Decapeptyl®	Ferring
Octreotide acetate	Sandostatin LAR® Depot	Novartis
Lanreotide	Somatuline® LA	Ipsen
Triptorelin pamoate	Trelstar® Depot	Pfizer
Minocycline	Arestin®	Orapharma
Risperidone	Risperidal® Consta	Johnson & Johnson

The biocompatibility of a PLGA may be affected by various factors including its chemical composition, molecular weight, crystallinity, and the environment in which it has been placed.

The tissue responses to the polymer materials are broadly divided into three time phases as shown in Table 1.7 (Anderson and Shive, 1997). The 50:50 PLGA NPs have a phase II response of 50 to 60 days, whereas for the PLA microspheres, it takes around 350 to 400+ days, thereby indicating its dependence on the rate of biodegradation of the NPs (Visscher *et al.,* 1985; Visscher *et al.,* 1986).

Table 1.7 Immune **r**esponses to polymer material

Phase	Duration	Response
I	1–2 week	Acute or chronic inflammatory responses that are independent of the degradation rate and the polymer composition
II	0–3 week	Response depends on the rate of polymer degradation and includes granular tissue development, foreign-body reaction, and fibrosis
III	3 week	Phagocytosis by macrophages and foreign-body giant cells

1.6.3. Methods of preparation of NPs

The use of a particular manufacturing technique in the preparation of NPs depends on various factors including the nature of the polymer employed, nature of the drug to be encapsulated, intended use of the system, and intended duration of the therapy. The parameters those can be controlled externally to yield NPs of desired particle characteristics include type and concentration of polymer, its molecular weight and copolymer composition, nature and solubility of the drug to be encapsulated, drug-loading concentrations, type and volume of the organic solvent, the aqueous phase volume, pH, temperature, concentration, types of surfactants, and the mechanical speed of agitation. Conventionally, NPs are prepared using one of the following techniques.

1.6.3.1. Phase separation (coacervation) in aqueous system

The coacervation technique for preparation of polyester microspheres was first reported by Fong in 1979 and modifications of the same are used now-a-days for the preparation of NPs. This method involves precipitation of the

drug-entrapping polymer by the addition of a third compound to the drug-polymer solution or by some other physical means (Fong, 1979). Generally, the process consists of two steps, first is the formation of liquid droplets of the polymer from the solution phase, which depends on the solubility parameters of the polymer and, subsequent hardening of the polymer droplets by extraction or evaporation of the polymer solvent. A number of organic solvents, such as dichloromethane, isopropanol, and heptanes, have been used as solvent, coacervating agent, and hardening agent. Phase separation could occur as a result of counterions (Rajaonarivony *et al.*, 1993), changes in pH (El-Samaligy and Rohdewald, 1983), or as a result of the aqueous phase acting as a nonsolvent for the polymer. Both, hydrophilic and hydrophobic drugs could be entrapped by this technique, although with different drug-entrapment efficiencies. For example, hydrophilic drugs can be solubilised in water and this aqueous phase can be added to an organic solution of the polymer to form w/o emulsion (Ruiz *et al.*, 1989), whereas lipophilic drugs can be dissolved/dispersed in the polymer solution. Entrapment efficiency of hydrophilic drug decreases significantly if a large volume of water is used in the process or water is used as a coacervating agent. Various process variables such as the aqueous phase/organic phase volume ratio, stirring rate, addition rate of the nonsolvent, polymer concentration, polymer solvent/nonsolvent ratio, and viscosity of the nonsolvent affect the characteristics of the NPs (Nihant *et al.*, 1994; Nihant *et al.*, 1995). The major advantage of coacervation method is that it protects active drugs from partitioning out into the dispersed phase. However, the residual solvent content is a major concern, especially when organic solvents are used as the hardening agent.

1.6.3.2. Single emulsion-solvent evaporation

This method is primarily used for entrapping lipophilic/water-insoluble drugs. In this technique, the drug and polymer are first dissolved in a water-immiscible, volatile organic solvent such as chloroform or dichloromethane. The drug-polymer solution then emulsified into an aqueous phase containing an emulsifier such as poly(vinyl alcohol) (PVA), gelatin, polysorbate 80, or polaxamer-188 to form an o/w emulsion. Subsequently, the formulation is subjected to organic solvent removal/extraction to harden the nanoemulsion droplets into solid NPs. Size reduction of the polymer droplets could be facilitated by high-speed homogenisation or sonication. For the removal of solvent, the stirring process may be continued for several hours at elevated-temperature/low-pressure conditions. The rate of solvent extraction or evaporation has significant effects on the porosity of the NPs, which, in turn, affects the drug release from the NPs. Since the solvent extraction is normally faster than the evaporation rate (the latter depends on the boiling point of the solvent), the resultant porosity of the particle matrix prepared by the solvent extraction method is usually greater than one prepared by the evaporation process (Arshady, 1991). One of the major challenges encountered in this method is the poor entrapment and burst release effect of water-soluble and hydrophilic drugs because of their diffusion and partitioning from the dispersed oil phase into the aqueous continuous phase.

1.6.3.3. Double emulsion (w/o/w)-solvent evaporation

The encapsulation efficiencies of the water-soluble drugs can be improved by using double (w/o/w) emulsion method. This technique involves

emulsification of an aqueous drug solution into organic polymer solution under vigorous stirring to form a w/o emulsion. This w/o emulsion is again emulsified into second aqueous phase containing stabiliser to form the w/o/w emulsion. The formulation is then subjected to solvent removal (Vandervoort and Ludwig, 2002). After evaporation of organic solvents, the polymer precipitates as NPs. There are several variables, which can affect the characteristics of NPs formed by this method. These include the amount of drug to be incorporated, type and concentration of stabiliser used, the polymer concentration, and the volume of external aqueous phase.

1.6.3.4. Emulsification solvent-diffusion method

This technique was developed by Doelker and coworker, which involved emulsification of drug-polymer solution in partially water-miscible solvent (such as ethyl acetate, benzyl alcohol, propylene carbonate) pre-saturated with water, in an aqueous solution containing stabiliser under vigorous stirring to form o/w emulsion. The process is followed by reduction in droplet-size using a high-speed homogeniser/sonicator (Quintanar-Guerrero *et al.,* 1996). Subsequently, water is added to the o/w emulsion system under constant stirring causing outward diffusion of the organic solvent from the internal phase, leading to the nanoprecipitation of the polymer. Finally, the solvent can be eliminated by evaporation or vacuum distillation.

1.6.3.5. Salting out method

The salting-out method for the production of NPs have been developed to meet the US FDA specification on the residual amount of organic solvents in injectable colloidal systems. Polymeric NPs can be prepared by using an

emulsion technique that avoids chlorinated solvents and involves a salting-out process between two miscible solvents to separate the phases (Allémann *et al.,* 1992). This technique involves addition of an electrolyte-saturated (usually magnesium chloride hexahydrate) or a non–electrolyte-saturated aqueous solution containing stabiliser to an oil phase composed of the polymer and the drug dissolved in acetone under continuous mechanical stirring at room temperature. The saturated aqueous solution prevents miscibility of both the phases by virtue of the high salt content. After the preparation of the initial water-in-oil emulsion (w/o), water is immediately added in sufficient quantity to cause a phase inversion from water-in-oil (w/o) to oil-in-water (o/w) type emulsion; which induces complete diffusion of acetone from the internal nonaqueous phase into the continuous external aqueous phase, thus leading to the formation of NPs. The formulation is then subjected to solvent removal.

1.6.3.6. Solid-in-oil-in water (s/o/w) emulsion-evaporation

Double emulsion (w/o/w) method has been often used for successfully encapsulating a wide range of proteins/water soluble drugd in the particles, because it allowed hydrophilic proteins to be dissolved in an aqueous phase before the encapsulation process. However, the structural properties of proteins might be altered to some extent by interfaces and agitation stress with this method. To overcome this, the s/o/w method is often employed as an alternative to the double emulsion method, in order to avoid the water-organic solvent interface during the first emulsification step. Briefly, proteins are directly suspended in ethyl acetate or methylene chloride and sonicated to obtain a finely dispersed solid-in oil (s/o) suspension. Subsequently, polymer is dissolved in the same. The resultant s/o dispersion is emulsified

into an aqueus phase containing stabiliser to form s/o/w emulsion, which is subjected to solvent removal to form the NPs (Qiu *et al.,* 2007). Since the proteins are formulated as solids (by dispersing directly into the organic solvents) avoiding the water-organic solvent interface and agitation, they can maintain their secondary and tertiary structure, which is the key advantage of the method. Another major advantage of this technique is higher entrapment efficiency of water soluble drug (Qiu *et al.,* 2007).

1.6.3.7. Solid-in-oil-in oil (s/o/o) emulsion

This is an important method for encapsulating water soluble/sensitive drugs as it uses anhydrous system for particle preparation (Herrmann and Bodmeier, 1998; Marinina *et al.,* 2000; Jiang and Schwendeman, 2001; Sanchez *et al.,* 2003). Briefly, dehydrated protein/drug powder is suspended in a polymer solution (inner oil phase consisting of N,N-dimethylformamide (DMF)), and then the suspension is emulsified into an oil continuous phase (outer oil phase consisting oils like corn oil containing surfactant) and homoginised to form an s/o/o emulsion. After that, ethyl ether is slowly added into the emulsion to extract DMF. Then the particles are collected by centrifugation, washed in turn by ethyl ether, ethanol and water, and lyophilised.

1.6.3.8. Nanoprecipitation

This technique is employed to incorporate lipophilic drugs into the polymeric carriers based on the interfacial deposition of a polymer following displacement of a semipolar solvent miscible with water from a lipophilic solution (Molpeceres *et al.,* 1996; Govender *et al.,* 1999). Drug and polymer

are dissolved in a semipolar water-miscible solvent, such as acetone, dimethyl sulfoxide (DMSO) or ethanol. The solution is then poured or injected into an aqueous solution containing stabiliser under magnetic stirring and due to rapid solvent diffusion NPs are formed instantaneously. The solvent is then removed from the NPs suspension under reduced pressure. The injection rate of the organic phase into the aqueous phase may affect the particle size. This technique gives particles with relatively narrow size distribution. The drug entrapment efficiency was found to be lower for the hydrophilic drugs compared to lipophilic drugs because of their poor interaction with the polymer leading to diffusion of the drug from the organic phase to the external aqueous environment. Improved bioavailability of proteins and peptides was demonstrated using PLGA NPs by the nanoprecipitation method (Barichello *et al.,* 1999). Govender *et al.* (1999) showed improved incorporation of the water-soluble drug (procaine hydrochloride) into PLGA NPs by increasing the aqueous phase pH and replacing procaine hydrochloride by procaine dihydrate base. The trouble faced in this method is the choice of drug/polymer/solvent/nonsolvent system in which the NPs would be formed and the drug efficient efficiency.

1.6.3.9. Spray drying

This method offers a relatively rapid and convenient production of NPs that is easy to scale-up, involves mild processing conditions, and has relatively less dependence on the solubility characteristics of the drug and the polymer. In spray drying technique, a solution of a drug in an organic solvent containing the polymer is sprayed from the sonicating nozzle of a spray dryer and subsequently dried to yield NPs. The process parameters those can be varied include the inlet and outlet air temperatures, spray flow, and

compressed spray air flow (represented as the volume of the air input). In a novel, low-temperature, freeze–spray-drying method (Johnson *et al.*, 1999), the drug either solibilised in an organic solvent containing the dissolved polymer is sprayed or atomised through an ultrasonic nozzle into a vessel containing liquid nitrogen overlaying frozen ethanol and frozen at −80°C and lyophilised. The liquid nitrogen is evaporated, upon which the melting liquefied ethanol extracts the organic solvent from the frozen droplets causing the particles to harden. The NPs are subsequently filtered and dried under vacuum. With an aim to avoid significant product loss due to nanoparticulate adhesion on to the interior wall of the spray dryer, as well as to prevent the aggregation of the NPs, a double-nozzle spray-drying method has been developed together with the use of mannitol as an anti-adherent (Takada *et al.*, 1995). In this method, drug solution or suspension in the polymer solution is sprayed from one nozzle, with the aqueous mannitol solution being simultaneously sprayed from another nozzle. In this process, the surface of the spray-dried NPs gets coated with mannitol and the agglomeration is reduced.

1.6.3.10. Supercritical fluid technology

Major advantage of the method is that the use of organic solvent/surfactant can be avoided/minimised and thus producing NPs, which are free from toxic impurities. CO_2 is nontoxic, nonflammable, and environmentally acceptable, and supercritical CO_2 can be easily obtained by pressurising and heating the CO_2 system to a minimum of 73.8 bars and 31.05°C, respectively. In the supercritical anti-solvent technique, both, the drug and the polymer, are dissolved in a suitable organic solvent and are atomised through a nozzle into supercritical CO_2 (Dixon *et al.*, 1993; Bodmeier *et al.*, 1995; Subramaniam

et al., 1997). Subsequently, the dispersed organic solvent phase and the anti-solvent CO_2 phase diffuse into each other. Since CO_2 is miscible only with the solvent and not with polymer, the solvent gets extracted causing the supercritical fluid–insoluble solid to precipitate as NPs. The rates of solvent diffusion are much faster than that for conventional organic anti-solvents. With decreasing density of CO_2, the atomisation of the spray is intensified leading to faster mass transfer rates, thus rapid nucleation and smaller particle sizes (Sassiat *et al.,* 1987). The dried NPs are then collected following the depressurisation of CO_2.

In the gas anti-solvent method (Randolph *et al.,* 1993), anti-solvent CO_2 is introduced into the organic solution containing the solutes of interest. As supercritical CO_2 is miscible only with the solvent and it does not solubilise the solutes, it causes the solvent concentration to be significantly lowered, resulting in the precipitation of the drug-polymer matrix as NPs. Later, the solid product is flushed with fresh CO_2 to remove the residual solvent. The rate of addition of CO_2 to the organic solution affects the final particle size. A major challenge in this technique is the need to filter the precipitate from the organic solvent solution without particle growth and aggregation.

In the rapid expansion of supercritical solutions technique (Matson *et al.,* 1987), the drug and polymer are dissolved in supercritical CO_2 and this solution is atomized through a nozzle into a collection chamber at atmospheric conditions. When expanded, CO_2 immediately evaporates and the solute precipitates as a coprecipitate of the drug embedded in the polymer matrix. Various parameters that affect the resulting particle size and morphology are the pre- and postexpansion temperature and pressure, nozzle geometry, and solution concentration (Philips and Stella, 1993; Subramaniam *et al.,* 1997). The disadvantages of this method include the use

of higher temperatures to form homogenous precipitates (which can cause degradation of thermally labile drugs) and the limited solubility of the polymers and drugs that results in low drug loading (Ting *et al.,* 1993).

1.6.4. Physical stability of the NPs (DLVO theory)

Particle collision in a suspension fromulation may occur due to Brownian motion. The collision would results either in the formation of aggregates or redispersion of the particles. The outcome of collision depends on the attractive or repulsive forces between the particles and determines the quality of the preparation. Zeta potential plays a very important role in suspension stability. A minimum, known as the critical zeta potential, is essential to prepare a stable suspension. A colloidal system with low critical zeta potential value signify that only a minute charge is required for stabilisation and it will show marked stability against the added electrolytes. The precipitation/destalisation of suspension can be brought about by adding electrolytes. The precipitating power increases rapidly with the valence of the ions, which is known as the Schulze-Hardy rule.

Derjaguin and Landau, and Verwey and Overbeek investigated independently using the knowledge from Schulze-Hardy rule to describe the stability of lyophobic colloids (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). This is called the classic DLVO theory that explains the result of particle interaction in lyophobic colloids. According to DLVO theory, the potential energy of interaction between particles, V_T is the result of repulsion due to electrical double layer, V_R and attraction due to van der Waals' force, V_A and can be shown by the following equation (Matthews and Rhodes, 1970):

$$V_T = V_R + V_A$$

V_R depends on several factors including the zeta potential of the system, the particle radius, the interparticular distance, the dielectric constant of the medium, whereas the factors that affect V_A include the particle radius, and the interparticular distance.

DLVO theory can be easily understood with Fig. 1.8, which shows net energy of interaction between two particles as a function of interparticulate distance. Electrostatic repulsion due to the electric double layer and the attraction due to van der Waals' force are shown in the opposite direction due to their opposite nature of force. At any distance from a particle (h), the net energy (V_T) can be calculated by subtracting the smaller value from the larger one. When the net energy curve remains above the baseline, it indicates repulsion. On the other hand, attraction can be shown by the curves below the baseline. The maximum repulsive value is known as the energy barrier. In order to agglomerate, two colliding particles must have sufficient momentum to cross this barrier. As the particles overcome the repulsion, they agglomerate due to the attractive force and can be considered trapped due to van der Waals'-London force.

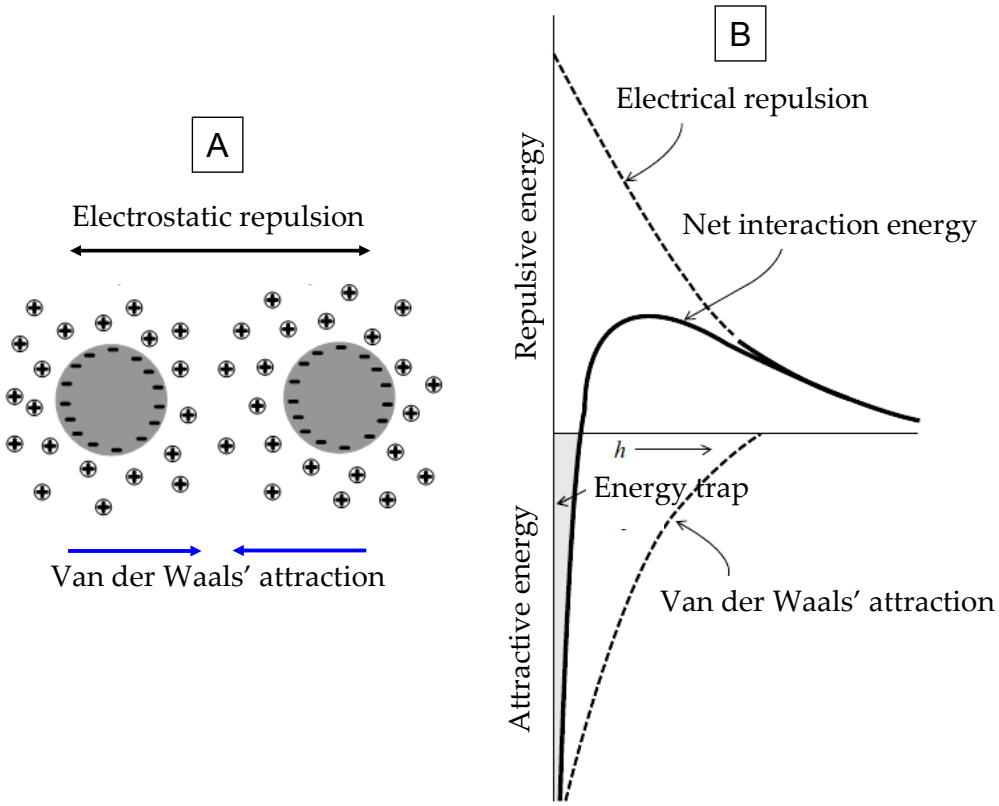


Fig. 1.8 Electric double layer (A) and net energy plot of interaction between two particles as a function of interparticulate distance (B).

The net energy interaction curves at different situations are shown in Fig. 1.9. Curve A exists when V_R is much larger than V_A ($V_R >> V_A$). In such situation, the dispersion will be highly stable due to the high net repulsive force. This dispersion is resistant to aggregation (i.e., flocculation or coagulation) as long as the particles do not sediment under gravity. Curve B explains a situation in which a high energy barrier, V_M, must be overcome by the particles to form aggregates. If V_M greatly exceeds the mean thermal energy of the particles, these particles will not enter P, the primary energy minimum (Tabibi and Rhodes, 1996). The minimum value of V_M that can create this situation corresponds to a zeta potential of more than 50 mV (Flory, 1953; Napper, 1967; Raghavan *et al.,* 2000). A very small interparticular distance is found at P. The high magnitude of energy at P causes the particles to bond tightly together. Consequently, it is possible that it will compact into a hard cake, which will be very hard to redisperse. Occasionally, a secondary minimum of curve B at S may occur. Loose aggregates can be created at this point, and this aggregate can usually be broken easily by shaking or dilution. The aggregation of solid particles in suspension systems can be termed either as "flocculation" or "coagulation." Flocculation occurs at the secondary energy minimum, S; and coagulation occurs at the primary energy minimum, P, of the potential energy curve of two interacting particles (Fig. 1.9). Curve C represents a situation where attractive forces predominate over repulsion forces all the time ($V_A >> V_R$); and rapid aggregation will occur.

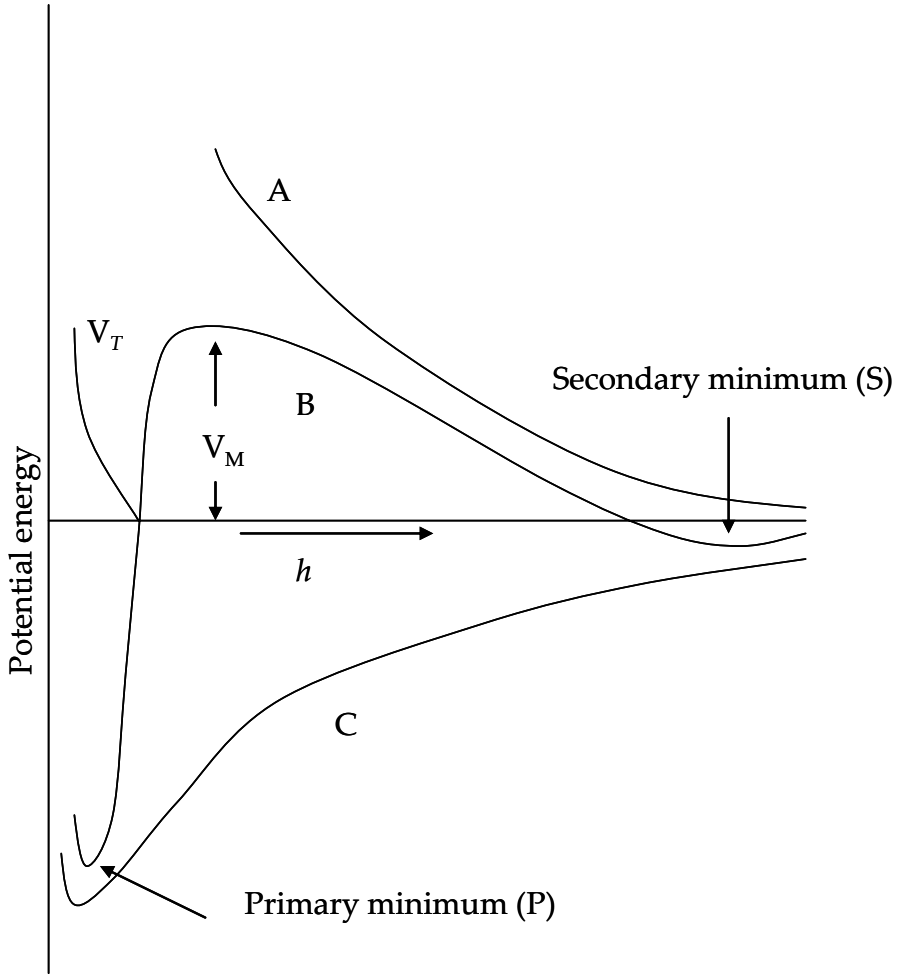


Fig. 1.9 Net energy interaction curves at different situations of attractive and repulsive forces. V_T, net energy of the particle; V_M, high energy barrier; P, primary energy minimum; S, secondary energy minimum; h, distance from a particle.

In addition to electrostatic stabilisation, steric stabilisation can also be applied to prepare a stable dispersion. Substances such as nonionic surfactants or polymers, when adsorbed at the particle surface (Fig. 1.10), can stabilise the dispersion, even in absence of significant zeta potential (Matthews and Rhodes, 1970). Therefore, the term for steric stabilisation, V_S, should be added to the equation obtained in DLVO theory, which gives rise to:

$$V_T = V_R + V_A + V_S$$

Steric stabilisation of colloids involves polymeric molecules/surfactants added to the dispersion medium in order to prevent the aggregation of the colloidal particles. The polymeric molecules create a repulsive force counterbalancing the attractive van der Waals force acting on a particle approaching another particle. Two types of steric stabilisation could be possible: (i) the steric stabilisation of colloids is achieved by polymer/surfactant molecules attached to the particle surface and forming a coating, which creates a repulsive force and separates the particle from another particle, and (ii) depletion stabilisation of colloids involves unanchored (free) polymer/surfactant molecules creating repulsive forces between the approaching particles.

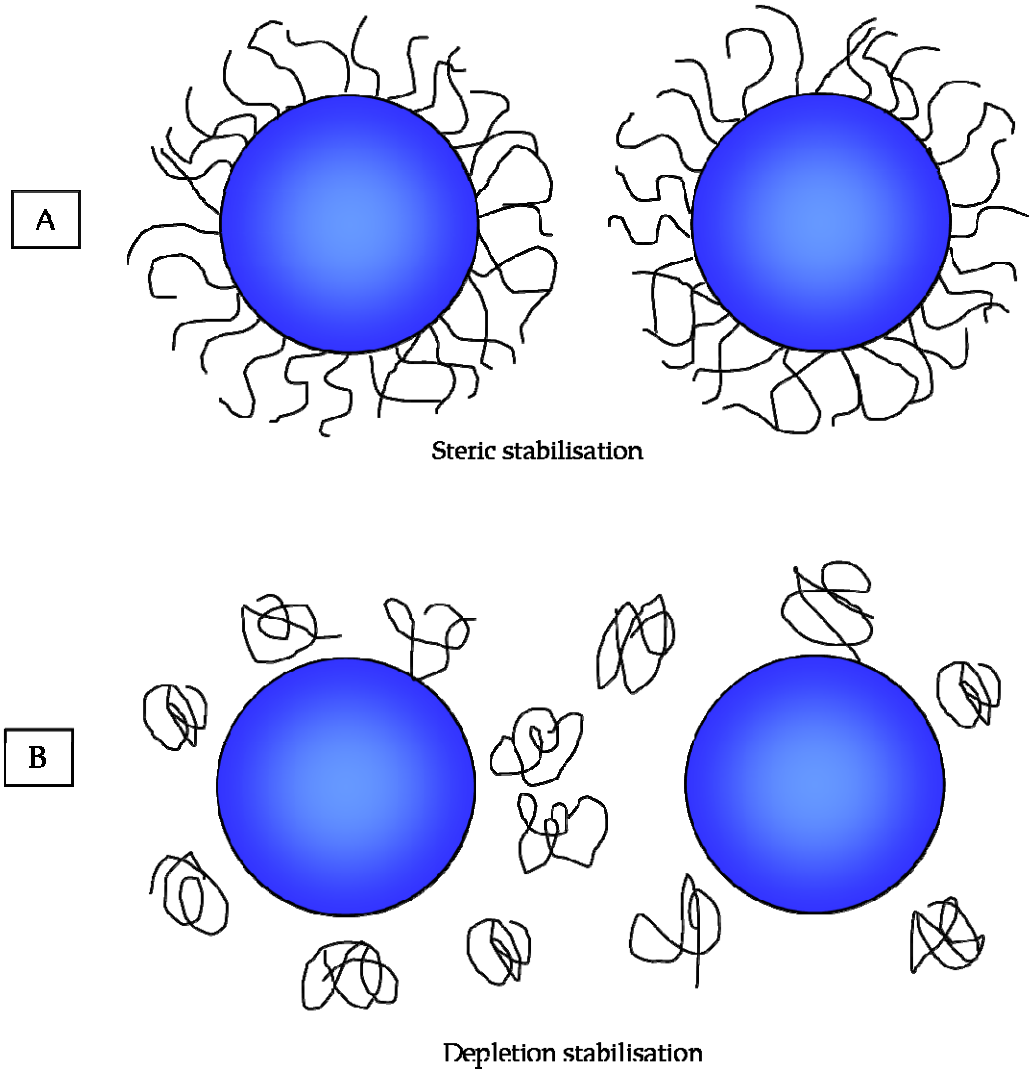


Fig. 1.10 Stabilisation of colloids: (A) steric stabilisation and (B) depletion stabilisation

1.7. Objective and specific aims

In the preliminary studies, we have reported preparation and evaluation of nanoparticulate formulation of AMB. The particle size of the formulation was 165.6±2.9 nm with 34.5±2.1% entrapment efficiency at 10% w/w (of polymer) initial drug loading. The studies rodents showed that this formulation has improved oral bioavailability of AMB and exhibited sustained *in vivo* release of AMB with significant reduction in the toxicities associated with the conventional AMB therapy (Italia *et al*., 2009).

Particle size is the most critical factor, which determines the extent of intestinal uptake and fate of the NPs in biological system (Jani *et al.,* 1990; Desai *et al.,* 1996; Desai *et al.,* 1997). Significantly higher intestinal uptake of small-sized (100 nm) particles has been observed compared to larger (1 and 10 μm) particles. Accordingly, the nanoparticulate formulation with smaller particle size around of ~100 nm would be better for oral delivery.

Apart from particle size, drug loading is another important parameter that affects the overall bioavailability of the nanoparticulate formulation. Previous experiments in our laboratory have shown that overall bioavailability of the nanoparticulate formulation is increased with increasing the drug loading as a result of more numbers of drug molecules passes into systemic circulation per particle absorbed.

Considering these, we hypothesized that nanoparticulate formulation with smaller particle size and higher drug loading would enhance the over all intestinal absorption of AMB and offer a potential means of oral therapy of systemic fungal infections and VL. The specific aims defined for the dissertation were:

1. To develop analytical methods for *in vitro* and *in vivo* analysis of AMB
2. To study the influence of process variables/parameters on the formulation characteristics.
3. To develop an efficient purification processes for the AMB-NPs and scale-up the process.
4. To freeze-drying the formulation for enhanced shelf life.
5. To evaluate the potential of oral AMB-NPs for treating systemic fungal infections and VL.

