Method development and stability of Pheroid™-based anti-retroviral formulations

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TABLE OF CONTENTS

LIST OF FIGURES/SCHEMES

LIST OF TABLES

ABREVIATIONS

API - Active Pharmaceutical Ingredient

ART – Anti-retroviral Therapy

ARV – Anti-retroviral

AUC – Area Under the Curve

BD – Twice a day

BHA – Butylated Hydroxyanisole

BHT - Butylated Hydroxytoluene

CDC – Centre for disease control

C_{max} – Maximum Concentration

C_{min} – Minimum Concentration

FDC – Fixed Dose Combination

HAART – Highly Active Anti-retroviral Therapy

HPLC – High Performance Liquid Chromatograms

QID – Four times a day

TDS – Three times a day

$T_{1/2}$ – Half life

UNAIDS – Joint United Nations Programme on HIV/AIDS

UNICEF – United Nations Childrens Fund

WHO – World Health Organisation

ABSTRACT

Southern Africa is the worst affected sub region in the world, with South Africa continuing to have the highest number of people living with HIV in the world. It is estimated that 5.3 million people in South Africa were living with HIV at the end of 2003. According to the WHO the total number of people living with HIV in 2004 was 39.4 million. The estimated total for children with HIV under 15 years was 2.2 million. From a total of 3.1 million AIDS deaths in 2004, 510 000 were children under 15 years.

Combination antiretroviral therapy has proven to be the most effective approach in treating HIV positive patients. This triple cocktail treatment is also known as highly active antiretroviral therapy (HAART). The key to its success lies in the drug combination's ability to disrupt HIV at different stages in its replication. Reverse transcriptase inhibitors, which usually make up two drugs in the HAART regimen, restrain an enzyme crucial to an early stage of HIV duplication. Protease inhibitors hold back another enzyme that functions near the end of the HIV replication process. This 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 amount of tablets. Therefore an alternative dosage form to the conventional fix dose combination tablets is desired and would be of importance for paediatric HIV patients, and for those like the elderly, who cannot swallow other oral dosage forms such as capsules and tablets.

According to the WHO should paediatric formulations consist of the following actives:

Stavudine/Lamivudine/Nevirapine;

Zidovudine/Lamivudine/Nevirapine; or

Zidovudine/Lamivudine/Abacavir.

The triple combination containing zidovudine, was omitted because of the toxicity and adverse effects, which is cardiomyopathy in children.

The aim of this study was therefore, to formulate a triple combination formulation containing stavudine/lamivudine/nevirapine in the Pheroid™ delivery system; to develop and validate a HPLC method for all the actives and preservatives; and to evaluate the physical and chemical stability of these products over a period of three months (12 weeks) at three conditions, namely 25°C / 60%RH, 30°C / 65%RH and 40°C / 70%RH.

Two types of Pheroid™ formulations were used in this study i.e., **Formulation A** which contains the water and oily phase of the Pheroid™ and **Formulation B** which consists of only the oil phase. The active ingredients, together with the preservatives and the anti-

oxidants are entrapped in each of these formulations. After the formulations were made, initial assays were done as well as pH and viscosity tests, and then these samples were kept in climate rooms for accelerated stability studies of three months.

A HPLC method were successful develop and validated which is suitable to analyse lamivudine, stavudine, nevirapine, sodium methyl hydroxybenzoate and propyl hydroxybenzoate simultaneously in the pheroid or the pro-pheroid solution for stability testing, quality control and batch release purposes. This method could be regarded as being stability indicating.

The development of an HPLC method for the anti-oxidants, BHT, BHA and Vitamin E simultaneously in the pheroid or the pro-pheroid were successful and can also be regarded as being stability indicating.

A pH test as well as a viscosity test were done on formulation A. The results for the pH and viscosity tests indicate that no significant changes took place during the accelerated stability testing. Viscosity and pH tests were not performed on formulation B, due to the high viscosity of the formulation.

The results for the microbial limit tests for formulation A and formulation B indicated the absence of micro-organisms for all practical purposes.

There was however, a change in the physical appearance of the product in formulation A and B. The product became a darker yellow at the higher temperatures. This could be due to the breakdown of stavudine or due to the free radicals present in the formulation. Results during the assay yielded a wide variation in the concentrations of the actives, and this indicated a major problem in the formulation/active combination. Nevirapine is highly insoluble in aqueous media and stavudine had stability problems. The assay values of lamivudine remain stable throughout the study.

