Nevirapine in pro-Pheroid: A preservative efficacy and stability study

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This dissertation is dedicated to my granddad, Eli Langenhoven, who died on the August, 2, 2009.

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ABSTRACT

Nevirapine in pro-Pheroid: A preservative efficacy and stability study

Human immunodeficiency virus (HIV) is a retrovirus that can lead to acquired immunodeficiency syndrome (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first identified on December 1, 1981, making it one of the most destructive pandemics in recorded history.

Pheroid™ technology is a patented delivery system that consists of both plant and essential fatty acids. It is often confused with lipid-based delivery system. Although there are some similarities, Pheroid™ technology owns its advantages in terms of absorption and/or efficacy of pharmacologically active compounds and other useful molecules. The Pheroid™ structure can be manipulated in terms of morphology, structure, size and function. The effectiveness of Pheroid™ technology has been illustrated by several national and international clinical trials with products based on this technology. Pro-Pheroid production is similar to that of Pheroid™ production, except that no aqueous phase is introduced; instead, the active compounds are dissolved in the oil phase.

This study was conducted to determine the stability of both nevirapine and butylparaben in the pro-Pheroid delivery system as well as the preservative efficacy of butylparaben. High performance liquid chromatography (HPLC) was used to determine the stability of nevirapine and butylparaben in pro-Pheroid. The formulation was stored under controlled conditions, i.e. 5°C, 25°C + 60% RH, 30°C + 65% RH and 40°C + 75% RH, for three months. The accelerated stability study was performed according to ICH guidelines. The preservative efficacy study was done by the EnviroCare Laboratory according to international specifications.

The various studies conducted on nevirapine in pro-Pheroid to determine the stability showed that nevirapine could successfully be formulated in the pro-Pheroid system and that butylparaben is an effective preservative in the formulation.

It is finally concluded that before nevirapine in pro-Pheroid is further formulated into viable products, the following issues will have to be addressed:

- The pro-Pheroid manufacturing process should be assessed and validated to ensure batch to batch uniformity.
- In order to establish a sound analytical method, stability of the pro-Pheroid system (without the addition of pharmaceutical actives) should be evaluated over a period of at least six months.
- The specific UV detection wavelengths of both nevirapine and butylparaben should be assessed and validated to get the optimum wavelength for HPLC assay analysis to ensure the integrity of the results obtained.

The physical properties (colour, smell and taste) of the pro-Pheroid system need to be addressed to make the product acceptable to the consumer.

UITTREKSEL

Nevirapien in pro-Pheroid: 'n Preserveringseffektiwiteits- en stabiliteitstudie

Die menslike immuungebrek virus (MIV) is 'n retrovirus wat lei tot verworwe immuungebrek sindroom (VIGS), 'n toestand waarin die mens se immuunstelsel begin faal, en uiteindelik lei tot lewensbedreigende opportunistiese infeksies. Die Joint United Nations Programme on HIV/AIDS (UNAIDS) en die Wêreld Gesondheidsorganisasie (WGO) het vanaf Januarie 2006 beraam dat VIGS die dood van meer as 25 miljoen mense veroorsaak het, vandat dit die eerste keer op 1 Desember 1981 geïdentifiseer is. Dit maak VIGS een van die dodelikste pandemies in *die geskiedenis*.

Pheroid™ tegnologie is 'n gepatenteerde afleweringsisteem wat bestaan uit beide plant en essensiële vetsure. Dit word dikwels verwar met lipied gebaseerde afleweringsisteme. Alhoewel daar sekere ooreenstemminge is, het Pheroid™ tegnologie sy eie voordele in terme van absorpsie en/of effektiwiteit van farmakologiese aktiewe verbindings en ander bruikbare molekules. Die Pheroid™ struktuur kan gemanipuleer word in terme van morfologie, struktuur, grootte en funksie. Die effektiwiteit van die Pheroid™ tegnologie is al geïllustreer deur menigte nasionale en internasionale kliniese proefstudies met produkte gebaseer op die tegnologie. Pro-Pheroid produksie is gelykstaande aan die van Pheroid™ produksie, behalwe dat daar geen waterfase word gebruik nie. Die aktiewe verbinding word dus in die oliefase opgelos.

Hierdie studie was uitgevoer om die stabiliteit van beide nevirapien en butielparabeen in die pro-Pheroid afleweringsisteem te bepaal, asook die preserveringseffektiwiteit van butielparabeen. Hoëdoeltreffendheidsvloeistofchromatografie (HDVC) is gebruik om die stabiliteit van nevirapien en butielparabeen in pro-Pheroid te bepaal. Die formulering is onder gekontroleerde toestande vir drie maande, 5°C, 25°C + 60% RH, 30°C + 65% RH en 40°C + 75% RH, geberg. Die versnelde stabiliteitstudie is volgens ICH riglyne uitgevoer. Die preserveringseffektiwiteitstudie is deur die EnviroCare Laboratorium behartig volgens internasionale spesifikasies.

Die verskillende studies wat op nevirapien in pro-Pheroid uitgevoer is om die stabiliteit te bepaal het getoon dat nevirapien suksesvol in die Pheroid™ sisteem geformuleer kan word en dat butielparabeen 'n effektiewe preserveermiddel is in die formulering.

Alvorens nevirapien in pro-Pheroid geformuleer kan word, moet die volgende twispunte aangespreek word:

- Die pro-Pheroid vervaardigingsproses moet geëvalueer en gevalideer word om loteenvormigheid te verseker.
- Om 'n gevestigde analitiese metode te vestig, moet die stabiliteit van die pro-Pheroid sisteem (sonder die byvoeging van farmaseutiese aktiewes) geëvalueer word oor 'n periode van ten minste ses maande.
- Die spesifieke UV deteksie golflengtes van beide nevirapien en butielparabeen moet geëvalueer en gevalideer word om die optimum golflengte vir HDVC analise te vind om die integriteit van die resultate verkry, te kan verseker.

Die fisiese eienskappe (kleur, reuk en smaak) van die pro-Pheroid sisteem moet aangespreek word om die produk vir die gebruiker aanvaarbaar te kan maak.

AIMS AND OBJECTIVES

This project aims to investigate the suitability of a novel delivery system by:

1. Entrapment of the drugs in the novel Pheroid™ carrier system;
2. To perform stability studies according to ICH guidelines to ensure product stability at different temperature and humidity conditions;
3. To test the samples at different wavelengths to determine the optimum detection wavelength for each compound tested; and
4. To perform a preservative efficacy study to determine the efficacy of the preservative incorporated in the formulation.

Methodology

- Formulation with Pheroid™ technology with nevirapine.
  This is done to determine the exact type and amount of each individual ingredient to get a stable, compatible and safe formulation.
- Determine the correct preservatives and ratio to be used in the formulation.
  Several preservatives are used to determine the most compatible and effective preservative to keep the final formulation stable and to adhere to British Pharmacopeial Standards. The different preservatives are added to the formulation and the formulations are diluted into different concentrations and spread out onto a petri-plate. The amount of micro-organism colonies is counted and the efficacy of the specific preservative is determined after a 28 day study with the preservative.
- Accelerated stability studies with the final formulation over a period of 3 months.
  The accelerated stability studies are designed to increase the rate of chemical degradation or physical change of a Drug Substance (DS) / Active Pharmaceutical Ingredient (API) or Drug Product (DP) using exaggerated storage conditions. The purpose of the study is to monitor any degradation reactions which than will help to predict the shelf life of a Drug Substance (DS) or Drug Product (DP) under the defined (ICH) storage conditions.
- HPLC method development for the specific formulation.
  The reason for developing a HPLC method for this specific formulation is to get adequate resolution and good quantitation in order to study the formulation. During the

method development, different steps need to be taken, i.e. literature search, selection of HPLC method, mobile phase selection and temperature effects need to be considered.

- HPLC analysis of the samples subjected to accelerated stability testing (initial, months 1, 2 and 3).
- Preservative efficacy studies of the samples initially, after months 1, 2 and 3.
  A preservative efficacy study is done on the samples using the five prescribed micro-organisms, i.e. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. The micro-organisms are inoculated into the samples and then spread out onto agar in petri-plates. The number of viable micro-organisms is determined by plate count.

CHAPTER 1

HIV / AIDS

1.1 Background

Human immunodeficiency virus (HIV) is a retrovirus that can lead to acquired immunodeficiency syndrome (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections. Previous names for the virus include human T-lymphotropic virus-III (HTLV-III), lymphadenopathy-associated virus (LAV), and AIDS-associated retrovirus (ARV) (Moyle, 2002; Plosker & Figgitt, 2003).

Infection with HIV occurs by the transfer of blood, semen, vaginal fluid, pre-ejaculate, or breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells. The four major routes of transmission are unprotected sexual intercourse, contaminated needles, breast milk, and transmission from an infected mother to her baby at birth. Screening of blood products for HIV has largely eliminated transmission through blood transfusions or infected blood products in the developed world (Moyle, 2002).

HIV infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognised on December 1, 1981, making it one of the most destructive pandemics in recorded history. It is estimated that about 0.6% of the world's population is infected with HIV (UNAIDS, 2006). In 2005 alone, AIDS claimed an estimated 2.4 - 3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth and increasing poverty (Greener, 2002). According to current estimates, HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans (UNAIDS, 2006). Antiretroviral treatment reduces both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries (Palella *et al.*, 1998).

HIV primarily infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages and dendritic cells. HIV infection leads to low levels of $CD4^+$ T cells through three main mechanisms: firstly, direct viral killing of

infected cells; secondly, increased rates of apoptosis in infected cells; and thirdly, killing of infected $CD4^+$ T cells by CD8 cytotoxic lymphocytes that recognise infected cells. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections. If untreated, eventually most HIV-infected individuals develop AIDS and die; however about one in ten remains healthy for many years, with no noticeable symptoms (Buchbinder *et al.*, 1994).

Treatment with anti-retrovirals, where available, increases the life expectancy of people infected with HIV. It is hoped that current and future treatments may allow HIV-infected individuals to achieve a life expectancy approaching that of the general public.

1.2 Development of the epidemic

The first documented cases of the acquired immune deficiency syndrome (AIDS) occurred in the summer of 1981 in America. *Pneumocystis carinii* pneumonia and Kaposi's sarcoma began to be reported in young men, whom it was afterwards realised were both homosexual and immunocompromised. Even though the condition became known early on as AIDS, its cause and modes of transmission were not immediately apparent. In 1983, the virus now known to cause AIDS in a proportion of those infected, was discovered and given various names. The internationally accepted term is now the human immunodeficiency virus (HIV). More recently a new variant has been isolated in patients with West African connections – HIV-2 (Adler, 1993).

The definition for AIDS was used for surveillance purposes by most other countries. It was subsequently slightly modified in the light of the discovery of the causal agent and the development of laboratory tests to detect antibody and to include additional serious conditions. A further change took place in September 1987; the latest definition now includes encephalopathy, HIV wasting syndrome, and a wider range of diseases indicative of AIDS (Adler, 1993).

Three groups of patients are defined by:

- Those without laboratory evidence of HIV infection. This includes patients who have not been tested for HIV and those on whom tests have been carried out but with inconclusive results and who have a positive diagnosis of

an indicator disease – for example, extrapulmonary cryptococcosis, oesophageal candidosis, progressive multifocal leucoencephalopathy – but no other cause of immunodeficiency.

- Those with laboratory evidence of HIV infection, notwithstanding the presence of other causes of immunodeficiency, or any of the specified indicator diseases, whether diagnosed definitively or presumptively.

- Those with laboratory evidence against HIV infection. In this group AIDS is diagnosed only when all the other major causes of immunodeficiency have been excluded – for example, a high dose of long term systemic corticosteroid or other immunosuppressive-cytotoxic disease – and the patient has unequivocal *Pneumocystis carinii* pneumonia or any other disease indicative of AIDS and a T helper-inducer (CD4) lymphocyte count of less than $0.4 \times 10^9/1$ (Adler, 1993).

1.3 Transmission of the virus

HIV has been isolated from semen, cervical secretions, cell free plasma, cerebrospinal fluid, lymphocytes, saliva, tears, urine, and breast milk. This does not mean, however, that these fluids all transmit infection since the concentration of virus in them varies considerably (Adler, 1993), particularly infectious are semen, blood, and possibly cervical secretions. The most regular mode of transmission is through the receipt of infected blood or blood products, donated organs and semen. Transmission also occurs through the sharing or reuse of contaminated needles by injecting drug abusers or for therapeutic procedures and from mother to child (Adler, 1993).

Since the beginning of the pandemic, three main transmission routes for HIV have been identified (Coovadia, 2004):

- Sexual route. The majority of HIV infections are acquired through unprotected sexual relations. Sexual transmission can occur when infected sexual secretions of one partner come into contact with the genital, oral, or rectal mucous membranes of another of (Coovadia, 2004).

- Blood or blood product route. This transmission route can account for infections in intravenous drug users, haemophiliacs and recipients of blood

transfusions (though most transfusions are checked for HIV in the developed world) and blood products. It is also of concern for persons receiving medical care in regions where there is prevalent substandard hygiene in the use of injection equipment, such as the reuse of needles in Third World countries. HIV can also be spread through the sharing of needles. Health care workers such as nurses, laboratory workers, and doctors, have also been infected, although this occurs more rarely. People who give and receive tattoos, piercings, and scarification procedures can also be at risk of infection (Coovadia, 2004).

- Mother-to-child transmission (MTCT). The transmission of the virus from the mother to the child can occur *in utero* during pregnancy and *intrapartum* at childbirth. In the absence of treatment, the transmission rate between the mother and child is around 25% (Coovadia, 2004). However, where combination antiretroviral drug treatment and Caesarian section are available, this risk can be reduced to as low as 1% (Coovadia, 2004). Breast feeding also presents a risk of infection for the baby.

1.3.1 Relation between the virus and the disease

The advent of an effective antibody test in 1984 has allowed for a clearer understanding of the varying prevalence and the natural history of HIV infection. For example, tests on stored samples of serum collected for other reasons from a cohort of homosexual men in San Francisco give as indication of how the epidemic evolved. In 1978, 4% were anti-HIV positive; by 1980 the proportion had increased six fold, to 24%. In London and British provincial centres the rate of seropositivity has also increased (Adler, 1993). These surveys show that the proportion of individuals infected needs to be high before cases of AIDS start to become apparent. It also underlines the importance of health education campaigns early in the epidemic, when the seroprevalence of HIV is low. Once cases of AIDS start to appear, the epidemic drives itself and a much greater effort is required in terms of control and medical care (Adler, 1993).

The rate of infection has also increased in groups other than homosexuals. In southern Italy the prevalence of HIV antibody among intravenous drug users increased from below 1% in 1980 to 76% in 1985. Similar large increases have been seen in European countries such as Switzerland. In the United Kingdom the

prevalence varies – from 10% in London to 54% in Edinburgh (Adler, 1993). This geographical variation is also seen in the United States, with low rates in San Francisco and New Orleans (less than 5%) and high rates in Manhattan and northern New Jersey (greater than 50%) (Adler, 1993). Haemophiliac patients are the final group with a high rate of infection in the United Kingdom (an average national figure of 44%). The level of infection in prostitutes tested in the United Kingdom and Europe is low, ranging from below 1% in Italy, France, and England to 6% in Greece. Once prostitutes who are also intravenous drug addicts are studied the rate is much higher – for example, 70% in Italy (Adler, 1993).

AIDS results in a considerable direct and indirect cost not only in human suffering but also to the health service. Other costs, including time off work, the effect of the deaths of young people on national productivity, and domiciliary services. AIDS represents the most major public health problem in the world this century. A clear understanding of the epidemiology forms the basis of developing a strategy of control ranging from health education to research (Adler, 1993).

1.3.2 Pathogenesis of HIV

HIV has spread in two epidemiologically distinct patterns. The first pattern primarily involves male homosexual intercourse or contact with infected blood; this pattern predominates in the US and Europe. In the second pattern, heterosexual intercourse is the major mode of transmission (thus affecting men and women nearly equally); this pattern predominates in Africa, South America, and southern Asia. In some countries like Brazil and Thailand, there is no predominant mode (Beers *et al.*, 2006).

1.4 Pathophysiology of HIV

Retroviruses are plus-strand RNA viruses, but they replicate differently than all other RNA viruses. The family name *retro* (Latin, meaning "backward") refers to the unique, seemingly backward biochemical step in the replication cycle of these viruses. All cellular organisms use DNA as the template to make RNA; retroviruses do the reverse: They use RNA as a template to make DNA, employing a unique enzyme called reverse transcriptase. Only retrovirus-infected animal cells produce this enzyme (Ingraham & Ingraham, 2004).

Superficially, a HIV virion is similar to the virions of most enveloped RNA viruses. Its capsid is surrounded by a membrane with embedded protein spikes. Just under the

membrane is a layer of structural protein called matrix. The capsid, which is shaped like a truncated cone, lies inside the matrix. The unique aspects of the retroviral virion are found in the central part. First, the core contains two copies of the same plus-strand RNA molecule. In other words, the virion is diploid. Second, the core contains molecules of three enzymes: reverse transcriptase, intergrase, and protease. Viruses use enzymes available in their host cell for the most part. They also direct the host to make additional enzymes that they need but the host normally lacks. If one of these enzymes is needed for gene expression, it must be included in the virion. Reverse transcriptase is such an enzyme. HIV virions contain reverse transcriptase for the same reason that minus-strand RNA viruses contain RNA-dependent RNA polymerase (Ingraham & Ingraham, 2004).

HIV attaches to and penetrates host T cells via $CD4^+$ molecules and chemokine receptors. After attachment, HIV RNA and enzymes are released into the host cell. Viral replication requires that reverse transcriptase copy HIV RNA, producing proviral DNA; this copying is prone to errors, resulting in frequent mutations. Proviral DNA enters the host cell's nucleus and is integrated into host DNA in a process that involves HIV integrase. With each cell division, the integrated proviral DNA is duplicated along with host DNA. Proviral HIV DNA is transcribed to viral RNA and translated to HIV proteins, including the envelope glycoproteins 40 and 120. The HIV proteins are assembled into HIV virions at the inner cell membrane and budded from the cell surface; each host cell may produce thousands of virions. Protease, another HIV enzyme, cleaves viral proteins after budding, converting the virion into an infectious form (Beers *et al.*, 2006).

1.5 Clinical presentation / symptoms and signs

At first primary HIV infection may be asymptomatic or cause momentary nonspecific symptoms (acute retroviral syndrome). Acute retroviral syndrome usually begins within 1 to 4 week of infection and lasts 3 to 14 days, with fever, malaise, rash, arthralgia, generalised lymphadenopathy, and sometimes aseptic meningitis. Symptoms are often mistaken for infectious mononucleosis or benign nonspecific viral syndromes (Wells *et al.*, 2003).

Most patients have a period of months to years during which other symptoms are few, mild, intermittent, and nonspecific. Symptoms reflect either direct effects of HIV or opportunistic infections. The most common are asymptomatic, diffuse

lymphadenopathy; oral candidiasis; herpes zoster; diarrhoea; fatigue; and fever. Some patients have progressive wasting. Other common symptoms are asymptomatic, mild-to-moderate cytopenias (e.g., leucopaenia, anaemia, and thrombocytopenia) (Beers *et al.*, 2006).

Eventually, when $CD4^+$ counts drop <200/µL, symptoms worsen and AIDS-defining illnesses, often many, develop. Evaluation may reveal infection by *Mycobacterium* sp, *Pneumocystis jiroveci* (formerly *P. carinii*), *Cryptococcus neoformans*, or other fungi. Other infections that are common but suggest AIDS by their unusual severity or recurrence include herpes zoster, herpes simplex, vaginal candidiasis, and recurrent *Salmonella* sepsis. Some patients present with cancers (e.g., Kaposi's sarcoma, B-cell lymphomas) that occur with increased frequency or severity or have unique features in patients with HIV infection. In others, neurologic dysfunction may occur (Beers *et al.*, 2006).

1.6 Diagnosis

Available options for diagnosis include:

Screening (antibody) tests should be offered periodically to those at risk. For those at highest risk, especially sexually active people with multiple partners who do not practice safe sex, testing should be repeated every six months. Such testing is confidential and available, often free of charge, in many public and private facilities throughout the world (Beers *et al.*, 2006).

The most commonly used screening method for HIV is an enzyme-linked immunosorbent assay (ELISA), which detects antibodies against HIV-1 and is both highly sensitive and specific. False positives can occur in multiparous women; in recent recipients of hepatitis B, HIV, influenza, or rabies vaccine; following multiple blood transfusions; and in those with liver disease, renal failure, or undergoing chronic hemodialysis. False negatives may occur if the patient is newly infected and the test is performed before antibody production is detectable. The minimum time to develop antibodies is 3 to 4 weeks from initial exposure (Wells *et al.*, 2003).

Positive ELISAs are repeated in duplicate and if one or both tests are reactive, a confirmatory test is performed for final diagnosis. Western blot assay is the most commonly used confirmatory test (Wells *et al.*, 2003).

The viral load test quantifies viremia by measuring the amount of viral RNA. There are four methods used for determining the amount of HIV RNA: reverse transcriptase-coupled polymerase chain reaction (RT-PCR), branched DNA (bDNA), nucleic acid sequence-based assay (NASBA), and transcription-mediated amplification. Each assay has its own lower limit of sensitivity, and results can vary from one assay method to the other; therefore, it is recommended that the same assay method be used consistently within patients (Wells *et al.*, 2003).

Viral load can be used as a prognostic factor to monitor disease progression and the effects of treatment (Wells *et al.*, 2003).

The number of CD4 lymphocytes in the blood is a surrogate marker of disease progression. The normal adult CD4 lymphocyte count ranges between 500 and 1600 cells/μL, or 40% to 70% of all lymphocytes (Wells *et al.*, 2003).

1.7 Prognosis

The prognosis protocols or indicators for a case-by-case basis include:

The risk of AIDS and/or death is predicted by $CD4^+$ count in the short term and by plasma viral RNA level in the longer term. For every 3-fold (0.5 $\log_{10}$) increase in viral load, mortality over the next 2 to 3 years increases about 50%. However, $CD4^+$ counts rise and HIV RNA levels fall dramatically with effective treatment. HIV-associated morbidity and mortality are uncommon when $CD4^+$ count is ≥ 500/μL; low with counts of 200 to 499/μL; moderate with counts of 50 to 200/μL; and high if counts fall < 50/μL (Beers *et al.*, 2006).