CHAPTER 2

DEVELOPMENT OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (RP-HPLC) METHODS FOR *IN VITRO* AND *IN VIVO* ANALYSIS OF AMB

2.1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) is perhaps the most versatile analytical technique available to the modern analysts. In a single step process, it can separate a mixture into its individual components and simultaneously provide a quantitative estimate of each constituent.

2.1.1. Principles of RP-HPLC

The retention mechanism in RP-HPLC is mainly based the competitive hydrophobic interactions of the analyte and eluent components with the adsorbent surface (stationary phase) (Dorsey and Cooper, 1994). The stronger the interactions of the analyte with the stationary phase, the longer its retention.

RP-HPLC is an adsorptive process, in which separation occurs due to partitioning of analytes between a stationary phase contained in a column and a liquid phase, which is pumped under pressure through this column. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase.

If we consider a two-component mixture, A and B, each of the components will have a certain affinity for the stationary phase and a certain affinity for the mobile phase. Provided there is sufficient difference between the analytes in their relative affinities for the two phases, i.e. in their partition coefficients, then in an HPLC system they will separate. For example, analyte B is more hydrophobic compared to analyte A and hence have more affinity for the

hydrophobic stationary phase compared to mobile phase and will thus spend, relatively, greater time on the surface of the stationary phase (Fig. 2.1). For analyte A, the reverse may be true and the compound will spend a shorter time bound to the stationary phase. In this case, one would expect the two compounds to separate (to be resolved) and the compound A with the lower affinity for the stationary phase to be eluted from the column first.

The analyte nature and its appearance (e.g., ionisation state) in the mobile phase are also factors that affect the retention mechanism. Mobile phase pH influences the analyte ionisation equilibrium. The type of mobile phase, its composition, and presence of counterions affect the analyte solvation.

Reversed-phase chromatography generally uses gradient elution instead of isocratic elution. While drug molecules strongly adsorb to the surface of a reversed phase matrix under aqueous conditions, they desorb from the matrix within a very narrow window of organic modifier concentration. Along with the drug molecules, the typical *in vivo* sample usually contains a broad mixture of biomolecules with a correspondingly diverse range of adsorption affinities. Gradient elution, therefore, is favorable method for reversed phase separation of complex biological samples.

In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase as shown in Fig. 2.1. Accordingly, the degree of solute molecule binding to the reversed phase medium can be controlled by manipulating the hydrophobic properties of the initial mobile phase. Because of its excellent resolving power, RP-HPLC is an indispensable technique for the high performance separation of complex biomolecules.

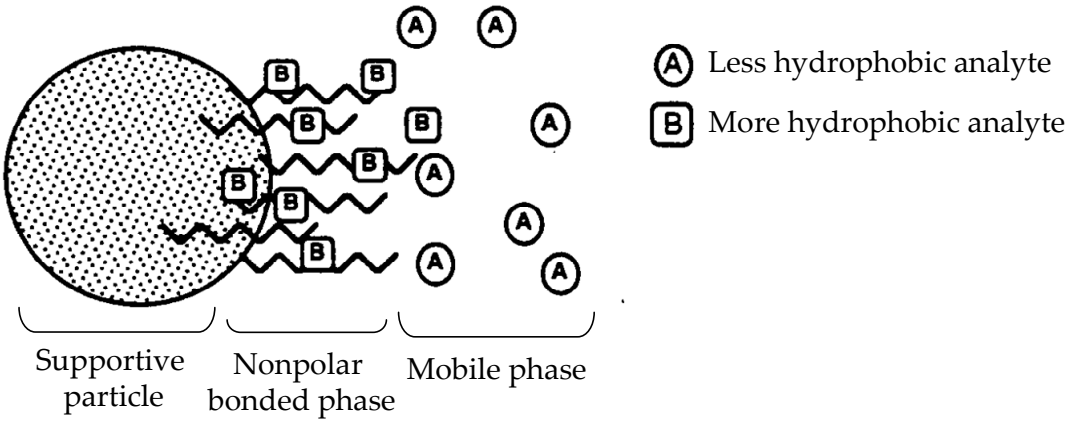


Fig. 2.1 Mechanism of separation in RP-HPLC. Less polar (more hydrophobic) analytes are more attracted to hydrophobic bonded phase, remain associated with the bonded phase for longer time and are eluted last.

2.1.3. Method validation

Validation of analytical method is the process used to confirm that the procedure employed for a specific test is suitable for its intended use. Results from validation process can be used to arbitrate the quality, reliability and consistency of analytical results; which is an integral part of any good analytical practice. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) provides guidelines for validation of an analytical method. ICH guidelines suggest few typical parameters, which should be considered for validation of analytical method include accuracy, precision, specificity, detection limit, quantitation limit, linearity and range.

2.1.3.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. The procedures used to demonstrate specificity would depend on the intended objective of the analytical procedure.

2.1.3.2. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration

or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be estimated. For the establishment of linearity, a minimum of 5 concentrations is recommended.

2.1.3.3. Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) 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.

2.1.3.4. Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the

analytical procedure. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

2.1.3.5. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability should be assessed using: (a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or (b) a minimum of 6 determinations at 100% of the test concentration.

- Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts,

equipment, etc. It is not considered necessary to study these effects individually.

- Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology). Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardisation of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias.

2.1.3.6. Detection limit (DL)

The DL of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the DL are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on visual evaluation: Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The DL is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on signal-to-noise: This approach can only be applied to analytical procedures, which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably

detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the DL.

Based on the SD of the response and the slope: The DL may be expressed as:

$$DL = 3.3\ \sigma/S$$

where, σ = the standard deviation of the response, S = the slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example: The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example: (1) based on the SD of the blank, where measurement of the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and the SD of these responses are calculated; and (2) based on the calibration curve, in which a specific calibration curve is studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the SD.

2.1.3.7. Quantitation limit (QL)

The QL of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The QL is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Several approaches for determining QL are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation: Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The QL is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise Approach: This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the SD of the response and the slope: The QL may be expressed as:

$$QL = 10\ \sigma / S$$

where, σ = the SD of the response, S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

Based on SD of the blank: Measurement of the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the calibration curve: A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the SD.

2.1.4. Analysis of AMB

Several HPLC methods have been reported for determination of AMB in biological fluids (Granich *et al.,* 1986; Shihabi *et al.,* 1988; Graves and Runyon, 1995; Lambros *et al.,* 1996; Echevarría *et al.,* 1998; Eldem and Arican-Cellat, 2001). The majority of the methods do not employ an internal standard (IS), and therefore, sample preparation has to be carefully controlled in order to minimise error, which can limit the repeatability, precision and the accuracy of the method (Graves and Runyon, 1995). Different IS have been used in AMB assays such as N-acetylamphotericin B (Granich *et al.,* 1986), piroxicam (Echevarría *et al.,* 1998) and natamycin (Lambros *et al.,* 1996), which are either expensive or not available easily. Moreover, most of these methods involve complex sample preparation like solid-phase extraction (Eldem and Arican-Cellat, 2001; Eldem *et al.,* 2001) and often require large volume of plasma (Eldem and Arican-Cellat, 2001; Mayhew *et al.,* 1983), which limits their application in pharmacokinetic studies in rodents. Therefore, a simple and effective method for *in vivo* analysis for AMB was developed using α-naphthol as an IS and validated according to ICH guidelines.

2.2. Materials

AMB was purchased from Fluorochem Ltd (Old Gossop, UK). α-aphthol was purchased from Sigma-Aldrich Co Ltd (Poole, UK). Acetonitrile (HPLC grade), DMSO (AR grade), sodium acetate trihydrate (AR grade), glacial acetic acid (AR grade) and water for HPLC were obtained from Fisher Scientific UK Ltd (Loughborough, UK).

2.3. Methods

2.3.1. *In vitro* analysis of AMB

2.3.1.1. Preparation of stock solutions

A stock solution of AMB (100 μg/ml) was prepared by dissolving 10 mg of AMB in 5 ml of DMSO. The volume was made up to 100 ml with methanol. The stock solution was diluted with methanol to obtain the calibration standards of 0.5, 1, 5, 10, 30 and 50 μg/ml.

2.3.1.2. Instrumentation and conditions

AMB was analysed using Thermo Finnigan HPLC system (Thermo Scientific, Hertfordshire, UK) consisting of Surveyor LC pump, Surveyor auto sampler, Surveyor photo-diode array detector (PDA) detector and a Hypersil Gold C_8 (4.6×150 mm, 5 μm) analytical column. The system was run on an isocratic mode. Mobile phase flow rate was kept at 1 ml/min and detection was carried out at the wavelength of 407 nm (λ_{max}), which was selected from the UV absorption spectra (Fig. 2.2).

The mobile phase composition was optimised by varying the ratio of acetonitrile and 10 mM sodium acetate buffer (pH 4). Different compositions of mobile phase (acetonitrile:sodium acetate buffer; 50:50, 35:65 and 25:75) were tried. The effect of pH of sodium acetate buffer was also evaluated using three pH value, pH 4, 6 and 7.

2.3.1.3. Method validation

The HPLC method was validated according to the ICH guidelines. For linearity, calibration curves were generated using six concentrations (in triplicates) of AMB (0.5, 1, 5, 10, 30, 50 μg/ml). Linearity was evaluated by linear regression analysis. Accuracy and precision of the method were determined with three replicates of quality control (QC) samples in the same day (intra-day) and different days (inter-day), respectively.

2.3.2. *In vivo* analysis of AMB

2.3.2.1. Preparation of stock solutions

A stock solution of AMB (100 μg/ml) was prepared by dissolving 10 mg of AMB in 5 ml of DMSO. The volume was made up to 100 ml with methanol. A stock solution of α-naphthol (100 μg/ml, used as IS) was prepared by dissolving 10 mg of α-naphthol in 100 ml of methanol.

2.3.2.2. Instrumentation and conditions

Samples were analysed using Waters HPLC system (Waters Corporation, Milford, USA), consisting of 717 plus auto sampler and 600 controller coupled to PDA detector and a Nucleosil® 100-5C_{18} (150X4.6 mm) analytical column (Macherey-Nagel, Dorean). The flow rate was adjusted to 1 ml/min and detection wavelengths were 407 and 294 nm for AMB and IS, respectively, as per the λ_{max} values obtained in the UV absorption spectra (Fig. 2.2).

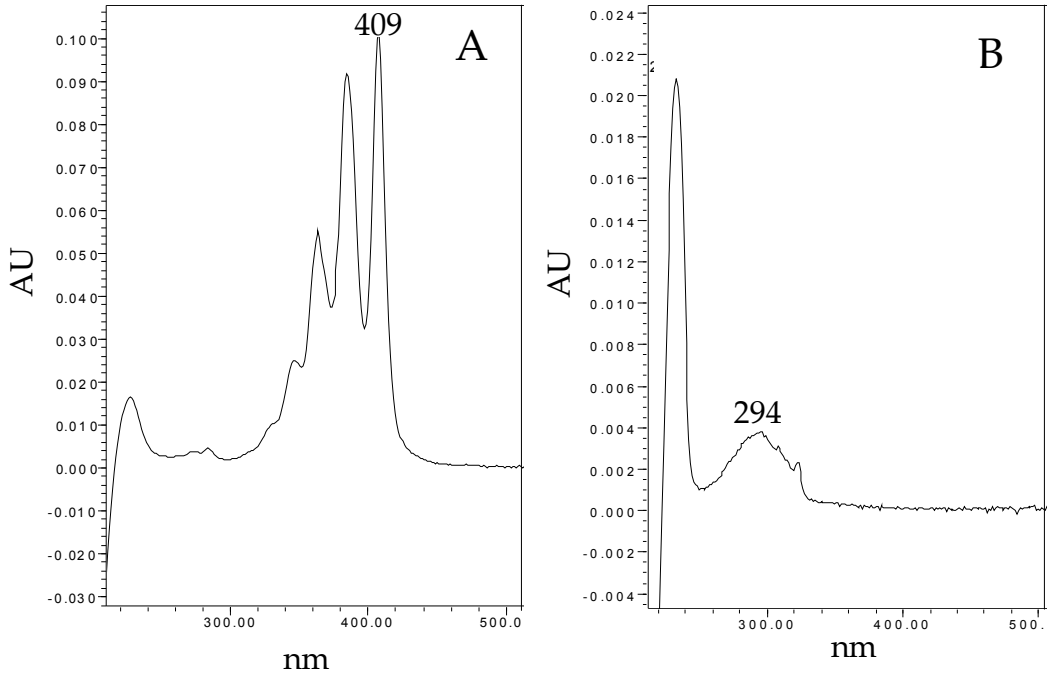


Fig. 2.2 UV absorption spectra of AMB and α-naphthol at 6.8 min (A) and 7.8 min (B) from the PDA detector showing λ_{max} values.

The effects of mobile phase composition on the retention behaviors of AMB and IS were investigated. Two types of mobile phases containing either methanol (methanol:10 mM sodium acetate buffer; 45:55; v/v) or acetonitrile (acetonitrile:10 mM sodium acetate buffer; 45:55; v/v) were compared under isocratic conditions. However, due to poor reolution under isocratic conditionsc, gradient elution was carrier out using three different mobile phase gradients A, B and C (Table 2.1) and their effects on separation of AMB and IS was evaluated.

Table 2.1 Mobile phase gradients evaluated for *in vivo* HPLC

Gradient	**Time (min)**	**Flow rate (ml/min)**	**Acetonitrile**	**% Buffer***	**Curve type†**
A	0.01	1.00	20.0	80.0	1
	4.00	1.00	80.0	20.0	1
	8.00	1.00	80.0	20.0	1
	9.00	1.00	20.0	80.0	1
B	0.01	1.00	30.0	70.0	1
	4.00	1.00	80.0	20.0	1
	8.00	1.00	80.0	20.0	1
	9.00	1.00	30.0	70.0	1
C	0.01	1.00	40.0	60.0	1
	4.00	1.00	80.0	20.0	1
	8.00	1.00	80.0	20.0	1
	9.00	1.00	40.0	60.0	1

* 10 mM sodium acetate buffer (pH 4).

† As specified in the Millennium software[32].

2.3.2.3. Sample preparation

Plasma samples (250 μl) were placed in micro-centrifuge tubes and spiked with different concentration of AMB standards (to obtain AMB concentrations of 10, 25, 50, 100, 500, 1000 and 2000 ng/ml), to which 1 ml of methanol containing 0.125 μg/ml of IS (α-naphthol) was added. Tubes were vortexed for 5 min, centrifuged at 15000 rpm for 5 min, the resulting clear supernatants were collected and 50 μl of each supernatant was injected into the HPLC.

2.3.2.4. Assessment of linearity, recovery and matrix effect (ME)

Four sets of five calibration plots were prepared to evaluate the linearity, recovery and ME.

Set-1 (Neat standards). Five calibration plots were constructed using neat solutions of AMB and IS in mobile phase and 50 μl aliquots were injected into HPLC.

Set-2 (Plasma samples spiked post-extraction). Five calibration plots were constructed using five different lots of plasma. 250 μl of plasma samples were placed in centrifuge tubes followed by the addition of 1 ml of methanol for protein precipitation. The tubes were then vortexed and centrifuged at 15000 rpm for 5 min. The clear extracts were collected, spiked with required amount of AMB and IS and 50 μl of the samples were injected into the HPLC.

Set-3 (Plasma samples spiked pre-extraction). Five calibration plots were constructed using five different lots of plasma (same plasmas used in set-2). 250 μl of plasma samples were placed in centrifuge tubes and spiked with required concentration of AMB. Then 1 ml of methanol containing 0.125 μg/ml of IS was added and the tubes were vortexed, centrifuged at 15000

rpm for 5 min. The clear supernatants were collected and 50 µl of each supernatant was injected into HPLC.

Set-4 (Plasma samples spiked pre-extraction without IS). Five calibration plots were constructed using same plasma as used in set-2. 250 µl of plasma samples were placed in centrifuge tubes and spiked with required concentration of AMB. Then 1 ml of methanol (without IS) was added and the tubes were vortexed and centrifuged for 5 min at 15000 rpm. The clear supernatants were collected and 50 µl of each supernatant was injected into HPLC.

The linearity of each standard curve was assessed by plotting the peak area ratio of AMB to IS *versus* AMB concentration. ME was expressed as the ratio of the mean peak area of AMB spiked post-extraction to the mean peak area of the same neat solutions of analyte multiplied by 100. The recovery of AMB and IS was expressed as ratio of mean peak area obtained in samples spiked pre-extraction and after extraction multiplied by 100.

2.3.2.5. Assessment of accuracy and intermediate precision

Plasma samples (n=5) were spiked with three different concentration of AMB (20, 500 or 2000 ng/ml) and then 1 ml of methanol containing 0.125 µg/ml of IS was added. The tubes were vortexed for 5 min, centrifuged at 15000 rpm for 5 min, the clear supernatants were collected and 50 µl of each supernatant was injected into HPLC. Three consecutive measurements were performed for each concentration within the same day and on three different days. A similar set of samples was also prepared omitting IS and analysed in the same manner.

2.3.2.6. Sensitivity

DL and QL for AMB were determined based on signal-to-noise concept, as the lowest concentrations at which signal-to-noise ratio is 3:1 and 10:1, respectively.

2.4. Results and discussion

2.4.1. *In vitro* analysis of AMB

The *in vitro* HPLC method of AMB was optimised by changing the ratio of mobile phase and pH of sodium acetate buffer. The optimal mobile phase composition was found to be 35:65 (acetonitrile:10 mM acetate buffer, pH 4). Decreasing the proportion of acetonitrile further in the mobile phase was resulted in the peak broadening while increasing the proportion of acetonitrile led to early elution of AMB along with other interfering peaks. The method was found to be specific for estimation of AMB extracted from AMB-NPs (Fig. 2.3) as no interfering peaks were fond to be co-eluted with the peak of AMB.

Calibration curves constructed in the concentration range of 0.5-50 μg/ml showed good linearity with correlation coefficient (r^2) value of >0.99 (Fig. 2.4). The method validation parameters including the intra-day and inter-day variability are shown in Table 2.2. The variability observed was in the range of 1.94-2.35 for intra-day and 2.94-4.64 for inter-day run. The accuracy of the method was in range of 96.4-103.1%.

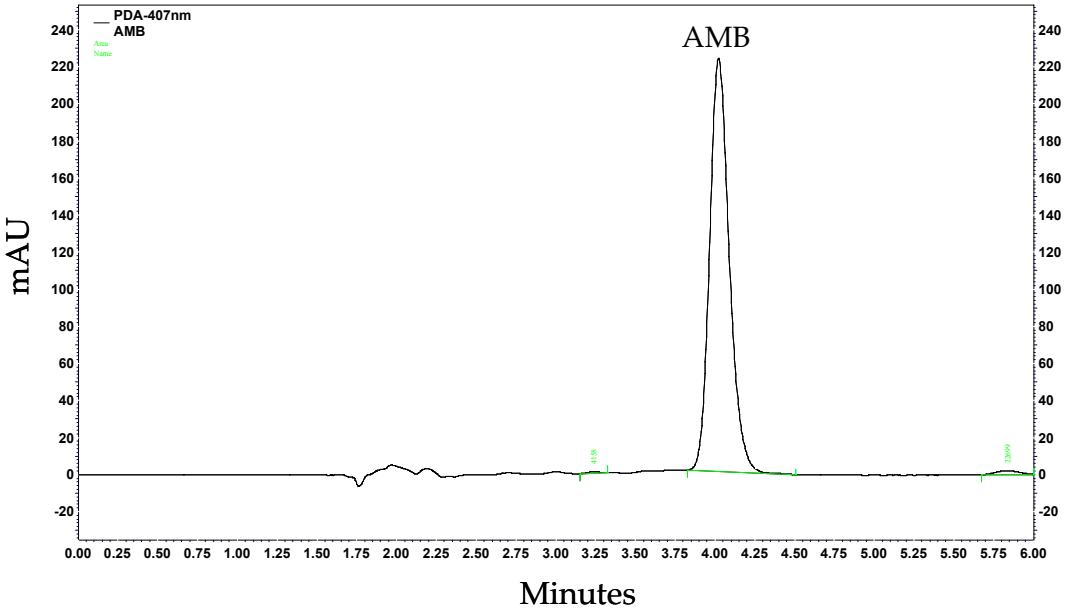


Fig. 2.3 Chromatogram of AMB extracted from AMB-NPs

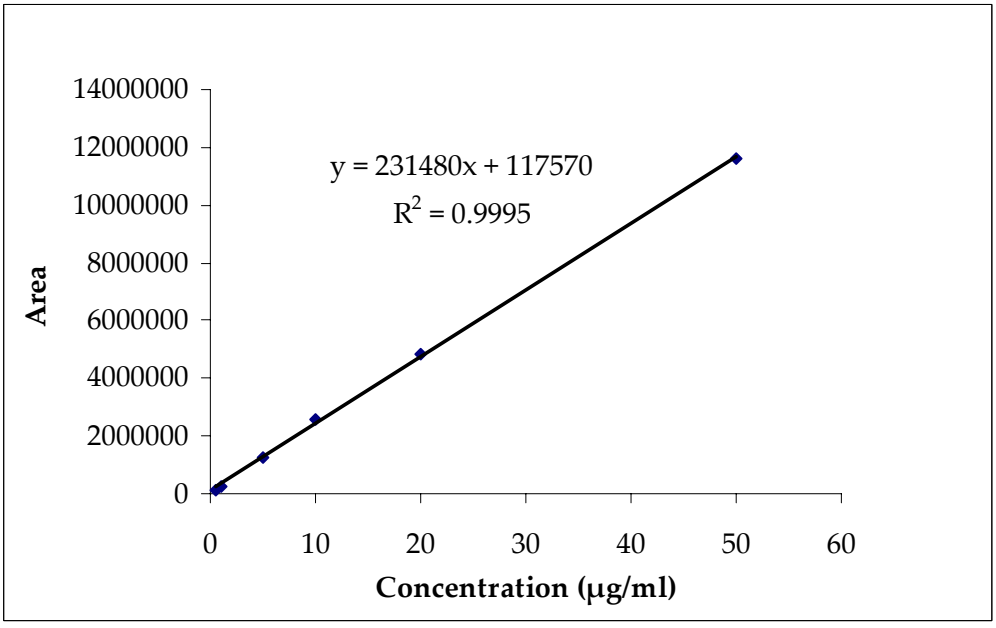


Fig. 2.4 Calibration curve for *in vitro* analysis of AMB

Table 2.2 Intermediate precision and accuracy of *in vitro* HPLC method

Concentration (µg/ml)	Intra-day*		Inter-day†	
	% Accuracy	Precision (%CV)	%Accuracy	Precision (%CV)
1	98.5	1.94	96.4	4.64
10	98.3	2.04	98.9	2.94
50	102.6	2.35	103.1	3.43

***** For intra-day variability triplicates were analysed three times on a single day.

† For inter-day variability triplicates of the concentrations specified were analysed on 3 consecutive days.

2.4.2. *In vivo* analysis of AMB

Unlike the reported methods using more complex sample preparation or expensive IS, the developed method provides a simple and reliable procedure for determination of AMB using low-cost and readily available IS. Although there is no structural similarity between AMB and α-naphthol, they share similarity in the solubility behavior in the mobile phase used and retention time, which makes α-naphthol an appropriate choice as the internal standard.

The effects of solvent composition of the mobile phase on the retention behavior of AMB and IS were investigated. Initially, methanol:sodium acetate buffer (45:55; v/v) and acetonitrile:sodium acetate buffer (45:55; v/v) were used as the two solvent systems and compared under the isocratic mode. The use of acetonitrile appeared to be better than that of methanol with respect to the peak symmetry of the analyte. However, even with the use of acetonitrile in the mobile phase there was peak broadening under isocratic conditions. With the use of gradient mode instead of isocratic mode, sharp peak of AMB was obtained. Among the diffent mobile phase gradients

tried, gradient C (Table 2.1) was found to be optimal. The retention times for AMB and the IS were 6.8 and 7.8 min, respectively (Fig. 2.5). The specificity of the method was confirmed by the UV spectra of the compounds (Fig. 2.2) and no change in the pattern of AMB and IS spectra was observed in the extracted plasma samples indicating that no interfering peaks were be co-eluted with AMB or IS. The chromatograms obtained for blank and spiked plasma samples with AMB and IS are shown in Fig. 2.5. No interfering peaks with retention times similar to AMB or IS were observed in blank plasma sample chromatogram further confirming the specificity of the method.

2.4.2.1 Assessment of linearity, recovery and ME

Linearity of the developed method was assessed with plasma samples spiked with known concentrations of AMB and IS over a concentration range of 10-2000 ng/ml. A correlation coefficient of >0.99 (Fig. 2.6) was obtained. The assessment of the ME on quantitative bioanalysis of drugs and metabolites is an important and often overlooked element of assay validation. It is not only required by the current validation standards but is also critical to generate reliable pharmacokinetic data (Shah *et al.,* 2000). In general, validation experiments and daily run-to-run standard curves are prepared in a single lot of a biofluid, which is then used to quantify an analyte in biofluid samples originating from a huge number of different subjects. Apparently, the same biofluid (for example, plasma) from these subjects may contain different endogenous compounds that were not present in the plasma lot used during assay validation, which can lead to error in estimation. However, the reliability of the method can be considerably improved through eliminating a ME by evaluating control biofluids (plasma, for example) from different sources or subjects (Matuszewski *et al.,* 2003).

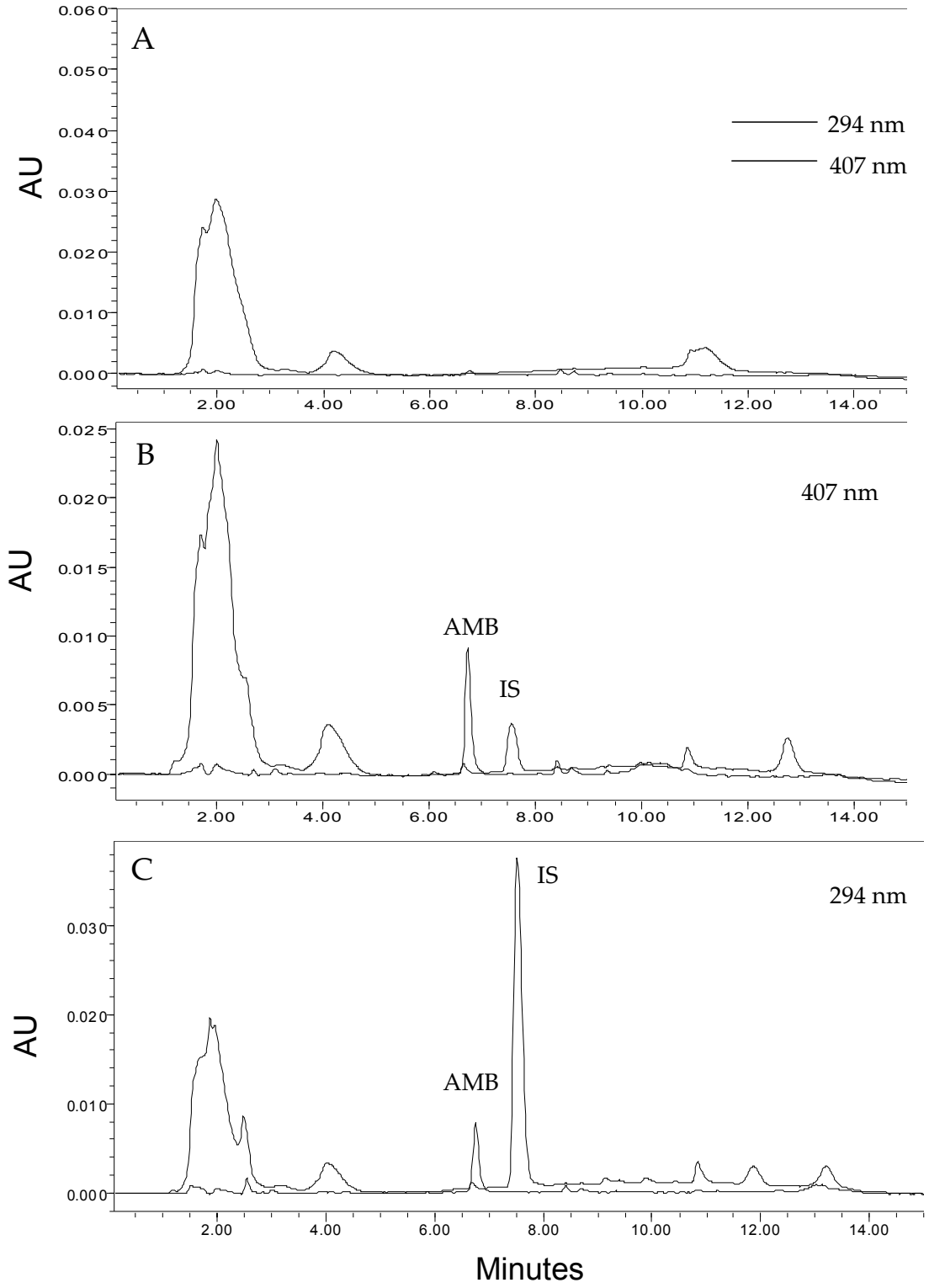


Fig. 2.5 Chromatograms of the methanolic extracts of blank plasma sample (A), plasma sample spiked with AMB and IS (chromatogram extracted at 294 nm, B) and plasma sample spiked with AMB and IS (chromatogram extracted at 294 nm, C).