Due to the variations in the assay results, this pilot study showed some critical formulation problems, but the pheroid and pro-pheroid formulations has definite marketing possibilities and could become an essential part in the fight against AIDS.

OPSOMMING

Suidelike Afrika is die streek in die wêreld wat die meeste deur MIV aangetas is en Suid-Afrika het steeds die hoogste persentasie mense wat hierdie infeksie het. Dit word beraam dat 5.3 miljoen mense in Suid-Afrika teen die einde van 2003 MIV gehad het. Volgens die WGO het 39.4 miljoen mense in 2004 wêreldwyd MIV gehad. Die beraamde totaal vir kinders jonger as 15 jaar met MIV was 2.2 miljoen. Van 'n totaal van 3.1 miljoen sterftes van VIGS in 2004 was 510 000 kinders jonger as 15 jaar.

Dit is getoon dat behandeling met 'n kombinasie van antiretrovirale middels die mees effektiewe benadering vir MIV-positiewe pasiënte is. Hierdie behandeling met drie middels staan ook as hoogsaktiewe antiretrovirale terapie (HAART) bekend. Die sleutel tot die sukses hiervan is die kombinasie se vermoë om verskillende stadiums in die replikasie van MIV te ontwrig. Omgekeerdetranskriptaseremmers, wat gewoonlik twee van die middels in die HAART-regimen is, beïnvloed 'n ensiem wat kritiek in 'n vroeë stadium van duplisering van MIV is. Proteaseremmers inhibeer 'n ander ensiem wat aan die einde van die replikasieproses van MIV werk. Hierdie behandeling met 'n kombinasie van middels veroorsaak 'n groot probleem met meewerkendheid van kinders en babas. Dit is moeilik vir kinders en babas om 'n groot hoeveelheid tablette te drink. Daarom is 'n ander doseervorm as die konvensionele kombinasie van tablette met 'n vaste dosis gewens en sal nuttig wees vir pediatriese MIV-pasiënte en vir diegene soos bejaardes wat nie ander orale doseervorme soos kapsules en tablette kan drink nie.

Volgens die WGO moet pediatriese formulerings uit die volgende kombinasies bestaan: stavudien/lamivudien/nevirapien of sidovudien/lamivudien/nevirapien of sidovudien/lamivudien/abakavir.

Die drievoudige kombinasies wat sidovudien bevat, is vir hierdie studie uitgelaat vanweë die toksisiteit en newe-effekte wat in kinders kardiomiopatie is.

Die doel van hierdie studie was om 'n drievoudige kombinasie te formuleer wat stavudien/lamivudien/nevirapien in die Pheroid™-afleweringstelsel bevat, om 'n HDVC-metode vir die analise van al die aktiewe bestanddele en preserveermiddels te ontwikkel en te valideer en om die fisiese en chemiese stabiliteit van hierdie produkte oor 'n periode van drie maande (12 weke) by drie toestande, naamlik 25 °C / 60 %RH, 30 °C / 65 %RH en 40°C / 70 %RH, te evalueer.

Twee tipes Pheroid™-formulerings is in hierdie studie gebruik, naamlik Formulering A wat die water- en oliefase van die Pheroid™ bevat en Formulering B wat net uit die oliefase

bestaan het. Die aktiewe bestanddele, stavudien, lamivudien en nevirapien en die preserveermiddels en antioksidante is in elk van hierdie formulerings vasgevang. Nadat die formulerings berei is, is aanvanklike bepalings van die drie aktiewe bestanddele, die preserveermiddels en antioksidante gedoen en die pH en viskositeit gemeet waarna die monsters vir die versnelde stabiliteitstoetse vir drie maande in klimaatkamers gehou is.

'n HDVC-metode is suksesvol ontwikkel en gevalideer wat geskik vir die gelyktydige analise van stavudien, lamivudien, nevirapien, natriummetielhidroksibensoaat en propiel-hidroksibensoaat in die Pheroid™ of pro-pheroidoplossing is en vir stabiliteitstoetse, kwaliteitskontrole en vrystelling van die lotte gebruik kan word. Hierdie metode is as stabiliteitsaanduidend beskou.

Die ontwikkeling van 'n HDVC-metode vir die gelyktydige kwantitatiewe analise van die antioksidante, BHT, BHA en vitamien E in die Pheroid™ of pro-pheroid was suksesvol en hierdie metode is ook as stabiliteitsaanduidend beskou.