Because adequate antiviral therapy can cause significant long-term morbidity, it is not recommended for everyone. Current indications include $CD4^+$ count of < 350/μL and HIV RNA level of > 55 000 copies/mL. Use of potent combinations of antiretroviral drugs for HIV therapy (highly active antiretroviral therapy [HAART]) aims to reduce plasma HIV RNA levels and increase $CD4^+$ lymphocyte counts (immune restoration or reconstitution). The lower the pre-treatment $CD4^+$ count and the higher the HIV RNA level, the less likely treatment is to succeed; however some improvement is likely even in those with advanced immunosuppression. The increase in $CD4^+$ count indicates a corresponding decrease in the risk of opportunistic infections, other complications, and death. With immune restoration, even complications for which no specific treatment exits (e.g., HIV-induced cognitive

dysfunction) or that were previously considered untreatable (e.g., progressive multifocal leucoencephalopathy) may improve. Cancers (e.g., lymphoma and Kaposi's sarcoma) and opportunistic infections have improved outcomes as well. Vaccines that may enhance immunity to HIV among infected patients have been under investigation for many years but are not yet available (Beers *et al.*, 2006).

1.8 Treatment of HIV

Whilst it is recognised that the treatment of HIV holds limited and varied outcomes, the following chemical treatments are available:

Antiretroviral therapy (ART) for treatment of Human Immunodeficiency Virus type 1 (HIV-1) infection has improved steadily since the advent of combination therapy in 1996. More recently, new drugs have been approved that offer new mechanisms of action, added potency, dosing convenience, and improved safety profiles, whereas some previously popular drugs are being used less often as their drawbacks become better defined. Resistance testing is used more commonly in clinical practice, and interactions among antiretroviral agents and other drugs have become more complex (Dept. of Health and Human Services, 2008).

1.8.1 Treatment goals

Eradication of HIV infection cannot be achieved with available antiretroviral regimens. This is chiefly because the pool of latently infected CD4 T-cells is established during the earliest stages of acute HIV infection (Chun *et al.*, 1998) and persists with a long half-life, even with prolonged suppression of plasma viremia (Chun *et al.*, 1997; Finzi *et al.*, 1999; Finzi *et al.*, 1997; Wong *et al.*, 1997). The primary goals driving the decision to initiate antiretroviral therapy therefore are to:

- reduce HIV-related morbidity and prolong survival,
- improve quality of life,
- restore and preserve immunologic function,
- maximally and durably suppress viral load, and
- prevent vertical HIV transmission.

Adoption of treatment strategies recommended in these guidelines has resulted in substantial reductions in HIV-related morbidity and mortality (Mocroft *et al.*, 1998;

Palella *et al.*, 1998; Vittinghoff *et al.*, 1999) and has reduced vertical transmission (Garcia *et al.*, 1999; Mofenson *et al.*, 1999).

Higher plasma HIV RNA levels (viral load) are associated with more rapid disease progression (Mellors *et al.*, 1996; Rodriguez *et al.*, 2006) although other factors likely contribute as well to the rate of CD4 T-cell decline (Rodriguez *et al.*, 2006). Maximal suppression of plasma viremia for as long as possible to delay the selection of drug resistance mutations, to preserve CD4 T-cell numbers, and to confer substantial clinical benefits are the most important goals of antiretroviral therapy (O'Brien *et al.*, 1996).

The goal of maximal viral suppression in initial therapy may be difficult in some cases of HIV with pre-existing resistance mutations. To be successful, antiretroviral regimens need to contain at least two, and preferably three, active drugs from multiple drug classes. When maximal initial suppression is not achieved or is lost, changing to a new regimen with at least two active drugs is required for this goal. If this is not possible in a clinically and immunologically stable patient, an interval of persisting viremia may be acceptable while waiting for arrival of potent new therapies (Dept. of Health and Human Services, 2008).

Viral load reduction to below limits of assay detection in a treatment-naïve patient usually occurs within the first 12 – 24 weeks of therapy. Predictors of virologic success include:

- high potency of antiretroviral regimen,
- excellent adherence to treatment regimen (Powderly *et al.*, 1999; Yamashita *et al.*, 2001),
- low baseline viremia,
- higher baseline CD4 T-cell count (Powderly *et al.*, 1999; Yamashita *et al.*, 2001) and
- rapid (i.e., $\geq$1 $\log_{10}$ in 1 to 4 months) reduction of viremia in response to treatment (Yamashita *et al.*, 2001).

Successful outcomes are not always observed. Viral suppression rates in clinical practice may be lower than the 80%–90% seen in clinical trials, although the use of current compact, potent, and well-tolerated regimens has probably decreased this

difference in outcomes between clinical trials and clinical practice (O'Brien *et al.*, 1996; Moore *et al.*, 2005).

1.9 Strategies to achieve treatment goals

Achieving treatment goals requires a balance of sometimes competing considerations, outlined below. Providers and patients must work together to define priorities and determine treatment goals and options (Dept. of Health and Human Services, 2008).

1.9.1 Selection of initial combination regimen

Several preferred and alternative antiretroviral regimens are recommended for use. They vary in efficacy, pill burden, and potential side effects. A regimen tailored to the patient may be more successful in fully suppressing the virus by allowing more complete medication adherence. Individual tailoring is based on such considerations as expected side effects, convenience, comorbidities, interactions with other required medications, and results of pretreatment genotypic drug resistance testing (Dept. of Health and Human Services, 2008).

1.9.2 Pretreatment drug resistance testing

Current studies suggest a prevalence of HIV drug resistance of 6%–16% in antiretroviral treatment-naïve patients, and some studies suggest that the presence of transmitted drug-resistant viruses, particularly those with non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations, may lead to suboptimal virologic responses. Therefore, pretreatment genotypic resistance testing should be used in guiding selection of the most optimal initial antiretroviral regimen (Dept. of Health and Human Services, 2008).

1.9.3 Improving adherence

Suboptimal adherence may result in reduced treatment response. Incomplete adherence can result from complex medication regimens; patient factors, such as active substance abuse and depression; and health system issues, including interruptions in medication access and inadequate treatment education and support. Conditions that promote adherence should be maximised prior to initiating antiretroviral therapy (Dept. of Health and Human Services, 2008).

1.9.4 Initial combination regimens for the antiretroviral-naïve patient

There are more than 20 approved antiretroviral drugs across six mechanistic classes, with which to design combination regimens. These six classes include the nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, CCR5 antagonists, and integrase inhibitors (Dept. of Health and Human Services, 2008).

1.9.5 Summary of recommended regimens

The most extensively studied combination antiretroviral regimens for treatment-naïve patients generally consist of one NNRTI with two NRTIs, or a PI (with or without ritonavir-boosting) with two NRTIs (Dept. of Health and Human Services, 2008).

The following example shows how the selection of a first antiretroviral regimen for treatment-naïve patients is considered:

In its deliberations, the Panel on Antiretroviral Guidelines for Adults and Adolescents (Dept. of Health and Human Services, 2008) reviews clinical trial data published in peer-reviewed journals and data prepared by manufacturers for FDA review. In selected cases, data presented in abstract format in major scientific meetings are also reviewed. The first criteria for selection are data from a randomised, prospective clinical trial with an adequate sample size, demonstrating durable viral suppression and immunologic enhancement (as evidenced by increased CD4 T-cell count). Few of these trials include clinical end points, such as development of AIDS-defining illness or death. Thus, assessment of regimen efficacy and potency is primarily based on surrogate marker endpoints (i.e., viral load and CD4 responses). Components are designated as preferred for use in treatment-naïve patients when clinical trial data have demonstrated optimal efficacy and durability with acceptable tolerability and ease of use. Alternative components refer to those for which clinical trial data show efficacy but also show disadvantages compared with preferred components. On the basis of individual patient characteristics and needs, a regimen listed as an alternative regimen may actually be the preferred regimen.

With the improved choices available for more effective and convenient regimens, some of the agents or combinations previously recommended by the Panel as alternative regimens have been removed from the list.

1.9.6 Factors to consider when selecting an initial regimen

Regimen selection should be individualised, and should consider a number of factors including:

- comorbidity (e.g., cardiovascular disease, chemical dependency, liver disease, psychiatric disease, pregnancy, renal diseases, or tuberculosis);
- patient adherence potential;
- convenience (e.g., pill burden, dosing frequency, and food and fluid considerations);
- potential adverse drug effects;
- potential drug interactions with other medications;
- pregnancy potential;
- results of genotypic drug resistance testing;
- gender and pre-treatment CD4 T-cell count if considering nevirapine; and
- HLA B*5701 testing if considering abacavir (Dept. of Health and Human Services, 2008).

1.10 HIV-infected adolescents

Older children and adolescents now make up the largest percentage of HIV-infected children cared for at U.S. sites. The CDC estimates that at least one-half of the 40,000 yearly new HIV-infected cases in the United States are in people 13 to 24 years of age (Dept. of Health and Human Services, 2002). HIV-infected adolescents represent a heterogeneous group in terms of sociodemographics, mode of HIV infection, sexual and substance abuse history, clinical and immunologic status, psychosocial development, and readiness to adhere to medications. Many of these factors may influence decisions concerning when to start and what antiretroviral medications should be used.

Most adolescents have been infected during their teenage years and are in an early stage of infection, making them ideal candidates for early intervention, such as prevention counselling. A limited but increasing number of HIV-infected adolescents are long-term survivors of HIV infection acquired perinatally or through blood

products as infants. Such adolescents may have a unique clinical course that differs from that of adolescents infected later in life (Grubman *et al.*, 1995).

1.10.1 Antiretroviral therapy considerations in adolescents

Adult guidelines for antiretroviral therapy are usually appropriate for postpubertal adolescents because HIV-infected adolescents who were infected sexually or through injecting drug use during adolescence follow a clinical course that is more similar to that of adults than to that of children.

Dosage for medications for HIV infection and opportunistic infections should be prescribed according to Tanner staging of puberty and not on the basis of age (Anon, 2008). Adolescents in early puberty (i.e., Tanner Stage I and II) should be administered doses using paediatric schedules, whereas those in late puberty (i.e., Tanner Stage V) should follow adult dosing schedules. Because puberty may be delayed in perinatally HIV-infected children (Buchacz *et al.*, 2003) continued use of paediatric doses in puberty-delayed adolescents can result in medication doses that are higher than the usual adult doses. Because data are not available to predict optimal medication doses for each antiretroviral medication for this group of children, issues such as toxicity, pill or liquid volume burden, adherence, and virologic and immunologic parameters should be considered in determining when to transition from paediatric to adult doses. Youth who are in their growth spurt (i.e., Tanner Stage III in females and Tanner Stage IV in males) using adult or paediatric dosing guidelines and those adolescents whose doses have been transitioned from paediatric to adult doses should be closely monitored for medication efficacy and toxicity (Dept. of Health and Human Services, 2008).

1.10.2 Adherence concerns in adolescents

HIV-infected adolescents have specific adherence problems. Comprehensive systems of care are required to serve both the medical and psychosocial needs of HIV-infected adolescents, who are frequently inexperienced with health care systems. Many HIV-infected adolescents face challenges in adhering to medical regimens for reasons that include:

- denial and fear of their HIV infection;
- misinformation;

- distrust of the medical establishment;
- fear and lack of belief in the effectiveness of medications;
- low self-esteem;
- unstructured and chaotic lifestyles; and
- lack of familial and social support (Dept. of Health and Human Services, 2008).

Treatment regimens for adolescents must balance the goal of prescribing a maximally potent antiretroviral regimen with realistic assessment of existing and potential support systems to facilitate adherence. Adolescents benefit from reminder systems (beepers, timers, and pill boxes) that are stylish and do not call attention to themselves. It is important to make medication adherence as user friendly and as little stigmatising as possible for the older child or adolescent. The concrete thought processes of adolescents make it difficult for them to take medications when they are asymptomatic, particularly if the medications have side effects. Adherence to complex regimens is particularly challenging at a time of life when adolescents do not want to be different from their peers. Direct observed therapy, although considered impractical for all adolescents, might be important for selected adolescents infected with HIV (Murphy *et al.*, 2001; Stenzel *et al.*, 2001).

Developmental issues make caring for adolescents unique. The adolescent's approach to illness is often different from that of an adult. The adolescent also faces difficulties in changing caretakers - graduating from a paediatrician to an adolescent care provider, then to an internist.

Given the lifelong infection with HIV and the need for treatment through several stages of growth and development, HIV care programs and providers need to support this appropriate transition in care for HIV-infected infants through adolescents.

1.11 Prevention

Vaccines against HIV have been difficult to develop because of the extreme mutability of HIV surface proteins that result in an enormous diversity of antigenic types. Nonetheless, many candidates are at various stages of investigation for their ability to prevent or ameliorate infection (Beers *et al.*, 2006).

1.11.1 Prevention of transmission

Public education is effective and appears to have decreased rates of infection in some countries, notably Thailand and Uganda. Because sexual contact accounts for most cases, education to avoid unsafe sex practices is the most relevant measure. Unless both partners are known to be free of HIV and remain monogamous, safe sex practices are essential. Condoms offer the best protection, but oil-based lubricants may dissolve latex, increasing the risk of latex condom failure. Antiretroviral therapy of HIV-infected people reduces the risk of sexual transmission, but the extent of reduction is unclear (Beers *et al.*, 2006).

Safe sex practices remain advisable to protect HIV-positive patients as well as their partners. For example, unprotected sex between HIV-infected people may expose an individual to resistant or more virulent strains of HIV and to other viruses (e.g., cytomegalovirus, Epstein-Barr virus, herpes simplex virus, hepatitis B) that cause severe disease in AIDS patients (Beers *et al.*, 2006).

Parenteral drug users should be counselled about the risk of sharing needles. Counselling is probably more effective if combined with provision of sterile needles and with treatment of drug dependence and rehabilitation (Beers *et al.*, 2006).

Confidential testing for HIV infection, which also mandates the availability of pre-test and post-test counselling, should be offered to anyone requesting it. Pregnant woman who test positive are advised of the risk of maternal-foetal transmission; risk is decreased by ⅔ using monotherapy with ZDV or nevirapine, and probably even more using combinations of 2 or 3 drugs. Therapy can be toxic to the foetus or mother and cannot be guaranteed to prevent transmission. Some women choose to terminate their pregnancy for this or other reasons (Beers *et al.*, 2006).

In parts of the world where donated blood and organs are screened universally using current methods (e.g., ELISA), the risk of transmitting HIV by blood transfusion is probably between 1/10000 and 1/100000 per unit transfused. Transmission is still possible, because antibody results may be falsely negative during early infection. Currently, screening of blood for both antibody and p24 antigen is mandated in the US and probably further reduces the risk of transmission. To reduce risk further, people with risk factors for HIV infection, even those with recent negative HIV antibody test results, are asked not to donate blood or organs for transplantation (Beers *et al.*, 2006).

To prevent HIV transmission from patients, medical and dental professionals should wear gloves in situations that may involve contact with any patient's mucous membranes or body fluids and be taught how to avoid needle-stick accidents. Home care-givers should wear gloves if their hands may be exposed to body fluids. Surfaces or instruments contaminated by blood or other body fluids should be cleaned and disinfected. Effective disinfectants include heat, peroxide, alcohols, phenolics, and hypochlorite (bleach). Isolation of HIV-infected patients is unnecessary unless indicated because of an opportunistic infection (e.g., TB). Consensus regarding measures to prevent transmission from infected professionals to patients has not been reached (Beers *et al.*, 2006).

1.12 Cancers common in HIV-infected patients and infectious complications of HIV

Kaposi's sarcoma, non-Hodgkin lymphoma, and cervical cancer are AIDS-defining neoplasms in HIV-infected patients. Other cancers that appear to be increased in incidence or severity include Hodgkin lymphoma (especially the mixed cellularity and lymphocyte-depleted subtypes), primary CNS lymphoma, anal cancer, testicular cancer, melanoma and other skin cancers, and lung cancer. Leiomyosarcoma is a rare complication of HIV infection in children (Beers *et al.*, 2006).

The development of certain opportunistic infections is directly or indirectly related to the level of CD4 lymphocytes. The most common opportunistic disease and their frequencies found before death in patients with AIDS between 1990 and 1994 were *Pneumocystis carinii* pneumonia, 45%; *Mycobacterium avium* complex, 25%; wasting syndrome, 25%; bacterial pneumonia, 24%; cytomegalovirus (CMV) disease, 23%; and candidiasis, 22% (Wells *et al.*, 2003).

1.13 Conclusion

It is stock knowledge that HIV and AIDS is a very serious disease. It's got an exponential devastating influence on not only the infected person's health, but also on that person's emotions, family, work, community and their country's economy. We have come a long way since 1981 to where we are today in terms of knowledge about HIV and AIDS. We now know that this disease is seen widely spread, not only amongst homosexual men or women, but also heterosexuals, not only in America or Africa, but across the entire world, not only amongst the poor and infamous, but

amongst everyone, not only amongst black or white people, but amongst any race or culture. With the population explosion of the world, statistics shows an enormous increase in the number of people across the world that became newly infected with HIV. Even more shocking is the fact that most of the people infected lives in countries already struggling with very tough economic times. This makes our work even more important. By continuing to promote safe sex, distributing protection against STD's and making sure that the infected uses their ARV's correctly, we can make a positive difference in the lives of everyone affected by the disease. It's also of extreme importance to note that people infected with HIV can live a full and often very normal life if they adhere to their treatment regimen and make a few minor lifestyle changes. However, the one group standing out is the paediatric patients, especially the ones that lost their parents and family members to complications due to HIV and AIDS. But by working together, creating a safe and secure environment for these children, educating them on the dangers of the disease and teaching them how to protect themselves from not contracting the disease, we can create a future generation that is stronger than HIV or AIDS. By creating a culture where people can stand up against the power of HIV and AIDS, fighting to prevent new infections and supporting those already infected, we can ultimately realise the dream of a world free of this terrible disease.

CHAPTER 2

PHEROID™ TECHNOLOGY

2.1 Introduction

Pheroid™ technology is a patented delivery system that consists of both plant and essential fatty acids. It is often confused with lipid-based delivery systems. Although there are some similarities, Pheroid™ technology owns its advantages in terms of absorption and/or efficacy of pharmacologically active compounds and other useful molecules. The Pheroid™ structure can be manipulated in terms of morphology, structure, size and function. The effectiveness of Pheroid™ technology has been illustrated by several national and international clinical trials with products based on this technology (Grobler *et al.*, 2008).

2.2 Structural characteristics of Pheroids™

Pheroids™ are unique and stable lipid-based submicron- and micron-sized structures inside a colloidal system and can be manipulated in terms of size, structure, morphology and function. The Pheroids™ are uniformly distributed in a dispersion medium that can be adapted to the indication. The Pheroid™ particles are usually between 1 – 100 nm in diameter, but can be formulated to have a larger diameter depending on the rate of delivery and administration route chosen (Grobler *et al.*, 2008). The intention in using colloidal systems as carriers of pharmacologically active compounds is to enhance the efficacy of the administered compounds while reducing the unwanted side effects (Grobler *et al.*, 2008).

2.3 Composition and molecular organisation of Pheroids™

Pheroids™ primarily consists of ethylated and pegylated polyunsaturated fatty acids, which includes both the omega-3 and omega-6 fatty acids, but excludes arachidonic acid. The omega-3 and omega-6 fatty acids are compatible with the orientation of fatty acids in humans because of the *cis*-formation they are in. These fatty acids can be formulated with various compounds for novel and innovative dosage forms. Colloidal dosage forms commonly used include liposomes, emulsions and microspheres, both micro-emulsions polymeric and macromolecular. By incorporating one or more features of each of these dosage forms, the Pheroid™ was designed (Grobler *et al.*, 2008).

Pheroids™ generally contain a lipid bilayer, but it contains no phospholipids or cholesterol, as is the case with liposomes. Pheroids™ are formed by a self-assembly process similar to that of a low-energy emulsion and micro-emulsion, but in contrast with liposomes, it's not necessary for lyophilisation or hydration of the lipid components (Grobler *et al.*, 2008).

As in the case of emulsions, Pheroids™ are dispersed in a dispersion medium, but it contains two liquid phases as well as a dispersed gas phase which is associated with the fatty acid dispersed phase. Some of the reservoir characteristics of the polymeric microspheres are added by the specific ratio of the pegylated to ethylated fatty acids used in the assembling of the Pheroids™, while the formulation of natural depots is reminiscent of the structure of macromolecular microspheres (Grobler *et al.*, 2008).

The one unique component of the Pheroid™ is nitrous oxide which is found distributed in association with the dispersed phase throughout the continuous phase. Another dimension is added to the basic Pheroid™ by the addition of a dispersed gas phase to the respective oil and water phases, thus the association of N_2O with the oil and water phases has been shown to have at least three functions:

- Contributing to the miscibility of the fatty acids in the dispersal medium;
- Contributing to the self-assembly process of the Pheroids™; and
- Contributing to the stability of the formed Pheroids™ (Grobler *et al.*, 2008).

N_2O is a volatile anaesthetic compound that is both water- and fat-soluble, which is the characteristic that enables the gas to move freely through the epidermal and dermal layers. N_2O has an average lipid solubility (compared to other volatile anaesthetics) which is indicated by the oil / gas partition coefficient of 1.4. An ideal site in which N_2O can concentrate is provided by the lipid-rich membrane. Membrane fluidity of specific cells is increased when sufficient accumulation occurs. The increase in fluidity brought on by N_2O and unsaturated fatty acids should increase the movement of hydrophobic molecules or hydrophilic compounds in association with essential fatty acids to move laterally in the membrane to the connecting cells (Grobler *et al.*, 2008).

There is some interaction between the fatty acids and the nitrous oxide, resulting in stable vesicular Pheroid™ structures, as indicated by studies. The nitrous oxide essential fatty acid (NOEFA) matrix thus provides a functional model for the transport

of hydrophobic and hydrophiiic drugs. The efficacy and stability of the formulation was decreased dramatically if either the N_2O or the essential fatty acids were absent from the formulations, according to Grobler *et al.* (2008).

2.4 Design of Pheroids™

The design of the Pheroid™ allows for manipulation of both its structural and functional features. By changing the degree of hydrogenation of the fatty acids, the surface charge of the Pheroid™ can be adapted. The mean particle size can be reproducibly manipulated by changing the composition and ratio of the fatty acids. The structural and functional characteristics of Pheroids™ can be manipulated by:

- changing the fatty acid composition or concentrations;
- the addition of non-fatty acids or phospholipids such as cholesterol;
- the addition of cryo-protectants;
- the addition of charge-inducing agents;
- changing the hydration medium;
- changing the method of preparation;
- changing the character and the concentration of the active compound;
- the addition of sunscreen formulations (Grobler *et al.*, 2008).

2.5 Classification of Pheroid™ system

Three main types of Pheroids™ can be formulated by changing the composition and method of manufacturing, for example:

i. lipid-bilayer vesicles in both the nano- and micrometer size range;

ii. micro sponges; and

iii. depots or reservoirs that contain pro-Pheroids (Grobler, 2004).