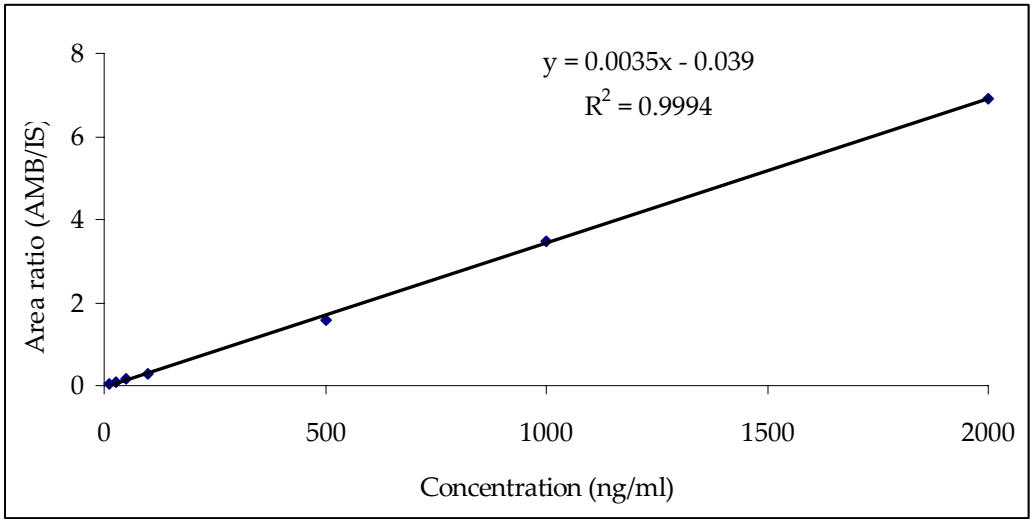


Fig. 2.6 Calibration curve for *in vivo* analysis of AMB

The ME or the possibility of peak suppression or enhancement for AMB and IS was evaluated by comparing the results of analysis of different sets of samples prepared as described in the experimental section 2.3.2.4. The results of analyses of set-1 (neat standards) provided an insight into detector performance in measuring the absolute peak areas on consecutive injections and the overall HPLC system reproducibility. In set-2, plasma samples originating from five different sources were first extracted and then spiked with the AMB and IS. Any additional variability in %CV of the peak areas for the analytes as compared to set-1, at each concentration, would indicate an effect of sample matrix as same concentrations of the anlaytes were spiked with plasma extracts. In set-3, same plasma samples (as in set-2) were spiked with AMB and IS before extraction. The variability in %CV values here would reflect a combined effect of a sample matrix and potential differences in recovery of analytes from different plasma lots.

The ME and recovery of the extraction procedure were calculated by comparing the absolute peak areas for AMB obtained in sets 1-3. If one depicts the peak areas obtained in set-1 as A, set-2 as B and set-3 as C, the ME and recovery values can be calculated as follows (Matuszewski *et al.,* 2003):

$$\text{ME (\%)} = (B/A) \times 100$$

$$\text{Recovery (\%)} = (C/B) \times 100$$

The ME calculated in this manner may be referred as "absolute" ME. Although the presence of this absolute ME may be of some concern, the more important parameter is the demonstration of the absence of a "relative" ME, the word relative referring to the comparison of ME values between different lots (sources) of biofluids in validation of a bioanalytical method. The ME values for AMB and IS are shown in Table 2.3.

Table 2.3 Assessment of ME and recovery

Nominal concentration (ng/ml)	ME (%)		Recovery (%)	
	AMB	IS	AMB	IS
10	100.6	99.5	98.5	94.4
25	108.5	106.0	93.6	100.9
50	105.2	104.7	98.2	98.6
100	99.1	101.1	100.0	97.8
500	98.4	103.2	99.2	99.3
1000	97.4	98.3	96.4	97.6
2000	101.1	99.6	99.8	93.4

The recovery of a compound extracted from plasma is usually determined by comparing the peak areas of a compound spiked before extraction with the corresponding peak areas of the neat solutions of the same compound. The recovery calculated by this way may not be correct, as it does not take the ME into account, which may greatly influence this ratio. Therefore, the recovery should be determined as a ratio of (C/BX100) (eq 2), a "true" recovery value, which is not affected by the ME. The "true" recovery values were 93.6 to 100% and 93.4 to 100.9 % for AMB and IS, respectively (Table 2.3).

The assessment of the presence or absence of a relative ME could be made based on direct comparison of the peak areas of an analyte from the post-extraction spiked samples originating from different lots of plasma (Table 2.3). The variability in these responses, expressed as %CV, can be considered as a measure of the relative ME for a given analyte (Matuszewski *et al.,* 2003). The %CV of the analysis of set 2 at various concentrations varied from 3.5 to 7.3 and 4.3 to 6.2 for AMB and IS, respectively (Table 2.4, columns B and E).

Table 2.4 Precision (%CV) of peak areas of AMB, IS and the peak area ratios (AMB/IS) in Sets 1-3

Nominal concentration (ng/ml)	Precision (%CV)								
	Peak area of AMB			Peak area of IS			Peak area ratios (AMB/IS)		
	Set 1*	Set 2†	Set 3‡	Set 1*	Set 2†	Set 3‡	Set 1*	Set 2†	Set 3‡
10	4.1	7.3	6.1	2.4	5.8	9.2	2.0	4.2	4.3
25	2.3	3.6	5.2	3.1	6.2	4.5	2.3	4.3	4.8
50	2.3	6.4	5.8	3.2	4.3	6.1	3.7	3.9	3.8
100	3.6	5.9	4.8	2.8	5.8	5.2	2.4	4.9	4.3
500	4.4	4.3	2.9	4.5	4.3	4.9	4.8	4.7	2.8
1000	2.6	3.5	4.5	5.3	7.6	4.3	3.0	3.9	2.9
2000	3.4	5.0	5.6	4.4	5.9	4.8	3.9	4.9	1.5
Column	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**I**	**J**

* Comprised of neat standards of AMB and IS in the mobile phase.

† Comprised of plasma samples originating from five different sources and spiked with AMB and IS post-extraction.

‡ Comprised of plasma samples originating from five different sources and spiked with AMB and IS pre-extraction.

This variability is comparable or only slightly higher to the precision of determination of neat standards injected directly (2.3-4.4 and 2.4-5.3 for AMB and IS, respectively, Table 2.4, columns A and D) indicating practical absence of relative ME.

The variability in the determination of peak area ratio of drug to IS, which is a measure of drug concentration is, can be best evaluated by comparing the slopes of standard curves constructed in different plasma lots. The high variability in %CV of these slopes (set-2) is indicative of the overall effect of the sample matrix on the drug/IS ratio rather than the variability of the individual responses. Any variability in the analogous slopes obtained in set 3 in five different lots of plasma may reflect the combined differences in the effect of matrix and a variable recovery in different plasma lots. In the present study, the %CV of the standard curve slopes (2.33 for set-2 and 3.69 for set-3, Table 2.5), were comparable to the %CV of similar five slopes constructed directly from neat standards (set-1, %CV, 3.20, Table 2.5), indicating the absence of any significant matrix effect on quantification.

Table 2.5 Correlation coefficient and slopes of the standard curves for neat standards (Set-1), in five different lots of plasma spiked post-extraction (Set-2), spiked pre-extraction (Set-3) and spiked pre-extraction omitting IS (Set-4)

Parameters	Set 1*		Set 2**		Set 3†		Set 4‡	
	Mean±SD	% CV	Mean±SD	% CV	Mean±SD	% CV	Mean±SD	% CV
Correlation coefficient (r2)	0.9997±0.0009	0.09	0.9995±0.0006	0.06	0.9996±0.0010	0.06	0.9961±0.0012	0.12
Slope	0.00390±0.00012	3.20	0.00358±0.00008	2.33	0.00314±0.00015	3.69	294.70±17.07	6.13

***** Comprised of neat standards of AMB and IS in the mobile phase.

****** Comprised of plasma samples originating from five different sources and spiked with AMB and IS post-extraction.

† Comprised of plasma samples originating from five different sources and spiked with AMB and IS pre-extraction.

‡ Comprised of plasma samples originating from five different sources and fortified with AMB pre-extraction but omitting IS.

2.4.2.2. Assessment of accuracy and intermediate precision

The intermediate precision of the assay was evaluated in the form of the intra-day and inter-day variability. The variability obtained was in the range of 1.22-3.71 % for intra-day run and 1.41-4.91 % for inter-day when assay was performed using IS. An increase in the variability (3.39-6.00% for intra-day and 4.04-8.25% for inter-day) was observed when the assay was performed without the use of IS, which clearly indicates the benefit of using internal standard. The percent accuracy reflects the difference between the nominal and observed concentration, which was found to be 96.3-100.2% when IS was used and 95.4-99.1% without use of IS (Table 2.6).

Table 2.6 Intermediate precision and accuracy of the *in vivo* method

Nominal conc. (ng/ml)	With IS				Without IS			
	Intra-day*		Inter-day†		Intra-day*		Inter-day†	
	% Accuracy	Precision (% CV)	% Accuracy	Precision (% CV)	% Accuracy	Precision (% CV)	% Accuracy	Precision (% CV)
20	97.3	3.71	96.3	4.91	93.5	6.00	92.4	8.25
1000	98.1	1.13	98.2	2.11	97.7	4.44	96.4	5.96
2000	100.2	1.22	99.8	1.41	99.1	3.39	98.2	4.04

* For intra-day variability triplicates were analysed three times on a single day.

† For inter-day variability triplicates of the concentrations specified were analysed on three consecutive days.

2.5. Conclusions

Simple, fast, accurate and reliable HPLC methods for measuring AMB in *in vitro* and *in vivo* samples have been developed. The *in vivo* method will also be favored for its use of low volume of plasma, cheap and readily available IS, simplicity of the sample preparation, short run time and more importantly, absence of ME. Intra-day and inter-day variability were less than 5%, which is very much within acceptable limit. The developed *in vivo* method was sensitive for AMB, could quantify as low as 10 ng/ml, and should be suitable for pharmacokinetic studies for sustained release formulations.

CHAPTER 3

FORMULATION OPTIMISATION, PURIFICATION, SCALE-UP, FREEZE-DRYING AND STABILITY OF AMB-NPs

3.1. Introduction

Exploration of polymeric NPs as drug delivery systems has become increasingly popular due to their ability to overcome many of the obstacles inherently associated with the administration of certain drugs. (Hariharan *et al.,* 2006; Italia *et al.,* 2007; Mittal *et al.,* 2007; Meena *et al.,* 2008). The nanoparticulate carriers have ability to cross the intestinal barrier intact and thus potential for enhancing the oral bioavailability via unique uptake mechanisms (McClean *et al.,* 1998).

3.1.1. Preparation and scale-up of NPs

A variety of techniques has been developed to prepare NPs for the drug delivery (Bala *et al.,* 2004). However, the successful implementation of these techniques for large-scale production is still in its infancy because of relative lack of information concerning the scale-up of technologies used for their production and unavailability of an efficient purification process.

The existing information related to the scale-up is restricted to very few studies (De Labouret *et al.,* 1995; Colombo *et al.,* 2001; Galindo-Rodríguez *et al.,* 2005). Another important requirement related to the production of the NPs at large-scale is an efficient purification process. The purification is of great relevance to ensure the biological tolerance for the product (Quintanar-Guerrero *et al.,* 1996; Quintanar-Guerrero *et al.,* 1998a). Raw NPs suspension often contains potentially toxic impurities such as residual organic solvents, stabilisers and unentrapped drug depending on the method of preparation. Therefore, an effective purification method is required, which can remove these impurities from the formulation. In previous studies, purification methods including dialysis (Rolland, 1989), gel filtration (Oppenheim *et al.,*

1978; Beck *et al.,* 1990), centrifugation (Krause *et al.,* 1986) and occasionally cross flow filtration (Allémann *et al.,* 1993; Quintanar-Guerrero *et al.,* 1998b) have been used. These methods have normally been used for the purification of NPs at the lab-scale and are inappropriate for large-scale manufacturing. Ultrafiltration, however, could easily be used at large-scale.

3.1.2. Stability of NPs

Another major obstacle that limits the use of the NPs is the physical (aggregation/particle fusion) and/or the chemical instability (hydrolysis of polymer materials forming the NPs and degradation of drug) of NPs aqueous suspensions stored for an extended period of time (Chacon *et al.,* 1999).

Many factors can affect stability of the NPs. In General, the homogenous colloidal suspension does not tend to separate since NPs sediment so slowly that the effect is obliterated by the mixing tendencies of diffusion and convection (Magneheim and Benita, 1991). Thermal motion of the colloidal particles is known as the Brownian motion. Suspended colloidal particles continuously change direction due to random collisions with the molecules of the suspending medium, other particles, and walls of the containing vessel. Because of thermal motion, colloidal particles diffuse from a region of high concentration to a region of lower concentration until the concentration is uniform. Gravitational forces, responsible for particle sedimentation and Brownian motion (diffusion forces) oppose each other. Both these forces are dependent on particle size. At colloidal size range, the Brownian forces dominate the gravitational forces, so colloidal particles tend to remain suspended.

NPs suspensions may be destabilised and aggregates when other components of the formulation are added. A suitable stabiliser can be used in the nanoparticulate formulation to avoid the aggregation phenomena. Nevertheless, the adsorption of active molecules on the NPs may induce particle agglomeration, probably by displacing at least part of the steric stabilising surfactant layer as noticed in poly(D,L-lactide) nanospheres before and after nifedipine adsorption (Magneheim and Benita, 1991).

Chemical stability of polymeric nano carriers is dependents on their storage conditions (temperature and pH of the medium) and on the polymer characteristics (type and molecular weight of the polymer used for preparing NPs).

The NPs made up of polyesters (hydrolytically degradable polymers) will undergo degradation (although at a lower rate if the temperature and pH are controlled) over time. Lemoine *et al.* (1996) reported that the stability of polymeric NPs depends on the type of polymer with the following increasing order of polymer stability: PLA25GA50 (PLGA containing 50% D,L-lactide acid and 50% Glycolide acid)<PLA37.5GA25 (PLGA containing 75% D,L-lactide acid and 25% Glycolide)<PLA50=poly(ε-caprolactone). An investigation of the *in-vitro* degradation of nanospheres made from of two different molecular weight PLA (Mw: 25000 and 95000) showed that the pH of nanospheres dispersion has major effects on the chemical stability of the polymer (Belbella *et al.,* 1996). The optimal stability in aqueous medium was observed in a buffered solution with a pH corresponding to the physiological conditions and a temperature of 4°C. The chemical stability of drugs entrapped in the NPs is another essential aspect of the overall stability evaluation of these formulations. Oppenheim *et al*. (1981) have reported that if the drug is water-sensitive, the time of contact with water will influence

the amount of drug incorporated into the NPs. Since many such drug molecules have a pH-dependent degradation profile, the pH needs to be closely controlled. The manufacturing procedure should minimise the time over which degradation may occur. A number of cytotoxic drugs are light-sensitive. Hence, during the manufacturing procedure, exposure to light should also be minimised. Since the final product usually consists of the drug incorporated within the bulk of a solid particle, light-induced degradation of the delivery system should not pose a serious problem (Magneheim and Benita, 1991).

The overall stability of the NPs dispersion could be improved by removing water from the formulation for example, by freeze-drying or lyophilisation of the product, which is known to enhance the stability and shelf life of pharmaceutical formulations (Franks, 1998). The freeze-drying process generates many stresses, which could destabilise nanoparticulate formulation, especially, the stress of freezing and dehydration and so additives, known as cryo/lyo-protectants, are often included in the formulation to protect it. With an optimal freeze-drying process, the shelf life of the NPs could be considerably improved.

In preliminary studies, we have formulated nanoparticulate formulation of AMB, using DMSO as cosolvent employing nano-precipitation method. The formulation exhibited improved oral bioavailability and reduced toxicity compared to Fungizone®, the conventional AMB formulation (Italia *et al.*, 2009). In the present study, further optimisation of the formulation was undertaken with the aim of enhancing the AMB loading with smaller particle size. In addition, a production method that would allow production of the AMB-NPs at pilot-scale was assessed by increasing the batch size upto 20-fold. Two purification methods, centrifugation and ultrafiltration, were

evaluated for their influence on the particle characteristics. Purified AMB-NPs were subjected to freeze-drying process and the effect of freeze-drying on particle characteristics in absence and presence of cryo/lyo-protectants was studied. The preliminary stability of the freeze-dried AMB-NPs was evaluated at the storage temperature of 4°C.

3.2. Materials

PLGA (Resomer RG 50:50 H; inherent viscosity 0.41 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany) and AMB was purchased from Fluorochem Ltd (Old Gossop, UK). Acetonitrile (HPLC grade), acetone (LR grade), DMSO and sucrose were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Ethanol (LR grade), mannitol, trehalose and glucose were obtained from Sigma-Aldrich Co Ltd (Poole, UK). Vitamin E-TPGS (VE-TPGS) was a gift sample from Peboc Division of Eastman Company UK Ltd (Llangefni, UK).

3.3. Methods

3.3.1. AMB-NPs formulation optimisation

AMB-NPs were prepared by adapting a method developed in our laboratory (Italia *et al.,* 2009). Briefly, 5 mg of AMB was dissolved in 0.65 ml of DMSO and 50 mg of PLGA was dissolved in 2 ml of acetone. The PLGA solution was slowly added to the DMSO solution containing AMB with stirring. The AMB-PLGA solution was then drop-wise added into dispersing phase consisting of 10 ml of 50% v/v of ethanol containing 1.4% w/v of VE-TPGS

with stirring. 25 ml of distilled water was added and the formulation was stirred overnight in a fume hood for removal of ethanol. The AMB-NPs formed from this process were purified by ultrafiltration method as explained in section 3.3.3.2.

Various factors can affect characteristics of the NPs prepared by nanoprecipitation method. The foremost parameter that may affect the particle characteristic is the nature and composition of dispersing medium. It has been previously demonstrated that the rate of diffusion of the organic solvent into the dispersing medium directly affects the particle characteristics and should be evaluated (Stainmesse *et al.,* 1995). It can be evaluated by varying the composition of dispersing phase. The other parameter that can alter the particle characteristics could be the nature and composition of the organic solvent used to dissolve the drug and polymer. This can be evaluated by changing the type and compostion of the solvents that constitutes the organic phase. Different composition of DMSO:acetone could be considered. The volume of the organic phase should also be considered as it direcly affects the polymer concentration and the viscosity of the organic phase. The polymer concentration and viscosity directly influence the solvent diffusion into the dispersing medium, which in tern affects the particle characteristics (Galindo-Rodriguez *et al.,* 2004). By varying the volume of the organic solvents, the effect of polymer concentration/viscosity on particle characteristics can be evaluated. The last thing, which is considered to affect the particle characteristics is the initial drug loading. Variation in intial drug loading affects the viscosity of the organic phase, which in tern affects the solvent diffusion and thus the particle characteristics.

3.3.1.1. Composition of dispersing phase

AMB-NPs were prepared by the same method as explained previously but using three different dispersing phases (10 ml of water, 50% v/v of ethanol or absolute ethanol). The effect of these dispersing phases on particle characteristics such as particle size, size distribution and entrapment efficiency were studied. The optimal dispersing phase was identified from the particle characteristics and was used for subsequent experiments.

3.3.1.2. Composition of solvent-mixture (DMSO:acetone)

AMB-NPs were prepared using 2 ml of solvent mixture (consisting of DMSO:acetone, 25:75, 50:50, 75:25 or 100:00, v/v). 10 ml of ethanol (optimal dispersing phase) containing 1 mg/ml of VE-TPGS was used as dispersing phase. The optimal solvent composition identified in this experiment was used in subsequent experiments.

3.3.1.3. Solvent volume

AMB-NPs were prepared using four different volumes (1, 2, 3 or 4 ml) of DMSO (optimal solvent) and 10 ml of ethanol containing 1 mg/ml of VE-TPGS as dispersing phase. The optimal solvent volume found in the experiment was used in subsequent experiments.

3.3.1.4. Initial AMB loading

AMB-NPs were prepared using 10, 20, 30 or 40% (w/w of polymer) of initial AMB loadings. 3 ml (optimal solvent volume) of DMSO was used as solvent and 10 ml of ethanol containing 1 mg/ml of VE-TPGS was used as dispersing

phase. AMB-NPs formulation with optimal particle characteristics obtained in this study was considered as lead formulation and was used for the subsequent *in vitro* and *in vivo* experiments.

3.3.2. Scale-up of AMB-NPs

3.3.2.1. Preparation of AMB-NPs at lab-scale

15 mg of AMB and 50 mg of PLGA were dissolved in 3 ml of DMSO. This drug-polymer solution was added drop-wise to 10 ml of ethanol containing 1 mg/ml of VE-TPGS. The preparation was stirred overnight in a fume hood for the removal of ethanol; alternatively, ethanol can be removed under vacuum.

3.3.2.2. Pilot-scale production of AMB-NPs

The effect of scale-up on AMB-NPs characteristics was determined by increasing the batch size upto 10 & 20-fold. Scale-up was performed using the continuous process shown in Fig. 3.1. The experimental set-up consisted of three reservoirs (R1, R2 and R3) and two reactors (RE1 and RE2, provided with magnetic stirring). The reservoirs R1, R2 and R3 contained the solvent (PLGA+AMB in DMSO), dispersing phase (ethanol containing 1 mg/ml of VE-TPGS) and distilled water, respectively. Both phases were continuously supplied by independent peristaltic pumps (P1 and P2) into the reactor RE1. Magnetic stirring served to mix both the phases. When both phases came into contact, the solvent diffused into dispersing phase immediately, forming the NPs. The raw NPs dispersion is continuously transferred from the RE1 to

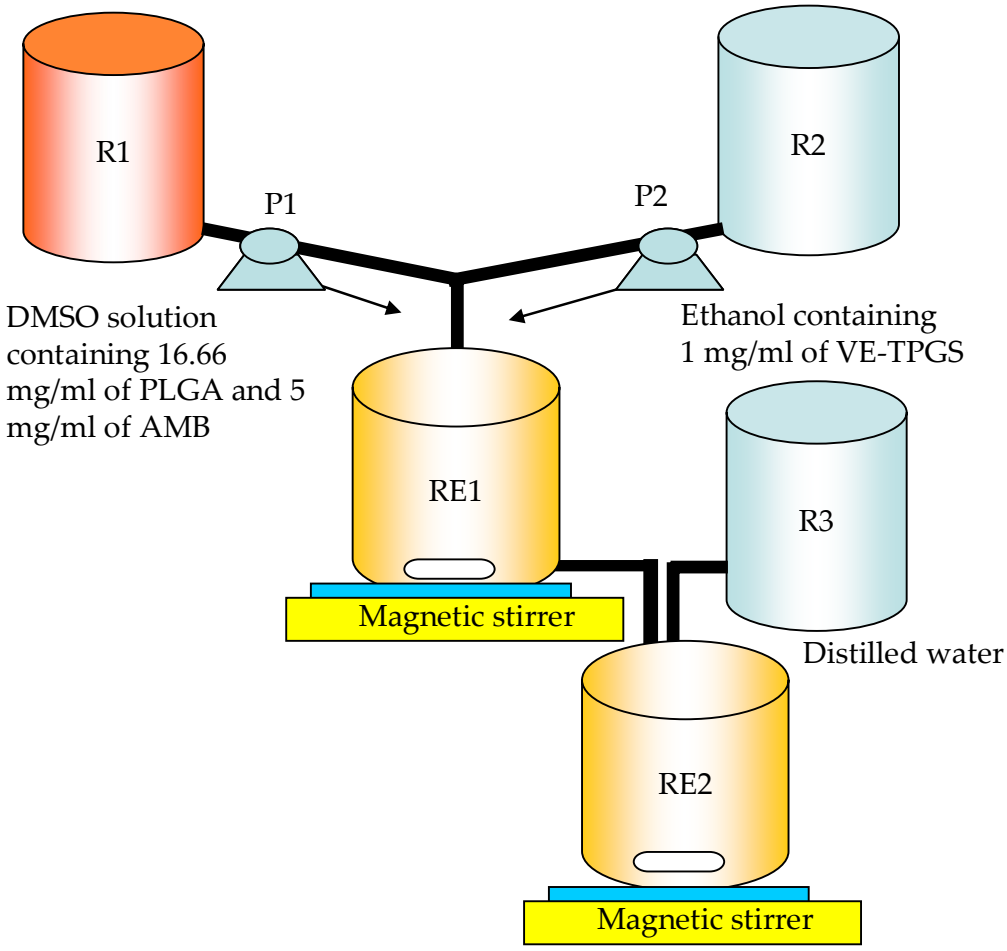


Fig. 3.1 Set-up for AMB-NPs production at pilot-scale. R1, R2 and R3 are the reservoirs; RE1 and RE2 are the reactors with magnetic stirring; P1 and P2 are peristaltic pumps, which transfer the drug-polymer solution and dispersing phase to RE1.

RE2 where water was added under stirring. The NPs were subsequently subjected to the ethanol removal by overnight stirring in the fume-hood or alternatively, vacuum could be applied to speed up the ethanol evaporation.

3.3.3. Purification of raw AMB-NPs suspension

Following the AMB-NPs preparation, removal of DMSO, unentrapped AMB and stabiliser was carried out by two methods, centrifugation or ultrafiltration.

3.3.3.1. Centrifugation

The centrifugation method could only be used for lab-scale batches. Total 9 batches of AMB-NPs (divided in 3 lots) were processed by centrifugation. Three lots of the AMB-NPs were centrifuged, each at a different speed (10000, 15000 or 20000 rpm) for 30 min. The pellets were collected and re-dispersed in 2 ml of the distilled water separately by vortexing for 2 min.

3.3.3.2. Ultrafiltration

The raw NPs dispersion was also purified by ultrafiltration method at lab- and pilot-scale using Vivaflow® 50 modules (Sartorius Stedim UK Ltd, Epsom, UK) equipped with polysulfonate membrane with a 30000 Da molecular cutoff. Three lab-scale batches were diluted to 50 ml and each was subjected to ultrafiltration separately. At the end of the each filtration cycle, the formulation was concentrated to approximately ~7 ml. Five subsequent washings (15 ml each) were given with distilled water. At the end of the

process, the NPs left in the assembly were recovered by washing up the assembly with 10 ml of distilled water.

For the pilot-scale batches of 500 and 1000 mg, the start up volumes were 500 and 1000 ml. Five interlocking filter modules, connected in a series with connectors, were used for the scale-up batch. The use of multiple filter modules reduce the time required for filtration. At the end of the filtration cycle, the volume of NPs suspension left was ~10 ml. Five washings (each of 25 ml) were given with distilled water. At the end of the filtration process, the assembly was washed up with 20 ml of distilled water to recover the residual particles from the assembly.

At the end of the experiment, purified AMB-NPs were characterised for particle size and size distribution. AMB contents and DMSO levels in purified particles and supernatants/filtrate were analysed by HPLC method and particle recovery was calculated as per described in Sections 3.3.6.2 & 3.3.6.3. Change in the particle recovery and size distribution after ultrafiltration process was compared with centrifugation process.

3.3.4. Freeze-drying of AMB-NPs

The effect of four different cryo/lyo-protectants viz. glucose, sucrose, trehalose and mannitol on the stability AMB-NPs was evaluated. All cryo/lyo- protectants were used at 5 and 10% w/v concentrations and AMB-NPs without cryo/lyo-protective were used as reference samples.

The cryoprotective ability of each of the additive in the process of freezing of AMB-NPs was determined by freeze-thawing. Aliquots of purified AMB-NPs (0.5 ml, 5 mg/ml) containing 5 and 10% w/v of mannitol, glucose, trehalose and sucrose were placed in vials and frozen at -80°C for 24 h.

Sample thawing was carried out at room temperature. Particle size and size distributions were measured and final (Sf) to initial (Si) size ratios (Sf/Si) were calculated.

The cyoprotectants, which were found effective in freeze-thawing study, were used for the freeze-drying of AMB-NPs. Samples (0.5 ml, 5 mg/ml) of AMB-NPs containing 5 and 10% w/v of trehalose and sucrose were placed in 5 ml semi-stoppered glass vials with rubber closures. Samples were frozen at -55°C for 6 h on the shelves of the lyophilisation chamber and then lyophilised (primary drying) for 48 h at shelf temperature of −50°C at a pressure of 0.01 mBar using CHRIST® freeze dryer (model: EPSILON 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Secondary drying was carried out at 25°C for 6 h at the same vacuum pressure. The freeze-dried formulations were resuspended in 1 ml of distilled water and characterised for particle size and size distribution. The lyo/cryo-protection provided by each additive was evaluated by determining Sf/Si values and size distribution profiles.

3.3.5. Stability study of AMB-NPs

The vials containing AMB-NPs were sealed immediately after freeze-drying and stored at 4°C. The stability of AMB-NPs was assessed by comparing the AMB content, particle size and size distribution of AMB-NPs obtained immediately after freeze-drying with those obtained at different time intervals during storage at 4°C for 3 months.

3.3.6. Characterisation of AMB-NPs

3.3.6.1. Analysis of particle size

The particle size and size distribution of the NPs were determined by zeta sizer (model: Nano ZS, Malvern Instruments, UK) based on dynamic light scattering and were taken as an average of 10 measurements.

3.3.6.2. Entrapment efficiency and particle recovery

The amount of the AMB entrapped in the NPs was determined by the validated HPLC method. Briefly, 20 µl of the NPs suspension (obtained after purification by ultrafiltration process) was dissolved in 1 ml of DMSO followed by dilution with 1 ml of methanol. The clear solutions were injected into HPLC. The entrapment efficiency was calculated based upon the following formula:

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of AMB entrapped}}{\text{Initial amount of AMB added}} X100$$

The particle recovery was calculated based upon the following formula:

$$\% \text{ Recovery} = \frac{\text{Actual amount of AMB found in purified AMB-NPs}}{\text{Theoretical amount of AMB entrapped in AMB-NPs based on entrapment efficiency}} X100$$

3.3.6.3. Estimation of residual DMSO

AMB-NPs were assayed for residual DMSO using an HPLC method. The HPLC system (Thermo Finnigan, consisting of Surveyor LC pump, Surveyor auto sampler and Surveyor PDA detector) with a Nucleosil® 100-5C_{18} column (150X4.6 mm, Macherey-Nagel, Dorean) was used. Elution of DMSO was performed at a flow rate of 1 ml/min using water as a solvent for the first 7 min followed by a linear gradient up to 70% acetonitrile in 6 min and back to pure water for another 5 min. The detection of DMSO was carried out at a wavelength of 191 nm. The DMSO was quantified in the ultrafiltered AMB-NPs, filtrate (obtained after ultrafiltration) and centrifuged AMB-NPs.