Die resultate van die meting van die pH en viskositeit het getoon dat geen beduidende veranderings tydens die versnelde stabiliteitstoetse oor 12 weke plaasgevind het nie. Die pH en viskositeit van Formulering B is vanweë die hoë viskositeit van die formulering nie gedoen nie.

Die uitslae van die grenstoets vir mikro-organismes in Formulering A en Formulering B het vir alle praktiese doeleindes die afwesigheid van mikro-organismes aangetoon.

Daar was egter 'n verandering in die fisiese voorkoms van die produk in Formulerings A en B. By hoër temperature het die produk donkerder geel verkleur. Dit kan vanweë die ontbinding van stavudien of vanweë vry radikale in die formulering wees. Kwantitatiewe analise het 'n groot variasie in die konsentrasies van die aktiewe bestanddele gelewer wat 'n groot probleem met die formulering/aktiewe kombinasie aantoon. Nevirapien is hoogs onoplosbaar in waterige medium en stavudien het stabiliteitsprobleme gehad. Die analisewaardes van lamivudien het dwarsdeur die studie stabiel gebly.

Vanweë die variasie in die uitslae van die analises het hierdie loodsstudie sekere kritiese probleme met die formulerings uitgewys, maar die formulerings met die Pheroid™ en pro-pheroid het besliste bemarkingspotensiaal en kan 'n wesenlike deel van die stryd teen VIGS word.

AIMS AND OBJECTIVES

There is a desperate need for a Fixed Dose Combination (FDC) product that combines lamivudine, stavudine and nevirapine in a single product that can easily be administered to paediatric, geriatric patients. The formulation should preferably be in a liquid dosage form as this would be easier for paediatric and geriatric patients to swallow. This would also increase patient compliance and cause less cases of resistance.

The aim of this study was to adhere to the need for FDC's for paediatric and geriatric use. The stability of lamivudine, stavudine and nevirapine in combination in the pheroid formulation has never been studied before.

The main objectives of this study were:

- Development and validation of a stability indicating assay method for the simultaneous determination of the three active pharmaceutical ingredients, lamivdine, stavudine and nevirapine, and the two preservatives present in the formulation.
- Development and validation of a stability indicating assay for the simultaneous determination of the three anti-oxidants present in the formulation.
- The determination of the physical and chemical stability of the formulated pheroid formulation during accelerated stability conditions.
- To establish the effectiveness of the preservatives chosen.

CHAPTER 1

HUMAN IMMUNODEFIENCY VIRUS (HIV) AND ACQUIRED IMMUNODEFIENCY SYNDROME (AIDS)

1.1 INTRODUCTION

Southern Africa is the sub region in the world worst affected by HIV, with South Africa continuing to have the highest number of people living with HIV in the world. It is estimated that 5.5 million people were living with HIV at the end of 2005 in South Africa. According to the WHO the total number of people living with HIV in 2004 was 39.4 million. The estimated total for children under 15 years was 240 000. From a total of 3.1 million AIDS deaths in 2004, 510 000 were children under 15 years (UNAIDS/WHO, AIDS epidemic update: December 2006).

Combination antiretroviral therapy has proven to be the most effective approach in treating HIV positive patients. The triple cocktail treatment, also known as highly active antiretroviral therapy (HAART) consists for example out of nevirapine, zidovudine and saquinavir. The key to its success lies in the drug combination's ability to disrupt HIV at different stages in its replication. Reverse transcriptase inhibitors, which usually make up two drugs in the HAART regimen, restrain an enzyme crucial to an early stage of HIV duplication. Protease inhibitors hold back another enzyme that functions near the end of the HIV replication process. Triple combination therapy can be used for all HIV-infected infants and children (Henkel, 2007).

This 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 amounts of tablets. Therefore an alternative dosage form to the conventional fix dose combination tablets is desired and would be of importance for paediatric HIV patients, and for those like the elderly, who cannot swallow solid oral dosage forms such as capsules and tablets.

There is currently a major need for alternative dosage forms to optimise AIDS therapy in babies and children.

The existing drugs or combinations of drugs used in the treatment of these infectious diseases will be formulated in novel and innovative delivery systems.

1.2 DISCOVERY OF HIV

According to the National Institute of Health (In their own words) from the time that there were reports about a new disease, scientists from around the world have tried to find the cause of it. They circulated information informally; they held meetings to exchange ideas; and they published promising findings. Dr. Robert Gallo of the National Cancer Institute was a pioneer in this effort. He recently discovered the first two human retroviruses, HTLV-I and HTLV-II. In 1984, the cause of AIDS was identified as a retrovirus by Dr. Gallo, Dr. Luc Montagnier at the Pasteur Institute in Paris, and Dr. Jay Levy at the University of California, San Francisco. Dr. Gallo called the virus HTLV-III, Dr. Montagnier called it LAV and Dr. Levy called it ARV.