Each type of Pheroid™ has a specific composition. The size and shape of the vesicles can be controlled to obtain reproducibility (typically between 0.5 – 1.5 μm), whereas it ranges between 1.5 – 5 μm with the micro sponges. Micro sponges are

ideal for combination therapies, as one drug can be entrapped in the interior volume and the other in the sponge spaces (Grobler, 2004).

Grobler (2004) explains that although all Pheroid™ systems contain a small polyethylene glycol (PEG) component, the use of increased concentrations and larger polymers has led to the development of the pro-Pheroid. This has only been possible when the resultant formulation has been treated to stabilise the Pheroid™ once it is formed. Polyethylene glycol is a relatively non-reactive and non-toxic polymer that is frequently used in food and pharmaceutical products. Pro-Pheroid systems were designed to have significant advantages over other delivery systems (Grobler, 2004).

2.6 Metabolism, targeting and distribution

According to Grobler *et al.* (2008), the distribution of Pheroids™ can be influenced, depending on the type and extent of the fatty acid modifications. The cellular uptake of the Pheroid™ is based on various native interactions between fatty acids and cells, amongst these the binding between fatty acids and the fatty acids binding proteins in the cell membrane and the interaction between Pheroids™ and the lipid rafts present in the cell membrane. The release of the active compound is the result of metabolism of the Pheroid™ in either the mitochondria or the peroxisomes of the cells, depending on the composition of the Pheroid™, as confirmed by co-localisation studies of Pheroids™ and various sub-cellular organelles.

2.7 Formulations

Nevirapine was chosen for formulation in the pro-Pheroid system to test the stability of this active pharmaceutical ingredient in the pro-Pheroid system as well as the efficacy of the preservative, butylparaben.

2.7.1 Physico-chemical and pharmacological properties

2.7.1.1 Nevirapine

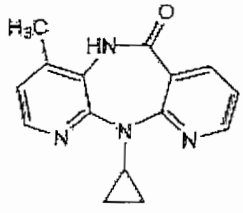


Figure 2.1 Nevirapine $C_{15}H_{14}N_4O$ (Pharmaceutical Press, 2009)

2.7.1.1.1 Chemical properties

According to the Pharmaceutical Press (2009) nevirapine has two polymorphic forms, i.e. anhydrous and a hemihydrate. Both polymorphic forms are white to an almost or off-white colour, which is odourless to nearly odourless.

- **Synonyms:** BI-RG-587; BIRG-0587
- **Chemical name:** 11-Cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-b:2',3'-*e*]-[1,4]diazepin-6-one
- **CAS registry number:** 129618-40-2
- **Molecular formula:** $C_{15}H_{14}N_4O$
- **Molecular weight:** 266.30 (anhydrous)
- **Dissociation Constant:** pK_a 2.8
- **Partition Coefficient:** Log *P* (octanol/water), 83.
- **Melting range:** 247 - 249°C.
- **Solubility:** It is highly soluble in water at pH<3 but solubility decreases to approximately, 0.1 g/L at neutral pH, lipophilic.
- **Percent composition:** C 67.65%; H 5.30%; N 21.04%; O 6.01%. (Moffat *et al.,* 2004).

2.7.1.1.2 Pharmacokinetics

- **Bioavailability:** Oral, about 93%.

- **Half-life:** 45 h which decreases on multiple dosing to 20 to 30 h over a 2- to 4-week period.
- **Volume of distribution:** Steady state, 1.2 to 1.5 L/kg. Reported as 1.54 L/kg in females and 1.38 L/kg in males.
- **Clearance:** Oral, 0.27 to 0.52 mL/kg/min. Plasma, 3.96 L/h (after a 200 mg dose).
- **Protein binding:** 60%.
- Nevirapine crosses the placenta and has been detected in breast milk. It undergoes extensive metabolism in the liver mainly be the cytochrome P450 isoenzymes of the CYP3A and CYP2B6 family and the major metabolite is hydroxymethyl-nevirapine. Auto induction of these enzymes results in a 1.5- to 2-fold increase in apparent oral clearance after 2 to 4 weeks at usual dose. It is excreted via urine as the glucuronide conjugates of the hydroxylated metabolites. The drug is widely distributed in body tissues and the CNS. (Moffat *et al.*, 2004; Pharmaceutical Press, 2009).

2.7.1.1.3 Paediatric and adult dose

Adult: 200 mg once daily which can be increased to 400 mg daily after 2 weeks provided that no rash is present. If treatment is interrupted for more than 7 days, it should be reintroduced using the lower dose for the first 14 days as for new treatment.

Children under 50 kg (2 months to 8 years): 4 mg/kg increased to 7 mg/kg twice daily (maximum 400 mg daily) if no rash is present.

Children under 50 kg (8 to 16 years): 4 mg/kg once daily increased to twice daily (maximum 400 mg).

Alternatively, the dose may be calculated according to body-surface; an oral dose of 150 mg/m^2 once daily for two weeks is given followed by 150 mg/m^2 twice daily thereafter. A total dose of 400 mg daily should not be exceeded.

Renal impairment: Dose adjustments are not required for patients with a creatinine clearance more than 20 mL/min. Patients on dialysis should receive an

additional 200 mg of nevirapine after each dialysis session (Moffat *et al.*, 2004; Pharmaceutical Press, 2009).

2.7.2 Nevirapine and butylparaben

Since nevirapine and butylparaben are highly soluble in a lipophilic medium, no problems were experienced with the formulation. The nevirapine concentration in the pro-Pheroid formulation was 0.024 mg/ml and that of butylparaben was 0.03 mg/ml as manufactured and supplied by the Pheroid™ experimental formulation facility.

2.8 Conclusion

The Pheroid™ delivery system has many advantages over its competitors, of which one of the most important being the fact that the Pheroids™ can be manipulated to be optimised for the active compound and indication of the drug. The fact that the Pheroid™ contains essential fatty acids, results in recognition by the immune system with a smooth delivery of the active compound.

The polyphilic character of the Pheroid™ enables us to entrap drugs with different solubilities, as well as insoluble drugs, within the Pheroid™ vesicles and micro sponges.

The effective delivery of the active compound makes the Pheroid™ delivery system very cost-effective, as lower dosages can be formulated with the same therapeutic effect. This being only theoretical, it is said that more of the active compound reaches the target and less "waste" reaches non-targeted areas.

Last of all, the Pheroid™ system has been shown to be very stable by various stability studies. It is thus safe to say that the Pheroid™ system is safe, effective and affordable (Elgar, 2008).

CHAPTER 3

INTRODUCTION TO MICROBIOLOGY

3.1 The general structure of a bacterial and fungal cell

Bacteria are unicellular and can exist as a discrete entity. In the fungi, whereas yeasts are unicellular organisms typically 10 μm in diameter, almost all, if not all, the contaminant molds grow as filaments or hyphae that may be cross-walled (septate) or a continuous tube (coenocytic). Both bacterial and fungal cells possess a cell wall that is a rigid structure but with differing chemical constitutions. Here any formal anatomical similarity ends (Baird & Denyer, 2007).

3.1.1 The bacterial cell

Bacteria are small, generally between 0.75 and 4 μm in length. They are characteristically shaped, and those responsible for spoilage come from groups that are either short cylinders with rounded ends (bacilli) or spherical (cocci). Figure 3.1 shows the main features. On, or toward, the outside there is a rigid cell wall that confers the characteristic shape. Chemically this is a complex polymer of sugars, amino sugars, and amino acids. Within that lies a nonrigid structure known as the cytoplasmic membrane. This consists of a raft of phospholipid molecules, which are fatty material containing a phosphate group. In this raft float protein molecules that have structural or enzymic functions (figure 3.2). The rest of the cell is known as the cytoplasm. It consists of a viscous fluid in which are embedded (a) the nucleus, made up of nucleic acids and responsible for directing enzymic and structural protein synthesis and thus controlling the basic characters of the cell, and (b) ribosomes, which are the sites of the buleic acid-directed protein synthesis. In addition, enzymes and metabolic reserves (often in polymeric form) are found in the cytoplasm (Baird & Denyer, 2007).

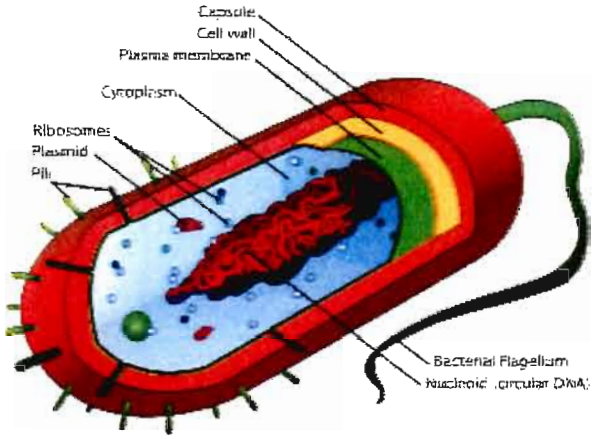


Figure 3.1 Diagram of the main features of the bacterial cell (Wikipedia, 2009a).

Figure 3.2 Phospholipid bilayer (Wikipedia, 2009b).

Bacterial cells occur in two structural types known as Gram-positive and Gram-negative (figures 3.3A and 3.3B, respectively), and no one reading a book on bacteriology will fail to find these terms. The terms positive and negative refer to a staining reaction and the word Gram refers to the discoverer of the method – Christian Gram. The simplified diagram (figure 3.3) shows that Gram-negative cells have an additional structure, the outer membrane, which is linked to the outside of the rigid cell wall by divalent cations; this is lacking in Gram-positive cells. This outer membrane confers the differential staining property and in many cases contains toxic material responsible, if ingested or injected, for disease and elevated temperature (pyrogen). It may also contribute to the resistance of some Gram-negative bacteria toward certain antibacterial agents (Baird & Denyer, 2007).

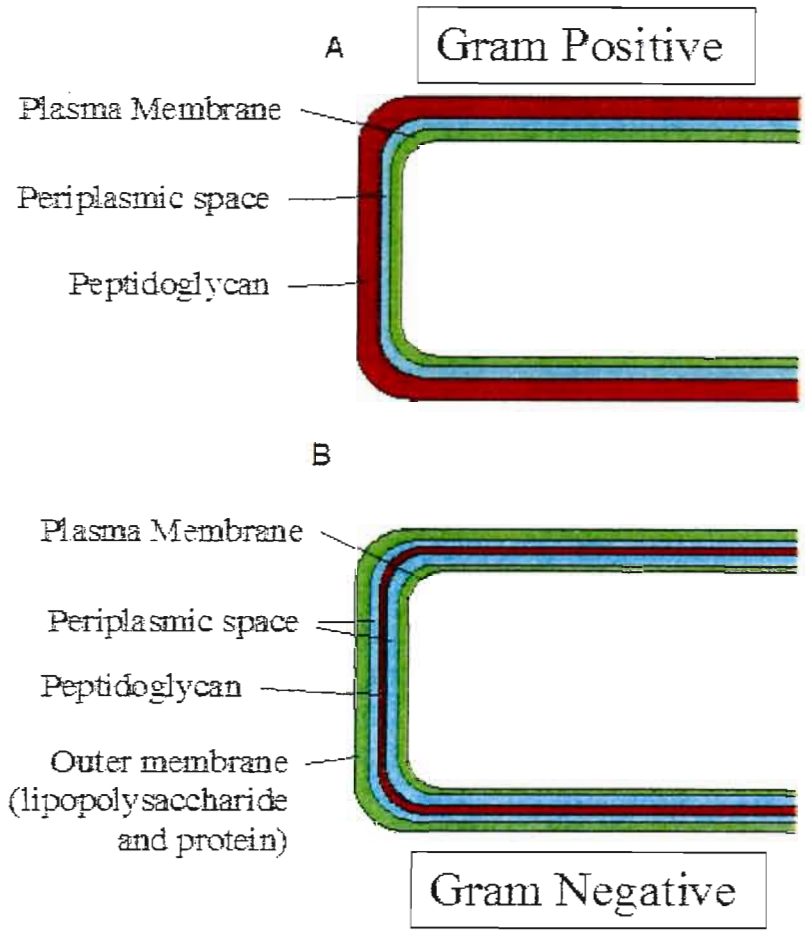


Figure 3.3 The difference between a gram positive and gram negative cell (Wikipedia, 2009c).

3.1.2 The fungal cell

Whether unicellular (yeasts) or filamentous with or without cross-walls (molds), both types of spoilage fungi have a rigid cell wall of cellulose and another polymeric material called chitin, chemically related to the shells of crustaceans. Within this rigid wall lies the cell membrane consisting of phospholipids and proteins. In addition, and here is a fundamental difference from bacteria, the fungal membrane contains sterols. Typical sterols found in fungal membranes are ergosterol and zymosterol (Baird & Denyer, 2007).

Lying within this membrane is the cytoplasm. This contains the nuclear material surrounded, unlike bacteria, by a pore-containing nuclear membrane. It directs protein synthesis as in the bacterial cell. Also within the cytoplasm are found the ribosomes, as before, sites of the directed protein synthesis. These ribosomes differ in size and structure from those in bacterial cells (Baird & Denyer, 2007).

3.2 Prokaryote and eukaryote

This very brief outline of the structure of a bacterial and fungal cell has drawn attention to fundamental differences between these cell types. These differences, detected by the techniques of sub cellular biology, have enabled biologists to suggest a fundamental division in the living world. Bacteria were named prokaryotic organisms or prokaryotes, a name derived from their unenclosed nucleus, and fungi (and, in fact, all other living plants and animals) were called eukaryotic or eukaryotes; they possessed a nuclear membrane. Some of these differences have been summarised in table 3.1 (Baird & Denyer, 2007).

Table 3.1 Differences between bacterial and fungal cells

Some Differences between Bacterial (Prokaryotic) Cells and Fungal (Eukaryotic) Cells		
Feature	**Prokaryotic**	**Eukaryotic**
Nucleus	No enclosing membrane	Enclosed by a membrane
Flagella	Simple	Complex
Cell Wall	Peptidoglycan	Cellulose, chitin, and other polymers
Cytoplasmic membrane	Generally does not contain sterols	Contains sterols
Ribosome	70S[a]	80S[a]
Oxidative phosphorylation	In cytoplasmic membrane	In mitochondria
Mitochondria	Absent	Present

[a] S is a measure of ribosome size (calculated from sedimentation in a centrifuge); 80S ribosomes are larger than 70S.

3.3 The bacterial and fungal spore

Spore is a word common to both bacterial and fungal morphology, but it is in the spore that the largest difference in function between the two groups can be seen. In brief, bacterial spore formation, which is limited to two genera important in contamination, constitutes a survival package that is formed under adverse conditions and from which; when conditions are again suitable, vegetative bacteria arise. Fungal spores, however, are part of the normal life cycle of these organisms (Baird & Denyer, 2007).

3.4 Bacterial and fungal growth

It is important to realise from the outset that a large number of bacteria and fungi, including many of those associated with contamination, can grow in what may appear to be nutritionally very simple systems and often at quite low temperatures. Cold is not lethal, although growth is slowed at low temperatures. The notion that bacteria and, to a lesser extent, molds require a rich and often exotic nutritional environment and careful and controlled incubation at 37°C (bacteria) or 25°C (molds) arose because of the dominance of medical bacteriology and mycology where such conditions were almost invariably mandatory (Baird & Denyer, 2007).

Process water is an acceptable culture medium for many bacteria and, to a lesser extent, molds, and can be a dangerous commodity in the pharmaceutical, food, and cosmetic industries unless carefully handled. The causal organism of Legionnaire's disease grows in water-cooled heat-exchangers (Anon, 1989).

For each bacterial and fungal species, a set of conditions is necessary for optimum growth and, if correctly balanced, will give maximum yields. It should be realised that although laboratory studies often seek to optimise growth, in the case of contamination, conditions may not be optimal but nevertheless may allow growth to proceed, giving rise to spoilage (Baird & Denyer, 2007).

3.4.1 Requirements for growth

These can be divided conceptually into two categories: first, the range of substrates, the consumables needed; and second, the nature of the environment, which is, temperature, pH, osmotic pressure (Baird & Denyer, 2007).

3.4.1.1 Consumables

A formula that supports the growth of many bacterial and fungal contaminants consists of an aqueous solution containing ($g.L^{-1}$): $(NH_4)_2HPO_4$, 0.6; KH_2PO_4, 0.4; glucose, 10.0. Growth will be slow and can be enhanced by the presence of trace elements (in addition to those present in the laboratory reagents) and carbohydrates, fats, proteinaceous material, amino acids, sugars, and vitamins such as nicotinic acid, riboflavin, and thiamine. Notwithstanding the simple nutritional requirements of some organisms, others exhibit specific needs that form the basis of selective and diagnostic media (Baird & Denyer, 2007).

3.4.1.2 Environmental factors

Water – The presence of water is essential and dry products or intrinsically anhydrous material are not liable to spoilage. From very early in history, man has exploited the drying of foods to preserve them (Baird & Denyer, 2007).

Gaseous nutrient – Some microorganisms grow in the absence of oxygen and are termed anaerobic; most, however, require oxygen and are called aerobic. Some bacteria possess the facility to grow in either the absence or presence of oxygen and are termed facultative organisms. In general terms, this means that the exclusion of oxygen (air) may not prevent some spoilage organisms from growing (Baird & Denyer, 2007).

pH – There is an optimum pH range over which microorganisms can grow. In particular, bacteria grow best around pH 7 but many molds can tolerate more acid conditions, pH 5 to 6. Some microorganisms are surprisingly tolerant of hostile pH environments and contamination can occur in products where pH is outside the optimum growth value although the growth may be slower (Baird & Denyer, 2007).

Temperature – As with pH, there is an optimum temperature range. Low temperatures will slow growth and rising temperatures will increase growth rate. As the temperatures rises above the optimum, however, growth is inhibited and microorganisms are killed, a phenomenon exploited in heat sterilisation (Baird & Denyer, 2007).

3.5 Pathogenicity

Historically, microorganisms have been grouped into pathogenic or non-pathogenic types, according to their ability to cause disease. In recent years, increasing interest has also been shown in the so-called opportunistic pathogens, which are capable of causing disease when given the opportunity to do so. These include the free-living Gram-negative bacteria such as pseudomonads and members of the Enterobacteriaceae, for example, *Klebsiella* and *Serratia* spp., all of which have simple nutritional requirements, thus enabling them to survive in some unlikely environments, including disinfectant and antiseptic solutions. Opportunist pathogens pose a particular threat to certain groups of patients at risk, especially neonates, the elderly, and those compromised by trauma, burns, or immunosuppressant therapy (Baird & Denyer, 2007).

True pathogens, such as *Clostridium tetani* and *Salmonella* spp., rarely occur in pharmaceutical products but inevitably cause serious problems when present. Opportunist pathogens are more common contaminants in these products, particularly in aqueous preparations where total viable counts may well exceed 10^6 $CFU.mL^{-1}$ (Baird & Denyer, 2007).

3.6 General properties of selected microorganisms

3.6.1 Gram-negative organisms

3.6.1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa has acquired some notoriety as a contaminant of pharmaceutical products. Widely distributed in the environment, it is a free-living opportunist pathogen and can cause particular problems in susceptible groups of patients, especially neonates, the elderly, and the immunocompromised. It is a flagellated rod-shaped organism that does not form spores and grows aerobically. It does not ferment carbohydrates and thus will not produce gaseous products when growing in the presence of carbohydrates. Under appropriate conditions of growth, it may produce a blue or green fluorescent pigment. *Pseudomonas aeruginosa* grows readily on standard laboratory media, but will grow even in distilled water provided trace amounts of organic matter are present. As a mesophile, *P. aeruginosa* will grow over a temperature range from 20 to 42°C, with optimal growth occurring at 37°C. It is commonly found in biofilm growth (Baird & Denyer, 2007).

3.6.1.2 *Escherichia coli*

Escherichia coli is a motile, nonsporing rod typically of dimensions 1 µm x 4 µm and is a member of the Enterobacteriaceae. It differs from *P. aeruginosa* in that it is able also to grow anaerobically. When it grows on carbohydrates, it does so by fermentation, producing gaseous products. Media with only a single carbon source are sufficient for promoting *E. coli* growth. It can grow at low temperatures and also at temperatures as high as 40°C. Optimal growth occurs at 37°C. It forms part of the gut flora of man; its presence is therefore indicative of faecal contamination and gross defects in hygiene. It has gained particular notoriety through the verotoxigenic strain *E. coli* 0157:H7 (Baird & Denyer, 2007).

3.6.2 Gram-positive organisms

3.6.2.1 *Staphylococcus aureus*

This is a spherical organism of approximately 1 µm in diameter. It is nonmotile and does not form spores. It is able to grow aerobically and anaerobically and will grow readily in a chemically defined medium containing glucose, essential salts, selected amino acids, thiamine, and nicotinic acid. It is relatively resistant to antimicrobial preservatives such as phenol, can remain alive at temperatures as cold as 4°C and as warm as 60°C, and will grow in media containing up to 10% sodium chloride (Baird & Denyer, 2007).

If present in pharmaceutical products, *S. aureus* and the closest related *S. epidermidis* may indicate contamination from a human source, for example, from the hands, skin, or hair. In particular, *S. epidermidis* is frequently implicated in medical-device-related infection. *Staphylococcus aureus* is a common cause of boils, middle ear infection, pneumonias, and osteomyelitis. It can proliferate in foods, secreting an exotoxin that can give rise to food poisoning (Baird & Denyer, 2007).

3.6.3 Fungi

3.6.3.1 *Aspergillus niger*

This organism grows only in the filamentous (mycelial) form and is familiar to most people as a white, turning to black, disk of growth on jams and other exposed foodstuffs. Colonies grow over a wide temperature range, up to 50°C, although optimal temperature for growth is 24°C. The characteristic fruiting bodies, which

when formed are responsible for the colour change from white to black, may be seen under the microscope (Baird & Denyer, 2007).

Spores of *Aspergillus* are commonly present in air and can infest and germinate in pharmaceutical and cosmetic products, causing discolouration and spoilage. They are generally not as resistant to antimicrobial agents as are bacterial spores. Some *Aspergillus* strains produce characteristic carcinogens, the aflatoxins (Baird & Denyer, 2007).

3.6.3.2 *Candida albicans*

This is a commensal yeast, which may cause oral and vaginal thrush. It has been occasionally implicated in infusion-related infections. It grows readily on conventional mycological media at room temperature (optimal growth at 25°C) or at 37°C. It is dimorphic, growing first as yeast cells, but with aging will form chlamydospores, which are more difficult to destroy. There are no temperature tolerance differences between the two forms. Viewed microscopically, it appears to possess septate hyphae, known as pseudomycelia, among the yeastlike cells. It is unpigmented and colonies have a creamy white appearance (Baird & Denyer, 2007).