3.3.6.4. Atomic force microscopy (AFM)

Freeze-dried AMB-NPs were re-suspended in distilled water. The AMB-NPs were further diluted to 50% v/v with distilled water and 200 µl of the diluted suspension was placed on a glass slide. The residual liquid was removed from the slide after 5 min and the AMB-NPs were rinsed with water to remove cryo/lyoprotectant and then air-dried. AFM measurements were performed using NanoWizard® (JPK instruments, Berlin, Germany), which was vibration-damped. Commercial pyramidal Si3N4 tips (NSC16 AlBS, Micromasch, Estonia) mounted to a cantilever with a length of 230 µm was used, with a resonance frequency of about 160-170 kHz and a nominal force constant of about 40 N/m. Measurements were performed in intermittent contact mode to avoid damage to the sample. The scanning speed was proportional to the scan size and the scan frequency was between 0.3 and 0.8 Hz. Image was obtained by displaying the height or amplitude signal in the trace direction (512 x 512 pixels).

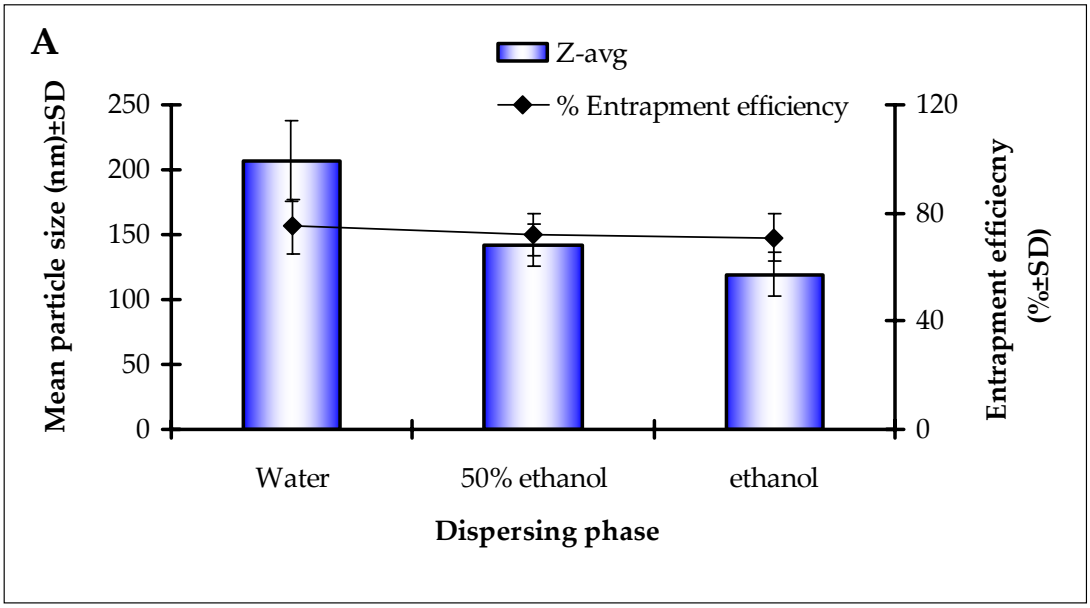
3.4. Results and discussion

3.4.1. AMB-NPs formulation optimisation

3.4.1.1. Composition of dispersing phase

The mean particle size was dependent on the composition of the dispersing phase used and decreased in the order, water>50% v/v ethanol>ethanol (Fig. 3.2A). Ethanol is presumably a "poorer" solvent for PLGA compared to water and it promotes the precipitation of the polymer more actively than water (Murakami *et al.,* 2000; Peltonen *et al.,* 2002). The size distribution was also dependent on dispersing phase composition, where particles made with ethanol show narrow distribution profile compared to water and 50% v/v ethanol (Fig. 3.2B). As the volume of DMSO remained constant, no considerable change was observed in the entrapment efficiency (Fig. 3.2A).

Addition of a surfactant (VE-TPGS) was necessary for stabilisation of AMB-NPs suspension as aggregation was observed without use of VE-TPGS. Considering the lowest particle size and narrow size distribution, ethanol was used as dispersing phase for the subsequent experiments.



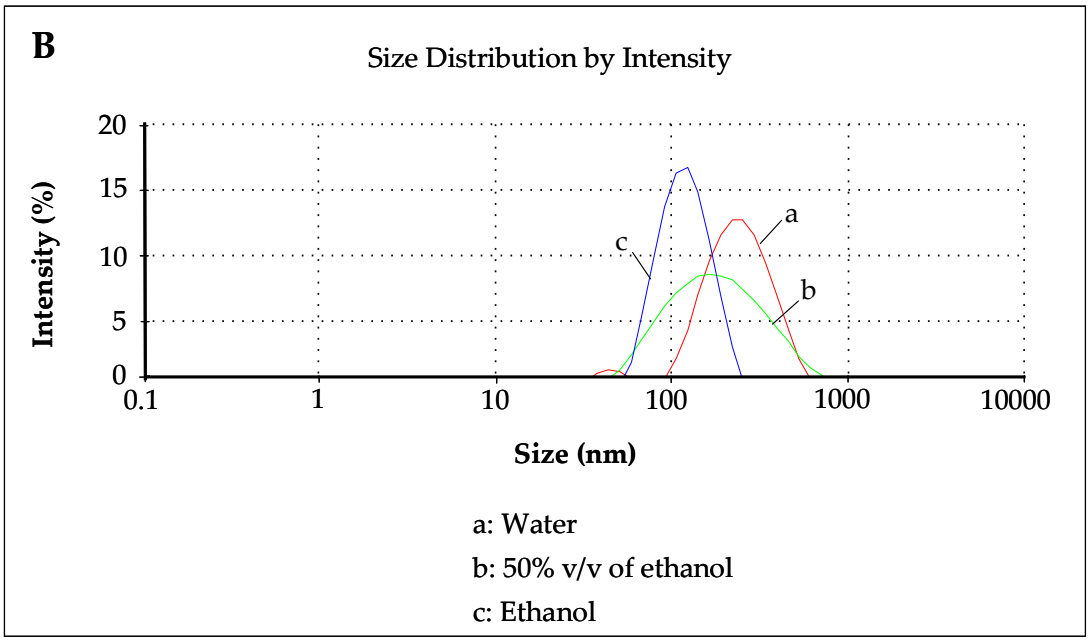


Fig. 3.2 The effect of dispersing phase composition on particle size, entrapment efficiency (n=3) (A) and size distribution (B). Briefly, 5 mg of AMB and 50 mg of PLGA were dissolved in 0.65 ml of DMSO and 2 ml of acetone, respectively. The PLGA solution was then slowly added to the AMB solution with stirring. The AMB-PLGA solution was then drop-wise added into dispersing phase (water, 50% v/v ethanol or absolute ethanol) followed by evaporation of ethanol wherever used.

3.4.1.2. Composition of solvent-mixture (DMSO:acetone)

Particle characteristics were also significantly affected by the composition of organic solvent. Mean particle size and entrapment efficiency were decreased as the proportion of DMSO in the solvent mixture increased (DMSO:acetone, 25:75>50:50>75:25>100:00), although there was a little change in the size distribution pattern (Fig 3.3A & B). The smallest particles were obtained using 100% DMSO as the solvent. The decrease in particle size, as the proportion of DMSO in the solvent increased, can be explained by the physico-chemical properties of the solvents and dispersing phase used in the preparation of AMB-NPs. The properties of the solvents and dispersing phase can be quantitatively characterised by solvent polarity/polarisability, solvent basicity and solvent acidity. Accordingly, the solvents or dispersing phase used in the present study can be divided into three types: (1) type I, strong electron pair donor (EPD) with high polarity, high basicity and low acidity; (2) type II, solvents with medium polarity and low acidity; (3) type III, solvents with strong electron pair accepter (EPA) with medium polarity and high acidity.

DMSO (type I) is a good electron pair donor (EPD) solvent, with high basicity due to the presence of its lone electron pairs (Xiong *et al.,* 2005). Acetone (type II) is a solvent with no EPD or EPA activity and having low acidity. Ethanol (type III) is a good EPA with high acidity. A strong EPD-EPA interaction arises between DMSO and ethanol molecules and thus DMSO molecules interacts with the ethanol molecules with higher affinity compared to acetone molecules, which could lead to faster diffusion of DMSO in the ethanol compared to acetone and thus smaller particle size (Galindo-Rodriguez *et al.,* 2004).

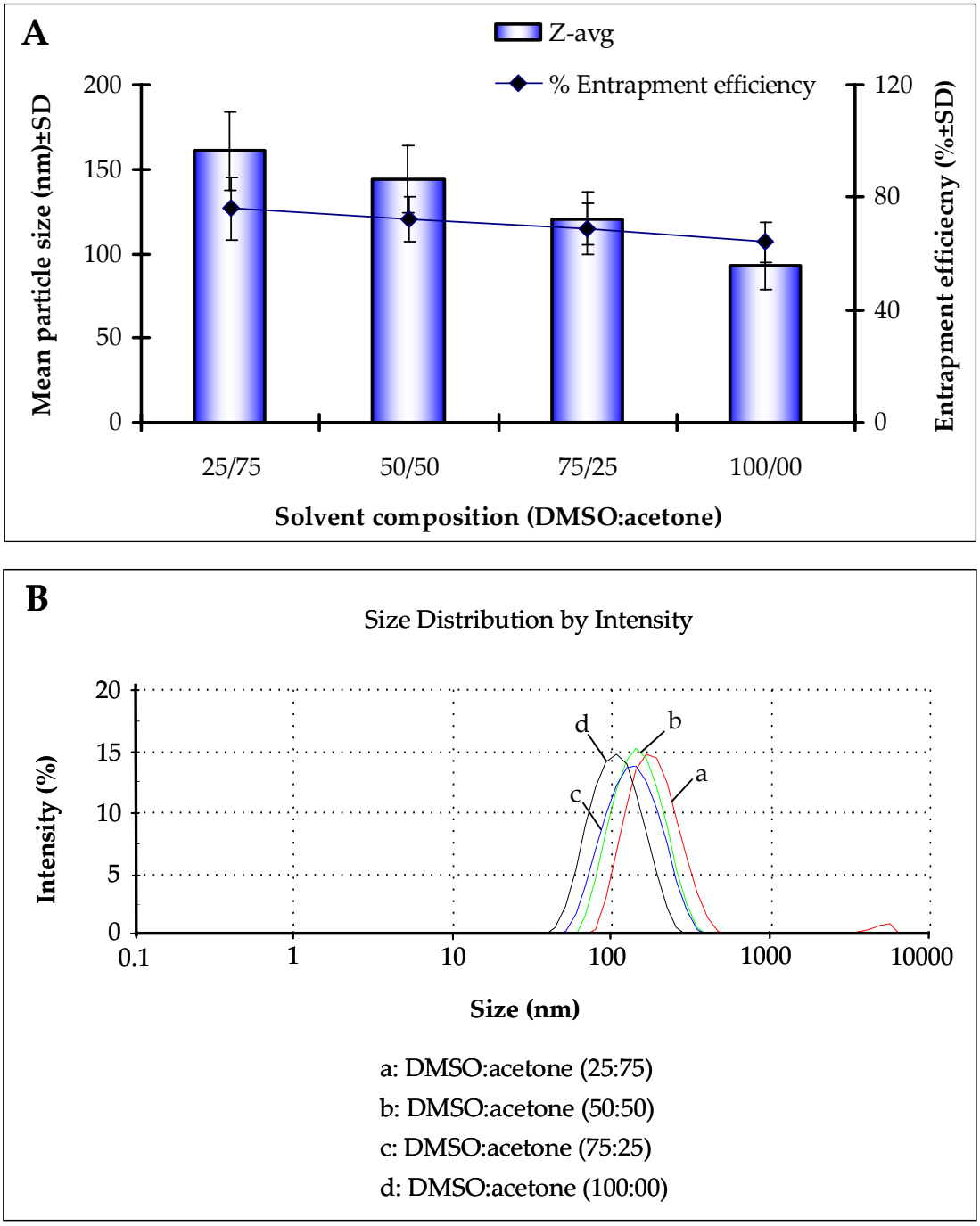


Fig. 3.3 The effect of solvent composition (DMSO:acetone) on particle size, entrapment efficiency (n=3) (A) and size distribution (B). Briefly, 5 mg of AMB and 50 mg of PLGA were dissolved in 2 ml of DMSO:acetone mixture (consisting of 25:75, 50:50, 75:25 or 100:00). The AMB-PLGA solution was then drop-wise added into dispersing phase (ethanol containing 1 mg/ml of VE-TPGS) followed by evaporation of ethanol.

Since AMB has very good solubility in the DMSO compared to acetone, there was a decrease in the entrapment efficiency with increase in the DMSO proportion in the solvent mixture (Fig 3.3A). 100% DMSO was used as solvent for future experiments, as AMB-NPs with a smaller particle size were required for the formulation.

3.4.1.3. Solvent volume

Four volumes of DMSO (1, 2, 3 and 4 ml) were used to prepare AMB-NPs and its effect on particle size, size distribution and entrapment efficiency was evaluated. There was a decrease in particle size and entrapment efficiency as solvent volume increased from 1 to 4 ml (Fig 3.4A & B). This trend could be explained by considering two facts: (I) the number of polymer chains per unit volume of solvent and (II) the influence of polymer concentration on the viscosity.

First, with the lowest volume of solvent, there are greater numbers of polymer chains per unit volume of solvent and consequently, the solvent diffusing into the dispersing phase carries out more polymer chains, which aggregate and form larger particles. This phenomenon is also favored by the fact that increasing polymer concentration increases polymer-polymer interactions so that more polymer chains remain associated during the diffusion process. The influence of polymer concentration on the viscosity of the organic phase can also be taken into account. As the polymer concentration increases, a more viscous organic phase is obtained, which provides a higher mass transfer resistance. Thus the distribution of polymer-solvent phase into the dispersing phase is reduced and larger particles are formed (Galindo-Rodriguez *et al.,* 2004).

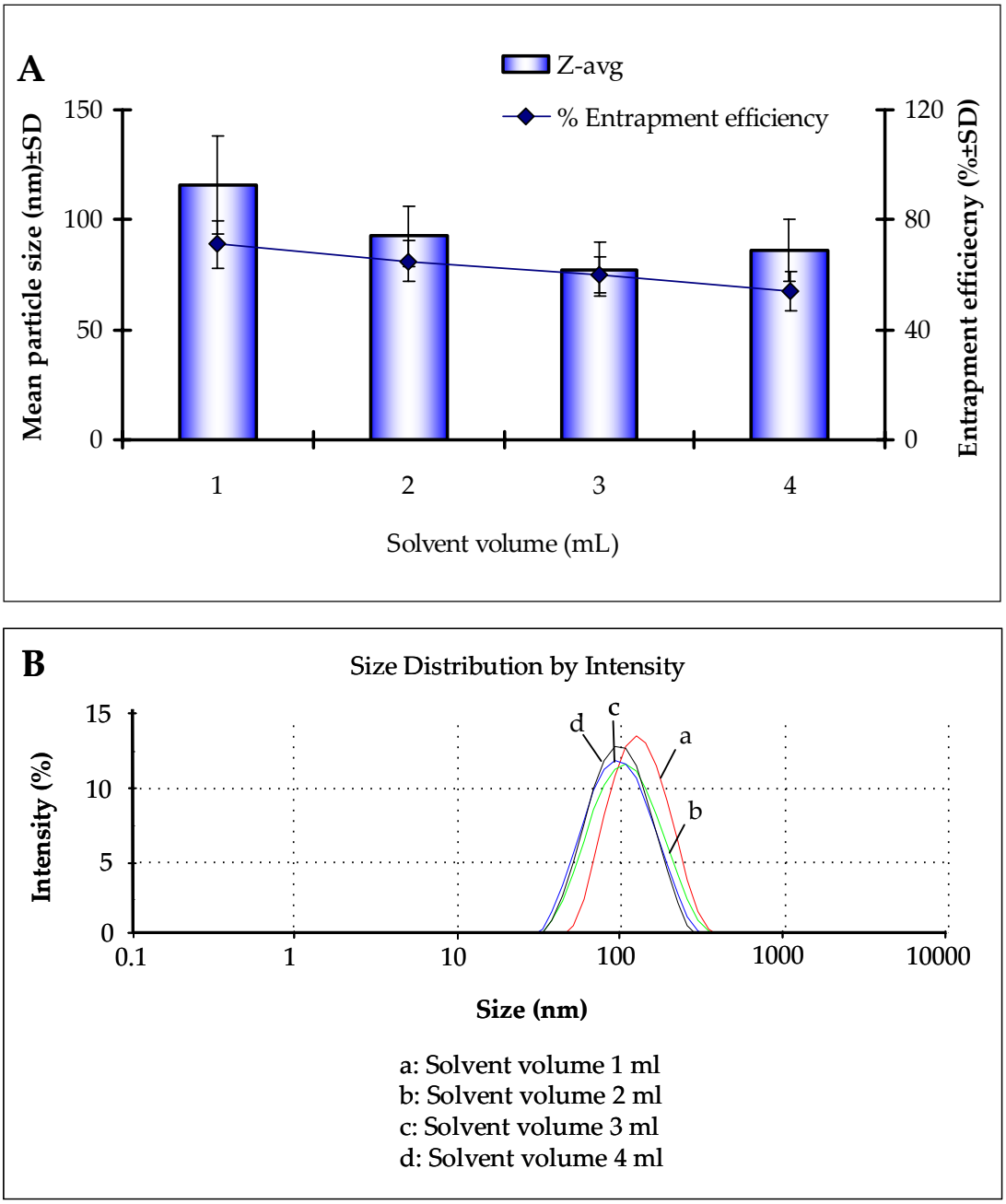


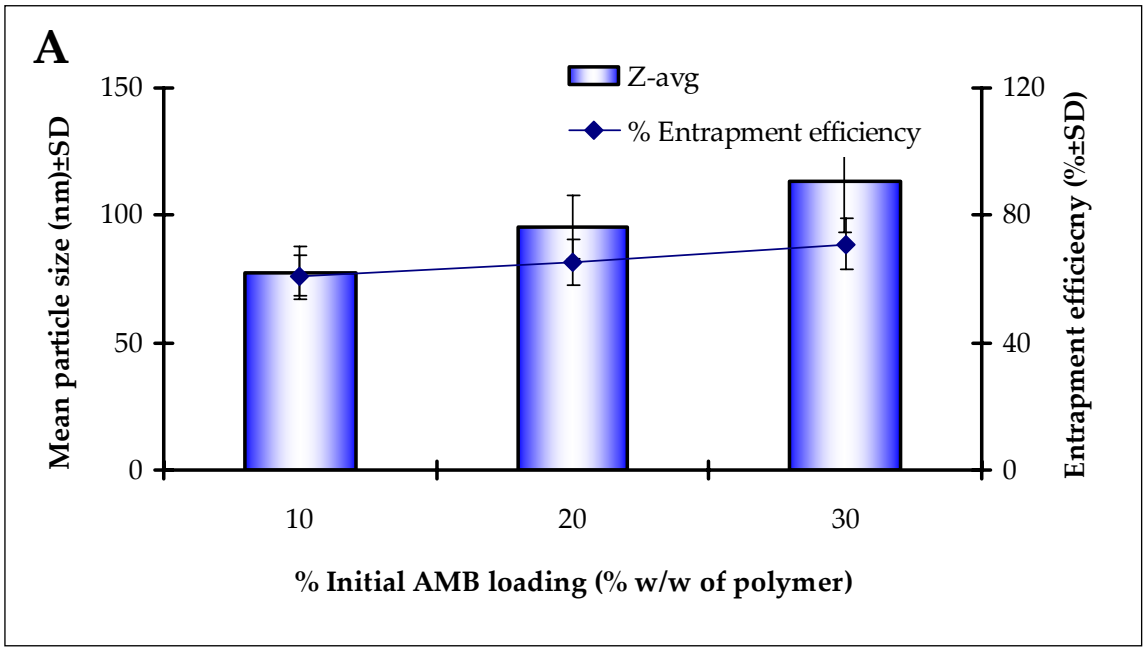
Fig. 3.4 The effect of solvent (DMSO) volume on particle size, entrapment efficiency (n=3) (A) and size distribution (B). Briefly, 5 mg of AMB and 50 mg of PLGA were dissolved in 1, 2, 3 or 4 ml of DMSO. The AMB-PLGA solution mixture was then drop-wise added into dispersing phase (ethanol containing 1 mg/ml of VE-TPGS) followed by evaporation of ethanol.

In contrast, a decrease in the polymer concentration, due to increase in the solvent volume, decreases the viscosity of the organic phase and increases the distribution efficiency of the polymer-solvent phase into the dispersing phase leading to formation of smaller particles. The decrease in AMB entrapment could be due to increase in solubility of AMB in the external phase as the DMSO proportion increased. Therefore, based on the results of particle size and entrapment efficiency, 3 ml of organic solvent was selected for future studies.

3.4.1.4. Initial AMB loading

Effect of initial AMB loading on particle size, size distribution and entrapment efficiency was evaluated using four different payloads (10, 20, 30 and 40% w/w of polymer). Increasing the initial loading from 10 to 30% w/w (of polymer) resulted in an increase in the particle size and entrapment efficiency and a little widening of the size distribution of the formulation (Fig. 3.5A & B).

The increase in the particle size could be due to the increase in the viscosity of the organic phase, which results in slower diffusion of the solvent into dispersing phase and thus higher particle size. Above 30% initial drug loading based on w/w of polymer, AMB and polymer were precipitated without formation of NPs, indicating that PLGA could not hold AMB above this concentration. Therefore, AMB-NPs with 30% (w/w of polymer) AMB loading were considered as lead formulation and used for future *in vitro* and *in vivo* studies.



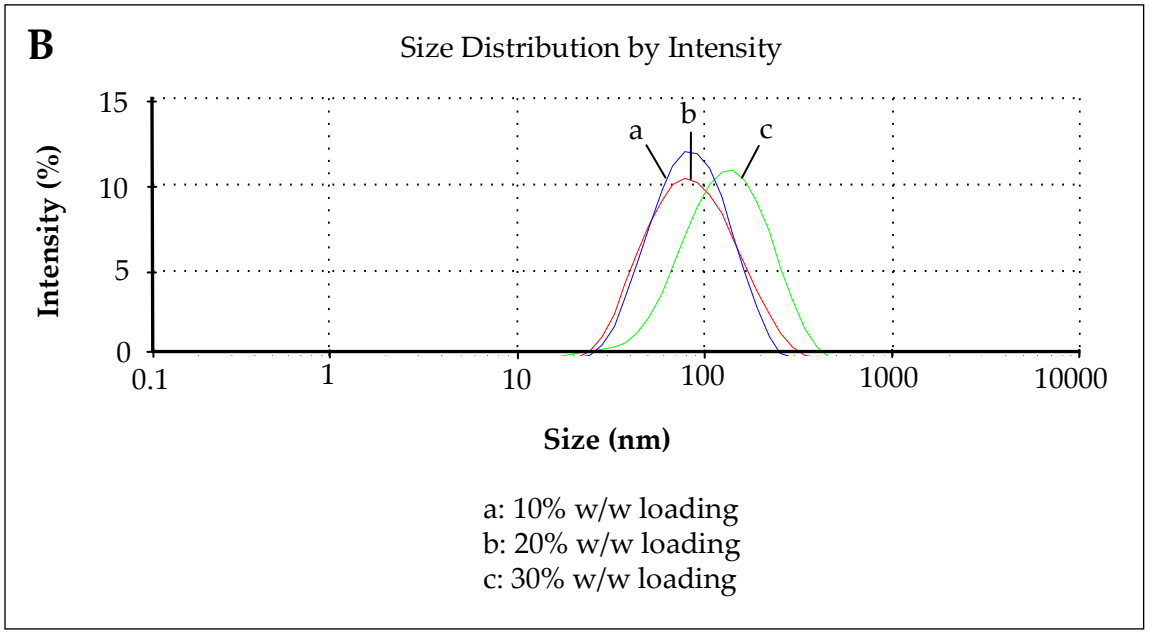


Fig. 3.5 The effect of initial AMB loading on particle size, entrapment efficiency (n=3) (A) and size distribution (B). Briefly, 5, 10 or 15 mg of AMB and 50 mg of PLGA were dissolved in 3 ml of DMSO. The AMB-PLGA solution was then drop-wise added into dispersing phase (ethanol containing 1 mg/ml of VE-TPGS) followed by evaporation of ethanol.

3.4.2. Scale-up of AMB-NPs

NPs formation during the nano-precipitation method is governed by the "diffusion-stranding phenomena." In the early stage of this process, solvent and polymer chains contained in the organic phase together diffuse into the dispersing phase. Further diffusion of solvent induces desolvation of polymer chains, which aggregate to form NPs. As reported previously (Venier-Julienne and Benoît, 1996), any change in the solvent-diffusion behavior leads to changes in the mean size of the NPs. AMB-NPs produced at lab-scale manifested homogeneous particle population with mean size ~110 nm with a low batch-to-batch variability (Table 3.1).

Table 3.1 Effect of batch size on the particle characteristics of AMB-NPs (n=3)

Batch size	Mean particle size (nm)±SD	Entrapment efficiency (%±SD)	Polydispersity index (PDI)
50 mg (Lab-scale)	108±17	71±9%	0.23±0.05
500 mg (Pilot-scale)	112±11	76±7%	0.25±0.07
1000 mg (Pilot-scale)	118±16	79±8%	0.26±0.05

A continuous method (Fig 3.1) was used in pilot-scale production of NPs. The drug-polymer solution and the dispersing phase were mixed in a ratio of 3:10 (v/v) by adjusting their flow rates to 30 ml/min and 100 ml/min, respectively. No significant change in the mean particle size, polydisersity index (PDI) and entrapment efficiency was observed for the pilot-scale batches compared to lab-scale batches (Table 3.1). Since the raw NPs

suspension was continuously removed from RE1, the solvent to dispersing phase ratio was maintained constant for all the time, which could for unaltered particle characteristics obtained with the pilot-scale batches compared to the lab-scale batches.

3.4.3. Purification of AMB-NPs

Centrifugation is the most commonly used method for the purification of NPs; however, it can be used only for the smaller batches at lab-scale and is not suitable for the large-scale production. Venier-Julienne and Benoît (1996) reported the preparation and purification of AMB loaded PLGA NPs. NPs were prepared by emulsion technique using CH_2Cl_2 and DMSO as solvents. The particle size distribution was bimodal (142±41 and 757±114 nm) and the second population was due to precipitation of unentrapped AMB. Precipitated AMB could not be removed from the formulation by centrifugation or ultrafiltration so the adsorbent polymer "Amberlite XAD16" was used. However, in this study, no precipitation of unentrapped AMB was observed and the size distribution of AMB-NPs was essentially unimodal (Table 3.1). All of the unentrapped AMB was solubilised in dispersing phase-DMSO mixture.

3.4.3.1. Centrifugation

The AMB-NPs were centrifuged at three different speeds, 10000, 15000 or 20000 rpm for 30 min and the recovered AMB-NPs were characterised for particle size, size distribution and particle recovery. The particle size and distribution were increased proportionally with centrifugation speed (Fig. 3.6).

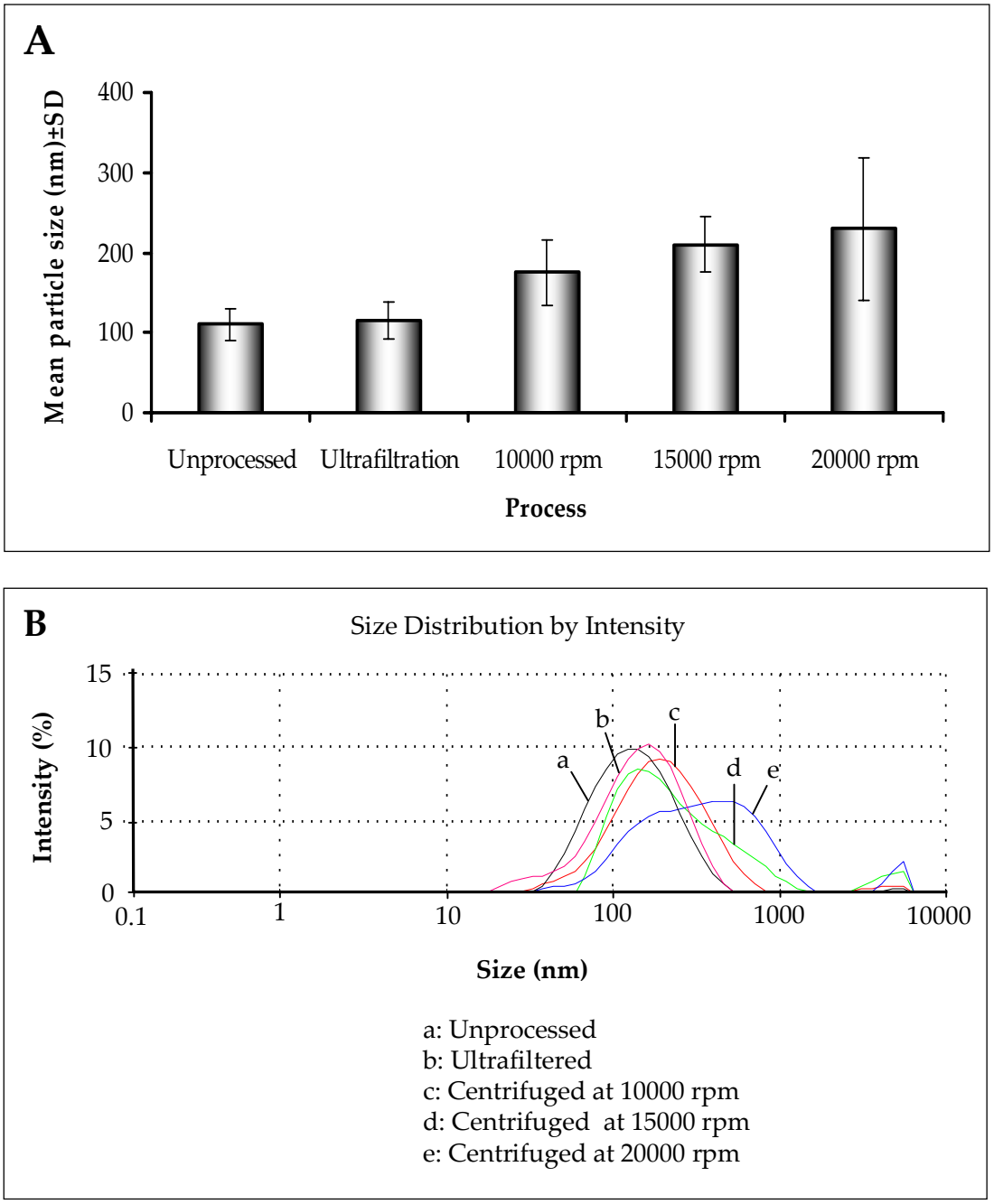


Fig. 3.6 Effect of ultrafiltration and centrifugation on particle size (n=3) (A) and size distribution (n=3) (B). In centrifugation process, AMB-NPs were centrifuged at a speed of 10000, 15000 or 20000 rpm for 30 min, while in case of ultrafiltration the particles were ultrafiltered and washed 5 times with 15 ml of distilled water.