3.7 Principles of preservation

3.7.1 The need for preservation of pharmaceutical products

Bacteria, yeasts, and molds have diverse metabolic requirements and are able to grow in aqueous pharmaceutical products when nutrients are available and when environmental conditions are suitable. The regulation of microbial growth by physical and chemical agents was presented by Moat & Foster (1988). An understanding of how these factors control microbial growth is necessary to determine the most suitable preservative system needed in any product (Orth, 2007).

Growth of bacteria, yeasts, or molds on, or in, products may make those products unsafe and unacceptable for use. The hazards of using contaminated products are due to the effect of microbial infections or harmful microbial by-products on human health. Several surveys conducted between 1969 and 1977 revealed contamination of cosmetic, toiletry, and pharmaceutical products (Baird, 1977; Bruch, 1971; Dunnigan & Evans, 1970). McCarthy (1980) reported that similar patterns of contamination for nonsterile products were observed in both the pharmaceutical and

cosmetic industries. Although most manufacturers have identified the critical control points in their processes and have implemented [validated] procedures to prevent microbial contamination, occasional contamination problems occur and have resulted in product recalls (Orth, 1999).

In the 1960s and 1970s, there were several reports of infections due to use of contaminated products. Hand lotions and creams were identified as sources of nonsocomial infections that resulted in septicaemia due to Gram-negative bacteria, particularly *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* spp., and *Serratia* spp. (Morse *et al.*, 1967; Morse & Schonbeck, 1968). Noble and Savin (1966) reported *Pseudomonas aeruginosa* contamination of a steroid cream preserved with chlorocresol, following modification of that cream by the addition of final concentration of chlorocresol of 0.1% w/v, which should have been sufficient to inactivate contaminating *P. aeruginosa*, this microorganism persisted in the product. (Note: Noble and Savin reported that 0.01% w/v chlorocresol was sufficient to inhibit several strains of *P. aeruginosa* on nutrient agar.) The cause of contamination was traced to a decrease in preservative level in the aqueous phase of the product, due to partitioning of the preservative into the oil phase. Contamination of the product (possibly with adapted organisms) was facilitated by the practice of refilling used containers. It had been assumed that use of the same preservative system in the modified product would be satisfactory, but unfortunately, it was not. This demonstrates why a preservative system must be tailored to a specific product (Cowen & Steiger, 1977).

The relative hazard created by microbiological contamination of cosmetic or pharmaceutical products may be related to the severity of infection or disease it causes. Dunnigan classified *Pseudomonas*, *Proteus*, *Staphylococcus*, *Serratia*, *Streptococcus*, *Penicillium*, *Aspergillus*, and *Candida* genera as health hazards (Dunnigan, 1968). Bruch (1972) refined the classification of objectionable microorganisms according to product type. Orth (1993) updated the list of objectionable microorganisms based on product type. The classification appearing in table 3.2 has been further updated by inclusion of *Burkholderia* spp (Orth, 2007).

Table 3.2 **Classification of Objectionable Microorganisms by Product Type (Orth, 1993).**

Sterile drugs	Any organism or pyrogen in a sterile product is **objectionable.**
Eye products	*Pseudomonas aeruginosa* is **always objectionable.** Other *Pseudomonas* spp., *Burkholderia* spp., *Staphylococcus aureus*, *Serratia marcescens*, and *S. liquifaciens* are **usually objectionable.**
Nonsterile oral products	Any enteric pathogen (i.e., *Salmonella* spp., *Yersinia* spp., *Campylobacter* spp.) and *Escherichia coli* are **always objectionable.** Other enteric organisms, such as *Enterobacter* spp., *Citrobacter* spp., *Pseudomonas* spp., *Burkholderia* spp., proteolytic *Clostridium* spp., enterotoxigenic *Staphylococcus aureus*, pathogenic yeasts (*Candida albicans*), and mycotoxin-producing fungi are **usually objectionable.**
Nonsterile topical products	*Pseudomonas aeruginosa*, *Klebsiella* spp., *Staphylococcus aureus*, *Serratia marcescens*, and *S. liquifaciens* are **always objectionable**; whereas *Pseudomonas putida*, *P. multivorans*, *Burkholderia cepacia*, *Clostridium perfringens*, *C. tetani*, and *C. novyi* are **usually objectionable.**
Genitourinary tract products	*Escherichia coli*, *Proteus* spp., *Serratia marcescens*, *Pseudomonas aeruginosa*, and *P. multivorans* are **always objectionable**; whereas *Klebsiella* spp., *Acinetobacter anitratus*, and *A. calcoaceticus* are **usually objectionable.**

Although the direct effects of microorganisms in infections and disease have been appreciated for many years, the insidious role they play in inflammation, immunomodulation, and altering human physiology is only beginning to be appreciated (Orth, 2007).

Products intended for use on, or in, the body must be safe. Even though the aerobic plate count and total viable counts of the finished product may reveal the presence of <10 colony-forming units (CFU) mL^{-1}, residual microbial by-products may produce undesirable reactions. The problems created by microbial contamination can be minimised by the use of raw materials that do not have a history of unacceptable microbial load, by adherence to validated manufacturing practices to reduce the risk

of microbial contamination during processing, and by sterilising products or using effective preservative systems in aqueous formulations (Orth, 2007).

3.7.2 Preservation of the product during use

In 1970, Halleck published the recommendations of the Preservation Subcommittee of the Toiletry Goods Association (TGA) Microbiology Committee (Halleck, 1970). These recommendations stated that preservation studies should consider product formulation, manufacturing conditions, packaging, product stability, and continued effectiveness of the preservative system during the intended use by the consumer (Orth, 2007).

In 1984, Eiermann noted that data obtained from surveys and during U.S. Food and Drug Administration (FDA) inspections of cosmetic manufacturers suggested that microbiological contamination of cosmetics during manufacturing was no longer a major regulatory issue (Eiermann, 1984). He indicated that the question of whether these products remain uncontaminated when used by consumers had not been resolved. This is addressed in the tentative final order regulating over-the-counter (OTC) antimicrobial drug products, in which the FDA used the phrase "effectively preserved" to include preservation during use by the consumer (Eiermann, 1984).

It is believed that normal use of some products by consumers repeatedly subjects these products to contamination. For example, hair care products (shampoos, conditioners, antidandruff products) are used while showering, which exposes these products to dilution with water and contamination with microorganisms. Repeated use of creams, which requires dipping a finger into a jar to obtain the product, may expose the cream to contamination and dilution with soil, microorganisms, and moisture on fingers. Also, adaptation may occur in product residues present on the threads of the cap or neck of the container if the residues present on the water or contaminated with body fluids (blood, urine, tissue fluids). These microorganisms may become adapted to the product and may be introduced into that product when the cap is next removed, resulting in microbial contamination of the remaining product (Orth, 2007).

3.7.3 The ideal preservative

Understanding the characteristics of an ideal preservative helps to provide the basis for rational selection of the most suitable agent(s) for a given formulation. The

desired characteristics of an ideal preservative have been discussed by many authors and include the following:

It should have a broad spectrum of activity. Ideally, a single preservative should be used as this will reduce costs and possibly may reduce the irritation or potential toxicity of the formula (Orth, 2007).

It should be effective and stable over the range of pH values encountered in cosmetic and pharmaceutical products. Ideally, the preservative should be able to function effectively at any pH compatible with any product applied topically or taken internally. In addition, it should be chemically stable so that there is no loss of preservative efficacy during the expected shelf life of the product (Orth, 2007).

It should be compatible with other ingredients in the formulation and with packaging materials. This attribute would prevent loss of preservative potency as a result of interactions with formula components or packaging material. It should not alter the therapeutic properties of a drug (i.e., loss of potency of active ingredients or alteration in the pharmacokinetic behaviour of the active ingredients), a phenomenon that may occur if the preservative reacted with formulation components (Orth, 2007).

It should not affect the physical properties of the product (i.e. colour, clarity, odour, flavour, viscosity, texture). Ideally, it should not produce any texture, aroma, or performance of the formulation (Orth, 2007).

It should have a suitable O/W partition coefficient to ensure an effective concentration of the preservative in the aqueous phase of the product. Biological reactions take place in aqueous systems or at the interface of oil-in-water systems; consequently, it is necessary to have sufficient preservative in the water phase to ensure adequate preservation of the product (Orth, 2007).

It should inactivate microorganisms quickly enough to prevent microbial adaptation to the preservative system. Preservatives are used in aqueous products to make them bactericidal and fungicidal in a short enough time to meet acceptance criteria, prevent adaptation and growth (Orth, 1997; Orth, 1999), and to reduce the likelihood of microbial persistence in anhydrous products that may be contaminated and moistened during use. It is believed that contaminating microorganisms may be able to develop resistance to a product if the preservative system does not inactivate them quickly enough to prevent genetic or biochemical modifications (i.e., enzyme

induction, modification of metabolic pathways, detoxification mediated by hydroperoxidases and oxygenases) that enable microorganisms to adapt to the product (Orth, 2007).

It should be safe to use. Safety includes handling of pure or concentrated materials in the manufacturing plant as well as the effect of preservatives in the finished formulation on the consumer. Ideally, the product should be used in accordance with permissible levels, where applicable (Orth, 2007).

It should be cost-effective to use. From a commercial perspective, an effective concentration should add little to the cost of the formulated product (Orth, 2007).

The parabens have been used more often than any other preservative in cosmetic products (Steinberg, 2004). However, no single preservative meets all the above characteristics of the ideal preservative for all formulations (Orth, 2007).

3.7.4 Rational development of a product preservative system

The first step is to review the product formula and type to determine what are the most likely to challenge organisms, then to decide which preservatives are indicated, and finally which preservative test method is the most appropriate. Samples of the product may then be prepared, with at least one sample containing an inadequate preservative system, one or two samples with the preservative level close to the expected target concentration, and at least one sample with excess preservative. This provides samples with a range of concentrations of the preservative under investigation and which may now be tested for preservative efficacy (Orth, 2007).

Several preservative efficacy test methods may be used (Lorenzetti, 1984; Orth, 1984; Parker, 1984). However, it is important to employ a test method that is reliable and is capable of indicating the concentration of preservative required for the preservative system of the product to meet acceptance criteria. The linear regression method is recommended because it provides quantitative data on the kinetics of inactivation. Thus, the *D* value for each concentration of the preservative used may be determined with each test organism. If the preservative concentrations were selected correctly, a family of curves will be obtained. It is necessary to select the concentration of preservative required to achieve the desired rate of death so that microorganisms are killed too quickly to allow them to adapt and grow. Orth *et al.* (1998) reported that Gram-negative bacteria may survive and grow if initial rates of

killing are too slow, when *D* values were about 30h or greater. This suggests that formulations that kill Gram-negative bacteria at rates approaching the maximum allowable limits of the United States Pharmacopoeia and Cosmetic, Toiletry, and Fragrance Association methods should be used only with special manufacturing precautions (aseptic filling) or with packaging that prevents water or microbial intrusion into the product (Orth, 2007).

3.7.5 Cross-resistance of preservatives with other antimicrobial agents

Antibiotic resistance is increasing and is a serious problem. Whereas antibiotics are known to have specific targets in microbial cells, biocides (including preservative agents) are believed to have multiple actions on the cell, including altering membrane permeability, inactivating enzymes, and interfering with nucleic acids. It has been assumed that bacteria cannot develop resistance to biocides because they do not have specific targets. This belief is being challenged by findings in recent years, and it has been demonstrated that bacteria have developed resistance to triclosan, chlorhexidine, quarternary ammonium compounds, and other biocides (Levy, 1998; McDonnell & Russell, 1999; McMurry *et al.*, 1998; Russell *et al.*, 1998). Increased resistance to antibiotics and disinfectants may be due to mutation or the acquisition of genetic material by horizontal gene transfer or plasmids (Orth, 2000). Growth conditions may affect resistance to antimicrobial agents. Exposure to aerobic conditions enables cells to develop tolerance to oxidative stress (i.e. the SOS response, which includes production of enzymes to detoxify reactive oxygen species and repair DNA lesions). Exposure of cells to subinhibitory doses of hydrogen peroxide was reported to increase resistance of *Escherichia coli* and *Salmonella* spp. to that agent (Demple & Halbrook, 1983; Winquist *et al.*, 1984). Starvation or low a_w resulted in slower rates of death (larger *D* values) for *P. aeruginosa* during preservative efficacy testing (Orth *et al.*, 1998). Although these workers did not demonstrate the presence of different levels of heat shock proteins, their findings showed that exposure to one stress helps prepare a population to survive another type of stress (Orth, 2007).

Pine oil disinfectant, salicylate, and other weak acids (benzoate) may induce multiple antibiotic resistances in a number of organisms (Cohen *et al.*, 1993; Gustafson *et al.*, 1999; Lambert *et al.*, 1997; McDonnell & Russell, 1999; Moken *et al.*, 1997). McDonnell and Russell (1999) reported increased cross-resistance to heat, ethanol, and hypochlorous acid. These reports suggest that exposure to sub-lethal

concentrations of biocides may foster the development of microorganisms with increased tolerance to biocides and other antimicrobial agents (Orth, 2000). At this time, we do not have sufficient data to state that development of antibiotic resistance caused by antimicrobials or biocides in laboratory studies occurs under actual product-use conditions. However, manufacturers of cosmetics and drugs should consider programs to ensure that their products will not encourage the development of drug-resistant microorganisms (Orth, 2000).

3.8 Conclusion

It is a fact that preservatives, disinfectants and antimicrobials play an important role in the pharmaceutical industry. They protect both the person using the product as well as the product itself from microorganisms. These microorganisms can, although very small, cause very serious to fatal infections if given the chance. It is therefore of the utmost importance to protect the consumer against infections by correctly preserving a product for human consumption or use. Although there is an important human protective role of the preservative, it is also extremely important in the formula to keep the product stable and therefore increase the shelf life of the product.

A wide range of preservatives are available on the market, each one different in its own way. Some preservatives work better against gram-positive bacteria, others against gram-negative bacteria, while a few works against both. You get hydrophilic, hydrophobic, lipophilic and lipophobic preservatives. Some preservatives are pH-sensitive, while others are not. Thorough research on the compatibility and suitability of the preservative is thus needed before it can be added as an ingredient in the formulation of the intended product.

By adding the appropriate preservative to a formulation, diseases can be treated safely and lives can be extended.

CHAPTER 4

SAMPLE ANALYSIS

4.1 Introduction

Everything made by human hand is subject to decay. Pharmaceuticals are no exception.

The rate at which drug products degrade varies dramatically. Some radiopharmaceuticals must be used within a day or so. Other products may, if properly stored and packaged, retain integrity for a decade or more, although in many jurisdictions the maximum shelf life that a regulatory agency will approve for a drug product is five years (Carstensen & Rhodes, 2000).

Stability testing includes long-term studies, where the products are stored at room temperature and humidity conditions, as well as accelerated studies where the product is stored under conditions of high heat and humidity. Proper design, implementation, monitoring and evaluation of the studies are crucial for obtaining useful and accurate stability data (Microbac Laboratories, 2005).

In this chapter the assay and the results for the nevirapine and butylparaben in the pro-Pheroid formulation are discussed.

4.2 Reasons for stability testing

Stability testing is performed to ensure that drug products retain their full efficacy up to the end of their expiration date. Stability in terms of both analytical and microbiological aspects is tested.

The information gathered from these tests is used to formulate the stability information, which ensures the quality, efficacy and safety of the drug product (Grimm & Krummen, 1993).

This means that all organoleptic, physic-chemical, chemical and microbial test results relevant to the stability of the product must be within the predefined tolerance ranges until the end of the expiration date. The stability information is thus an important element in the quality assurance of a drug product (Grimm & Krummen, 1993).

Stability testing accompanies the development of a drug product from the experiments with the drug substance over formulation development up to the final dose and formula for introduction, but it continues during production and necessary major changes (Grimm & Krummen, 1993).

Reasons for stability testing include:

- Concerns for patients' welfare and safety;
- To protect the reputation of the manufacturer;
- To obey to the requirements of the regulatory agencies; and
- To provide a database that may be of value in the formulation of other products (Carstensen & Rhodes, 2000).

In order that the quality of the drug product can be assured by the stability information, the following aspects must be taken into account:

- Expiration date or shelf-life: The expiration date must always refer to the climatic zone or zones in which the drug product is to be distributed.
- Storage instructions: Shelf-life can only be guaranteed if certain specific storage instructions are complied with.
- Use life, stability in opened container: It is necessary to specify a period within which the product must be used after the container has been opened.
- Overage: For certain drug products an overage is necessary in order to achieve an acceptable shelf-life. And overage may only be added if the formulation is already optimised (Grimm & Krummen, 1993).

4.3 Problems/ adverse effects due to instability

There is a variety of mechanisms by which drug products may degrade, and thus a quite wide range of adverse effects that can occur:

- Loss of active;
- Increase in concentration of active;
- Alteration in bioavailability;
- Loss of content uniformity;

- Decline of microbiological status;
- Loss of pharmaceutical elegance and patient acceptability;
- Formation of toxic degradation products;
- Loss of package integrity;
- Reduction of label quality; and
- Modification of any factor of functional relevance (Carstensen & Rhodes, 2000).

All reactions and the changes resulting there from can be influenced by:

- Factors relating to manufacture: Batch size, equipment sequence in which constituents of the formulation are added or different quality of active ingredients, excipients, packaging materials, although they were all analysed before use and complied with the tolerances.
- External factors: Temperature, humidity, light, oxygen and pH (Grimm & Krummen, 1993).

There are four major modes of degradation. Firstly, chemical degradation which is very common and includes solvolysis and oxidation of chemicals. Secondly, physical degradation which can be caused by a range of factors for example impact, vibration, abrasion, and temperature fluctuations such as freezing, thawing or shearing. Biological, especially microbiological, degradation is very likely to cause stability problems. And finally the limitations of this classification which may over-compartmentalise the approach to drug product stability (Carstensen & Rhodes, 2000).

4.4 Stability programme

The stability of both nevirapine and butylparaben in the pro-Pheroid formulation was tested. This formulation includes the anti-oxidant, tert-butylhydroquinone (TBHQ).

Stability testing was performed according to the International Conference and Harmonisation (ICH) guidelines. The stability of the nevirapine and the butylparaben in the pro-Pheroid formulation was conducted over a period of three months, in order to fulfil the technical requirements for the registration of pharmaceuticals for human use (ICH Q6A, 1999).

4.4.1 Storage conditions

Controlled storage facilities were used during the stability period. The formulation was stored at four different temperature and humidity conditions according to ICH guidelines:

- 5°C;
- 25°C ± 60% RH;
- 30°C ± 65% RH; and
- 40°C ± 75% RH.

4.4.2 Stability tests conducted

The master batch was divided into smaller containers, labelled and stored under the four different conditions for the three different time periods. At months 1, 2 and 3 the samples were removed from the stability environment for testing. The samples were left to reach room temperature before the assay tests for nevirapine and butylparaben would be performed according to the methods described.

The samples were analysed in duplicate and the averages are tabulated under the assay results below.

4.5 Method for HPLC analysis

4.5.1 Method for nevirapine and butylparaben in pro-Pheroid

The method for nevirapine in pro-Pheroid was developed and validated by Kühn, 2008.

Chromatographic conditions

The analytical procedure was performed under the following isocratic conditions:

Analytical instrument: Shimadzu Prominence series HPLC equipped with a LC-20AD quarternary pump, SIL-20AC auto sampler, SPD-M20A diode array detector and Shimadzu LC Solution data acquisition and analysis software.

Column: Phenomenex Luna C18 (2), 250 x 4.6 mm, 5 µm

Mobile phase: Methanol: Water (60:40)

Flow rate:	1.0 mL/min
Injection volume:	20 µℓ
Detection:	UV at 215 nm and 254 nm
Retention time:	± 4.5 minutes
Stop time:	30 minutes
Solvents:	Assay: methanol (100%) for standard as well as for sample preparation.

Standard preparations

Dissolve an accurately weighed quantity of nevirapine and butylparaben in the mobile phase and dilute quantitatively with the mobile phase to obtain a solution having a known concentration of about 0.024 mg/mL and 0.03 mg/mL, respectively.

Sample preparation

Samples of each formulation were stored at the stability conditions: 5°C; 25°C+60%RH; 30°C+65%RH and 40°C+75%RH. Samples were prepared for analysis in duplicate. Dissolve and dilute each of the samples tested for nevirapine and butylparaben with methanol to a known concentration of about 0.072 mg/mL and 0.09 mg/mL, respectively. Sonicate for approximately 15 minutes.

4.6 Assay results

4.6.1 Nevirapine in pro-Pheroid

The samples were measured at two different wavelengths as to see which wavelength is more ideal for which ingredient analysed. The two wavelengths used were 215 nm and 254 nm.

4.6.1.1 Results for nevirapine measured at 215 nm

The assay results for nevirapine measured at 215 nm are given in table 4.1 and illustrated in figure 4.1. The acceptance criteria for this product are 90.0 – 110.0% (USP, 2009).

Table 4.1 **Assay results of nevirapine in pro-Pheroid at 215 nm**

Initial (%) (%RSD)	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
100.13 (1.38)	5°C	94.04 (5.72)	118.69 (0.43)	58.50 (1.58)
	25°C + 60% RH	90.47 (3.78)	115.60 (0.29)	63.38 (1.47)
	30°C + 65% RH	91.00 (0.45)	119.94 (0.79)	66.04 (7.09)
	40°C + 75% RH	91.21 (0.75)	115.60 (0.25)	63.60 (0.63)

The percentage difference in the assay results of the samples in relation to the initial assay results was calculated according to the following equation:

$$\% \text{ Difference} = \frac{(\text{Value obtained} - \text{initial value})}{(\text{Initial value})} \times 100$$

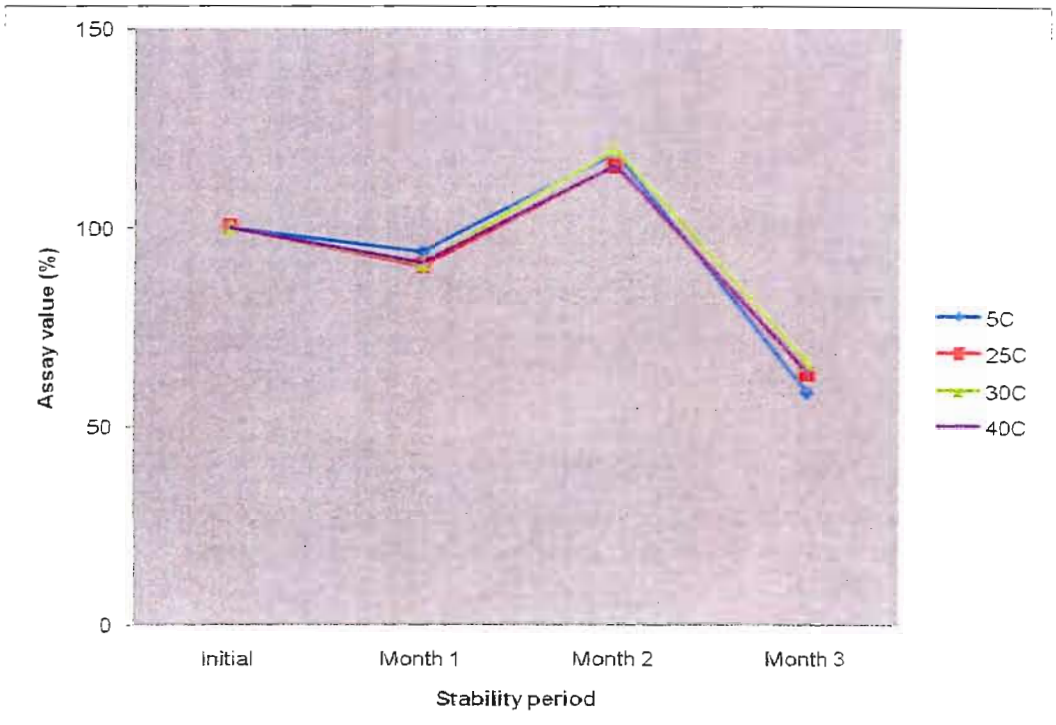


Figure 4.1 Nevirapine in pro-Pheroid measured at 215 nm

The results obtained for nevirapine samples measured at 215 nm are summarised in table 4.2.

Table 4.2 Percentage difference in assay results of nevirapine (215 nm) in pro-Pheroid

Temperature & Humidity	Month 1 (%)	Month 2 (%)	Month 3 (%)
5°C	-6.08	18.54	-41.58
25°C + 60% RH	-9.65	15.45	-36.70
30°C + 65% RH	-9.12	19.78	-34.05
40°C + 75% RH	-8.91	15.45	-36.48

4.6.1.2 Results for nevirapine measured at 254 nm

The assay results for nevirapine measured at 254 nm are given in table 4.3 and illustrated in figure 4.2. The acceptance criteria for this product are 90.0 – 110.0% (USP, 2009).

Table 4.3 **Assay results of nevirapine in pro-Pheroid at 254 nm**

Initial (%) (%RSD)	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
99.32 (0.15)	5°C	112.49 (2.43)	132.15 (8.30)	90.25 (4.49)
	25°C + 60% RH	103.49 (1.21)	121.61 (1.93)	124.69 (27.18)
	30°C + 65% RH	100.79 (0.62)	129.14 (3.22)	211.31 (30.07)
	40°C + 75% RH	103.47 (2.35)	139.44 (14.54)	187.92 (43.98)

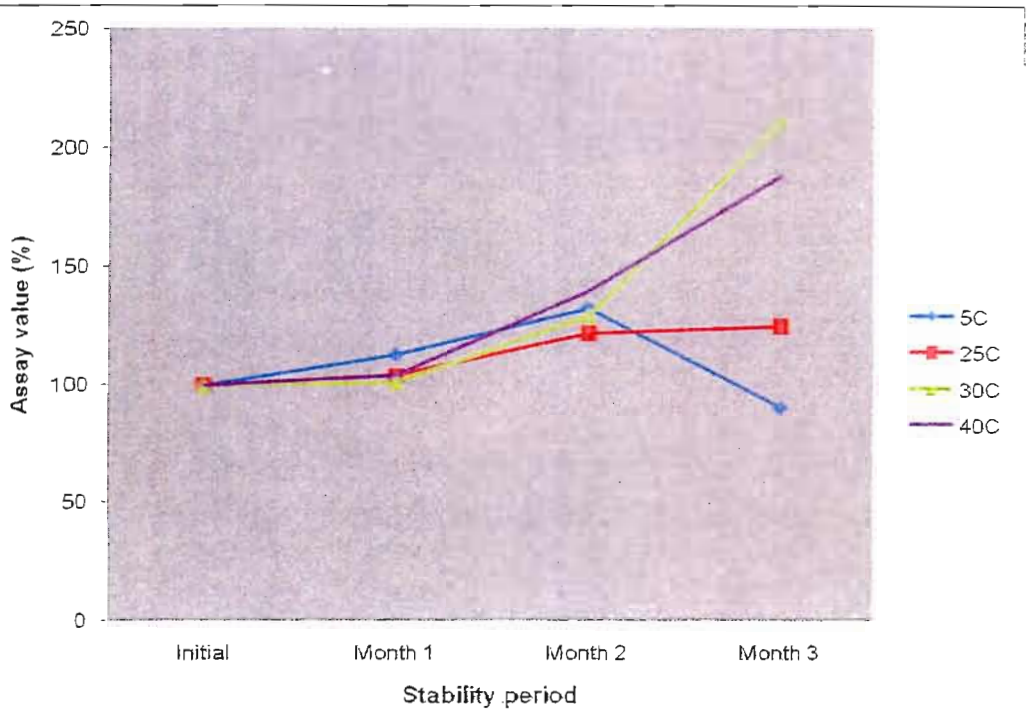


Figure 4.2 Nevirapine in pro-Pheroid measured at 254 nm

The percentage differences in the assay results of the nevirapine samples measured at 254 nm in relation to the initial assay results are given in table 4.4.

Table 4.4 Percentage difference in assay results of nevirapine (254 nm) in pro-Pheroid

Temperature & Humidity	Month 1 (%)	Month 2 (%)	Month 3 (%)
5°C	13.26	33.05	-9.13
25°C + 60% RH	4.20	22.44	25.54
30°C + 65% RH	1.48	30.02	112.76
40°C + 75% RH	4.18	40.39	89.21

4.6.1.3 Discussion

The assay results for nevirapine in pro-Pheroid measured at 215 nm showed that nevirapine was within the specification range during month 1 of the stability program. The assay results of the four samples after one month were between 90.47% and 94.04%. When the percentage relative standard deviation of each sample is evaluated, one can see that it is relative small fractions, between 0.45 for 30°C + 65% RH and 5.72 for 5°C, which illustrates good repeatability of the peaks obtained during month one of stability testing.

An increase in concentration was observed in month 2. The samples had percentage differences of between 15.45% and 19.78% when compared to the initial sample. Although slightly above the range that is specified in the acceptance criteria for this product, the peaks had very good repeatability because the percentage relative standard deviation for all the samples was <1%.

A drastic decrease in concentration for all four samples was the result of sample testing during month 3. The concentrations of the four samples analysed were between 58.5% for 5°C and 66.04% for 30°C + 65% RH. Repeatability of the peaks was still good with %RSD of between 0.63 – 7.09. A reason for this decrease could be degradation of nevirapine in this system.

The assay results for the samples measured at 254 nm had some differences compared to the results for 215 nm. During month 1 the concentrations of all four samples increased with comparison to the initial sample. Three of the four samples were within the specification range, only 5°C was 2.5% out of specification. Repeatability was excellent, the %RSD ranged from 0.62 for 30°C + 65% RH to 2.43 for 5°C, which reveals little interference of other peaks.

Month 2 had, as with 215 nm, an increase in concentration. An increase of between 22.44% for 25°C + 60% RH and 40.39% for 40°C + 75% RH when compared to the initial sample was obtained. Relative good repeatability was achieved with the three lower temperatures, but 40°C + 75% RH revealed a %RSD of 14.54, which gives the idea that some meddling with other peaks started.

During month 3 of the stability testing of nevirapine in pro-Pheroid measured at 254 nm random results was obtained. 5°C had a decrease in concentration, but was still within the specification range. It had a %RSD of 4.49 which demonstrates good repeatability for the peaks and only little interference, but good and reliable results.

The three higher temperatures had an increase in concentration which ranges from 25.54% to 112.76% when compared to the initial sample. Poor repeatability of the peaks was obtained with percentage relative standard deviations of between 27.18 for 25°C + 60% RH and 43.98 for 40°C + 75% RH. The assumption is made that there might have been a great deal of interferences from other peaks and that the results obtained does not reveal the real concentration of nevirapine.

When the two different wavelengths are compared, one sees much bigger differences in month to month results at 254 nm. Where the nevirapine concentration decreased with <10% in month 1 at 215 nm, the concentration increased at 254 nm. In month 2 nevirapine concentration increased with <20% at 215 nm, whilst at 254 nm it increased with 22.44 – 40.39%. The results of month 3 measured at 215 nm showed that all four conditions had a noticeable decrease in concentration, whilst only the 5°C sample had a decrease at 254 nm and the other three samples had in increase in concentration. This off course raises some questions about the stability of the product, but also opens hidden truths about chromatography and wavelengths. As mentioned in this discussion, there might have been interference from other peaks that could also be picked up at the higher wavelength and so could increase the AUC and thus increase the concentrations of the samples. What is seen is that the more ideal wavelength for measuring nevirapine is supposedly 215 nm as it has better repeatability of peaks. This should however be confirmed with more advanced analytical techniques.

4.6.2 Butylparaben in pro-Pheroid

As with nevirapine, the butylparaben was also analysed at both 215 nm and 254 nm.

4.6.2.1 Results for butylparaben measured at 215 nm

The assay results for butylparaben measured at 215 nm are given in table 4.5 and illustrated in figure 4.3. The acceptance criteria for this product are 90.0 – 120.0% (BP, 2009).

Table 4.5 **Assay results of butylparaben in pro-Pheroid at 215 nm**

Initial (%) (%RSD)	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
85.17 (3.63)	5°C	86.50 (2.08)	188.48 (0.43)	60.20 (1.79)
	25°C + 60% RH	85.13 (1.24)	83.18 (0.91)	59.50 (2.83)
	30°C + 65% RH	91.10 (11.33)	86.07 (1.46)	61.78 (12.84)
	40°C + 75% RH	99.08 (0.36)	85.87 (1.07)	65.82 (10.76)

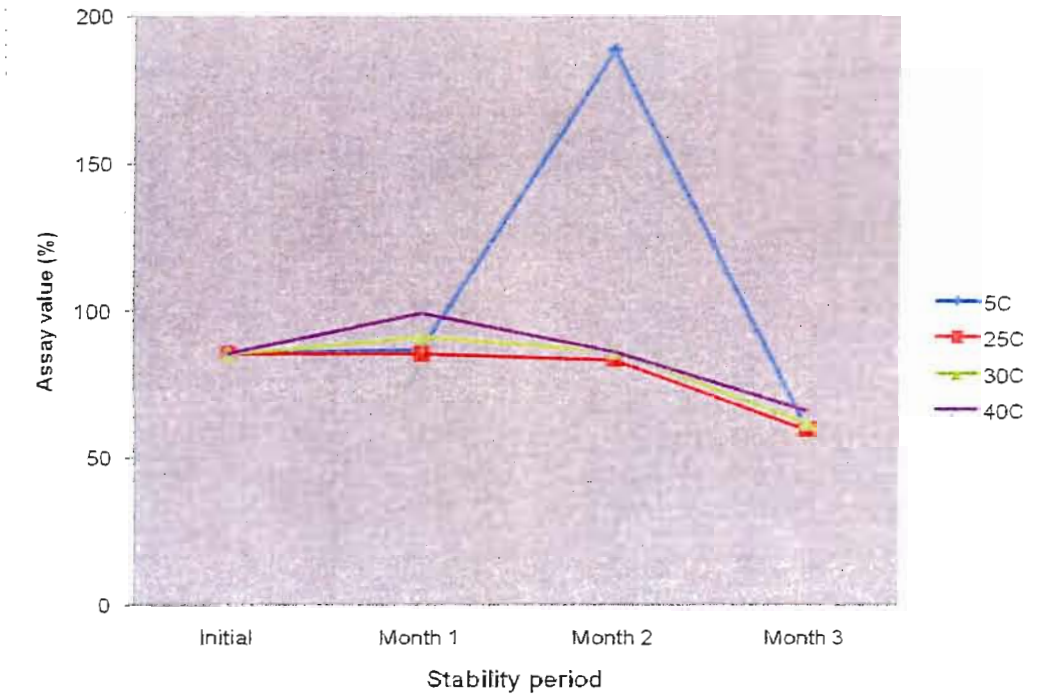


Figure 4.3 **Butylparaben in nevirapine pro-Pheroid formulation measured at 215 nm**

The percentage difference in the assay results of the butylparaben samples measured at 215 nm in relation to the initial assay results is given in table 4.6.

Table 4.6 **Percentage difference in assay results of butylparaben (215 nm) in pro-Pheroid**

Temperature & Humidity	Month 1 (%)	Month 2 (%)	Month 3 (%)
5°C	1.56	121.30	-29.32
25°C + 60% RH	-0.05	-2.34	-30.14
30°C + 65% RH	6.96	1.06	-27.46
40°C + 75% RH	16.33	0.82	-22.72

4.6.2.2 Results for butylparaben measured at 254 nm

The assay results for butylparaben measured at 254 nm are given in table 4.7 and illustrated in figure 4.4. The acceptance criteria for this product are 90.0 – 120.0% (BP, 2009).

Table 4.7 **Assay results of butylparaben in pro-Pheroid at 254 nm**

Initial (%) (%RSD)	Temperature & Humidity	Month 1 (%) (%RSD)	Month 2 (%) (%RSD)	Month 3 (%) (%RSD)
99.30 (12.80)	5°C	88.44 (3.13)	95.89 (0.48)	61.86 (3.20)
	25°C + 60% RH	91.18 (0.89)	115.36 (17.97)	71.70 (9.96)
	30°C + 65% RH	80.74 (5.04)	98.88 (0.75)	70.97 (9.49)
	40°C + 75% RH	81.24 (3.00)	95.64 (1.08)	66.92 (2.14)

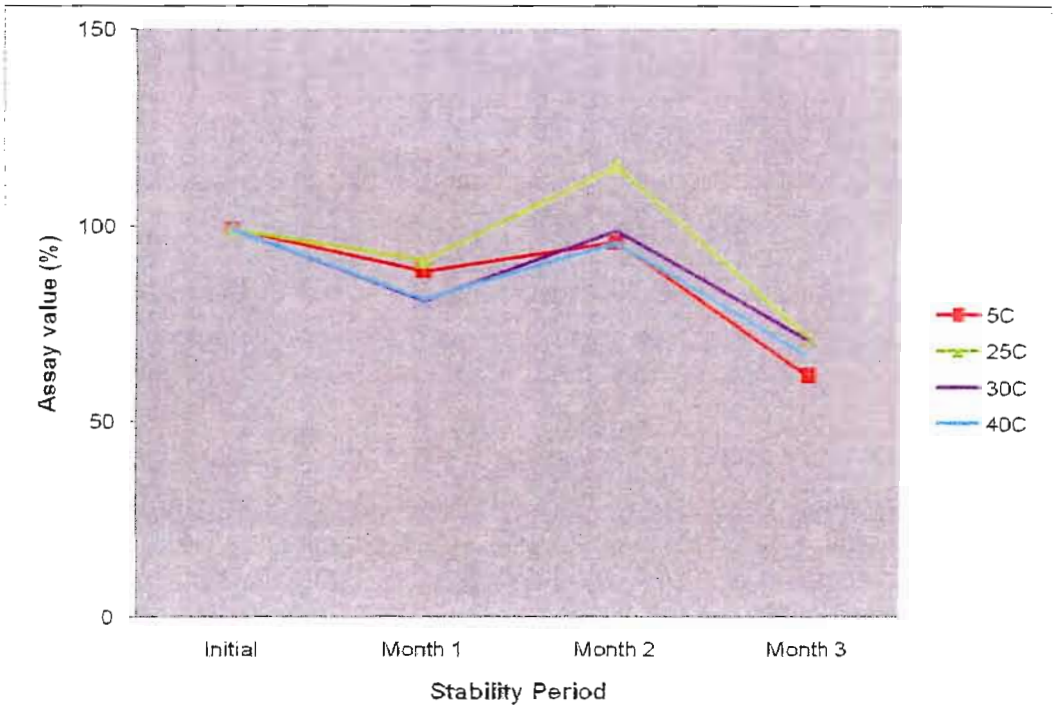


Figure 4.4 **Butylparaben in nevirapine pro-Pheroid formulation measured at 254 nm**

The percentage difference in the assay results of the butylparaben samples measured at 254 nm in relation to the initial assay results is given in table 4.8.

Table 4.8 **Percentage difference in assay results of butylparaben (254 nm) in pro-Pheroid**

Temperature & Humidity	Month 1 (%)	Month 2 (%)	Month 3 (%)
5°C	-10.94	-3.43	-37.70
25°C + 60% RH	-8.18	16.17	-27.79
30°C + 65% RH	-18.69	-0.42	-28.53
40°C + 75% RH	-18.19	-3.69	-32.61

4.6.2.3 Discussion

The assay done on butylparaben in the nevirapine / pro-Pheroid formulation measured at 215 nm had relatively stable assay results. Month 1 showed an increase in sample concentrations, except for 25°C + 60% RH, that had a decrease of 0.05% compared to the initial sample. The percentage relative standard deviation of each sample was reasonably good for all except the 30°C + 65% RH sample with a %RSD of 11.33. It can thus be assumed that little to none interference from other peaks happened during the HPLC analysis of the samples in month 1.

During month 2 of sample testing the three higher conditions had differences of between -2.34 to 1.06% compared to the initial sample. This gives one the idea that the 5°C sample's assay result might be an outlier as it is not in trend with the other assay results. It is assumed that the results obtained for the 5°C sample are due to a mistake made by the analyst. All four samples had very good peak repeatability as they all had excellent %RSD's.

As expected, the sample concentrations of all four stability conditions decreased during month 3 of the stability period. Although the sample concentrations decreased, ranging in persentage from 22.72% for 40°C + 75% RH to 30.14% for 25°C + 60% RH, and was well beneath the minimum specification range of 90%, it seemed to be still effective enough to prevent micro-organism growth (the preservative efficacy study is discussed in chapter 5). The decrease is suspected to be degradation of the butylparaben in this system.

When the assay results of butylparaben measured at 254 nm is analysed, one will notice a decrease in concentration for all samples during analysis in month 1. The decrease in concentration ranged between 8.18 – 18.69%. The percentage relative standard deviation of the four samples ranged from 0.89 for 25°C + 60% RH to 5.04 for 30°C + 65% RH, and shows good repeatability and only minor interferences from other peaks.

The assay results for month 2 revealed that all four samples were within the specification range of 90 – 120%. The 25°C + 60% RH had a concentration increase of 16.17% compared to the initial sample, but had a %RSD of 17.97 which shows possible interference from other peaks and could possibly have an incorrect concentration. The other three temperatures and humidities all had a decrease in

concentration and had excellent percentage relative standard deviations. This shows good repeatability and trustworthiness of the assay results.

Sample concentrations of butylparaben for month 3 uncovered a decrease of between 27.79% for 25°C + 60% RH and 37.70% for 5°C compared to the initial sample. The percentage relative standard deviations of the four samples ranged between 2.14 – 9.96. Fairly good repeatability is thus revealed, although the 9.49 and 9.96 %RSD's of 30°C + 65% RH and 25°C + 60% RH, respectively, is questionable.

When the two wavelengths are compared, one will again notice huge differences amongst them. With the exception of the 25°C + 60% RH sample, there was an increase of butylparaben concentration during month 1 at 215 nm, but at 254 nm a decrease within all four samples. During month 2 the 215 nm assay results showed that the 5°C sample had an increase of 121.3% (which is explained to possibly be an outlier), whilst the 30°C + 65% RH and 40°C + 75% RH samples had an increase of 1.06 and 0.82%, respectively, and the 25°C + 60% RH sample a decrease of 2.34% in concentration compared to the initial sample. The decrease in concentration during month 3 was relatively the same for both wavelengths.

4.7 Physico-chemical analysis

4.7.1 pH-values

The pH-value of the initial sample as well as all four of the stability conditions after each month was determined to further investigate the stability of the nevirapine / pro-Pheroid formulation in terms of physical stability. The results are given in table 4.9 and are shown in figure 4.5.

Table 4.9 pH-values

Initial	Temperature & Humidity	Month 1	Month 2	Month 3
7.58	5°C	8.47	8.44	8.57
	25°C + 60% RH	8.49	8.59	9.40
	30°C + 65% RH	8.37	8.51	9.27
	40°C + 75% RH	8.23	8.25	9.00

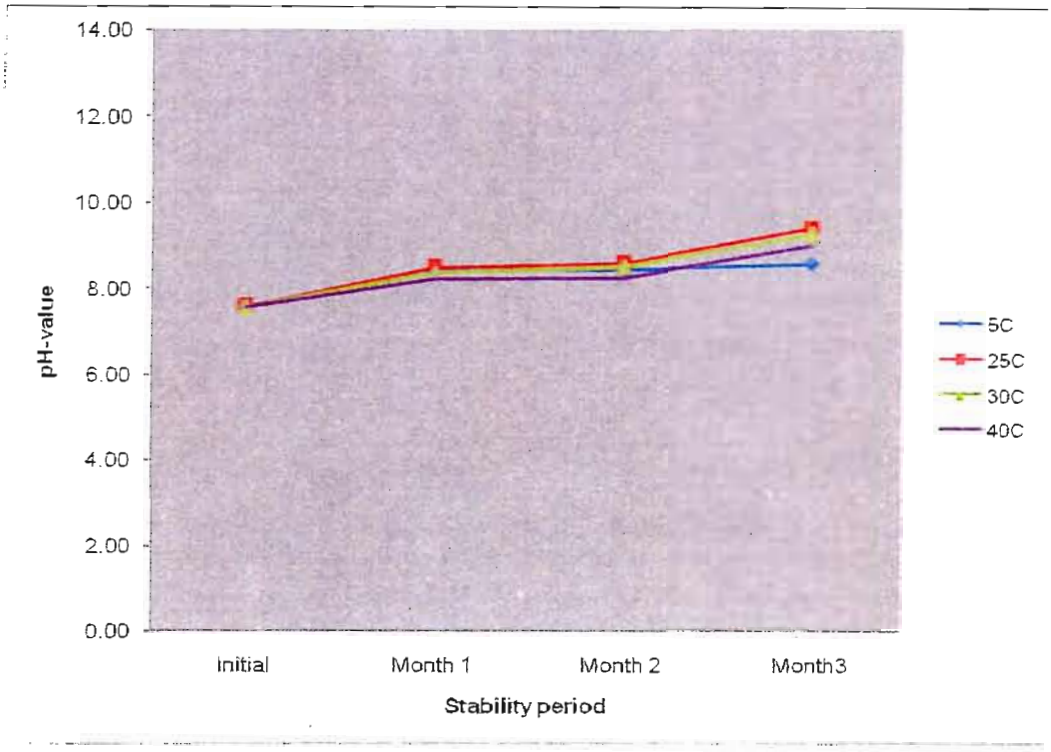


Figure 4.5 pH-values of the pro-Pheroid samples measured over three months

4.7.1.1 Discussion

As one can clearly see in figure 4.5, the pH-values of the sample remained relatively constant during the stability period of three months. The difference between the smallest pH-value (Initial sample: 7.58) and the greatest pH-value (Month 3, 25°C + 60% RH: 9.40) is only an increase of 1.82 over a period of three months. The standard deviation of all the pH-values is 0.47 and the relative standard deviation is 5.49%.This shows that the pro-Pheroid system is very stable in terms of pH, and that neither nevirapine nor butylparaben has any influence on the system to bring about that the pH of the system will be altered.