The change in the particle size and size distribution could be attributed to the particle aggregation upon the centrifugation, which would have increased as the centrifugation speed increased. Particle recovery was essentially low at all speeds used due to smaller particle size, so that most of the AMB-NPs remained in the supernatant. With increase in the centrifugation speed from 10000 to 15000 rpm, a little increase in the particle recovery was observed (Fig. 3.7). However, further increase in the centrifugation speed to 20000 rpm led to decrease in the particle recovery (Fig. 3.7). Ideally, particle recovery should be increased with the increase in the centrifugation speed. However, in this study, after centrifugation at 20000 rpm, a hard pellet was formed from the centrifuged particles and a fraction of the pellet could not be re-dispersed, which accounted for the lower particle recovery.

3.4.3.2. Ultrafiltration

Since, centrifugation method has limitations in the particle purification including alteration in particle size and size distribution as well as low particle recovery; an ultrafiltration method was attempted for AMB-NPs purification. The ultrafiltration process efficiently removed unentrapped AMB to unquantifiable levels. More importantly, the particle size and size distribution remained essentially unaltered after ultrafiltration (Fig. 3.6) and a higher particle recovery was obtained (Fig. 3.7).

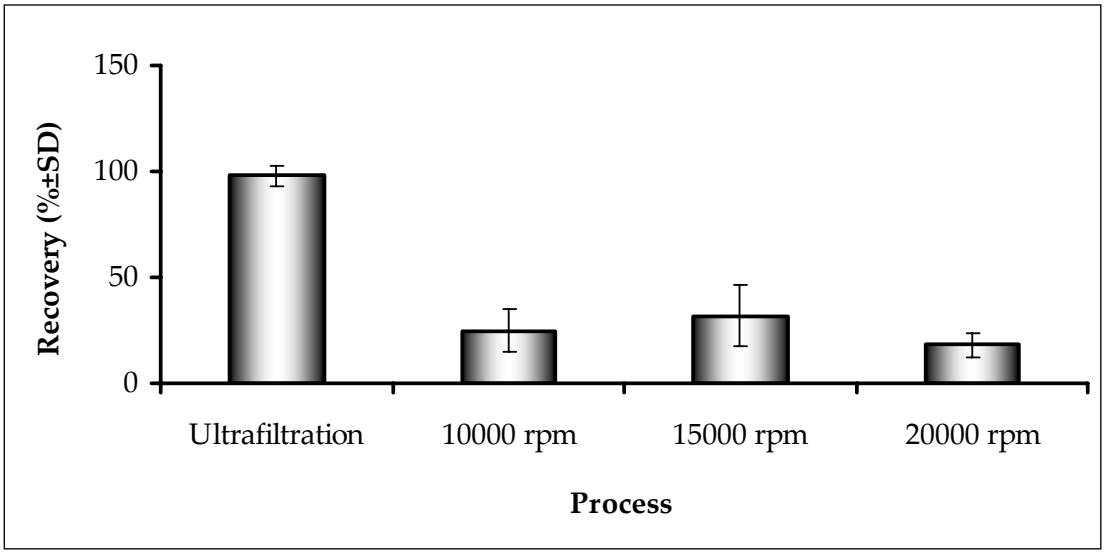


Fig. 3.7 Effect of ultrafiltration and centrifugation on particle recovery (n=3). In centrifugation process, AMB-NPs were centrifuged at a speed of 10000, 15000 or 20000 rpm for 30 min, while in case of ultrafiltration, the particles were ultrafiltered and washed 5 times with 15 ml of distilled water.

The ultrafiltration method was used for purification of 3 different sized (50, 500 and 1000 mg) batches of AMB-NPs. No significant change in particle characteristics was observed in each case irrespective of batch size (Table 3.2) indicating that this method is suitable for large-scale production of AMB-NPs.

Table 3.2 Particle characteristics of lab- and pilot-scale batches (50, 500 and 1000 mg) of AMB-NPs purified by ultrafiltration method (n=3).

Batch size	Mean particle size (nm)±SD	Entrapment efficiency (%±SD)	PDI
50 mg (Lab-scale)	122±17	70±8%	0.23±0.05
500 mg (Pilot-scale)	126±18	74±4%	0.26±0.07
1000 mg (Pilot-scale)	130±15	78±6%	0.24±0.04

Apart from unentrapped AMB, removal of DMSO from the formulation was also essential due to its potential for causing toxicity and its tendency for solubilisation of PLGA. Since DMSO has a high boiling point of 189°C (Windholz *et al.*, 1983), it can not be removed even under vacuum at ambient temperature. Ultrafiltration was found to be effective in removing DMSO from the formulation without altering the NPs characteristics. The concentration of DMSO in the formulations was reduced to undetectable levels after 5th washing step (Fig. 3.8). The residual DMSO levels measured in AMB-NPs purified by centrifugation or ultrafiltration method are shown in the Fig. 3.9. No detectable levels of residual DMSO were found in the formulations purified by the ultrafiltration method. However, when formulations were purified by centrifugation, quantifiable levels of residual DMSO were observed.

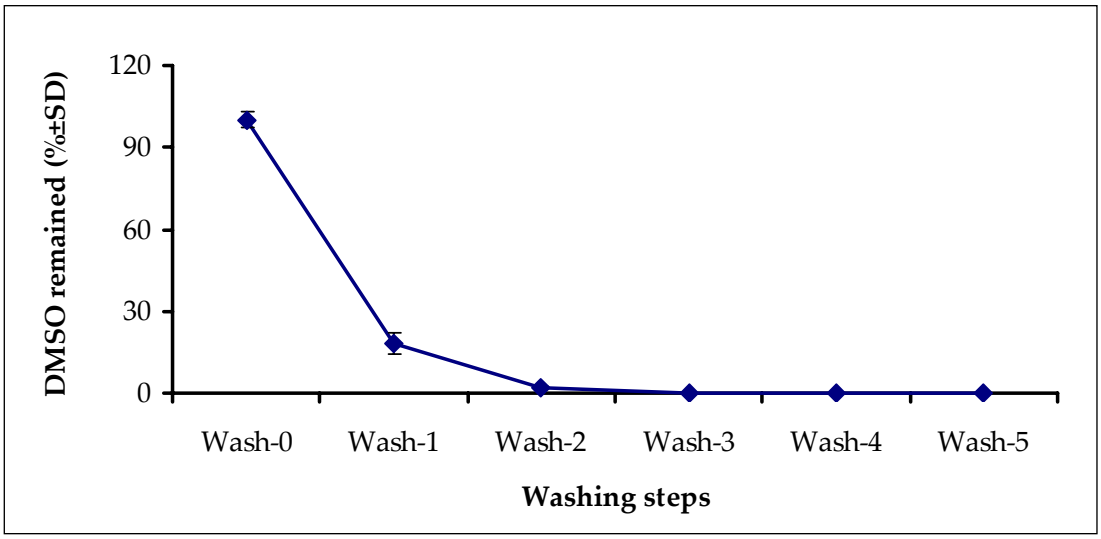


Fig. 3.8 Effect of washings on the DMSO content of the formulation in the ultrafiltration process (n=3).

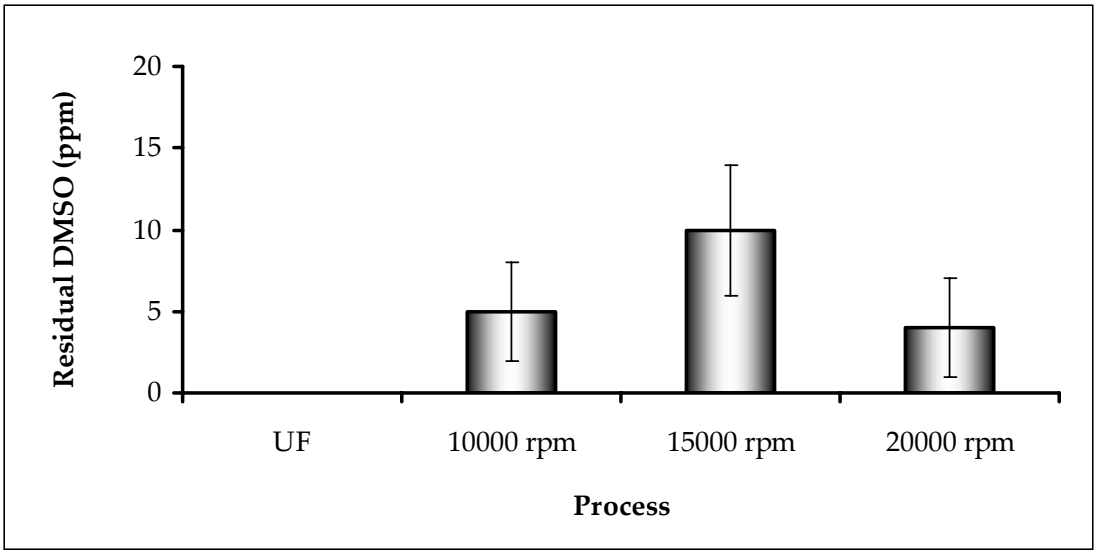


Fig. 3.9 Residual DMSO levels in AMB-NPs purified by ultrafiltration or centrifugation process. In centrifugation process, AMB-NPs were centrifuged at a speed of 10000, 15000 or 20000 rpm for 30 min, while in case of ultrafiltration, the particles were ultrafiltered and washed 5 times with 15 ml of distilled water. Subsequently, residual DMSO levels were analysed by HPLC method (Data presented as mean±SD, n=3).

3.4.4. Freeze-drying of AMB-NPs

Freeze-drying is an extensively used process for improving the stability of various pharmaceutical products including vaccines, proteins, and colloidal carriers like liposomes and NPs. Freeze-drying cycle is consisted of four steps: freezing (solidification), primary drying (ice sublimation), secondary drying (desorption of unfrozen water) and final conditioning and storage.

Freezing is the first step of freeze-drying cycle during which the liquid suspension is cooled and ice crystals of pure water forms. As the prosess progess, more and more water contained in the liquid freezes leading to increasing concentration of the NPs in the remaining liquid. As the suspension becomes more concentrated, its viscosity increases, which induces inhibition of further crystallisation. This highly concentrated and viscous liquid solidifies and yields, either crystalline, amorphous, combined amorphous-crystalline or glass phase (Franks, 1990). The small amount of water that does not freeze and remains in the liquid state is called bound water. Freezing often induces many destabilising stresses for the NPs. An increase in the concentration of NPs during freezing enhances the interaction between particles leading to their aggregation or fusion. The understanding of freezing stress is aided by the phase diagram of water–sucrose binary system (Fig. 3.10) (Franks, 1998). During freezing, a dilute sucrose solution will increase in concentration during the temperature reaches Tg' value (Tg': glass transition temperature of maximally cryo-concentrated solution). At Tg', the sucrose concentration is of 80% and further cooling will not increase the concentration.

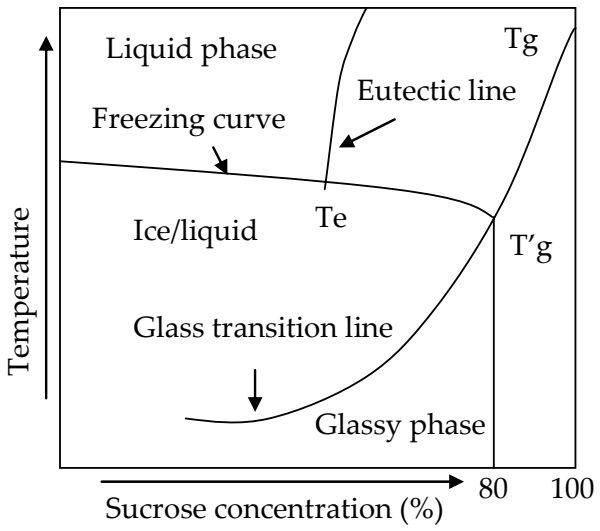


Fig. 3.10 Phase diagram for a binary system of sucrose–water showing Tg′.

Sucrose does not crystallise during freezing concentration but the eutectic point is shown in Fig. 3.10 if crystallising solutions (e.g. mannitol). To ensure the total solidification of a frozen sample, the formulation should be cooled to below the Tg′ of the formulation. This condition requires the shelf temperature for freezing be set below Tg' of the formulation. The frozen NPs samples should be kept at the set temperature for a sufficient time-period so that all the suspension transforms into solid. Therefore, it is very important to know the Tg′ of the NPs formulation before starting the freeze-drying study. The reported values of Tg′ for the cryoprotectants used in the present study are shown in Table 3.3. Considering these values, the freezing temperature of -55°C (which is well above the reported Tg′ values of cryoprotectants) was selected for the process of freeze-drying.

Table 3.3 Reported Tg′ values of the cryoprotective agents in aqueous solutions and when added to NPs formulations (Abdelwahed *et al.,* 2006; Saez *et al.,* 2000

Cryoprotective agents (% w/v)	Aqueous solutions (°C)	PLGA-NPs (°C)
Glucose 5%	-41.92	-
Glucose 30%	-43.6	-44.0
Sucrose 5%	-30.78	-
Sucrose 30%	-32.7	-33.5
Trehalose 30%	-29.8	-30.7
Mannitol 15%	-28.9	-31.1

Annealing is a process in which samples are maintained at a specified subfreezing temperature above the Tg for a period of time (Searles *et al.,* 2001). It has remarkable effects on the size distribution of ice crystals. Annealing process can lead to the growth of ice crystals. Searles *et al.* (2001)

reported that an increase in size of ice crystals caused by annealing could accelerate primary drying by increasing pores diameter in the plug structure, which were occupied by ice crystals. Such thermal treatment can also reduce the drying rate heterogeneity between samples to give a more homogenous structure. The annealing treatment can eliminate the thin skin layer formed at the top surface of freeze-dried cake. This skin is formed by the migration of cryoprotectants and NPs during the crystallisation of ice, which may prevent the transfer of water vapor during sublimation and slow down the sublimation rate resulting in product heating its fusion.

The primary drying or sublimation phase will follow when the frozen material, placed under vacuum, is progressively heated to deliver enough energy for the ice to sublimate. During this very critical period, a correct balance has to be adjusted between heat input (heat transfer) and water sublimation (mass transfer) so that drying can proceed without inducing adverse reactions in the frozen material such as back melting, puffing, or collapse. A continuous and precise adjustment of the operating pressure is then compulsory in order to link the heat input to the ''evaporative possibilities'' of the frozen material. In sublimation process, heat is transferred from the shelf to the frozen solution through the tray and the vial, and conducted to the sublimation front. The ice then sublimes and the water vapor formed passes through the dried portion of the product to the surface of the sample. The water vapor is then transferred from the surface of the product through the chamber and condenses on the condenser. At the end of sublimation step a porous plug is formed. Its pores correspond to the spaces that were occupied by ice crystals (Williams and Polli, 1984).

Secondary drying or desorption phase starts when ice is being distilled away and a higher vacuum allows the progressive extraction of bound water at

above zero temperatures. The bound is the water, which did not separate out as ice during the freezing, and did not sublimate off (Pikal *et al.,* 1990). The bound water may be adsorbed on the surface of the crystalline product or is in the solute phase either as water of hydration in a crystalline hydrate or dissolved in an amorphous solid to form a solid solution (Pikal and Shah, 1990). If not removed, bound water can cause rapid decomposition of the product when it is stored at room temperature. Secondary drying begins locally when all ice has been removed from that region. Removal of bound water is a difficult task since overdrying might be as bad as underdrying. For each product, appropriate residual moisture has to be reached under given temperatures and pressures.

Final conditioning and storage begins with the extraction of the product from the equipment. During this operation, great care has to be taken not to lose the refined qualities that have been achieved during the preceding steps. Thus, for vials, stoppering under vacuum or neutral gas within the chamber is the current practice. For products in bulk or in ampoules, extraction might be done in a tight gas chamber by remote operation. Water, oxygen, light, and contaminants are all important parameters and must be monitored and controlled.

Freeze-drying process generates many stresses that could destabilise NPs suspension, especially, the stress of freezing and dehydration. Freezing is considered to be the most aggressive and critical step during lyophilisation process and it can cause aggregation or destruction of NPs. The type of cryoprotectant and its concentration should be optimised to ensure a maximum stabilisation of formulation. To select the best cryoprotectant to preserve AMB-NPs characteristics, a freeze-thawing study was conducted prior to freeze-drying. The AMB-NPs samples were frozen at -80°C in the

presence of 4 different cryoprotectants (at the concentrations of 5 & 10% w/v) and Sf/Si and size distributions were measured after thawing the samples at room temperature. A visible particle aggregation was observed in the absence of cryoprotectant and the value of Sf/Si exceeded 3. Addtion of cryoprotectants led to a variable protective effect on the particle characteristics (Fig. 3.11A & B). Mannitol and glucose only partially protected AMB-NPs from aggregation. Trehalose and sucrose exhibited good cryoprotective action and successfully protected AMB-NPs against aggregation at the concentration of 5 and 10% w/v as indicated by the Sf/Si values (~1) and unaltered size distribution (Fig. 3.11A & B).

The successful cryoprotectants, trehalose and sucrose, identified in the freeze-thawing study were further evaluated for the lyoprotective efficacy during lyophilisation. The effect of trehalose and sucrose (5 and 10% w/v) on particle size and size distribution of freeze-dried AMB-NPs are shown in Fig. 3.12A & B. Freeze-dried AMB-NPs containing trehalose and sucrose (5 & 10% w/v) were porous cakes. AMB-NPs, freeze-dried without any cryo/lyo-protectant, showed a drastic change in the particle size and size distribution. Tehalose at the concentrations of 5 & 10% w/v exerted partial protection to the AMB-NPs, whereas, 5 or 10% w/v of sucrose gave good protection.

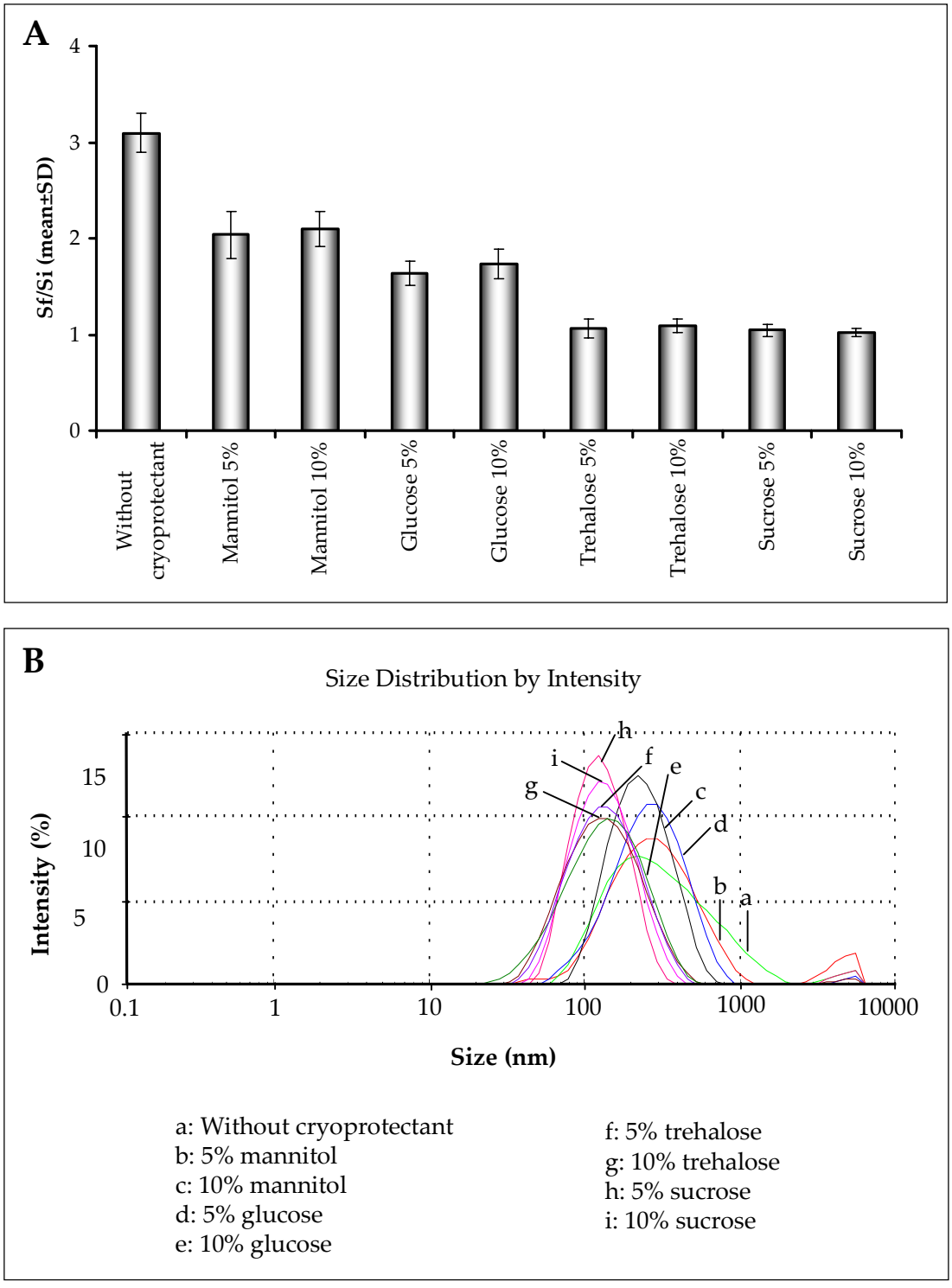


Fig. 3.11 Final to initial size ratio (Sf/Si) (n=3) (A) and size distribution (B) of AMB-NPs frozen at -80°C for 24 h followed by thawing at room temperature in presence of 5 and 10% w/v of mannitol, glucose, trehalose and sucrose.

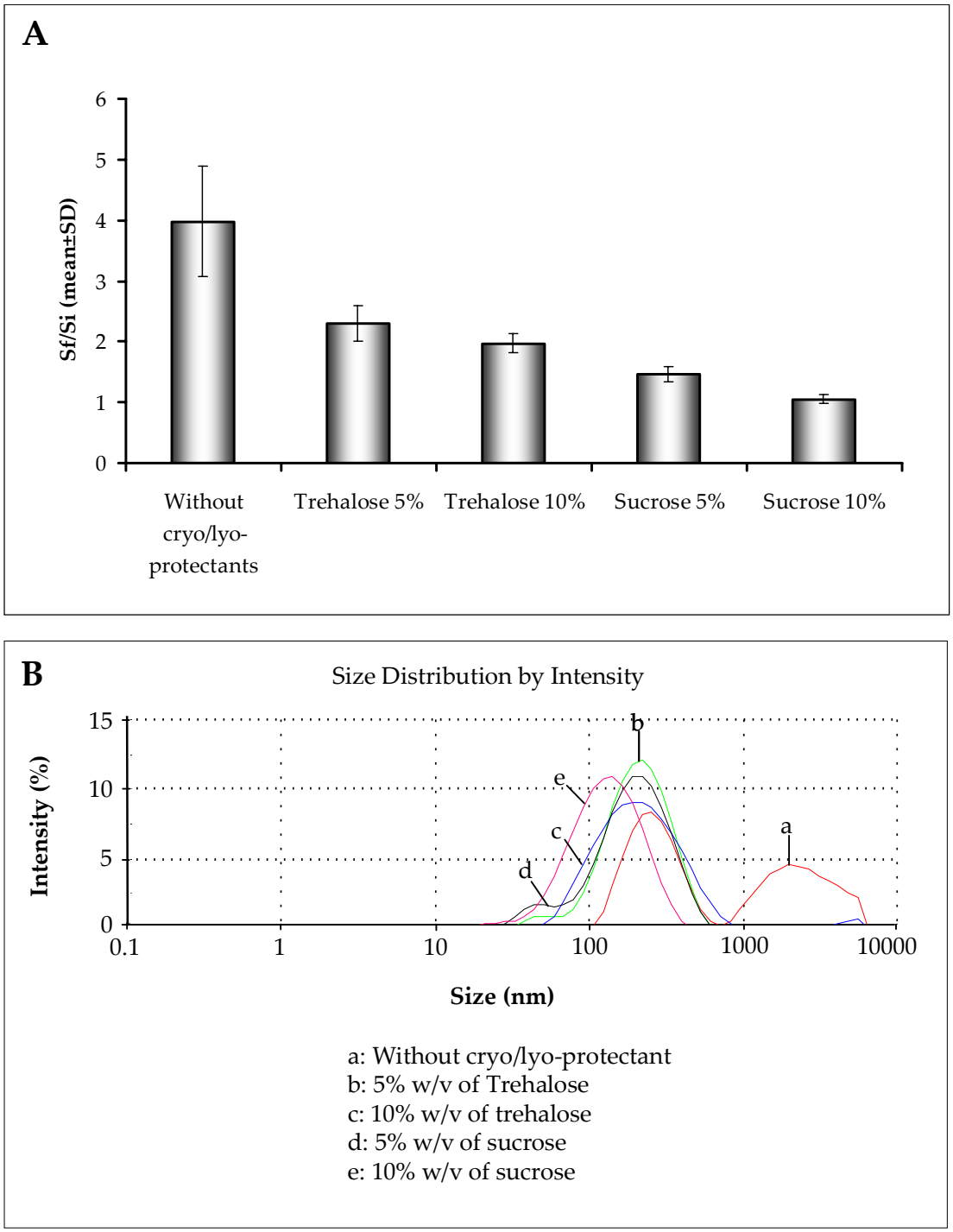


Fig. 3.12 Final to initial size ratio (Sf/Si)(n=3) (A) and size distribution (B) of freeze-dried AMB-NPs. AMB-NPs were mixed with 5 and 10% w/v of cry/lyo-protectants (trehalose or sucrose) and frozen at -55°C for 6 h and freeze-dried for 48 h at shelf temperature of -50°C and a pressure of 0.01 mBar. Secondary drying was carried out at 25°C for 6 h at the same vacuum pressure.

The results suggested that 10% w/v of sucrose was the most effective cryo/lyo-protectant for AMB-NPs (Fig. 3.12A & B).

The freeze-drying cycle can be divided into three stages: freezing, main drying (ice sublimation) and secondary drying (desorption of unfrozen water). Freezing results in a phase separation of the NPs suspension into ice and a cryo-concentrated solution of NPs. This high concentration of a particulate system can lead to aggregation and in some cases irreversible fusion of NPs. Furthermore, crystallisation of ice can induce a mechanical stress on NPs leading to their destabilisation. Therefore, cryoprotectants are added to the NPs suspension prior to freezing to protect these fragile systems from freezing stress (cryoprotectant) or drying stress (lyoprotectant) (Abdelwahed *et al.,* 2006). Sugars including trehalose, sucrose, glucose and mannitol are a few of the most popular cryoprotectants encountered in the literature for freeze-drying of NPs. The immobilisation of NPs within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. Another explanation of the possible mechanism of NPs stabilisation by cryoprotectants during the freezing step is the particle isolation hypothesis. It has been proposed that sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing (Allison *et al.,* 2000).

The dehydration steps involve the removing of ice and unfrozen water. This unfrozen water remains dissolved or adsorbed on the solid phase. Such process may destabilise unprotected NPs. A suggested stabilisation mechanism of NPs by lyoprotectants during drying steps is the water replacement hypothesis, which involves formation of hydrogen bonds between a lyoprotectant and the polar groups at the surface of NPs. These lyoprotectants preserve the native structures of NPs by serving as water

substitutes. The amorphous state of NPs and a lyoprotectant allows maximal H-bonding.

The atomic force microscopic image of AMB-NPs purified by the ultrafiltration process and freeze-dried using 10% w/v of sucrose is shown in the Fig. 3.13. The particles were nearly spherical and the size correlated well with that obtained by zeta-sizer studies indicating that freeze-drying process was successful in the stabilizing the formulation. In addition, absence of any major aggregation in the AFM image suggests that the freeze-dried formulation could easily be reconstituted with water.

3.4.5. Stability of AMB-NPs

Freeze-dried AMB-NPs formulation was found to be stable at 4°C. No significant change in AMB content of the formulation was observed at the end of 3 months (Fig. 3.14). The Sf/Si ratio for AMB-NPs at the end of 3 months was ~1 indicating that no significant particle aggregation occurred during the storage (Fig. 3.15A). The size distribution of the AMB-NPs was also unaltered during the storage period (Fig. 3.15B). Overall, the formulation was stable over a period of 3 months at the storage temperature of 4°C.