4.7.2 Particle size and confocal

The particle size of the samples was measured at the same intervals at which the HPLC analysis was done, initially, after 1, 2 and 3 months. The samples were stored under the exact same conditions, i.e. 5°C; 25°C+60%RH; 30°C+65%RH and 40°C+75%RH. The average distribution of all the samples troughout the stability

period was as follows: d(0.1) was 0.144 µm; d(0.5) was 0.199 µm; and d(0.9) was 0.293 µm. The particle sizes ranges from 0.112 µm to 0.564 µm. The particle size measured is supposedly only the pro-Pheroid particles as nevirapine is dissolved in the pro-Pheroid. The particle size of the nevirapine powder used was 25 µm. The size of the particles is ideal for the pro-Pheroid system as it falls in the ideal range for the Pheroid™ system described in chapter 2. The histogram of the particle size distribution of the initial sample is given in figure 4.6 as an example. The other particle size analysis reports are given in annexure 1.

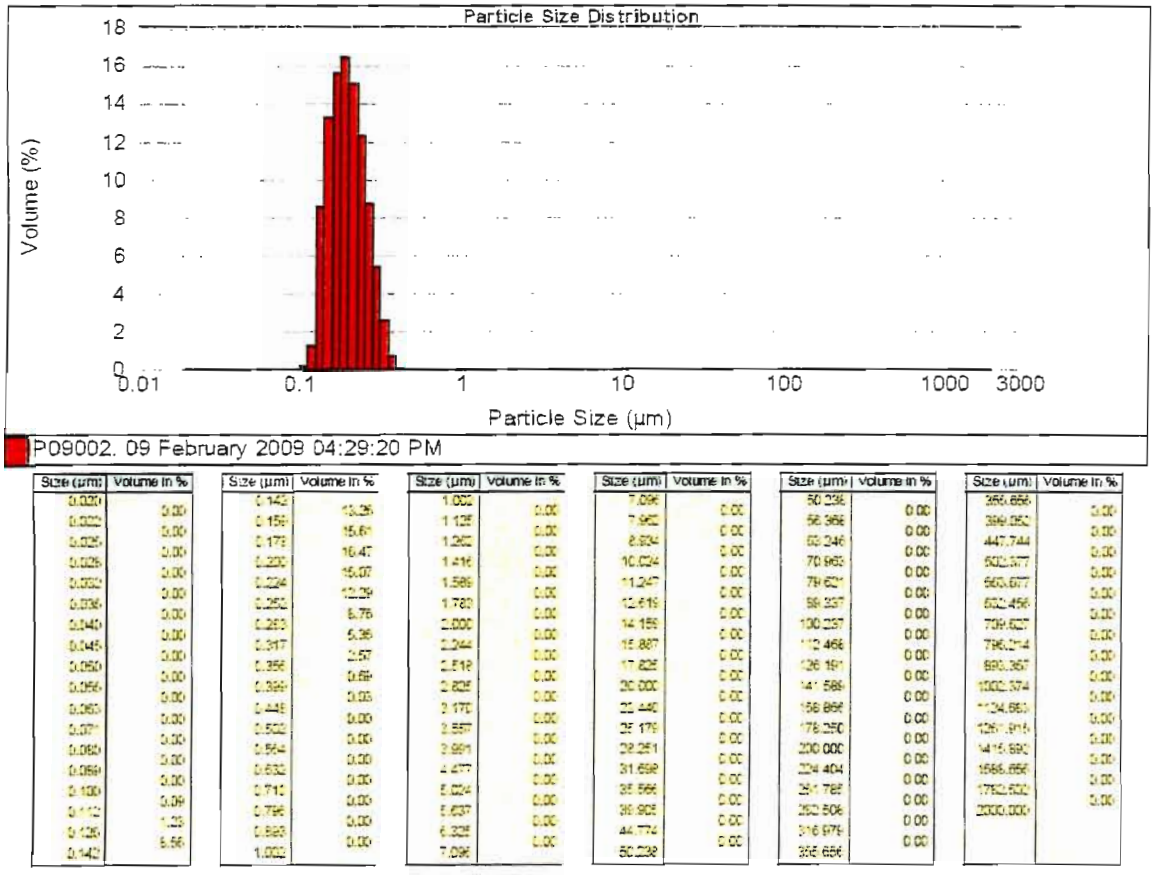


Figure 4.6 Particle size distribution analysis report for initial sample of nevirapine in pro-Pheroid

The particle size of each sample is summarised in figure 4.7.

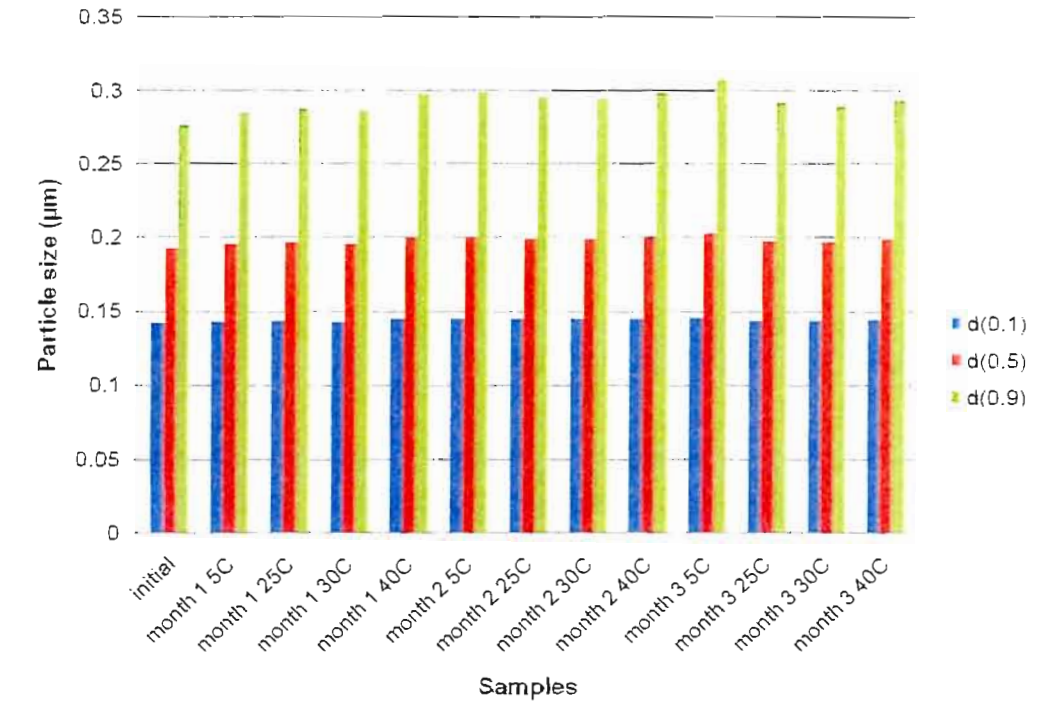


Figure 4.7 Particle size distribution of each sample over a period of three months

On the confocal pictures taken from the samples during the stability study, one can see how small the particles of the pro-Pheroid system is. The picture taken of the initial sample is shown in figure 4.8.

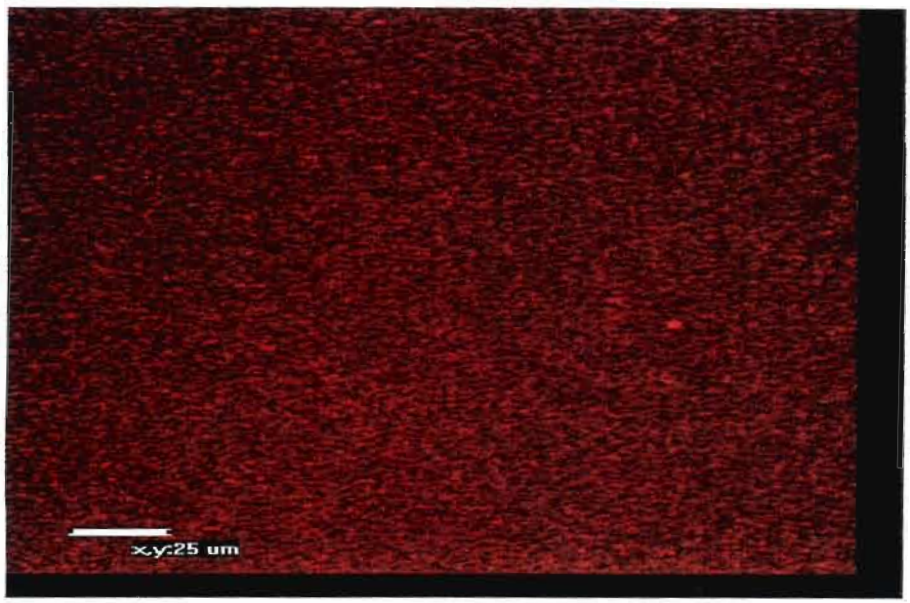


Figure 4.8 Confocal picture taken of initial sample

4.7.2.1 Discussion

The particle size of the samples stayed stable throughout the whole stability period. This shows that the nevirapine and butylparaben included in the formulation did not have any effects on the pro-Pheroid system in terms of particle size.

4.7.3 Colour

The colour of each sample was examined by taking photo's of each sample at time zero, month 1, month 2 and month 3. These pictures were compared and it was noticed that the colour of the samples changed both over time and because of temperature and humidity. The colour changed from a transparent yellow initially, to a darker yellow at 5°C and a very dark yellow at 40°C + 75% RH and became more and more intensified as time went by. These colour changes could possibly be the result of oxidation that the product underwent. Further studies could probably find a way to prevent oxidation from occurring.

4.8 Conclusion

The assays done on the pro-Pheroid samples showed random and sometimes unexplainable results. This confusion was exacerbated by measuring the samples at two different wavelengths. By carefully examining the results, it was noticed that

there might be hidden results at the lower wavelength that was possibly shown at the higher wavelength. This can possibly be the reason for the confusion and the random results obtained from the assay. With this said, it is also now very clear that each chemical structure has its own ideal wavelength where optimum assay results can be obtained.

Both the nevirapine as active ingredient and the butylparaben as the preservative did not comply with the specifications in the official pharmacopoeias, and therefore it is recommended that the formulation for the pro-Pheroid product should be reconsidered or should be investigated further in the future.

Although the chemical analysis of the nevirapine in pro-Pheroid revealed some instabilities and uncertainties, the physico-chemical analysis showed excellent results. Both the pH and the particle size of the product remained constant throughout the stability period, showing great stabilities in terms of the physico-chemical properties of the product.

The preservative efficacy of butylparaben is discussed in chapter 5.

CHAPTER 5

PRESERVATIVE EFFICACY STUDY

5.1 Introduction

Disinfectants, antiseptics and preservatives are chemicals that have the ability to destroy or inhibit the growth of micro-organisms and that are used for this purpose.

Preservatives are included in pharmaceutical preparations to prevent microbial spoilage of the product and to minimise the risk of the consumer acquiring an infection when the preparation is administered. Preservatives must be able to limit proliferation of micro-organisms that may be introduced unavoidably into non-sterile products such as oral and topical medications during their manufacture and use. It is essential that a preservative is not toxic in relation to the intended route of administration of the preserved preparation. Preservatives therefore tend to be employed at low concentrations, and consequently levels of antimicrobial action also tend to be of a lower order than for disinfectants or antiseptics (Hugo & Russell, 2004).

Preservation of the pharmaceutical product is therefore of utmost importance. According to Hugo and Russell (2004) micro-organisms can source from the following during the manufacturing process:

- the atmosphere;
- water;
- skin and respiratory tract flora;
- raw materials;
- packaging;
- buildings;
- equipment; and
- cleaning equipment and utensils.

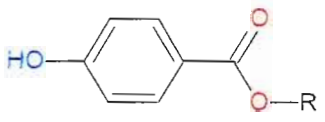
Pharmaceutical products used in the prevention, treatment and diagnosis of disease contain a wide variety of ingredients, often in quite complex physicochemical states. Such products must not only meet current pharmaceutical Good Manufacturing Practice (GMP) requirements for quality, safety and efficacy, but also must be stable and sufficiently elegant to be acceptable to patients. Products made in the

pharmaceutical industry must meet high microbiological specifications, i.e. if not sterile, they are expected to have no more than a minimal microbial population at the time of products release (Hugo & Russell, 2004).

Nevertheless, from time to time a few rogue products with an unacceptable level and type of contamination will occasionally escape the quality assurance net. The consequences of such contamination may be serious and far-reaching on several accounts, particularly if contaminants have had the opportunity to multiply to high levels. Firstly, the product may be spoiled, rendering it unfit for use through chemical and physicochemical deterioration of the formulation. Spoilage and subsequent wastage of individual batches usually results in major financial problems for the manufacturer through direct loss of faulty product. Secondly, the threat of litigation and the unwanted, damaging publicity of recalls may have serious economic implications for the manufacturer. Thirdly, inadvertent use of contaminated products may present a potential health hazard to patients, perhaps resulting in outbreaks of medicament-related infections, and ironically therefore contributing to the spread of disease. Most commonly, heavy contamination of product with opportunist pathogens, such as *Pseudomonas* spp., has resulted in the spread of nonsocomial infections in compromised patients; less frequently, low levels of contamination with pathogenic organisms, such as *Salmonella*, have attracted considerable attention, as have products contaminated with toxic microbial metabolites (Hugo & Russell, 2004).

A series of alkyl esters (figure 5.1) of *p*-hydroxybenzoic acid was originally prepared to overcome the marked pH dependence on activity of the acids. There parabens, the methyl, ethyl, propyl and butyl esters, are less readily ionised, having pK_a values in the range 8-8.5, and exhibit good preservative activity even at pH levels of 7-8, although optimum activity is again displayed in acidic solutions. This broader pH range allows extensive and successful use of the parabens as pharmaceutical preservatives. They are active against a wide range of fungi but are less so against bacteria, especially the pseudomonads, which may utilise the parabens as a carbon source. They are frequently used as preservatives of emulsions, creams and lotions where two phases exist. Combinations of esters are most successful for this type of product in that the more water-soluble methyl ester (0.25%) protects the aqueous phase, whereas the propyl or butyl esters (0.02%) give protection to the oil phase. Such combinations are also considered to extend the range of activity. As inactivation of parabens occurs with non-ionic surfactants due care should be taken

in formulation with both materials (Hugo & Russell, 2004). Butylparaben was used as the preservative in the pro-Pheroid formulation and was tested to determine the capability of this preservative to protect the product against microbial growth during accelerated stability testing.



R= CH_3; C_2H_5; C_3H_7; C_4H_9

Figure 5.1 Alkyl esters of *p*-hydroxybenzoic acid

The samples were sent to EnviroCare Laboratories to undergo the necessary and prescribed testing to determine the adequacy of the incorporated preservative. The guidelines of the tests are specified by the British Pharmacopoeia (BP, 2005). All tests were done by using calibrated and validated apparatus and standard operating procedures. The five micro-organisms used were *Staphylococcus aureus*, *Escherichia coli*, *Pseudomona aeruginosa*, *Candida albicans* and *Aspergillus niger* as specified by the British Pharmacopoeia.

5.2 Method for the preservative efficacy test

Preparation of inoculums

Preparatory to the test, inoculate the surface of agar medium TSA (Triptic soy agar) for bacteria and SDA (Sabouraud dextrose 4% agar) for fungi, with the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30-35°C for 18-24 h, the culture of *C. albicans* at 20-25°C for 48 h, and the culture of *A. niger* at 20-25°C for 1 week or until good sporulation is obtained. Subcultures may be needed after revival before the micro-organism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, use a sterile suspending fluid, containing 9 g/l of *sodium chloride* R, for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 10^8 micro-organisms per millilitre.

To harvest the *A. niger* culture, use a sterile suspending fluid containing 9 g/l of *sodium chloride* R and 0.5 g/l of *polysorbate 80* R and adjust the spore count to about 10^8 per millilitre by adding the same solution.

Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per millilitre in each suspension by plate count or membrane filtration. This value serves to determine the inoculums and the baseline to use in the test. The suspension shall be used immediately.

Method

To count the viable micro-organisms in the inoculated products, use the agar medium used for the initial cultivation of the respective micro-organisms.

Inoculate a series of containers of the product to be examined, each with a suspension of one of the test organisms to give an inoculum of 10^5 to 10^6 micro-organisms per millilitre or per gram of the preparation. The volume of the suspension of inoculum does not exceed 1 percent of the volume of the product. Mix thoroughly to ensure homogeneous distribution.

Maintain the inoculated product at 20 - 25°C, protected from light. Remove a suitable sample from each container, typically 1 ml or 1 g, at zero hour and at appropriate intervals according to the type of the product and determine the number of viable micro-organisms by plate count or membrane filtration. Ensure that any residual antimicrobial activity of the product is eliminated by dilution, by filtration or by the use of a specific inactivator. When dilution procedures are used, due to allowance is made for the reduced sensitivity in the recovery of small numbers of viable micro-organisms. When a specific inactivator is used, the ability of the system to support the growth of the test organisms is confirmed by the use of appropriate controls.

The procedure is validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

5.3 Results and discussion

The criteria for the evaluation of antimicrobial activity are shown in table 5.1, expressed in terms of the log reduction in the number of viable microorganisms and compared to the values obtained for the inoculum. The criteria in table 5.1 express the recommended efficacy to be achieved.

Table 5.1 **Oral preparation acceptance criteria**

	Log reduction	
	14 days	28 days
Bacteria	3	NI
Fungi	1	NI

NI: No increase

In this preservative study conducted, butylparaben acted as the preservative. It appeared efficient in the specific formulation, with the exception of a few border cases. For example, the preservative efficacy test of butylparaben in the nevirapine in pro-Pheroid formulation complied with the requirements of the BP, except for *Pseudomona aeruginosa* where the log reduction values was smaller than the required 3 during month 1 and 2 at the four stability conditions (table 5.2).

Table 5.2 Preservative efficacy test results of butylparaben in the nevirapine/pro-Pheroid formulation (initial and month 1)

Test organism	Total viable cell count (Cfu/mL)				
	0 hours	14 days	28 days	Log Reduction	
				0h – 14 days	0h – 28 days
Initial					
S. aureus	25×10^4	8×10^2	ND	>3	NI
P. aeruginosa	5×10^5	ND	ND	>3	NI
E. coli	2×10^5	ND	ND	>3	NI
A. niger	36×10^5	ND	ND	>1	NI
C. albicans	2×10^3	ND	ND	>1	NI
Month 1					
5°C					
S. aureus	15×10^5	13×10^1	6	>3	NI
P. aeruginosa	19×10^3	13×10^3	ND	1.7	NI
E. coli	19×10^3	3×10^2	ND	>3	NI
A. niger	4×10^3	ND	2×10^1	>1	NI
C. albicans	7×10^3	ND	ND	>1	NI
25°C + 60% RH					
S. aureus	19×10^5	2×10^1	5×10^1	>3	NI
P. aeruginosa	47×10^3	11×10^2	ND	2.3	NI
E. coli	48×10^3	ND	ND	>3	NI
A. niger	5×10^3	10×10^1	ND	>1	NI
C. albicans	7×10^3	ND	ND	>1	NI
30°C + 65% RH					
S. aureus	27×10^5	15	ND	>3	NI
P. aeruginosa	29×10^3	18×10^2	ND	2.2	NI
E. coli	4×10^4	ND	ND	>3	NI
A. niger	12×10^3	8×10^1	ND	>1	NI
C. albicans	55×10^2	ND	ND	>1	NI
40°C + 75% RH					
S. aureus	23×10^5	1×10^1	1×10^1	>3	NI
P. aeruginosa	33×10^3	12×10^2	7×10^1	2.3	NI
E. coli	15×10^4	ND	ND	>3	NI
A. niger	$>10^3$	3×10^1	ND	>1	NI
C. albicans	7×10^3	66×10^2	6×10^2	>1	NI

*ND: None detected

Table 5.3 Preservative efficacy test results of butylparaben in the nevirapine/pro-Pheroid formulation (month 2)

Test organism	Total viable cell count (Cfu/mL)				
	0 hours	14 days	28 days	Log Reduction	
				0h – 14 days	0h – 28 days
5°C					
S. aureus	25×10^4	ND	ND	>3	NI
P. aeruginosa	87×10^5	11×10^3	12×10^2	2.3	NI
E. coli	273×10^5	ND	ND	>3	NI
A. niger	83×10^1	2×10^1	ND	>1	NI
C. albicans	2×10^3	ND	ND	>1	NI
25°C ÷ 60% RH					
S. aureus	243×10^3	ND	ND	>3	NI
P. aeruginosa	103×10^3	11×10^3	85×10^2	2.3	2.9
E. coli	75×10^4	ND	ND	>3	NI
A. niger	17×10^2	ND	ND	>1	NI
C. albicans	1×10^3	ND	ND	>1	NI
30°C ÷ 65% RH					
S. aureus	394×10^3	ND	ND	>3	NI
P. aeruginosa	73×10^4	53×10^2	7×10^3	2.5	2.4
E. coli	65×10^3	ND	ND	>3	NI
A. niger	9×10^2	1×10^1	ND	>1	NI
C. albicans	3×10^3	ND	ND	>1	NI
40°C ÷ 75% RH					
S. aureus	395×10^3	ND	ND	>3	NI
P. aeruginosa	166×10^4	115×10^1	12×10^3	2	1.5
E. coli	210×10^5	ND	ND	>3	NI
A. niger	10×10^2	1×10^1	ND	>1	NI
C. albicans	5×10^3	ND	ND	>1	NI

*ND: None detected

During months 1 and 2 of the stability program, butylparaben seemed inadequate to preserve the pro-Pheroid formulation against *P. aeruginosa* at all four different conditions. The possible explanation for these unexpected results is that the amount of inoculated *Pseudomonas aeruginosa* was much higher than the other months. It is thus due to a human mistake that butylparaben seemed inadequate to preserve the pro-Pheroid formulation against *P. aeruginosa*. The recommendation is to increase the concentration of butylparaben in the formulation. Butylparaben was successful in preventing growth of any of the other four micro-organisms during month 2.

Table 5.4 Preservative efficacy test results of butylparaben in the nevirapine/pro-Pheroid formulation (month 3)

Test organism	Total viable cell count (Cfu/mL)				
	0 hours	14 days	28 days	Log Reduction	
				0h – 14 days	0h – 28 days
5°C					
S. aureus	117×10^4	ND	ND	>3	NI
P. aeruginosa	274×10^4	ND	ND	>3	NI
E. coli	42×10^4	ND	ND	>3	NI
A. niger	66×10^2	5×10^2	86	>1	NI
C. albicans	3×10^4	ND	ND	>1	NI
25°C + 60% RH					
S. aureus	174×10^4	ND	ND	>3	NI
P. aeruginosa	77×10^4	ND	ND	>3	NI
E. coli	6×10^4	ND	ND	>3	NI
A. niger	33×10^2	2×10^2	ND	>1	NI
C. albicans	5×10^4	ND	ND	>1	NI
30°C + 65% RH					
S. aureus	41×10^4	ND	ND	>3	NI
P. aeruginosa	468×10^4	ND	ND	>3	NI
E. coli	30×10^4	ND	ND	>3	NI
A. niger	46×10^2	13×10^2	ND	>1	NI
C. albicans	133×10^2	ND	ND	>1	NI
40°C + 75% RH					
S. aureus	71×10^4	ND	ND	>3	NI
P. aeruginosa	676×10^3	ND	ND	>3	NI
E. coli	429×10^4	ND	ND	>3	NI
A. niger	5×10^3	16×10^2	53	>1	NI
C. albicans	106×10^2	ND	ND	>1	NI

*ND: None detected

The results received from the EnviroCare Laboratory for the preservative efficacy test for month 3 of the stability process showed that butylparaben was successful in preventing growth in any of the micro-organisms, as it complied with the requirements of the British Pharmacopoeia.

5.4 Conclusion

Preservation of any pharmaceutical product is of utmost importance. The preservative efficacy study done by the EnviroCare Laboratory showed that butylparaben is a very suitable preservative in a formulation that is lipophilic. It is also recommended that either the concentration of the butylparaben is increased or a second preservative is used to secure the stability of the product against microbiological damage. When increasing the concentration of the preservative, one should be very careful not to increase it to such an extent that it will become harmful to the patient's health when using the product. It is important to remember that the preservative is an additive and should not be the alpha and omega of a formulation, but it should rather "protect" the product without being noticed.

In light of the possibility that a human error might have occurred during the inoculation with *Pseudomonas aeruginosa* during months 1 and 2, it would be wise to repeat the preservative study to gather necessary information to make a well-thought decision and to verify the results.

CHAPTER 6

CONCLUSION

After the completion of this study, various conclusions could be made. HIV / AIDS is most definitely one of the most problematic and destructive diseases known to mankind in the twenty-first century. It affects all of us, from the homeless to the rich and famous, any race, any culture, any gender and every age. Millions, if not billions, of dollars are spent on research and treatment of this disease each and every year. Governments spend a significant amount on informing communities and their people on the prevention and treatment of HIV and AIDS. It is thus clear that it is the responsibility of each and every one of us to try to prevent further spreading of this virus by practising safe sex, informing friends, colleagues, children and family members on the dangers of the disease, and to make sure that those infected with the virus are not treated differently and that they get the necessary support to help them lead a normal and full life. If everybody works together, we can make it possible to have an HIV free future.

The Pheroid™ delivery system has shown various exciting and promising characteristics to make it a very important factor to consider when formulating a product. It promises to change the way future pharmaceutical products will be formulated. The Pheroid™ technology has various advantages in terms of absorption and/or efficacy of pharmaceutical active compounds and other useful molecules. As described in chapter 2, the Pheroid™ system allows for manipulation of both its structural and functional features, which makes it a delivery system to be reckoned with in the pharmaceutical industry. The product that was formulated for this study manipulated the Pheroid™ delivery system with increased concentrations and larger polymers, i.e. pro-Pheroid, during the manufacturing process. Nevirapine is the active ingredient in the product and was formulated to be used as an oral antiretroviral for paediatric patients. The formulation included a suitable preservative to keep the product safe against the growth of micro-organisms and to protect the consumer against the harmful effects of the consumption of micro-organisms in large quantities.

The pharmacological and physico-chemical properties of nevirapine were discussed in chapter 2. The solubility of nevirapine was taken into consideration when it was formulated into the pro-Pheroid delivery system as nevirapine in lipophilic. As the

product is lipophilic, it was only apparent to use butylparaben as the preservative because of its solubility in an oil-based formula.

The formulated product, from here on further referred to as nevirapine in pro-Pheroid, was stored at four different temperature-humidity conditions and underwent an accelerated stability study over a period of three months. Initially (time=0) the following studies were performed: HPLC assay analysis, pH determination, particle size analysis and a preservative efficacy study. The confocal microscope picture as well as the physical colour of the various samples were also taken. After months 1, 2 and 3, these studies were repeated, the results processed and compared with other results in the same category.

During the HPLC assay analysis of nevirapine in pro-Pheroid the samples were analysed for nevirapine as the active ingredient and butylparaben as the preservative. Both were analysed at two separate wavelengths, i.e. 215 and 254 nm. Thus, the assay results could be compared with each other in terms of time of analysis, wavelength and temperature-humidity conditions. The following conclusions can thus be made regarding the use of nevirapine in pro-Pheroid:

- Nevirapine can successfully be formulated in the pro-Pheroid system.
- It seems that butylparaben can be used together with nevirapine as preservative.
- 5°C, 25°C + 60% RH, 30°C + 65% RH and 40°C + 75% RH all showed similar results throughout the stability program for both wavelengths and both ingredients, however, 25°C + 60% RH, 30°C + 65% RH and 40°C + 75% RH had a different pattern during month 3 of testing for nevirapine at 254 nm and 5°C revealed an outlier during month 2 of testing for butylparaben at 215 nm.
- The optimum and ideal wavelength for the UV detection of nevirapine seems to be 215 nm.
- Both 215 nm and 254 nm can be used to detect butylparaben in the pro-Pheroid system.

The preservative efficacy study showed that butylparaben is effective in pro-Pheroid, although it is suggested that the concentration of butylparaben be increased and/or butylparaben be combined with another preservative to secure the efficacy of the preservative(s). The HPLC assay analysis and preservative efficacy had contrasting results because HPLC analysis showed that butylparaben had a concentration short

of that of the specification by the British Pharmacopoeia, but the preservative efficacy study showed that the low concentration of butylparaben was still sufficient enough to prevent the growth of the five inoculated micro-organisms. It can thus be suggested that the specifications should be reconsidered.

The stability of both the pH-value and the particle size of nevirapine in pro-Pheroid show that the product is very stable in terms of the physico-chemical properties of the product. The extremely small particles of the pro-Pheroid system make this product very valuable in the delivery of nevirapine.

It is finally concluded that before nevirapine in pro-Pheroid is further formulated into viable products, the following issues will have to be addressed:

- The pro-Pheroid manufacturing process should be assessed and validated to ensure batch to batch uniformity.
- The pro-Pheroid analytical method should be validated and be evaluated over a period of six months to confirm the stability of the system, without any added active compounds.
- The specific UV detection wavelengths of both nevirapine and butylparaben should be assessed and validated to get the optimum wavelength for HPLC assay analysis to ensure the integrity of the results obtained.
- The physical properties (colour, smell and taste) of the pro-Pheroid system need to be addressed to make the product acceptable to the consumer.

Future studies on the pro-Pheroid delivery system would thus focus on formulating a product that is affordable, effective, save and have excellent quality to treat HIV in paediatric patients.

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ANNEXURE A

PARTICLE SIZE DISTRIBUTION ANALYSIS REPORTS

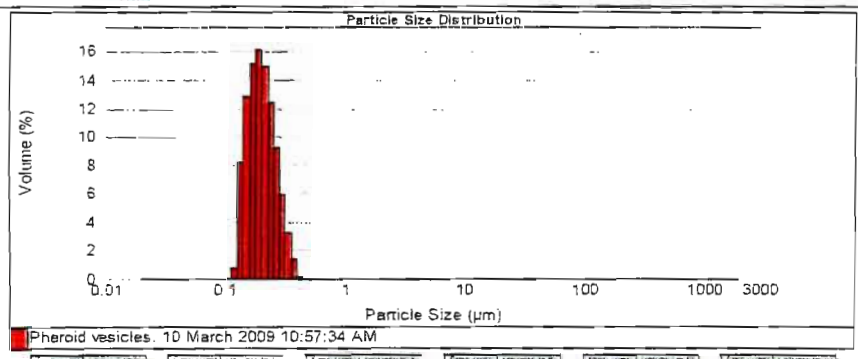
Month 1 5°C

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pheroid vesicles	**SOP Name:** Pheroid vesicles usmp Hydro 2000 MU	**Measured:** 10 March 2009 10:57:34 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marie	**Analysed:** 10 March 2009 10:57:35 AM	
Sample bulk lot ref: 1 month 5 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 12.64 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 22.001 %	**Result Emulation:** Off
Concentration: 0.0027 %Vol	**Span :** 0.726	**Uniformity:** 0.229	**Result units:** Volume
Specific Surface Area: 31.1 m²/g	**Surface Weighted Mean D[3,2]:** 0.193 um	**Vol. Weighted Mean D[4,3]:** 0.207 um	

d(0.1): 0.143 um d(0.5): 0.196 um d(0.9): 0.285 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007642

File name: 04 Mar 09
Record Number: 46

Month 1 25°C + 60% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pheroid vesicles
Sample Source & type: P08002
Sample bulk lot ref: 1 month 25 C

SOP Name: Pheroid vesicles using Hydro 2000 MU
Measured by: Liezl-Marié
Result Source: Measurement

Measured: 10 March 2009 11:01:29 AM
Analysed: 10 March 2009 11:01:30 AM

Particle Name: Oleic Acid
Particle RI: 1.458
Dispersant Name: Water

Accessory Name: Hydro 2000MU (A)
Absorption: 0.1
Dispersant RI: 1.330

Analysis model: General purpose
Size range: 0.020 to 2000.000 um
Weighted Residual: 21.720 %

Sensitivity: Enhanced
Obscuration: 10.91 %
Result Emulation: Off

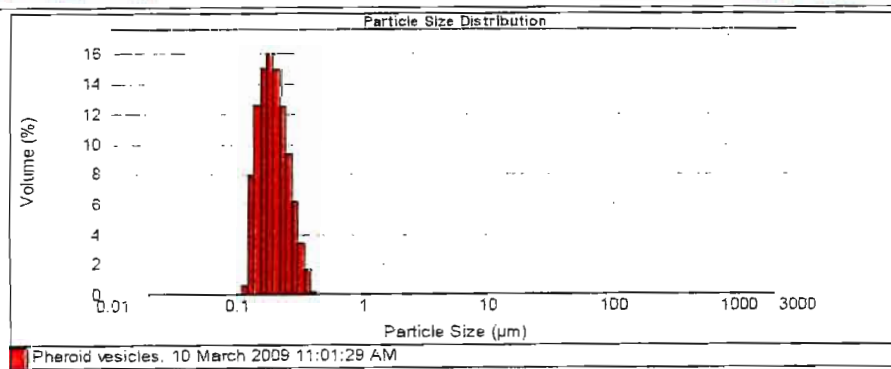
Concentration: 0.0023 %Vol
Specific Surface Area: 30.9 m²/g

Span : 0.733
Surface Weighted Mean D[3,2]: 0.194 um

Uniformity: 0.23
Vol. Weighted Mean D[4,3]: 0.208 um

Result units: Volume

d(0.1): 0.144 um d(0.5): 0.187 um d(0.9): 0.258 um



Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %
0.020	0.00	0.144	12.54	1.152	0.00	9.224	0.00	73.855	0.00	591.406	0.00
0.023	0.00	0.165	15.00	1.322	0.00	10.594	0.00	84.806	0.00	678.912	0.00
0.026	0.00	0.177	16.02	1.518	0.00	12.170	0.00	97.402	0.00	779.317	0.00
0.030	0.00	0.203	14.90	1.743	0.00	13.976	0.00	111.848	0.00	894.963	0.00
0.034	0.00	0.234	12.48	2.002	0.00	16.050	0.00	128.433	0.00	1027.126	0.00
0.039	0.00	0.269	9.29	2.299	0.00	18.430	0.00	147.446	0.00	1179.744	0.00
0.045	0.00	0.309	6.04	2.640	0.00	21.164	0.00	169.275	0.00	1354.126	0.00
0.052	0.00	0.355	3.43	3.031	0.00	24.300	0.00	194.328	0.00	1554.883	0.00
0.059	0.00	0.408	1.59	3.481	0.00	27.900	0.00	223.094	0.00	1784.821	0.00
0.068	0.00	0.468	0.55	3.997	0.00	32.031	0.00	256.090	0.00	2048.400	0.00
0.078	0.00	0.538	0.10	4.590	0.00	36.771	0.00	293.969	0.00		
0.090	0.00	0.617	0.00	5.271	0.00	42.218	0.00	337.500	0.00		
0.103	0.00	0.709	0.00	6.052	0.00	48.472	0.00	387.570	0.00		
0.119	0.00	0.813	0.00	6.950	0.00	55.657	0.00	445.172	0.00		
0.136	0.00	0.934	0.00	7.980	0.00	63.913	0.00	511.414	0.00		
0.157	0.00	1.072	0.00	9.162	0.00	73.404	0.00	587.515	0.00		

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number: : MAL1007542

File name: 04 Mth 05
Record Number: 47

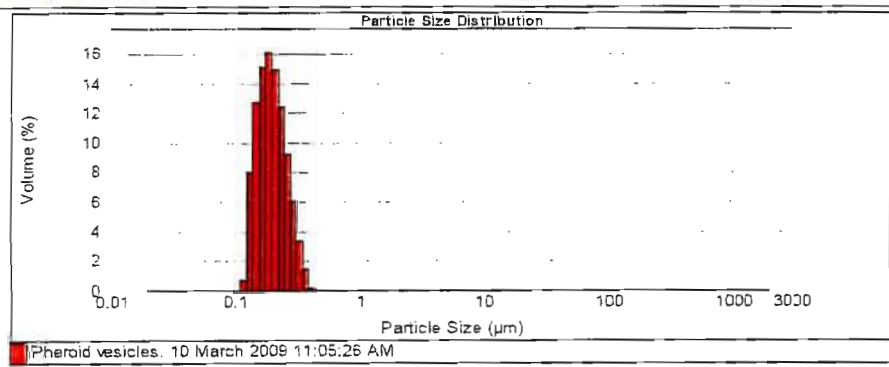
Month 1 30°C + 65% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pheroid vesicles	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 10 March 2009 11:05:26 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 10 March 2009 11:05:27 AM	
Sample bulk lot ref: 1 month 30 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 11.22 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.621 %	**Result Emulation:** Off
Concentration: 0.0024 %Vol	**Span :** 0.729	**Uniformity:** 0.229	**Result units:** Volume
Specific Surface Area: 31 m²/g	**Surface Weighted Mean D[3,2]:** 0.194 um	**Vol. Weighted Mean D[4,3]:** 0.207 um	

d(0.1): 0.143 um d(0.5): 0.196 um d(0.9): 0.267 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number: MAL1007542

File name: 04 Mth 05
Record Number: 49

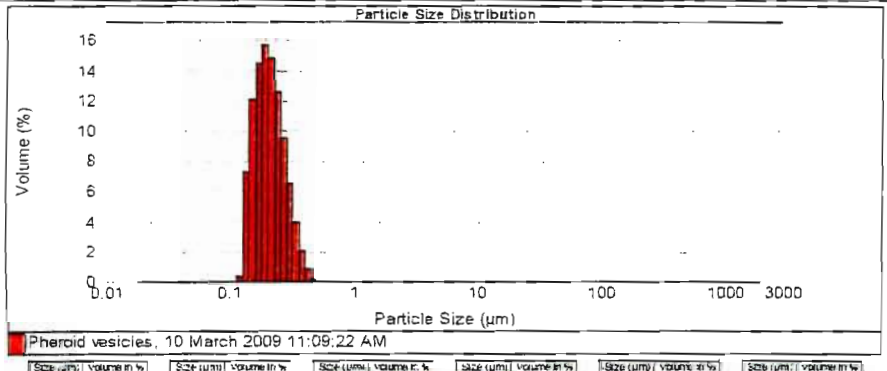
Month 1 40°C + 75% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pheroid vesicles	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 10 March 2009 11:09:22 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 10 March 2009 11:09:23 AM	
Sample bulk lot ref: 1 month 40 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 18.51 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 20.546 %	**Result Emulation:** Off
Concentration: 0.0034 %Vol	**Span :** 0.764	**Uniformity:** 0.24	**Result units:** Volume
Specific Surface Area: 30.3 m²/g	**Surface Weighted Mean D[3,2]:** 0.198 um	**Vol. Weighted Mean D[4,3]:** 0.213 um	

d(0.1): 0.145 um d(0.5): 0.200 um d(0.9): 0.298 um



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.000	0.04	7.054	0.00	49.766	0.00	351.056	0.00
0.022	0.00	0.154	0.00	1.107	0.00	7.952	0.00	56.356	0.00	396.052	0.00
0.024	0.00	0.170	0.00	1.263	0.00	8.974	0.00	63.246	0.00	447.744	0.00
0.026	0.00	0.190	0.00	1.418	0.00	10.024	0.00	70.963	0.00	505.387	0.00
0.028	0.00	0.214	0.00	1.581	0.00	11.247	0.00	79.621	0.00	570.455	0.00
0.031	0.00	0.242	0.00	1.783	0.00	12.611	0.00	89.337	0.00	643.454	0.00
0.034	0.00	0.273	0.00	2.000	0.00	14.155	0.00	100.337	0.00	726.027	0.00
0.038	0.00	0.307	0.00	2.244	0.00	15.887	0.00	112.466	0.00	819.214	0.00
0.043	0.00	0.347	0.00	2.518	0.00	17.829	0.00	126.191	0.00	924.387	0.00
0.048	0.00	0.389	0.00	2.825	0.00	20.000	0.00	141.586	0.00	1042.374	0.00
0.054	0.00	0.438	0.00	3.170	0.00	22.440	0.00	158.856	0.00	1174.886	0.00
0.060	0.00	0.492	0.00	3.557	0.00	25.179	0.00	178.250	0.00	1323.315	0.00
0.067	0.00	0.552	0.00	3.991	0.00	28.251	0.00	200.000	0.00	1491.990	0.00
0.076	0.00	0.619	0.00	4.477	0.00	31.698	0.00	224.404	0.00	1681.856	0.00
0.085	0.00	0.695	0.00	5.024	0.00	35.566	0.00	251.785	0.00	1895.522	0.00
0.095	0.00	0.780	0.00	5.637	0.00	39.905	0.00	282.508	0.00	2000.000	0.00
0.107	0.00	0.875	0.00	6.325	0.00	44.774	0.00	316.979	0.00		
0.120	0.00	0.982	0.00	7.096	0.00	50.234	0.00	355.656	0.00		

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

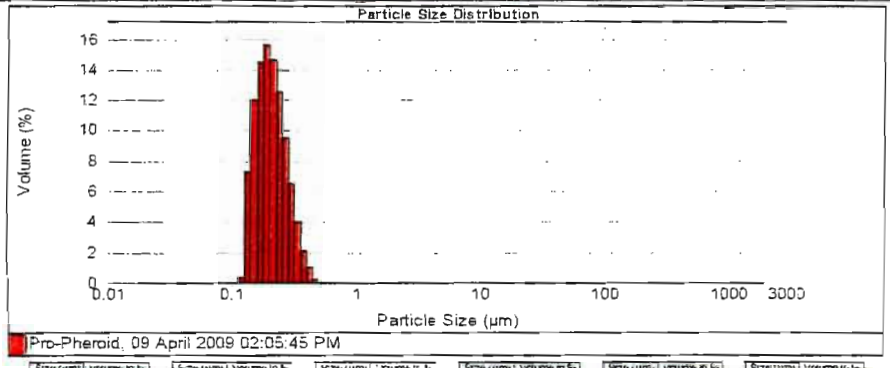
Month 2 5°C

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 09 April 2009 02:05:45 PM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 09 April 2009 02:05:46 PM	
Sample bulk lot ref: 5C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.456	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 11.11 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 20.662 %	**Result Emulation:** Off
Concentration: 0.0023 %Vol	**Span :** 0.772	**Uniformity:** 0.243	**Result units:** Volume
Specific Surface Area: 30.3 m²/g	**Surface Weighted Mean D[3,2]:** 0.198 um	**Vol. Weighted Mean D[4,3]:** 0.214 um	

d(0.1): 0.145 um d(0.5): 0.201 um d(0.9): 0.300 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1037548

File name: 04 Mn 05
Record Number: 84

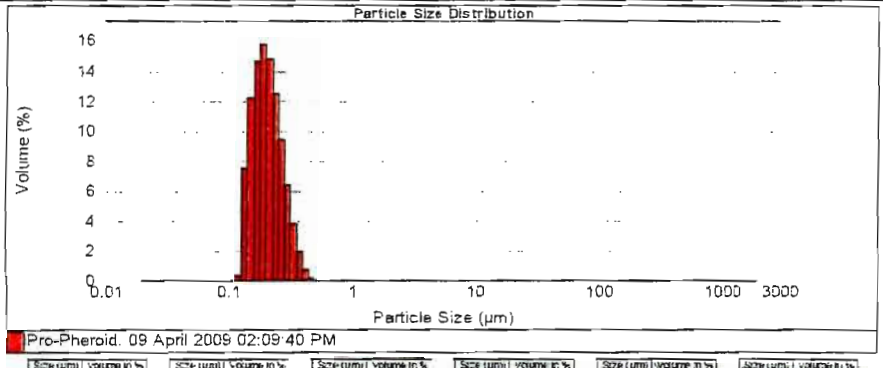
Month 2 25°C + 60% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 09 April 2009 02:09:40 PM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 09 April 2009 02:09:41 PM	
Sample bulk lot ref: 25C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 12.50 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.108 %	**Result Emulation:** Off
Concentration: 0.0027 %Vol	**Span :** 0.758	**Uniformity:** 0.238	**Result units:** Volume
Specific Surface Area: 30.5 m²/g	**Surface Weighted Mean D[3,2]:** 0.197 um	**Vol. Weighted Mean D[4,3]:** 0.212 um	

d(0.1): 0.145 um d(0.5): 0.199 um d(0.9): 0.296 um



Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %
0.020	0.00	0.142	0.00	1.000	0.00	7.054	0.00	49.770	0.00	351.130	0.00
0.022	0.00	0.154	14.62	1.125	0.00	7.942	0.00	56.058	0.00	395.352	0.00
0.025	0.00	0.173	15.71	1.265	0.00	8.934	0.00	63.096	0.00	445.044	0.00
0.028	0.00	0.195	14.77	1.416	0.00	10.054	0.00	70.963	0.00	500.917	0.00
0.032	0.00	0.219	12.47	1.585	0.00	11.314	0.00	79.870	0.00	563.677	0.00
0.036	0.00	0.250	9.42	1.782	0.00	12.735	0.00	89.937	0.00	632.494	0.00
0.040	0.00	0.283	6.36	2.000	0.00	14.338	0.00	100.237	0.00	709.627	0.00
0.045	0.00	0.317	3.83	2.244	0.00	16.137	0.00	112.868	0.00	795.214	0.00
0.050	0.00	0.355	2.02	2.512	0.00	17.825	0.00	126.791	0.00	890.367	0.00
0.056	0.00	0.399	0.71	2.825	0.00	20.000	0.00	141.589	0.00	1000.274	0.00
0.063	0.00	0.448	0.09	3.170	0.00	22.440	0.00	159.666	0.00	1124.681	0.00
0.071	0.00	0.502	0.00	3.557	0.00	25.179	0.00	178.250	0.00	1265.815	0.00
0.080	0.00	0.564	0.00	3.991	0.00	28.251	0.00	200.000	0.00	1415.982	0.00
0.089	0.00	0.633	0.00	4.477	0.00	31.690	0.00	224.404	0.00	1586.850	0.00
0.100	0.00	0.710	0.00	5.024	0.00	35.566	0.00	251.785	0.00	1779.520	0.00
0.112	0.00	0.796	0.00	5.637	0.00	39.905	0.00	282.506	0.00	2000.000	0.00
0.126	0.00	0.893	0.00	6.325	0.00	44.774	0.00	316.979	0.00		
0.142	0.00	1.002	0.00	7.094	0.00	50.238	0.00	355.656	0.00		