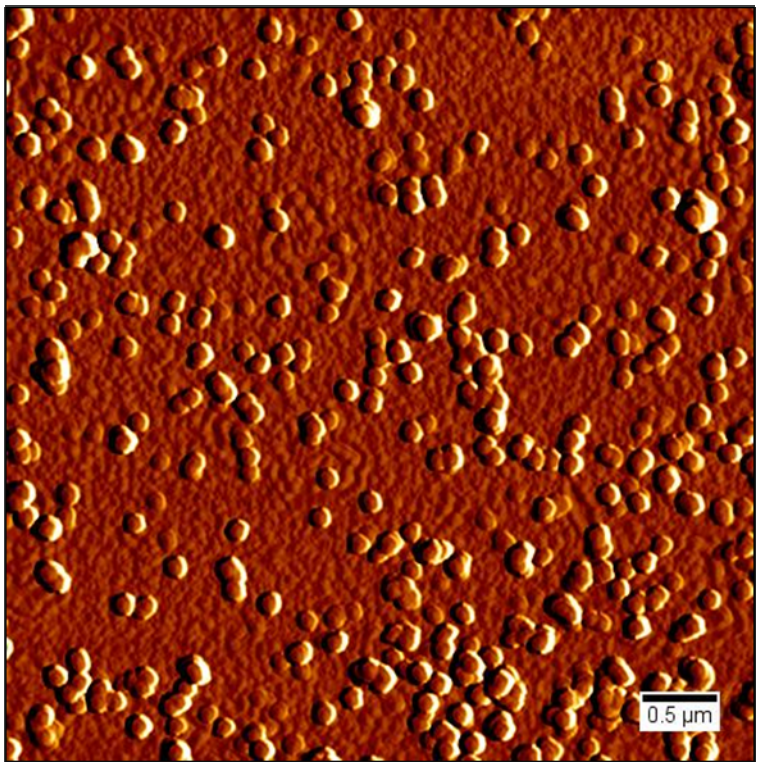


Fig. 3.13 AFM image of freeze-dried AMB-NPs

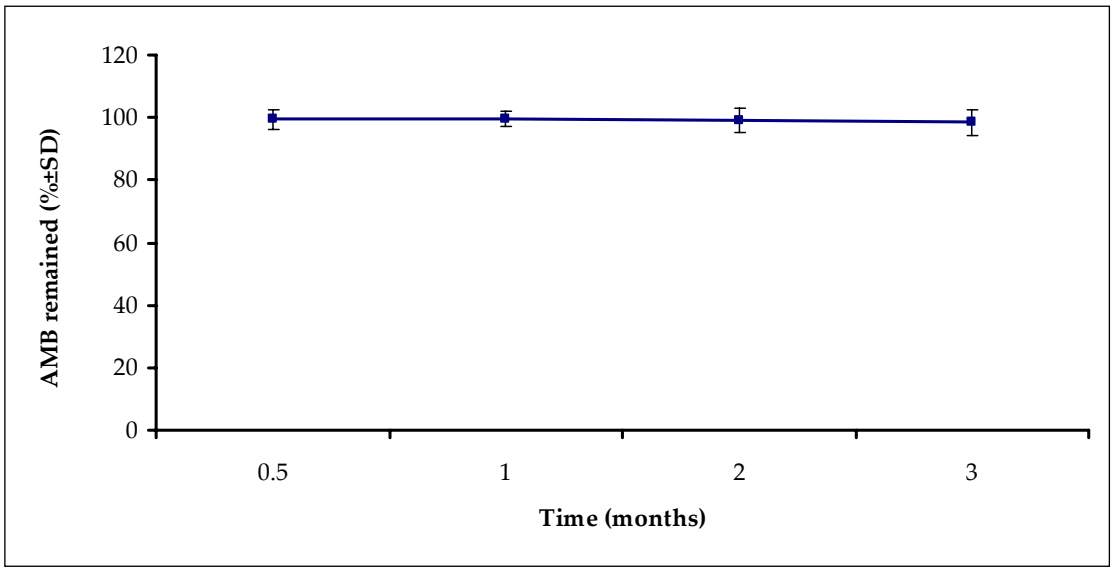


Fig. 3.14 AMB content of the formulation at different time points during storage at 4°C (n=3).

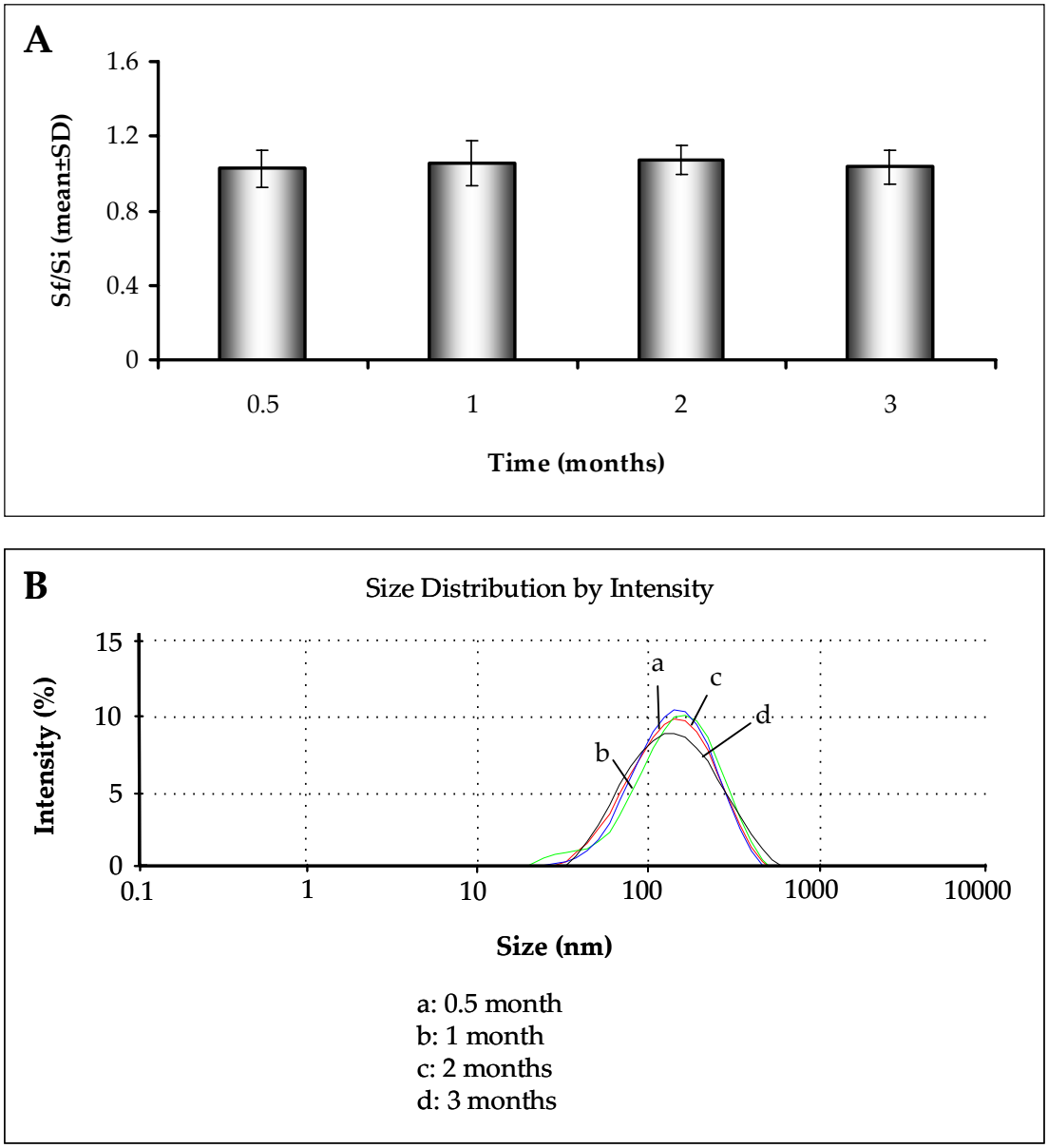


Fig. 3.15 Final to initial size ratio (Sf/Si) (n=3) (A) and size distribution (B) of AMB-NPs at different time points during storage at 4°C.

4.5. Conclusions

The studies identified an efficient way of producing AMB-NPs with a high AMB payload and entrapment efficiency using DMSO as solvent and ethanol as dispersing phase. This method was scaled-up to produce upto 1000 mg batches of AMB-NPs using a process, which is mild, rapid and reproducible. Ultrafiltration of AMB-NPs was found to be superior to centrifugation for removal of unentrapped AMB and DMSO with a little effect on particle size and size distribution. AMB-NPs could be successfully freeze-dried employing 10% w/v of sucrose as cryo/lyo-protectant and freeze-dried formulation was found to be stable after storage at 4°C for 3 months.

CHAPTER 4

EVALUATION OF *IN VIVO* ANTI-FUNGAL ACTIVITY OF AMB-NPs

4.1. Introduction

Aspergillus fumigatus is the most prevalent airborne fungal pathogen in developed countries and is responsible for causing one of the most devastating and life threatening invasive mycoses like IPA in the immunocompromised patients. Despite the advances made in the diagnosis and treatment of invasive IPA, the rate of incidence is still rising with stubbornly high mortality rates. Though AMB has been the main therapy for IPA, it is only marginally effective at improving the survival rate due to a combination of both, poor efficacy and toxicity.

Animal models of aspergillosis have been widely used to study various aspects of pathogenesis, innate and acquired host-response, transmition of the disease and treatment. The factors those should be considered before any particular model is employed. First, the choice of model and animal should strive to mimic the human disease. The model needs to have high reproducibility, which requires standardisation of all methods needed to execute the model, and should be economical. The parameters of infection, such as fungal burden in the tissues and mortality, should be controllable. Accordingly, the model needs to be carefully defined.

Several different animal models of aspergillosis have been developed including avian models, guinea pigs, rabbits and murine models. Of these models, murine models prevailed as the model of choice over the years to evaluate the efficacy of treatment of invasive and disseminated aspergillosis. Murine models have several advantages including availability of the genetically defined strains of mice, immunological reagents, low cost and ease of handling. Moreoever, due to their small size, larger numbers of animals can be utilised.

Murine models involve the induction of neutropenia or corticosteroid-induced immunosuppression to mimic human infection. Neutropenia may be induced by cyclophosphamide or other chemotherapeutic agents, whereas corticosteroids treatment impairs the functional ability of phagocytes to kill *A. fumigatus* conidia and hyphae (Waldorf *et al.,* 1984; Roilides *et al.,* 1993a; Roilides *et al.,* 1993b; Brummer *et al.,* 2001; Kamberi *et al.,* 2002).

Disseminated infection can be established in both, normal and immunosuppressed animals by intravenous inoculation. However, IPA can can only be induced in immunosuppressed mice after intranasal/intratracheal inoculation or inhalation, which closely mimics the human bronchopneumonia.

Mouse models have been used for a variety of studies on aspergillosis, including examination of the comparative virulence of different isolates of *Aspergillus*, which genes are involved in virulence, comparative susceptibility to infection with *Aspergillus*, and preclinical anti-fungal drug efficacy. Since the susceptibility of different strains of mice is about the same, regardless of genetic background of the mice or of the model of aspergillosis being used, both outbred and inbred animals can be used for experiments. Establishment of disease may depend somewhat on the strain of *A. fumigatus* used, as there appears to be a correlation between virulence and the presence of an undefined 0.95 kb fragment of genomic DNA (Mondon *et al.,* 1995; Mondon *et al.,* 1996).

The severity of the induced disease is directly proportional to numbers of conidia present in the inoculam chosen for use in the initiation of the infection regardless of the model being studied and strain of *Aspergillus* being used (Dixon *et al.,* 1989; Hector *et al.,* 1990; Clemons *et al.,* 2000; Chiller *et al.,* 2002). It is critical to know what outcome to expect with different inocula,

which allows the investigator to control the course of disease in the model. This is particularly important when performing models for preclinical drug efficacy, where too severe an infection may result in early deaths before therapy has begun or after a minimal number of doses have been administered. In contrast, an inoculum that is too low in conidial numbers may result in no mortality or even clearance of the infection without therapy. Numerous investigations have been carried out using a murine model of aspergillosis for the drug/formulation efficacy study (Hanson *et al.,* 1995; Polak, 1998; Clemons *et al.,* 2000; Chiller *et al.,* 2003; Warn *et al.,* 2006; Kagoshima *et al.,* 2010; Olson *et al.,* 2010).

In the present study, two types of murine models representing disseminated as well as invasive aspergillosis were used. In disseminated model, mice were immunosuppressed with a single dose of cyclophosphamide (causing temporary neutropenia) and then infected by intravenous administration of *A. fumigatus* conidia (Fig 4.1). In the absence of neutrophil recruitment due to neutropenia, the intravenously administered conidia colonise with uncontrolled hyphal growth in the different tissues mimicking the disseminated infection in humans. In IPA model, immunosuppression is induced by multiple doses of cyclophosphamide every 3 days along with glucocorticoid to give a persistent neutropenia with dysfunction of alveolar macrophages (Fig. 4.2). Mice are then infected with aerosolised *A. fumigatus* conidia to cause germination and uncontrolled hyphal growth of conidia in the alveolar spaces due to dysfunctional macrophages. The absence of neutrophils results in angioinvasion and dissemination of the fungus to other organs via blood, so that the model mimics IPA in humans.

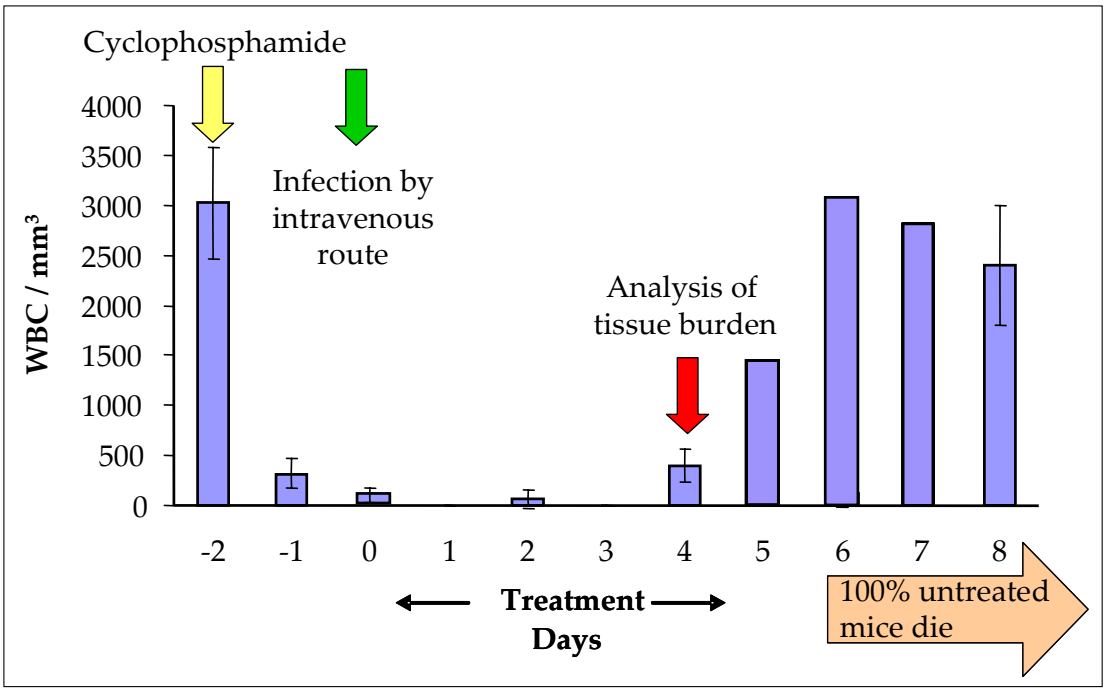


Fig. 4.1 Time course of disseminated aspergillosis model

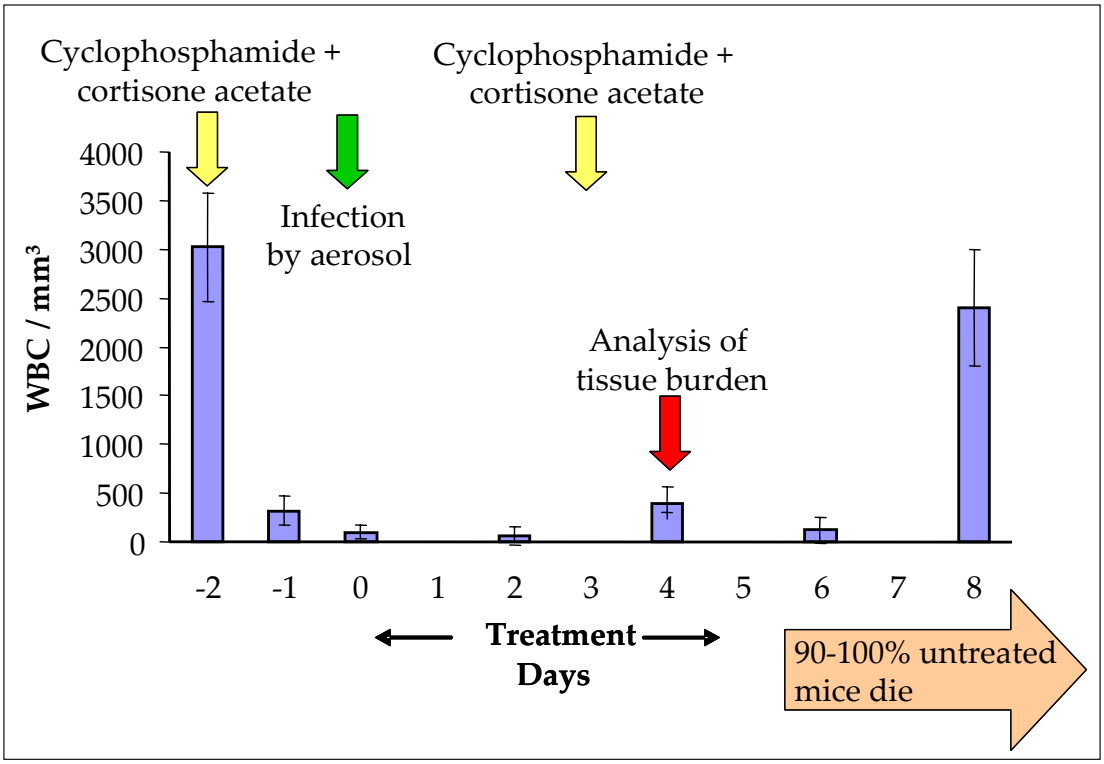


Fig. 4.2 Time course of IPA model

4.2. Methods

4.2.1. Animals

Male CD1 mice (age, 4 to 5 weeks; weight, between 18 and 20 g) purchased from Charles River UK Ltd (Margate, United Kingdom) were used for both, disseminated and IPA model studies. Mice were allowed free access to food and water, the cages were inspected twice daily and any infected animals unable to reach the water drinker were culled. The experiments were conducted in accordance with United Kingdom Home Office regulations (under project license PPL 40/3101) at the University of Manchester.

4.2.2. Activity of AMB-NPs in neutropenic murine model of disseminated aspergillosis

4.2.2.1. Immunosuppressive treatment and infection of mice

Mice were immunosuppressed by intraperitoneal administration of cyclophosphamide at a dose of 200 mg/kg. A state of profound neutropenia was achieved after 3 days of cyclophosphamide and lasted for 4 days. On day 3 following cyclophosphamide treatment, all mice were infected by intravenous injection of 0.2 ml of *A. fumigatus* (strain: A1163) conidia suspension containing, 4.0×10^5 colony forming units (CFU)/ml.

4.2.2.2. Drug treatment and organ cultures

Two sets of experiments were performed covering single dose and multiple dose administration of AMB-NPs. The treatment plan is shown in Tables 4.1 & 4.2.

Table 4.1 Treatment regimen in disseminated aspergillosis model (Experiment -I)

Group	Treatment	Dose mg/kg	Route	Regime	No. of doses
I	Blank NP	-	Intravenous	Once daily	4
II	Fungizone®	0.1	Intraperitoneal	Once daily	4
III	Fungizone®	0.3	Intraperitoneal	Once daily	4
IV	Posaconazole	2.5	Oral	Once daily	4
V	AMB-NPs	0.3	Intravenous	Once daily	4
VI	AMB-NPs	2	Oral	Once	1
VII	AMB-NPs	5	Oral	Once	1

All the treatments were started 4 h post-infection. The animals were culled 96 h post-infection and kidneys were aseptically removed and transferred to vials containing 2 ml of phosphate-buffered saline. The kidney samples were homogenised in a tissue grinder (Polytron; Kinematica AG, Lucerne, Switzerland) for approximately 30 s and diluted to 10^{-2}. The diluted suspensions (0.1 ml) were then transferred to Petri dishes containing sabouraud dextrose agar (Oxoid) and spread over the surfaces of the plates. Petri dishes were incubated at 37°C in a moist atmosphere and were examined daily for 5 days. Colony counts were recorded from all plates that showed growth. Single colonies were accorded a negative result, because of the possibility of airborne contamination.

Table 4.2 Treatment regimen in disseminated aspergillosis model (Experment-II)

Group	Treatment	Dose mg/kg	Route	Regime	No. of doses
I	Blank NPs	-	Intravenous	Once daily	4
II	Fungizone®	0.2	Intraperitoneal	Once daily	4
III	Fungizone®	0.5	Intraperitoneal	Once daily	4
IV	AMB-NPs	0.2	Intravenous	Once daily	4
V	AMB-NPs	0.5	Intravenous	Once daily	4
VI	AMB-NPs	2	Oral	Once daily	4
VII	AMB-NPs	5	Oral	Once daily	4

4.2.3. Activity of AMB-NPs in murine model of IPA

4.2.3.1. Immunosuppressive treatment and infection of mice

All the mice were immunosuppressed by intraperitoneal administration of cyclophosphamide at a dose of 200 mg/kg and subcutaneous administration of cortisone acetate at 250 mg/kg. Profound neutropenia was achieved after 3 days of immunosuppressive treatment. On day 3 post-immunosuppressive treatment, all mice were infected by exposing to 0.5 ml of *A. fumigatus* conidia (strain: A1163) aerosol, containing $1x10^9$ CFU/ml. The mice were given the same immunosuppressive treatment 3 days after infection.

4.2.3.2. Drug treatment and organ cultures

Mice were treated using the regimen given in Table 4.3. Drug treatments were started 4 h post-infection. The animals were culled 96 h post-infection

and lungs were transferred to sterile vials containing 1 ml of phosphate-buffered saline. The samples were homogenised in a tissue grinder for approximately 30 s and the homogenate was diluted to 10^{-2}. 0.1 ml of the diluted suspensions were transferred to Petri dishes containg sabouraud dextrose agar and spread over the surfaces of the plates. The Petri dishes were incubated at 37°C in a moist atmosphere and examined daily for 5 days. Colony counts were recorded from all plates that showed growth. Single colonies were accorded a negative result, because of the possibility of airborne contamination.

Table 4.3 Treatment regimen for IPA model.

Group	Treatment	Dose mg/kg	Route	Regime	No. of doses
I	Blank NPs	-	Intravenous	Once Daily	4
II	Fungizone®	1.5	Intraperitoneal	Once Daily	4
III	AmBisome®	5	Intravenous	Once daily	4
IV	Posaconazole	2.5	Oral	Once daily	4
III	AMB-NPs	5	Intravenous	Once	1
IV	AMB-NPs	5	Oral	Once	1
V	AMB-NPs	5	Intravenous	Once daily	4
VI	AMB-NPs	5	Oral	Once daily	4

4.2.3. Statistical analysis

There are two groups of statistical tests, parametric and non-parametric. The parametric tests include Student's t-test, Paired t-test and one way analysis of variance (ANOVA). Parametric tests make certain assumptions about the

underlying population distributions of the data on which they are used; for example that they are normal. Such test are called parametric because these assumptions are about population parameters (Parameters are measures computed from all the observations in a population – examples are the population mean and SD). Parametric tests are often robust, in that they are relatively unaffected by violations of these assumptions. However, some situations arise when there are markedly non-normal distributions or when the data collected are in the form of rankings rather than scores. When the data are not normally distributed, the parametric tests often perform poorly, resulting in a greater chance of committing an error.

A range of tests, commenly referred to as non-parametric (or rank order) tests, have been developed, which can be used in these situations (data is non-normal). Non-parametric tests are designed to have desirable statistical properties when few assumptions can be made about the underlying distribution of the data. In other words, when the data are obtained from a non-normal distribution or one containing outliers, a non-parametric test is often a more powerful statistical tool than its parametric 'normal theory' equivalent. The commenly used non-parametric tests include Kruskal Wallis test, Mann Whitney U test and Wilcoxon Sum Rank test.

In the present studies, normality of the data was first analysed using Minitab 15 statistical software using Kolmogorov-Smornov Test. Since the data was found to be non-nomally distributed, Kruskal Wallis test (non-parametric equivalent test of ANOVA) was applied.

4.3. Results and discussion

4.3.1. Activity of AMB-NPs in neutropenic murine model of disseminated aspergillosis

The *in vivo* efficacy of orally administered AMB-NPs was evaluated in two separate experiments of the neutropenic murine model of disseminated aspergillosis. The fungal burdens observed in the kidneys of the infected animals of the first experiment detailed in Table 4.1 are shown in Fig. 4.3. The control animals treated with blank NPs showed the highest fungal burden indicating that NPs had no inherent anti-fungal activity. Intraperitoneal administration of Fungizone® at the dose of 0.1 or 0.3 mg/kg daily for 4 days significantly ($p=0.0451$ and $p=0016$) suppressed the fungal burdens in the kidney of infected mice by 80.3±2.5 and 87.4±5.5%, respectively. Oral treatment with posaconazole at the dose of 2.5 mg/kg for 4 days resulted in 94.8±2.8% suppression of kidney fungal burdens. Intravenous administration of AMB-NPs at the dose of 0.3 mg/kg for 4 days resulted in a higher ($p<0.0001$) suppression of fungal burden (98.0±1.3%) compared to Fungizone®. Interestingly, single dose oral treatment with AMB-NPs at 2 or 5 mg/kg also significantly ($p=0093$ and $p<0.0001$) suppressed the fungal burdens by 78.1±9.7 and 98.3±1.5%, respectively. Oral treatment wih single dose of AMB-NPs (5 mg/kg) was more efficient than intraperitoneal treatment with Fungizone® (0.1 mg/kg for 4 days).

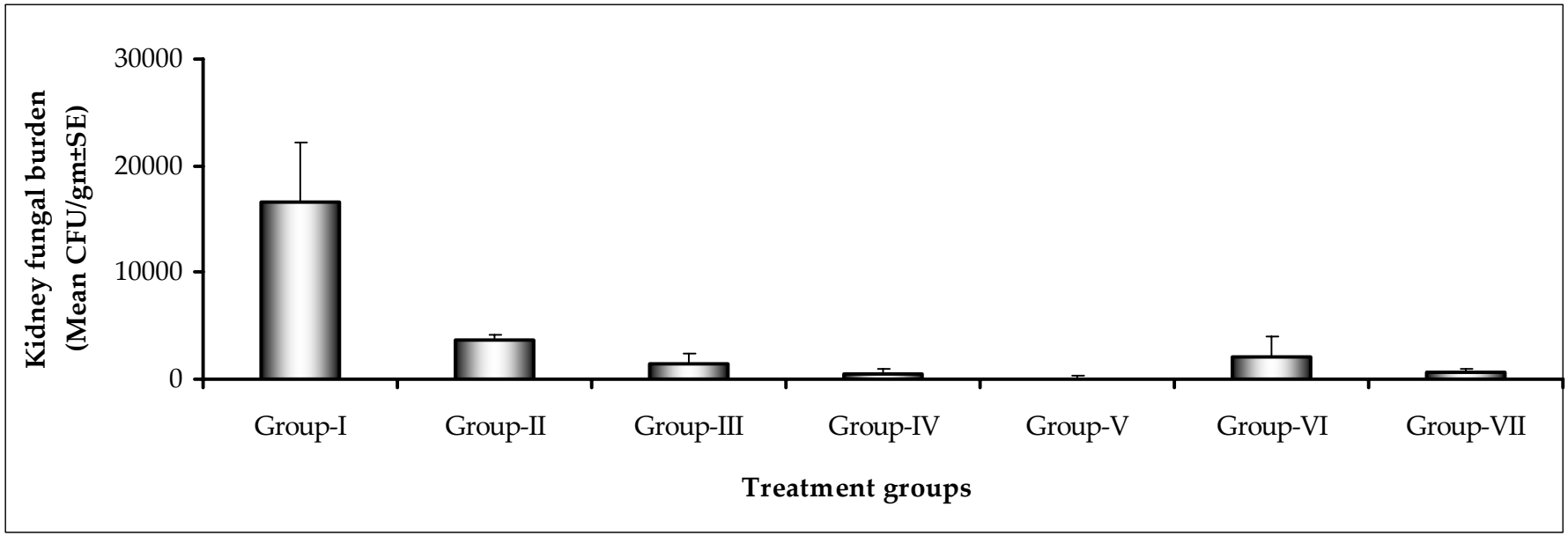


Fig. 4.3 The effect of treatment with different formulations of AMB on the kidney fungal burdens of mice infected with *A. fumigatus* (disseminated aspergillosis model, experiment-I). Group-I: IV blank NPs daily for 4 days, Group-II: intraperitoneal Fungizone® 0.1 mg/kg daily for 4 days (p=0.0451), Group-III: intraperitoneal Fungizone® 0.3 mg/kg daily for 4 days (p=0016), Group-IV: oral posaconazole 2.5 mg/kg daily for 4 days (p<0.0001), Group-V: intravenous AMB-NPs 0.3 mg/kg daily for 4 days (p<0.0001), Group-VI: oral AMB-NPs 2 mg/kg single dose (p=0093), Group-VII: oral AMB-NPs 5 mg/kg single dose (p<0.0001). The p values are from a Kruskal Wallis multiple comparison test.

A similar trend was observed in the results of the experiment-II (Fig. 4.4), where daily treatment with (0.2 mg/kg) or intravenous AMB-NPs (0.2 mg/kg) caused numerical reductions in fungal burden (89.8±5.5 and 86.5±3.5%) but the difference was not statistically significant. Daily treament with intraperitoneal Fungizone® (0.5 mg/kg for 4 days) and intravenous AMB-NPs (0.5 mg/kg for 4 days) significantly (p=0004 and p=0031) suppressed the kidney fungal burden by 97.8±0.4 and 97.4±0.4%, respectively. Of note oral treatment with AMB-NPs at the doses of 5 mg/kg for 4 days caused significant (p=0008) suppression of fungal burdens by 97.5±0.4%, which was comparable to the intraperitoneally administered Fungizone® 0.5 mg/kg for 4 days.