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place.*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number: MAL1007548

File name: 14 Mar 09
Record Number: 86

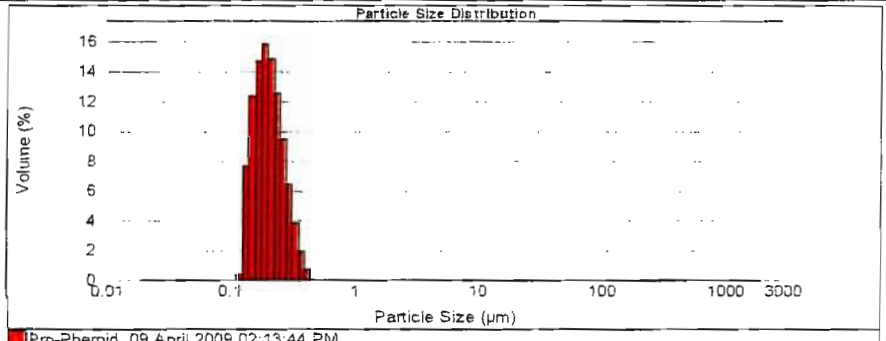
Month 2 30°C + 65% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 09 April 2009 02:13:44 PM	
Sample Source & type: P09002	**Measured by:** Liezl-Mare	**Analysed:** 09 April 2009 02:13:45 PM	
Sample bulk lot ref: 30 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 10.50 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.267 %	**Result Emulation:** Off
Concentration: 0.0022 %Vol	**Span :** 0.755	**Uniformity:** 0.238	**Result units:** Volume
Specific Surface Area: 30.5 m²/g	**Surface Weighted Mean D[3,2]:** 0.197 um	**Vol. Weighted Mean D[4,3]:** 0.211 um	

d(0.1): 0.145 um d(0.5): 0.199 um d(0.9): 0.295 um



Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %	Size (µm)	Volume in %
0.020	0.00	0.142	12.28	1.000	0.00	7.054	0.00	49.796	0.00	351.556	0.00
0.022	0.00	0.150	14.64	1.062	0.00	7.942	0.00	56.356	0.00	396.062	0.00
0.024	0.00	0.159	15.79	1.128	0.00	8.944	0.00	63.246	0.00	446.244	0.00
0.025	0.00	0.169	14.79	1.197	0.00	10.074	0.00	70.945	0.00	502.677	0.00
0.028	0.00	0.179	12.47	1.271	0.00	11.345	0.00	79.527	0.00	566.027	0.00
0.030	0.00	0.190	9.42	1.349	0.00	12.775	0.00	89.337	0.00	637.456	0.00
0.032	0.00	0.202	6.37	1.432	0.00	14.384	0.00	100.227	0.00	717.827	0.00
0.034	0.00	0.214	3.77	1.520	0.00	16.197	0.00	112.466	0.00	808.214	0.00
0.036	0.00	0.227	1.91	1.613	0.00	18.237	0.00	126.191	0.00	910.357	0.00
0.038	0.00	0.241	0.86	1.712	0.00	20.535	0.00	141.588	0.00	1025.374	0.00
0.041	0.00	0.255	0.26	1.817	0.00	23.119	0.00	158.866	0.00	1154.662	0.00
0.043	0.00	0.271	0.06	1.928	0.00	26.021	0.00	178.255	0.00	1300.315	0.00
0.046	0.00	0.287	0.00	2.046	0.00	29.273	0.00	200.000	0.00	1464.662	0.00
0.049	0.00	0.304	0.00	2.171	0.00	32.911	0.00	224.404	0.00	1649.899	0.00
0.052	0.00	0.323	0.00	2.304	0.00	36.974	0.00	251.785	0.00	1858.662	0.00
0.055	0.00	0.342	0.00	2.445	0.00	41.506	0.00	282.506	0.00	2093.000	0.00
0.059	0.00	0.363	0.00	2.594	0.00	46.557	0.00	316.979	0.00		
0.062	0.00	0.385	0.00	2.753	0.00	52.183	0.00	355.656	0.00		

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer: 2000 Ver. 5.31
Serial Number : MAL1007542

File name: 04 Mn 09
Record Number: 92

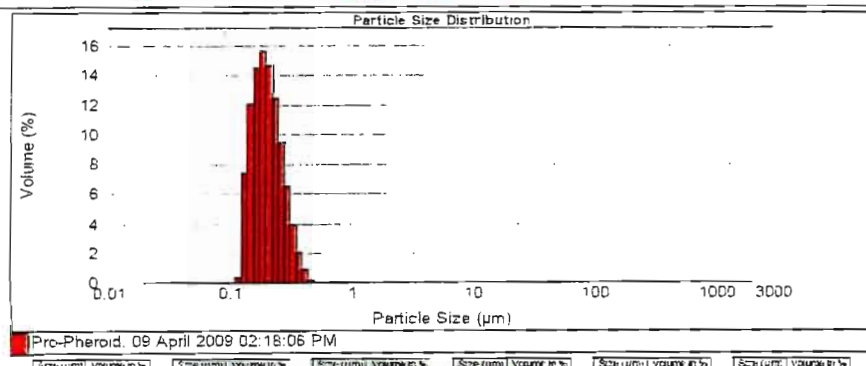
Month 2 40°C + 75% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 09 April 2009 02:18:06 PM	
Sample Source & type P09002	**Measured by:** Micron Scientific	**Analysed:** 09 April 2009 02:18:07 PM	
Sample bulk lot ref: 40 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.456	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 13.86 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 20.667 %	**Result Emulation:** Off
Concentration: 0.0030 %Vol	**Span :** 0.767	**Uniformity:** 0.241	**Result units:** Volume
Specific Surface Area: 30.3 m²/g	**Surface Weighted Mean D[3,2]:** 0.198 um	**Vol. Weighted Mean D[4,3]:** 0.213 um	

d(0.1): 0.145 um d(0.5): 0.200 um d(0.9): 0.299 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd.
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number: MAL1007548

File name: 04 Mar 09
Record Number: 58

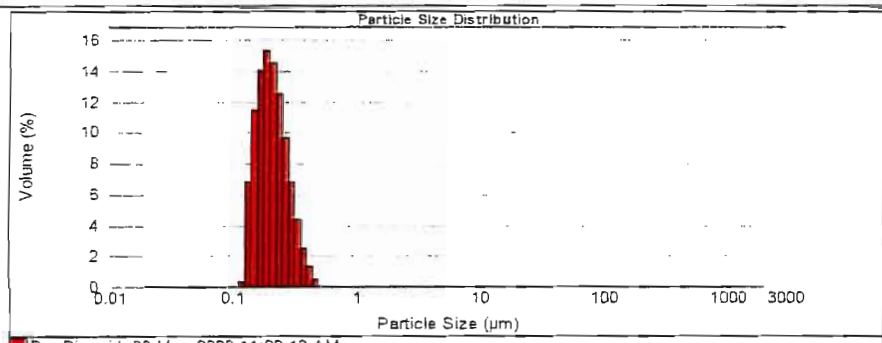
Month 3 5°C

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 08 May 2009 11:00:12 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 08 May 2009 11:00:13 AM	
Sample bulk lot ref: 3 month 5C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 12.69 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 19.913 %	**Result Emulation:** Off
Concentration: 0.0027 %Vol	**Span :** 0.797	**Uniformity:** 0.25	**Result units:** Volume
Specific Surface Area: 29.9 m²/g	**Surface Weighted Mean D[3,2]:** 0.201 um	**Vol. Weighted Mean D[4,3]:** 0.218 um	

d(0.1): 0.146 um d(0.5): 0.203 um d(0.9): 0.308 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007548

File name: 04 Mn 09
Record Number: 103

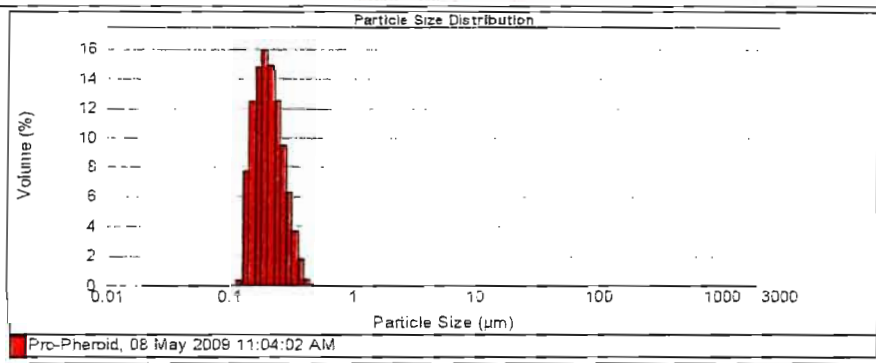
Month 3 25°C + 60% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles usmp Hydro 2000 MU	**Measured:** 08 May 2009 11:04:02 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marié	**Analysed:** 08 May 2009 11:04:08 AM	
Sample bulk lot ref: 3 month 25 C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 10.06 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.464 %	**Result Emulation:** Off
Concentration: 0.0021 %Vol	**Span :** 0.745	**Uniformity:** 0.234	**Result units:** Volume
Specific Surface Area: 30.6 m^2/g	**Surface Weighted Mean D[3,2]:** 0.196 um	**Vol. Weighted Mean D[4,3]:** 0.210 um	

d(0.1): 0.144 um d(0.5): 0.196 um d(0.9): 0.282 um



Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %	Size (um)	Volume in %
0.020	0.00	2.742	12.43	112	0.00	766	0.00	5242	0.00	35874	0.00
0.023	0.00	3.150	41.76	129	0.00	881	0.00	6026	0.00	41234	0.00
0.026	0.00	3.619	15.84	148	0.00	1012	0.00	6924	0.00	47389	0.00
0.030	0.00	4.157	4.86	170	0.00	1163	0.00	7956	0.00	54447	0.00
0.035	0.00	4.775	12.49	195	0.00	1336	0.00	9141	0.00	62561	0.00
0.040	0.00	5.485	9.40	224	0.00	1535	0.00	10502	0.00	71879	0.00
0.046	0.00	6.301	6.28	258	0.00	1764	0.00	12067	0.00	82577	0.00
0.053	0.00	7.239	3.86	296	0.00	2026	0.00	13864	0.00	94868	0.00
0.061	0.00	8.315	1.82	340	0.00	2328	0.00	15929	0.00	109000	0.00
0.070	0.00	9.550	0.38	391	0.00	2675	0.00	18302	0.00	125236	0.00
0.080	0.00	10.968	0.05	449	0.00	3073	0.00	21027	0.00	143887	0.00
0.092	0.00	12.599	0.00	516	0.00	3530	0.00	24156	0.00	165317	0.00
0.105	0.00	14.473	0.00	593	0.00	4055	0.00	27749	0.00	189917	0.00
0.121	0.00	16.625	0.00	681	0.00	4658	0.00	31879	0.00	218213	0.00
0.138	0.00	19.097	0.00	783	0.00	5352	0.00	36621	0.00	250737	0.00
0.159	0.00	21.938	0.00	899	0.00	6149	0.00	42072	0.00	288126	0.00
0.182	0.00	25.202	0.00	1033	0.00	7065	0.00	48337	0.00	331000	0.00
0.210	0.00	28.951	0.00	1187	0.00	8119	0.00	55532	0.00	380.054	0.00
0.241	0.00	33.256	0.00	1364	0.00	9329	0.00	63794	0.00	436.744	0.00
0.277	0.00	38.199	0.00	1567	0.00	10720	0.00	73.280	0.00	501.377	0.00
0.318	0.00	43.874	0.00	1801	0.00	12317	0.00	84.168	0.00	575.877	0.00
0.366	0.00	50.392	0.00	2069	0.00	14151	0.00	96.717	0.00	661.457	0.00
0.420	0.00	57.884	0.00	2377	0.00	16258	0.00	111.107	0.00	759.627	0.00
0.483	0.00	66.499	0.00	2731	0.00	18677	0.00	127.648	0.00	872.214	0.00
0.555	0.00	76.405	0.00	3137	0.00	21457	0.00	146.667	0.00	1001.367	0.00
0.637	0.00	87.796	0.00	3604	0.00	24652	0.00	168.537	0.00	1149.974	0.00
0.732	0.00	100.890	0.00	4140	0.00	28324	0.00	193.665	0.00	1320.000	0.00
0.841	0.00	115.911	0.00	4756	0.00	32541	0.00	222.500	0.00		
0.966	0.00	133.124	0.00	5464	0.00	37387	0.00	255.556	0.00		
1.110	0.00	152.861	0.00	6278	0.00	42947	0.00	293.379	0.00		
1.275	0.00	175.499	0.00	7212	0.00	49336	0.00	336.650	0.00		
1.465	0.00	201.500	0.00	8285	0.00	56690	0.00				

Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007542

File name: 04 Mth 05
Record Number: 105

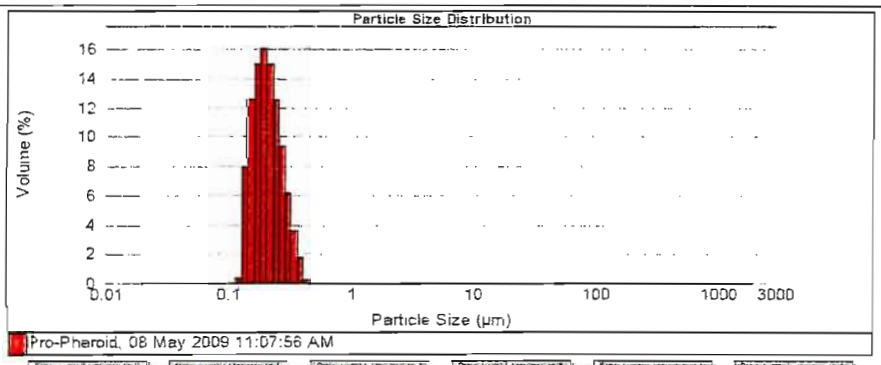
Month 3 30°C + 65% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pro-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 08 May 2009 11:07:56 AM	
Sample Source & type: P09002	**Measured by:** Liezl-Marie	**Analysed:** 08 May 2009 11:07:57 AM	
Sample bulk lot ref: 3 month 30C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 10.81 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.605 %	**Result Emulation:** Off
Concentration: 0.0023 %Vol	**Span :** 0.736	**Uniformity:** 0.231	**Result units:** Volume
Specific Surface Area: 30.8 m²/g	**Surface Weighted Mean D[3,2]:** 0.195 um	**Vol. Weighted Mean D[4,3]:** 0.209 um	

d(0.1): 0.144 um d(0.5): 0.198 um d(0.9): 0.290 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement. The obscuration should be between 10 - 30 % before measurement takes place.*

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Malvern, UK

Mastersize: 2000 Ver. 5.21
Serial Number : MAL1007542

File name: 04 Mar 09
Record Number: 107

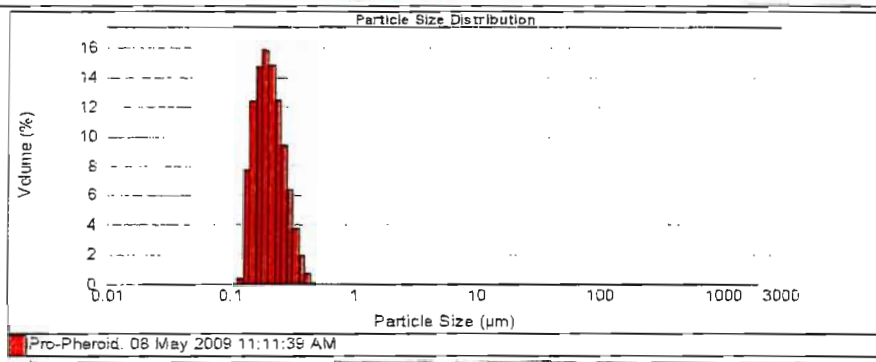
Month 3 40°C + 75% RH

MASTERSIZER 2000

Result Analysis Report

Sample Name: Pre-Pheroid	**SOP Name:** Pheroid vesicles using Hydro 2000 MU	**Measured:** 08 May 2009 11:11:39 AM	
Sample Source & type: PD9002	**Measured by:** Liezl-Marié	**Analysed:** 08 May 2009 11:11:40 AM	
Sample bulk lot ref: 3 month 40C	**Result Source:** Measurement		
Particle Name: Oleic Acid	**Accessory Name:** Hydro 2000MU (A)	**Analysis model:** General purpose	**Sensitivity:** Enhanced
Particle RI: 1.458	**Absorption:** 0.1	**Size range:** 0.020 to 2000.000 um	**Obscuration:** 10.76 %
Dispersant Name: Water	**Dispersant RI:** 1.330	**Weighted Residual:** 21.393 %	**Result Emulation:** Off
Concentration: 0.0023 %Vol	**Span :** 0.752	**Uniformity:** 0.237	**Result units:** Volume
Specific Surface Area: 30.6 m²/g	**Surface Weighted Mean D[3,2]:** 0.196 um	**Vol. Weighted Mean D[4,3]:** 0.211 um	

d(0.1): 0.145 um d(0.5): 0.199 um d(0.9): 0.264 um



Operator notes: *Add 2ml sample and allow the Obscuration level to stabilize before starting measurement The obscuration should be between 10 - 30 % before measurement takes place*

Malvern Instruments Ltd
Malvern, UK

Mastersizer 2000 Ver. 5.31
Serial Number : MAL1007542

File name: 04 Apr 09
Record Number: 109

Poster presented at the 5th International Conference on Pharmaceutical and Pharmacological Sciences

Nevirapine in pro-Pheroid: A Preservative efficacy and stability study

Carlie Britz, Wilna Liebenberg, Anita Wessels

Unit for Drug Research and Development, Faculty of Health Sciences, North-West University, Potchefstroom, 2520

Introduction

In 2008, about 2.5 million people died of AIDS, 5.6 million were living with HIV in South Africa. HIV / AIDS is one of the greatest challenges in developing countries with a huge impact on social-economics and resources. The impact on children is a severe and growing problem. In 2008, 64 000 babies under the age of 2 were infected with HIV and 370 000 died of AIDS. Combination antiretroviral therapy has proven to be the most effective approach in treating HIV positive patients. The combination therapy leaves us with a major patient compliance problem for children and babies. It would be difficult for children and babies to swallow large and many tablets. Therefore, an alternative dosage form to the conventional fixed dose combination tablets is desired and would be of importance for paediatric HIV patients. It may also prove to be advantageous for those like the elderly, who cannot swallow oral dosage forms such as capsules and tablets.

Method

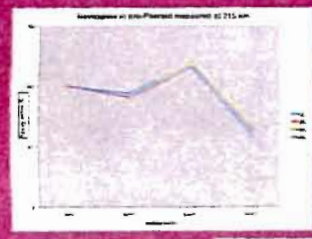
The formulation was subjected to an accelerated stability study over a period of three months. The ARV drug was constituted in the pro-Pheroid and stored at 25°C+60%RH, 30°C+65%RH and 40°C+75%RH for three months. HPLC analysis was done at 215 nm and 245 nm after each month according to the assay method. The method was developed by Köhn (2008).

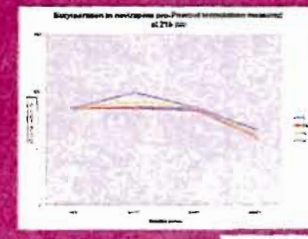
Objectives

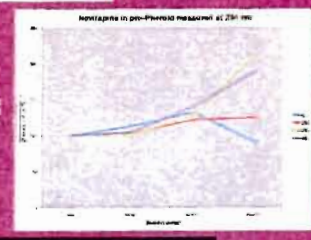
The aim of this study was to evaluate the stability of nevirapine and butylparaben when formulated in single API dosage forms containing the pro-Pheroid delivery system, and to determine whether the wave length played a role in the results obtained. The stability testing was done at different conditions over a three month period. A preservative efficacy study was also conducted on the samples under the same conditions, but the results for the preservative efficacy study are not shown in the poster.

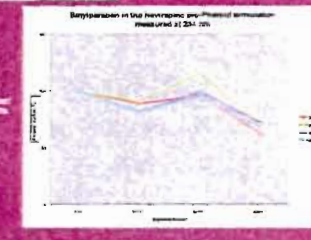
Results

The content of nevirapine and butylparaben remained stable throughout the first two months and decreased after month 3. Different results were obtained for both chemicals at the two different wavelengths showing that the wavelength does play a significant role in the result of the assay. The value for the 5°C sample of month 2 in the butylparaben in nevirapine pro-Pheroid formulation measured at 215 nm has been left out because compared with all the other results it is an outlier.









Conclusion

Preliminary stability studies showed that nevirapine could be successfully formulated in the pro-Pheroid delivery system. The results presented clearly shows that the optimum wavelength for nevirapine is 215 nm, as there is less interference and more convincing peaks are obtained. For butylparaben it really does not matter which one of the two wavelengths are used, as both shows clear results. Further studies should be conducted to determine whether there aren't any false positives presented in the results. The concentration of butylparaben drastically decreased during month three of stability testing, which made it fall out of the specification range, although the preservative efficacy study showed it was still enough to preserve the sample according to the specifications (Results for the preservative efficacy study are not presented in this poster).

References

TREATMENT ACTION CAMPAIGN. 2009. Key HIV Statistics. [Online] Available: [illegible] Date Accessed: 4 June 2009.

KÖHN, A. 2008. Pro-Pheroid Based Antiretroviral Formulations: HPLC Method Development and Stability Studies. Potchefstroom: Ohio.

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