Overall, oral treatment with AMB-NPs as either a single or multiple dose exhibited anti-fungal activity comparable to intraperitoneal therapy with Fungizone® and oral posaconazole. Another advantage with the AMB-NPs was its suitability of administration by intravenous bolus injection, offering possibility of alternate dosage form, which could provide convenience in switching between intravenous and oral formulations. Risovic *et al.,* (2007) evaluated the anti-fungal efficacy of an oral lipid based formulation of AMB made with Peceol in male albino Sprague-Dawley rats infected with *A. fumigatus*. Oral treatment with Peceol-AMB at the dose of 50 mg/kg/day for 4 days significantly reduced fungal burdens only in the brain and spleen but had no effect on fungal burdens in the kidneys, heart, liver or lungs of treated animals compared to control values.

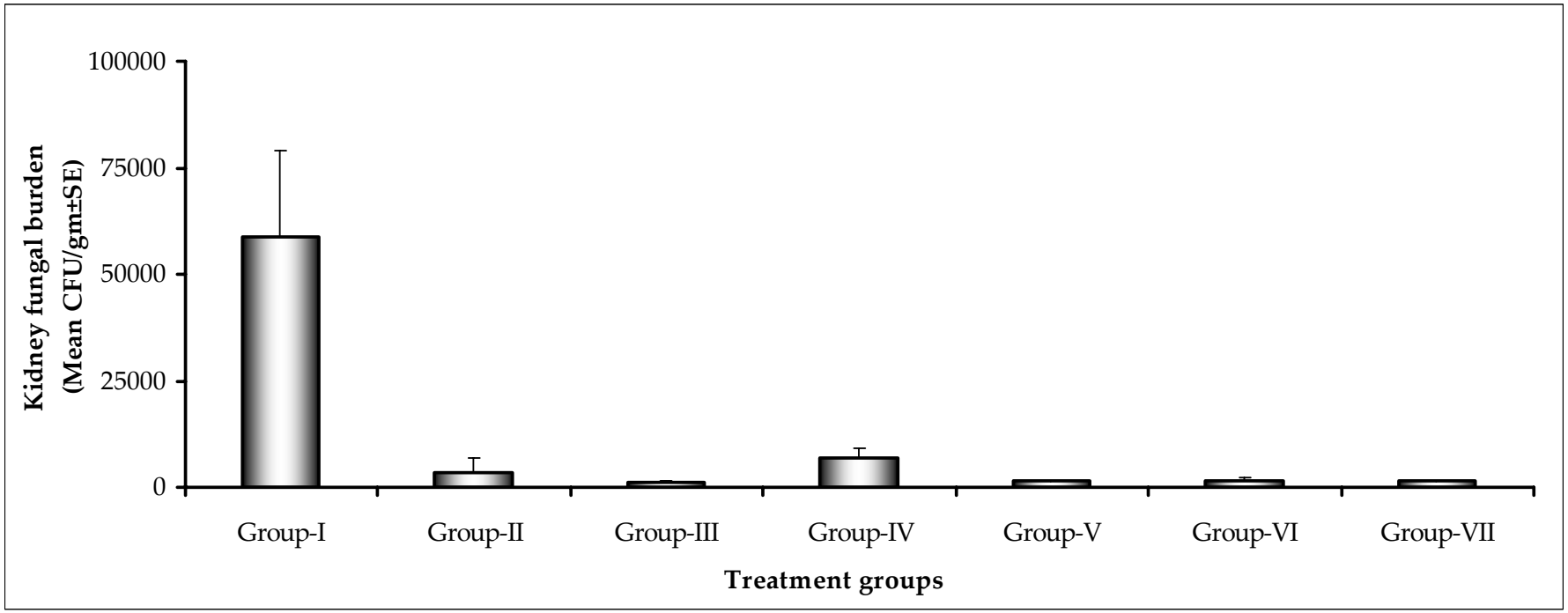


Fig. 4.4 The effect of treatment with different formulations of AMB on the kidney fungal burdens of mice infected with *A. fumigatus* (disseminated aspergillosis model, experiment-II). Group-I: intravenous blank NPs daily for 4 days, Group-II: intraperitoneal Fungizone® 0.2 mg/kg daily for 4 days (NS), Group-III: intraperitoneal Fungizone® 0.5 mg/kg daily for 4 days (p=0004), Group-IV: intravenous AMB-NPs 0.2 mg/kg daily for 4 days (NS), Group-V: intravenous AMB-NPs 0.5 mg/kg daily for 4 days (p=0031), Group-VI: oral AMB-NPs 2 mg/kg daily for 4 days (p=0016), Group-VII: oral AMB-NPs 5 mg/kg daily for 4 days (p=0008). The p values are from a Kruskal Wallis multiple comparison test; NS – Non-significant.

Recently, Wasan *et al.,* (2009a) determined the anti-fungal efficacy of a Peceol/ distearoylphosphatidylethanolamine-poly(ethylene glycol)$_{2000}$ based oral formulation of AMB in immunosuppressed male albino Sprague–Dawley rats infected intravenously with the *A. fumigatus* conidia. Oral administration of the formulation at a total dose of 20 mg/kg (administered in 4 divided doses, 5 mg/kg twice a day for 2 days) resulted in ~75% suppression of kidney fugal burdens compared to controls. In the present study, a higher suppression (more than 97%) of fungal burdens were achieved following oral treatment with AMB-NPs at equivalent dose.

4.3.2. Activity of AMB-NPs in murine model of IPA

The effect of different AMB formulations on the fungal burdens in lungs of infected mice is shown in Fig. 4.5. Control animals treated with blank NPs showed highest fungal burdens. Fungizone® administered intraperitoneally at a dose of 1.5 mg/kg for 4 days numerically suppressed fungal burdens by 86.3±11.3% compared to the control values, which is lower compared to 97.8±0.4% suppression obtained at the dose of 0.5 mg/kg for 4 days in disseminated model (Fig. 4.4). Lower efficacy of Fungizone® even at 3 times higher dose indicates that conventional formulation of AMB was not much effective at treating IPA. Intravenous treatment with AmBisome® (5 mg/kg for 4 days) and oral treatment with posaconazole (2.5 mg/kg for 4 days) numerically suppressed the lung fungal burden by (98.1±1.1 and 95.8±3.3%) but none of which were statistically significant ($p>0.05$).

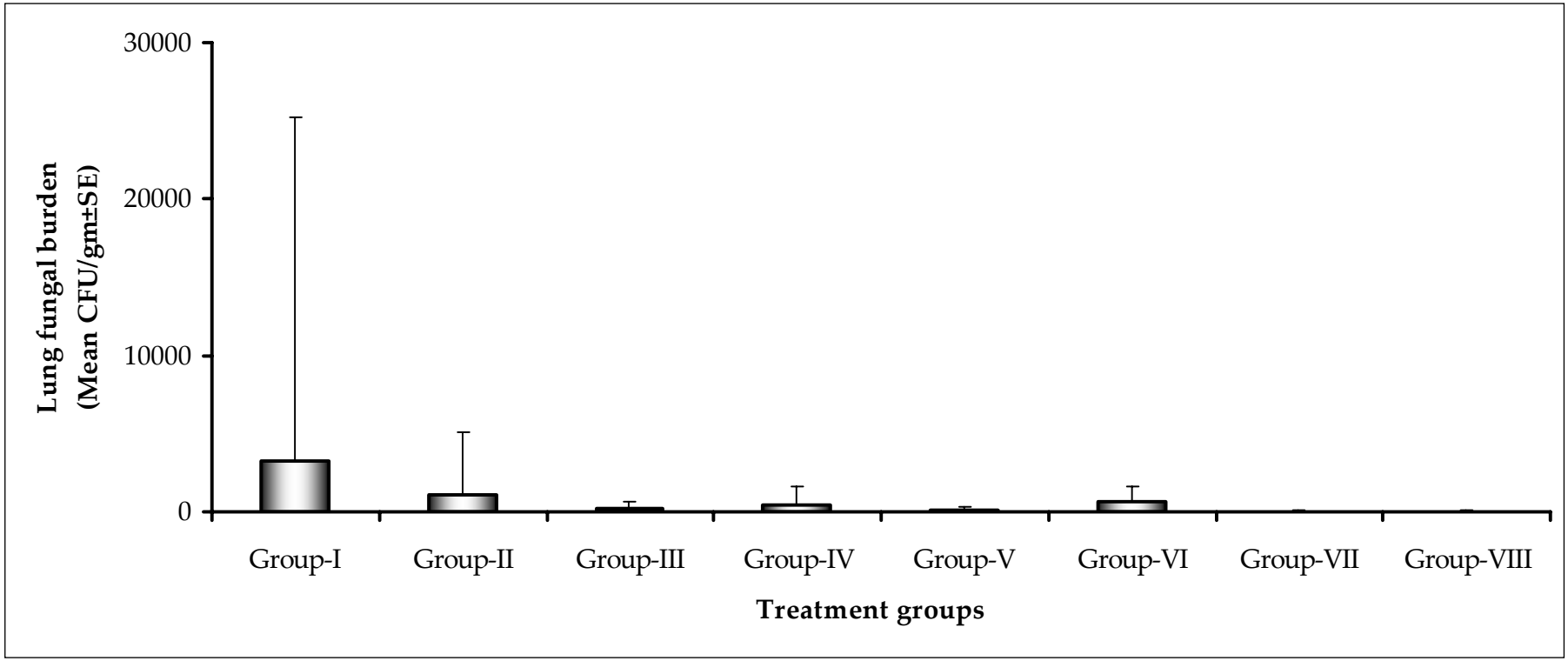


Fig. 4.5 The effect of treatment with different formulations of AMB on the fungal burdens in the lungs of neutropenic mice infected with *A. fumigatus* (IPA model). Group-I: intravenous blank NPs daily for 4 days, Group-II: intraperitoneal Fungizone® 1.5 mg/kg daily for 4 days (NS), Group-III: intravenous AmBisome® 5 mg/kg daily for 4 days (NS), Group-IV: oral posaconazole 2.5 mg/kg daily for 4 days (NS), Group-V: intravenous AMB-NPs 5 mg/kg single dose (p=0.0077), Group-VI: oral AMB-NPs 5 mg/kg single dose (p=0.0009), Group-VII: intravenous AMB-NPs 5 mg/kg daily for 4 days (p=0.0009), Group-VIII: oral AMB-NPs 5 mg/kg daily for 4 days (p=0.0001). The p values are from a Kruskal Wallis multiple comparison test; NS – non-significant.

Treatment with 5 mg/kg AMB-NPs either administered as single intravenous dose (p=0.0077) or daily intravenous dosing (p=0.0009) or orally (p=0.0001) caused large statistically significant reductions (98.9±0.8, 99.8±0.1 and 99.8±0.1%, respectively) in lung fungal burden indicating that AMB-NPs were highly effective at treating IPA. The results from this study are consistent the results from disseminated model studies. AMB-NPs administered orally exhibited comparable *in vivo* anti-fungal activity to parenterally administered commercial formulations of AMB, AmBisome® and Fungizone®. The IPA model used in the present study is a very severe model and the therapy with conventional AMB is not very effective at treating this model. In the present study, the oral and intravenously administered AMB-NPs were highly effective for treating the infected animals. NPs are known to enhance the intestinal uptake of the encapsulated drugs thereby bioavailability (Hariharan *et al.,* 2006; Italia *et al.,* 2009). Thus, improved efficacy of AMB-NPs could be primarily attributed to enhanced oral bioavailability.

4.4. Conclusions

Oral treatment with single or multiple doses of AMB-NPs was efficient at treating the disseminated aspergillosis in mice and the efficacy was comparable to parenterally administered Fungizone®. Moreover, oral AMB-NPs were also very effective at treating IPA, which is more difficult to treat. Together, the data strongly indicates the potential of oral AMB-NPs for treating systemic fungal infections.

CHAPTER 5

EVALUATION OF THE ANTI-LEISHMANIAL ACTIVITY OF AMB-NPs *IN VITRO* AND *IN VIVO*

5.1. Introduction

Leishmaniasis is neglected disease, which has forced itself upon medical attention as an increasingly significant threat over the last decade with a more widespread and steady increase in the reported incidence (Baily and Nandy, 1994). The treatment options for VL are limited to a few numbers of drugs, most of which require parenteral administration with long treatment regimens. The new drug discovery for this orphan disease is hampered by severe financial constrains. Thus, reformulation of previously approved drug could be a viable alternative. With the advancement in the field of novel drug delivery, it may be possible to reformulate AMB as oral formulation, which would significantly improve its access to patients and improve compliance.

Several *in vitro* assays systems have been developed to test *Leishmania* susceptibility to new drugs/formulations using two life stages of *Leishmania*; namely promastigotes and amastigotes. Promastigotes and amastigotes are morphologically and biochemically different and have different susceptibilities to anti-leishmanial drugs. Studies have shown that promastigotes are generally less sensitive to drugs than amastigotes (Coombs *et al.,* 1983; Roberts and Rainey, 1993). It has also been demonstrated that *in vitro* assays using intracellular amastigotes correlate better to *in vivo* responses to treatments than using extracellular promastigotes (Lira *et al.,* 1999). In *in vitro* systems, drug/formulation is typically added to the culture medium 24 h after parasite infection then the effect of treatment on amastigotes i.e., reduction in infection or number of parasites/host cell is determined after different time intervals.

In vivo assays/animal models enable drug/formulation activity to be determined in relation to absorption, distribution (different sites of infection),

metabolism, excretion and give an early indication of drug's inherent toxicity. Many experimental models of leishmaniasis have been developed. These models have the major attraction of allowing control over the genetics of both the parasite and the host, but none of the animal models entirely mimics the disease in humans. One of the key factors contributing to differences between humans and animal models is the size and nature of the parasite inoculum. In case of natural infections, the sandfly introduces a very small number (possibly as few as 100 to 1,000) metacyclic promastigotes together with strongly bioactive saliva into the skin, whereas in laboratory infections thousands to millions of culture-derived promastigotes or tissue-derived amastigotes are injected. The sandfly is a blood pool feeder, which uses its mandibles to cut a wound in the skin and sucks up the blood that accumulates. The infective parasite inoculum is deposited in this superficial pool, most probably in a very small volume. In contrast, the laboratory infection is commonly done in relatively large volumes of around 50 μL or more. In addition, in the laboratory the syringe-delivered parasites are deposited mostly subcutaneously or, in visceral leishmaniasis models, intravenously.

Experimental models of VL have been developed mainly in rodents such as hamsters and mice (Carter *et al.,* 1988; Gifawesen and Farrel, 1989). The golden hamster was used in earlier animal models for the study of VL. Infection with *L. donovani* in hamsters leads to visceral disease and death. This model mimics everal aspects of human disease such as hepatosplenomegaly, anemia, hyperglobulinemia, and progressive cachexia, which makes it a useful tool for the characterisation of molecules and mechanisms involved in pathogenesis (Hommel *et al.,* 1995). However, in recent years, interest in hamster model has declined and it is now used

primarily as a source of *L. donovani* amastigotes, which seem to be the required life cycle stage for infection of mice, the currently preferred model animal for VL.

BALB/c mice infected with *Leishmania* species have been the most widely used animal model for screening the *in vivo* anti-leishmanial activity of the drugs/formulations since this mouse strain is susceptible to most *Leishmania* species (Carter *et al.,* 1988; Mullen *et al.,* 1998; Carter *et al.,* 2001; Carter *et al.,* 2003; Wasan *et al.,* 2009b). Disseminated granulomes, with parasitised macrophages, are found during infection, especially in the liver and spleen of infected with *L. donovani* (Barbosa *et al.,* 1985; Squires *et al.,* 1989). In infected mice, there is an early increase of parasite burden, but the infection spontaneously declines when an anti-leishmania cellular immune response, involving both CD4+ and CD8+ T cells, is mounted (Stern *et al.,* 1988). A high parasite load is observed in the liver and is associated with interleukin (IL)-10, IL-6 and no interferon (IFN)-γ production. In the spleen, the parasite load is less pronounced and, accordingly, splenocytes produce IL-4, IL-6, IL-10 and IFN-γ (Kaye *et al.,* 1991; Wilson *et al.,* 1996). One of the major advantages of using mouse model is that it develops highly reproducible levels of infection when infected intravenously with an amastigote inoculum (Croft *et al.,* 2006) although this route of parasite administration does not mimic the natural infection by the sandfly. In this study, the *in vitro* and *in vivo* anti-leishmanial activity of AMB-NPs was determined using *L. donovani*.

5.2. Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium and fetal calf serum, penicillin/streptomycin and L-glutamine were purchased from

Gibco Ltd (Uxbridge, UK). AmBisome® was purchased form Glasgow Royal Infirmary (Glasgow, UK). Giemsa stain was obtained form BDH Chemicals Ltd (Poole, UK). DPX Mountant was purchased from Sigma-Aldrich Co Limited (Poole, UK).

5.3. Methods

5.3.1. Activity of AMB-NPs in bone marrow macrophages (BMMs) infected with *L. donovani*

5.3.1.1. Preparation of BMMs

Bone marrow cells isolated from the bone marrow of 6-8 week old female inbred BALB/c mice were incubated in the Petri dishes for 7 days with culture medium containing DMEM supplemented with 20% v/v of heat-inactivated fetal calf serum (HI-FCS), 30% v/v of L-cell conditioned supernatant, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at the standard conditions of 37°C in 5% CO_2 to produce BMMs (Carter *et al.,* 2005). Cells were harvested by scrapping the Petri dishes and counted using a hemocytometer using Tryphan blue exclusion method to differentiate live and dead cells. Cells were diluted to $5X10^6$ cells/ml in complete medium (RPMI-1640 supplemented with 10% v/v of HI-FCS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin). All animal experiments were carried out in accordance with United Kingdom Home Office regulations (license PPL 60/3740) and under clearance from the University of Strathclyde's Ethical Committee.

5.3.1.2. Parasite preparation

L. donovani (strain LV82) was maintained by serial passage through Golden Syrian hamsters. The spleen of an infected hamster was removed aseptically and broken up in supplemented RPMI 1640 medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine. The amastigotes were washed and re-suspended in 10 to 15 ml of medium and amastigotes concentration/ml was determined using a hemocytometer and microscope at 400X magnification.

5.3.1.3. Infection of BMMs culture with L. donovani and drug treatments

Briefly, 100 μl of BMMs ($1X10^6$ cells/ml) in complete medium were added to each well of a 24-well tissue culture containing round 13-mm cover slips. In one set of BMMs plates, the cells were treated with AMB solution or AMB-NPs diluted in complete medium (2, 20, 35, 50 or 100 ng/ml) 24 h pre-infection or 72 h pre-infection for 6 h. The drug was removed from the cells, the cells were washed with 200 μl medium and then infected at appropriate time by adding 100 μl of *L. donovani* promastigotes (strain 200016) at a parasite:host cell ratio of 1:20 followed by addition of 100 μl of complete medium. The plates were then incubated under standard conditions for 24 h and any unattached parasites were removed by replacing the well contents with 500 μl fresh medium. The plates were then incubated for 72 h at the standard conditions.

In another set of experiment, the cells were infected by adding 100 μl of *L. donovani* promastigotes (strain 200016) at a parasite:host cell ratio of 1:20 followed by addition of 100 μl of complete medium. The plates were then incubated under standard conditions for 24 h and any unattached parasites

were then removed by replacing well contents with fresh medium. The cells were then treated with 500 µl of AMB solution or AMB-NPs diluted in complete medium (2, 20, 35, 50 and 100 ng/ml) for 6 h. The cells were then washed with 200 µl of fresh medium and then 500 µl complete medium was added to each well before incubated at standard conditions for up to 72 h. At the end of experiment, the medium was removed from the wells and the cells were fixed for 2-3 min with methanol. The cells were then stained with 10% w/v Giemsa stain for 20 min. The staining solution was then removed from the wells and the cells were washed with water. The cover slips were taken out from the wells, air-dried and then mounted on the glass slides using DPX mountant. The mean percentage of cells infected was determined for control and drug treated cells from 200 randomly selected cells. The mean suppression in infection (mean percent±SE) was calculated by comparing each mean experimental value with the mean control value.

5.3.2. Activity of AMB-NPs in murine model of VL

5.3.2.1. Animals and parasites

Age-matched 8-10 week old BALB/c mice (in-house, in-bred females) were used in this study. Mice were infected by intravenous injection (tail vein) with 1×10^7 *L. donovani* (strain LV82) amastigotes in 0.2 ml without anesthesia. The day of parasite administration to the mice was designated day 0 of the experiment.

5.3.2.2. Drug treatment and parasite counting

Mice infected with *L. donovani* amastigotes were divided into 3 groups of 4 animals. Group-I (control) was treated with 0.2 ml water orally. Group-II was treated intravenously with AmBisome® at the dose of 2.5 mg/kg (in 0.2 ml) on day 7 post-infection. Group-III was treated orally with 0.2 ml of AMB-NPs on day 7-11 (50 mg/kg loading dose followed by 10 mg/kg for 4 days). On the day 14 post-infection, mice were sacrificed by CO_2 euthanasia and impression smears of the liver were obtained after weighing the organ. The smears were fixed in methanol, stained in 10% Giemsa for 20 min, washed and air-dried. The parasite burdens were determined microscopically by blindly counting the number of parasites per 1,000 host nuclei from randomly chosen smears. Leishman-Donovan units (LDU) were calculated for liver using the following formula: LDU = amastigote number per 1,000 host cell nuclei X organ weight in grams (Carter *et al.,* 2001).

5.3.2.3. Determination of blood urea nitrogen (BUN) and plasma creatinine (PC) levels

The blood samples were collected in centrifuge tubes containing 10 µl of heparin solution (1000 intenational units/ml). The samples were centrifuged 5000 rpm for 5 min to obtain the plasma samples, which were stored at -20°C until analysis. BUN was analysed using QuantiChrom™ urea assay kit (CA, USA) while PC was analysed by Creatinine assay kit (BioVision Research Products, CA, USA).

5.3.2.4. Analysis of AMB in plasma and tissues

Plasma and tissues samples (liver, spleen, kidneys and lungs) were collected and analysed for AMB using the HPLC method. Plasma samples were prepared and analysed by the method described in Section 2.3.2. The tissue samples (200 mg) were homogenised in 1.2 ml of 90% methanol containing 0.5 μg/ml of IS at 25000 rpm and then centrifuged at 15000 rpm for 5 min. The resulting clear supernatants were transferred to the sample vials and 50 μl of each supernatant was injected into the HPLC using same conditions as described in Section 2.3.2.

5.3.2.5. Statistical analysis

Normality of the data was first analysed using Minitab 15 statistical software using Kolmogorov-Smornov Test and was found to be normally distributed. If the data was found to be normally distributed. Hence, one way ANOVA (parametric test) was applied and $p<0.05$ was considered as statistically significant.

5.4. Results and discussion

5.4.1. Activity of AMB-NPs in BMMs infected with *L. donovani*

24 or 72 h pre-infection exposure (prophylactic) studies showed that AMB-NPs were more effective at suppressing infection levels in the *L. donovani* infected BMMs compared to free AMB (Fig. 5.1 & Fig. 5.2).

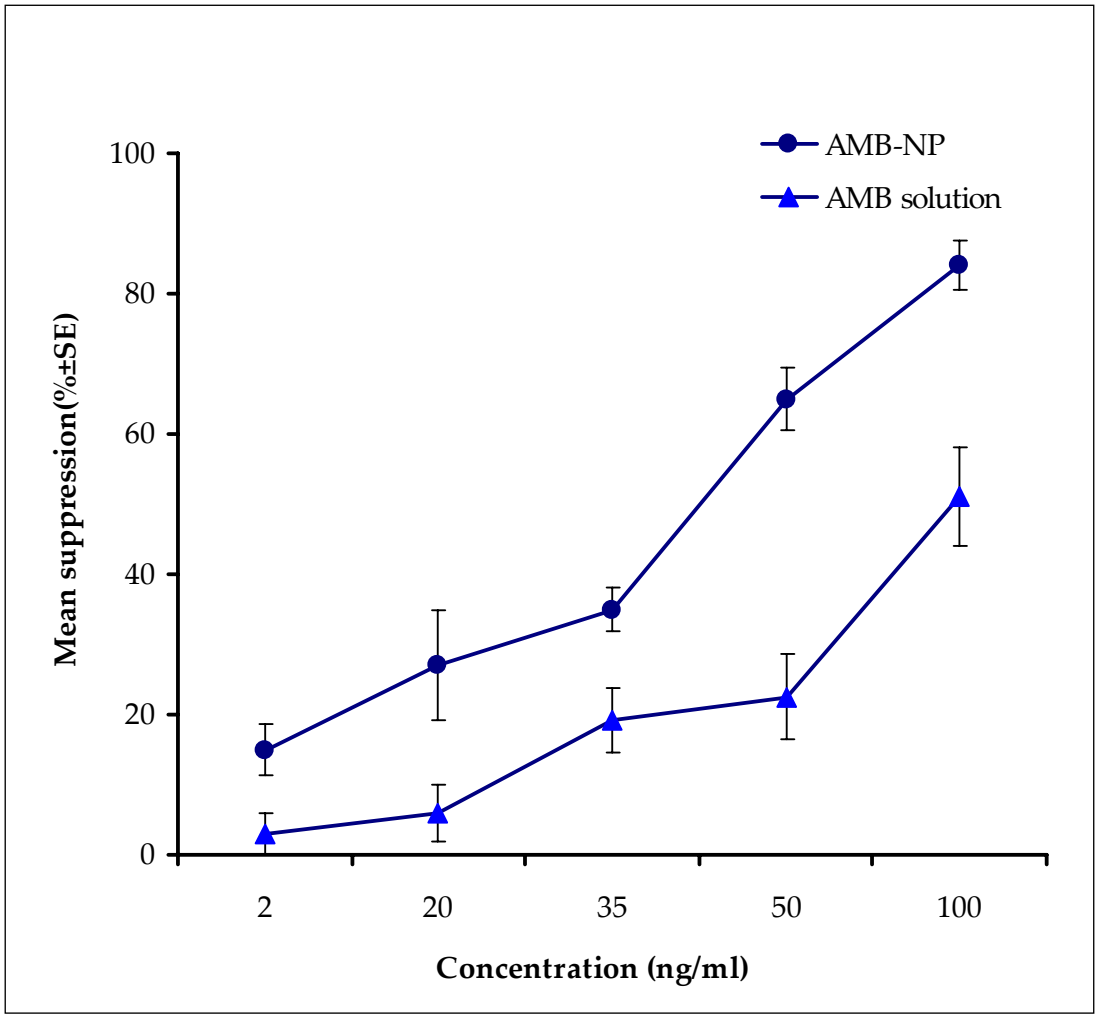


Fig. 5.1 *In vitro* anti-leishmanial activity of AMB solution and AMB-NPs following 24 h pre-infection exposure (n=4). BMMs were treated with different concentrations of AMB solution and AMB-NPs for 6 h, washed and incubated for 24 h in fresh medium. Cells were then infected with *L. donovani* amastigotes for 24 h and unattached parasites were removed by replacing well contents with fresh medium. The cells were then incubated for 72 h and parasite burdens were determined by staining the cells with Giemsa.

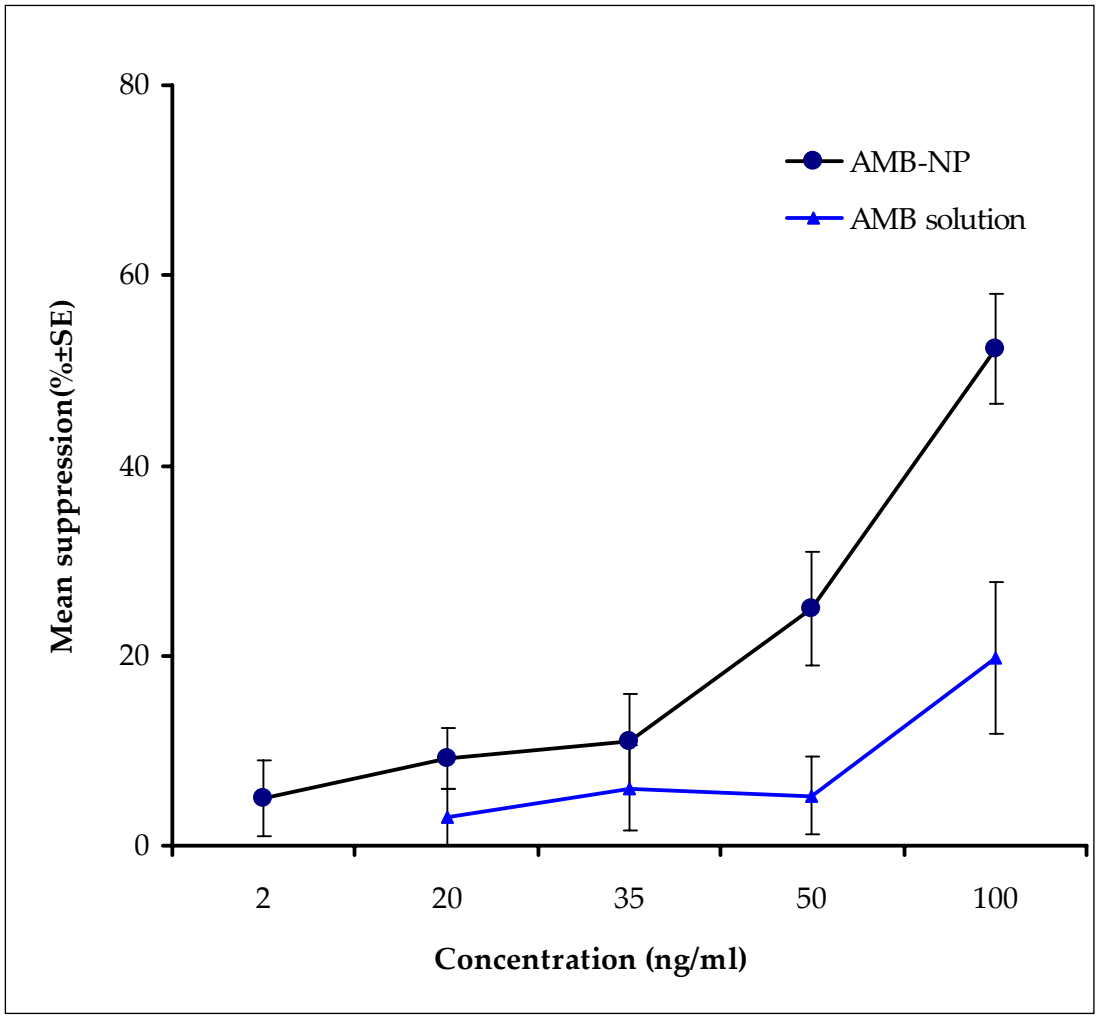


Fig. 5.2 *In vitro* anti-leishmanial activity of AMB solution and AMB-NPs following 72 h pre-infection exposure (n=4). BMMs were treated with different concentrations of AMB solution and AMB-NPs for 6 h, washed and incubated for 72 h in fresh medium. Cells were then infected with *L. donovani* amastigotes for 24 h and unattached parasites were removed by replacing well contents with fresh medium. The cells were then incubated for 72 h and parasite burdens were determined by staining the cells with Giemsa.

24 h pre-infection exposure gave maximum suppression of 85.0±3.5 and 51.0±7.0% for AMB-NPs and AMB solution, respectively at 100 ng/ml of concentration. Pre-treating the cells at the same drug concentrations (72 h pre-infection exposure) gave lower suppressions of 52.0±5.8 and 20.0±8.0%, respectively for AMB NPs and AMB solution at 100 ng/ml. A similar trend was obtained for cells treated with AMB formulations in therapeutic studies (24 h post-infection exposure). AMB-NPs were superior in suppressing infection levels in infected BMMs compared to AMB solution at all the concentrations tested except 100 ng/ml where similar results were obtained for both formulations (Fig. 5.3).

Nahar and Jain (2009) have determined *in vitro* anti-leishmanial activity of AMB incorporated into the PLGA NPs in macrophages J774A infected with *L. donovani* amastigotes. Maximum suppression of 71.77±1.23% and 87.10±2.48% was observed at 0.924 μg/ml concentration with AMB solution and nanoparticulate formulation, respectively. Comparable parasites suppression was observed with AMB-NPs at 100 ng/ml concentration in the present study indicating higher activity of the AMB-NPs.

5.4.2. Activity of AMB-NPs in murine model of VL

Oral administration of AMB-NPs at a single first dose of 50 mg/kg followed by 10 mg/kg on 4 consecutive days caused significant ($p=0.005$) reduction (58±6%) in liver parasite burden compared to controls (Fig. 5.4 & 5.5). On the other hand, intravenous AmBisome® at 2.5 mg/Kg resulted in higher ($p<0.001$) suppression (90±3%) of liver parasites. No increase in the BUN or PC levels was observed in the mice treated with AMB-NPs compared to control animals, indicating that treatments did not induce any nephrotoxicity (Fig. 5.6).

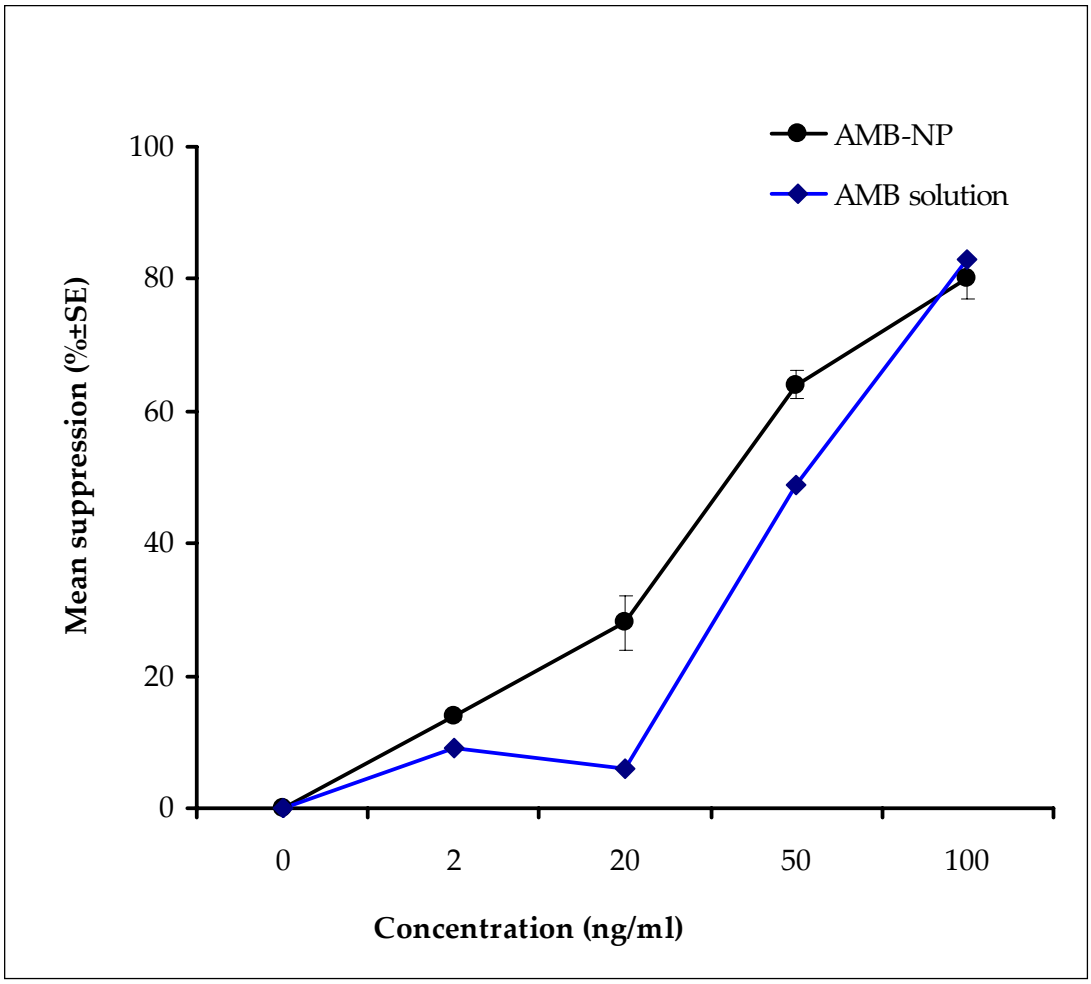


Fig. 5.3 *In vitro* anti-leishmanial activity of AMB solution and AMB-NPs following 24 h post-infection exposure (n=4). The macrophages were infected with *L. donovani* amastigotes for 24 h and unattached parasites were removed by replacing the well contents with fresh medium. The cells were then treated with different concentrations of AMB solution or AMB-NPs for 6 h. The cells were washed and incubated for 72 h. The parasite burdens were determined by staining the cells with Giemsa.

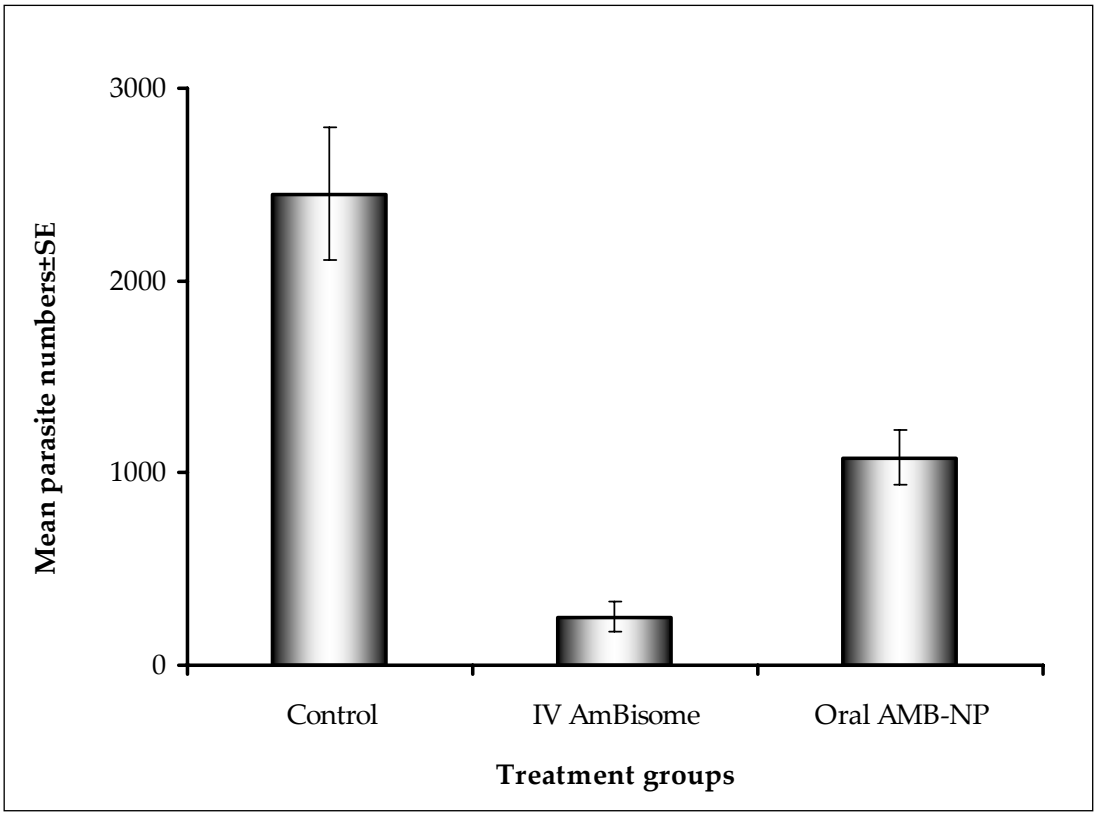


Fig. 5.4 The effect of treatment with AMB-NPs or AmBisome® on the parasite burdens in the liver (n=4). Mice were infected with 1X 10^7 *L. donovani* amastigotes and treated on either day 7 with a single intravenous bolus dose of AmBisome® or oral AMB-NPs 50 mg/kg followed by 10 mg/kg for 4 consecutive days. On day 14 parasite burdens in liver were determined. p=0.005, AMB-NPs vs. control, p<0.001, AmBisome® vs. control.
The p values are from one way ANOVA test.

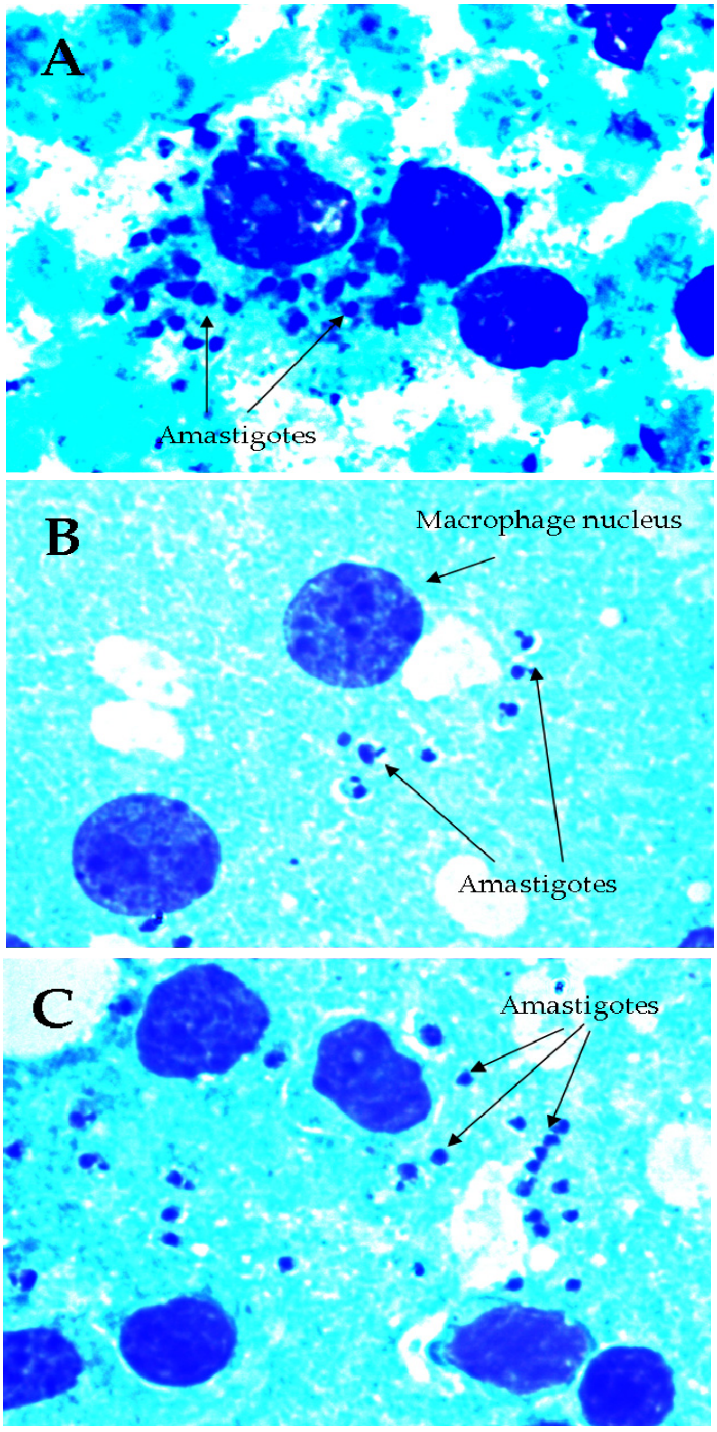


Fig. 5.5 Giemsa-stained liver smears obtained from *L. donovani* infected mice on day 14 post-infection. Mice treated with (A) water (control), (B) AmBisome® intravenously at a dose of 2.5 mg/kg on day 7 post-infection or (C) oral AMB-NPs at dose of 50 mg/kg on day 7 followed by 10 mg/kg on days 8-11.

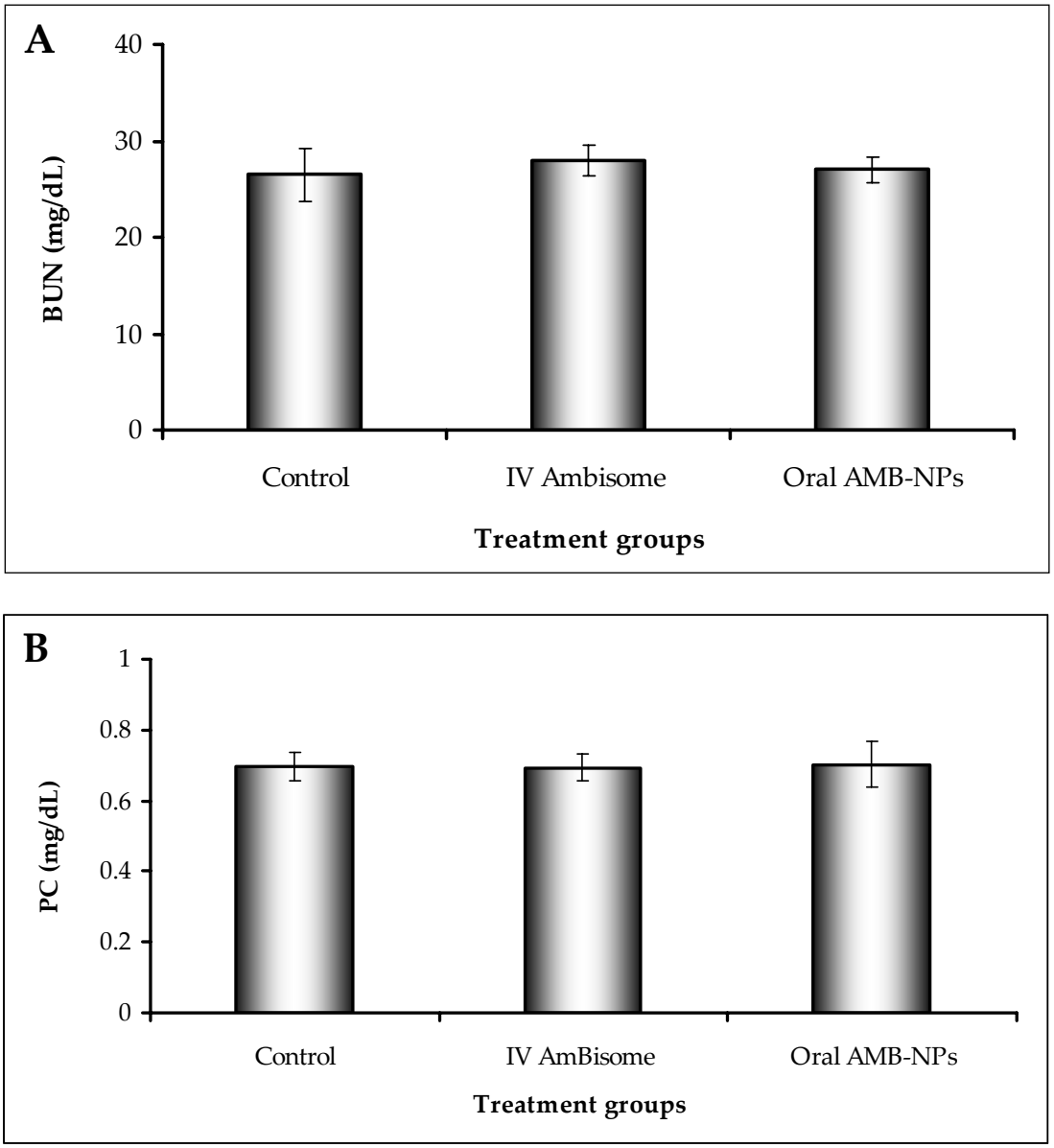


Fig. 5.6 The impact of oral AMB-NPs treatment on the BUN (A) and PC (B) levels in infected mice (Data presented as mean±SE n=4). Mice were infected with $1X10^7$ *L. donovani* amastigotes and treated on either day 7 with a single intravenous bolus dose of AmBisome® or oral AMB-NPs 50 mg/kg followed by 10 mg/kg for 4 consecutive days. On day 14, BUN and PC levels were determined.

The plasma and tissue levels of AMB on the day 14 post-infection are shown in Table 5.1. AMB was not detectable in any of the tissues in the AmBisome® group, where as quantifiable levels were present in the tissues treated with the AMB-NPs. The plasma levels were approximately 5 times lower than the tissues indicating AMB-NPs were sequestered in the tissues.

Table 5.1 Plasma and tissue concentrations of AMB in mice on the day 14 post-infection (Data presented as mean±SD, n=4)

Plasma/Tissue	Intravenous AmBisome®	Oral AMB-NPs ng/gm
Plasma	ND	55±10*
Liver	ND	243±35
Spleen	ND	200±31
Kidneys	ND	214±51

ND=Non-detectable; * ng/ml

Relatively lower anti-leishmanial activity of AMB-NPs compared to AmBisome® could be explained by the pharmacokinetics/pharmacodynamic differences following oral and intravenous administration. AmBisome® upon intravenous bolus injection leads to instant availability of much higher concentrations of AMB in the system to exert anti-leishmanial activity. Since anti-leishmanial activity of AMB is concentration-dependent and not time-dependent, the availability of higher concentration of AMB upon intravenous injection could lead to more efficient killing of the parasites and higher therapeutic efficacy. On the other hand, AMB-NPs upon oral administration resulted in relatively lower AMB concentration in plasma over a long period of time due to combined effect of both, oral route of administration and

sustained release of AMB from the absorbed NPs. Consequence of this could be the lower anti-leshmanial activity of AMB-NPs compared to AmBisome®.

Wasan *et al.,* (2009b) conducted a study using a lipid-based formulation of AMB for oral treatment of VL. The formulation, given at a dose of 40 mg/kg/day for 5 days (total dose of 200 mg/kg), starting on day 7 post-infection, reduced the liver parasite burdens by 99.8±0.2%. This was higher than parasite suppression levels obtained with AMB-NPs in the present study. However, the total dose administered was more than double compared to AMB-NPs. On the other hand, the *in vivo* anti-fungal activity of AMB-NPs (>97% suppression) was considerably higher than this lipid formulation (~75% suppression) in the disseminated aspergillosis model as discussed in Chapter 4, Section 4.3.1. Even higher activity (upto 99.8±0.1% suppression) of oral AMB-NPs was observed in the IPA model.

The difference in the anti-leishmanial and anti-fungal activity of AMB-NPs could be attributed to the difference in localisation of the fungal cells and *Leishmania* parasites. In case of systemic fungal infections, the fungal cells colonise in the tissues extracellularly and thus, the AMB released from absorbed AMB-NPs could directly reach the fungal cells. However, in case of VL infection, the parasites reside intracellularly within the phagolysosomes or parasitophorous vacuoles (Croft and Coombs, 2003). Therefore, the anti-leishmanial activity of AMB is governed by the drug concentration achieved in the microenvironment of the intracellular parasite rather than that in the host circulation. Systemically absorbed AMB-NPs or released AMB has to enter the macrophage to reach the parasites residing in the parasitophorous vacuoles (Fig. 5.7).

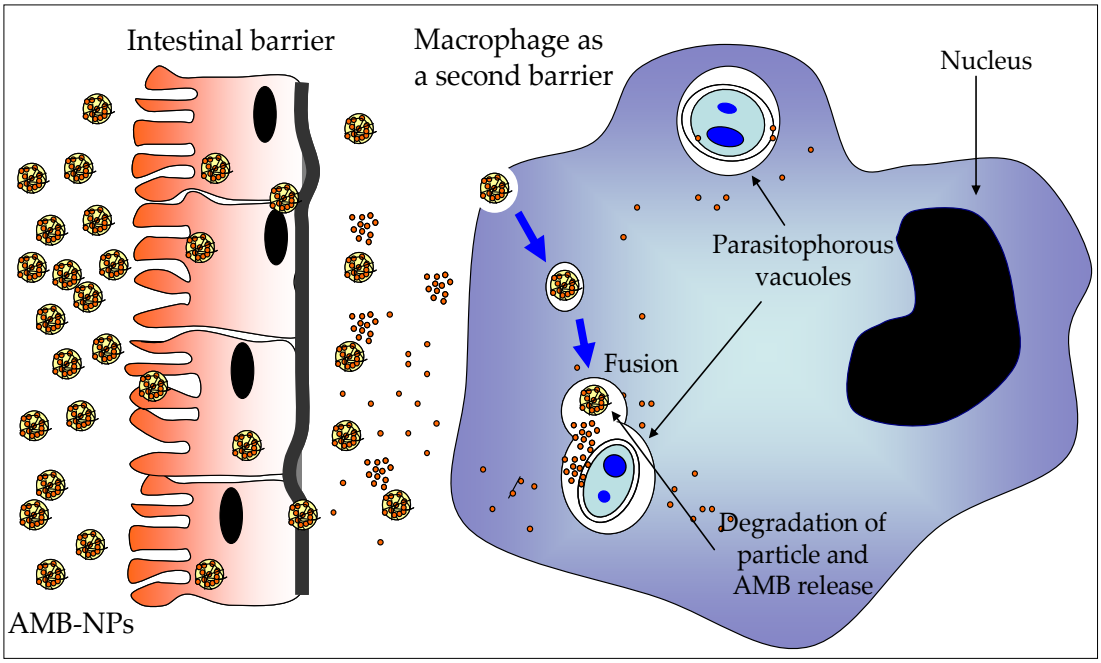


Fig. 5.7 Biological barriers for AMB-NPs to reach the intracellular *Leishmania* parasites.

The intracellular nature of *Leishmania* parasites thus presents a second barrier for the AMB-NPs after reaching to systemic circulation, which could be the primary reason for moderate efficacy of AMB-NPs in treating leishmaniasis compared to systemic fungal infections.

The other factor could be the sustained *in vivo* release profile of AMB-NPs. The preliminary pharmacokinetic studies in the rats following a single oral dose of AMB-NPs showed a sustained *in vivo* release of AMB over 5 days with T_{max} of ~24 h (Italia *et al.,* 2009), thus making relatively lower concentration of AMB available for killing the parasites over a period of time. However, in case of oral lipid formulation (T_{max}, ~6 h; Gershkovich *et al.,* 2009) or intravenous AmBisome®, higher concentration of AMB is available (due to faster release) at a time for killing the parasites leading to higher anti-leishmanial activity.

5.5. Conclusions

The present study showed improved *in vitro* anti-leishmanial activity of AMB-NPs as compared to AMB solution. The *in vivo* study demonstrated moderate efficacy of oral AMB-NPs in the treatment of VL. However, the formulations at such high doses did not cause any nephrotoxicity indicating the promise of the AMB-NPs and warrants further investigations to improve the anti-leishmanial activity.

SUMMARY

The developed HPLC methods were accurate and sensitive for analysis of AMB in *in vitro* and *in vivo* samples. The *in vivo* method is preferential due to requirement of low volume of plasma, easy sample preparation method, use of easily available IS, short analysis time and absence of ME. The method was precise with low intra-day and inter-day variability of less than 5%. The developed *in vivo* method was sensitive enough to quantify as low as 10 ng/ml concentration of AMB, indicating its suitability for pharmacokinetic studies for sustained/controlled release formulations.

The method of preparation of AMB-NPs could be successfully optimised by varying process parameters to obtain smaller particle size and higher AMB payload and entrapment efficiency. The optimised preparation method employed nanoprecipitation technique with DMSO as solvent to solubilise polymer and AMB while using ethanol as dispersing phase. The optimised formulation was ~115 nm in size with ~70% entrapment efficiency at 30% (w/w of polymer) AMB loading.

The process of making AMB-NPs could be scaled-up to a continous, mild and reproducible method. The batch sizes up to 1000 mg of polymer could be reproducibly prepared with the scaled-up method.

Centrifugation method employed for purification of AMB-NPs suffered from several drawbacks including considerable change in particle size, particle size distribution and typically low recoveries of AMB-NPs after purification due to aggregation and cake formuation of NPs during centrifugation. These drawbacks could be overcome using the ultrafiltration method. Ultrafiltration method was superior for purification of AMB-NPs in terms of unaltered particle characteristics and particle recovery. Moreover, it could be

successfully employed for the purification of pilot-scale batch of AMB-NPs offering possibility of its use at large scale.

The long-term stability of the AMB-NPs could be achieved with freeze-drying. The AMB-NPs could be freeze-dried with 10% w/v of sucrose (was found to be optimal cryo/lyo-protective) and the freeze-dried formulation was stable at 4°C over the study period of 3 months.

Oral AMB-NPs exhibited *in vivo* anti-fungal activity comparable to intraperitoneally administered Fungizone® in disseminated aspergillosis model. The anti-fungal efficacy of oral AMB-NPs was even better in IPA model, which was comparable to intravenously administered AmBisome® and better than oral posaconazole. AMB-NPs showed improved *in vitro* anti-leishmanial activity compared to AMB solution. Oral AMB-NPs demonstrated about 58% suppression of liver parasites in murine model of VL without causing alteration in renal functions. Although the anti-leishmanial activity of AMB-NPs was not as good as anti-fungal activity due to intracellular location of *Leishmnia* parasites, the data is promising considering absence of nephrotoxicity. Together, the data indicates potential of AMB-NPs in treating systemic fungal infections and VL and offers possibility of its development as an alternative treatment to the existing parenteral formulations.

FUTURE DIRECTIONS

The present investigation has shown that oral AMB-NPs were highly effective at treating systemic fungal infections and the efficacy was comparable to parenterally administered Fungizone® and AmBisome®. Oral AMB-NPs also exhibited moderate *in vivo* anti-leishmanial activity; however, the efficacy was limited by the intracellular nature of the parasites, which acts as a second barrier for the absorbed AMB-NPs. Several strategies can be considered for enhancing the anti-leishmanial activity of AMB-NPs. Firstly, the anti-leishmanial activity can be improved by enhancing particle uptake by macrophages. The particle uptake by macrophages is mostly dependent on two main factors, the particle size and the hydrophobicity/hydrophilicity. Increasing the particle size can improve the particle uptake by macrophages; however, it adversely affects its intestinal uptake. Altering the hydrophobicity of the particles may enhance the particle opsonisation and subsequent uptake by macrophages (Allen, 1981; Illum *et al.,* 1986; Prior *et al.,* 2002). The other strategy could be the altering of release profile of AMB-NPs. Increase in rate of AMB release from AMB-NPs can increase the concentration of free AMB available for killing the parasites, which may lead to improved anti-leishmanial activity. PLGA is a copolymer, made up of lactic acid and glycolic acid. As lactic acid being more hydrophobic than glycolic acid, hydrophobicity and degradation rate of PLGA mainly depends on the molar ratio of lactic and glycolic acid in the polymer chain. By varying copolymer composition of PLGA, degradation rate (and thus drug release) or hydrophobicity of the AMB-NPs can be optimised to obtain higher anti-leishmanial activity. On the other hand, NPs can actively be targeted to macrophages by functionalizing the particles using specific ligands like mannose.

The developed formulation exhibited excellent efficacy in the treatment of invasive and disseminated aspergillosis upon oral administration. However, as different fungal strains/species show different sensitivity to the anti-fungal therapy, the therapeutic efficacy of AMB-NPs may also be confirmed in the models of systemic/invasive fungal infections caused by other fungal species including *Candida albicans*, *Cryptococcus neofomans*.

The present studies demonstrated pharmacodynamic proof of concept in feasibility of the oral delivery of AMB as nanoparticulate formulation with considerable therapeutic efficacy. A detailed evaluation of pharmacokinetics and tissue distribution of AMB-NPs should be considered in normal animals and disease models of aspergillosis and VL.

The scale-up process was feasible and produced pilot-particle batches up to 1000 mg of polymer; however, at industrial levels, larger batches of AMB-NPs would be required. Hence, applicability of scale-up process for production of larger batches of AMB-NPs should be considered for future perspective.

Preliminary studies have shown that freeze-dried AMB-NPs were stable at 4°C for the study period of 3 months. The long-term stability studies (as per ICH guidelines) should be considered to determine the influence of a variety of environmental factors such as temperature, humidity and light to establish a shelf life for the drug product and recommended storage conditions.

Since the formulation is intended to be administered orally, the formulation should be essentially palatable. To improve the palatability, various additional excipients such as sweetening and flavoring agents need to be incorporated in the formulation. Since these additives can affect the stability

of the formulation, their effects on the stability of the final formulation should be evaluated.

Regulatory toxicology studies of the formulations are essential before the formulation is taken forward to clinical studies. In our case, the AMB-NPs formulstion demonstrated significant anti-fungal activity and is ready for a regulatory toxicology studies.

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