During the summer of 1984, an intensive study of the AIDS retrovirus was launched, resulting in findings such as: the CD4 molecules on T4 helper lymphocytes was identified as one receptor by which the AIDS virus entered cells. Genetic sequences of HTLV-III and LAV were determined (National Institute of Health, 2001).

In September 1984, a meeting between NCI investigators and Burroughs Wellcome pharmaceutical company was held to discuss plans to test potential drugs as retrovirus inhibitors. The outcome of this meeting was research and development of AZT, the first anti-retroviral drug approved to treat AIDS (National Institute of Health, 2001).

1.3 DEFINITION OF AIDS

AIDS is the result of damage to the immune system after infection with the most advanced stage of HIV (Human Immunodefiency Virus). It involves the infection and destroyal of critical immune cells (especially the CD4 cells) which causes the diminished function of the immune system. The immune system is now no longer able to guard against illness, making the person vulnerable to other infections (including opportunistic infections) and cancers. People with HIV may experience different clinical problems, depending on which specific infections or cancers they may develop.

In the United States, the Centres for Disease Control and Prevention (CDC) defines AIDS as a CD4 cell count below 200 cells/mm^3, or the presence of at least one oppportunistic illness in an HIV-positive individual (Elizabeth Glaser Pediatric AIDS Foundation, 2007).

1.4 LIFE CYCLE OF THE HIV-VIRUS

Step 1

Attachment to the Lymphocyte Membrane

On the surface membrane of all living cells are complex protein structures called "receptors". A receptor is often compared to a lock into which a key or "ligand" will fit. There are at least two receptors on T-lymphocytes to which the HIV sticks. The primary receptor is called the "CD4", but a second receptor that loops through the cell membrane 7 times is critical for infection to occur. HIV infection of a lymphocyte requires attachment of the virus to the cell membrane through both of these "ligand-receptor" links. In cells whose "7-transmembrane receptor" is different, the HIV "key" no longer matches the lymphocyte "lock" and attachment is incomplete. Those cells may avoid infection by HIV.

Step 2

Entry of the viral RNA

Tight attachment of the viral particle to receptors on the lymphocyte membrane enables fusion with the cell membrane. The viral contents, including viral RNA then empty into the cell's cytoplasm. Like other viruses that infect human cells, HIV commandeers the host's machinery to make multiple copies of itself.

Step 3

Reverse transcription: Converting viral RNA into DNA

An enzyme (protein) that's part of the human immunodeficiency virus reads the sequence of viral RNA nucleic acids that have entered the host cell and transcribes the sequence into a complementary DNA sequence. That enzyme is called "reverse transcriptase". Without reverse transcriptase, the viral genome couldn't become incorporated into the host cell, and couldn't reproduce. Reverse transcriptase sometimes makes mistakes reading the RNA sequence. The result is that not all viruses produced in a single infected cell are alike. Instead, they end up with a variety of subtle molecular differences in their surface coat and enzymes. Vaccines, which induce the production of antibodies that recognize and bind to very specific viral surface molecules, are an unlikely player in fighting HIV, because throughout infection, HIV surface molecules are continually changing.

Step 4

Integration of Viral DNA

Once the viral RNA has been reverse-transcribed into a strand of DNA, the DNA can then be integrated (inserted) into the DNA of the lymphocyte. The virus has its own enzyme called "integrase" that facilitates incorporation of the viral DNA into the host cells DNA. The integrated DNA is called a provirus.

Step 5

Transcription: Back to RNA

As long as the lymphocyte is not activated or "turned-on", nothing happens to the viral DNA. But if the lymphocyte is activated, transcription of the viral DNA begins, resulting in the production of multiple copies of viral RNA. This RNA codes for the production of the viral proteins and enzymes (translation) and will also be packaged later as new viruses.

Step 6

Translation: RNA→ Proteins

There are only 9 genes in the HIV RNA. Those genes have the code necessary to produce structural proteins such as the viral envelope and core plus enzymes like reverse transcriptase, intergrase, and a crucial enzyme called protease.

Step 7

Viral protease

When the viral RNA is translated into a polypeptide sequence, that sequence is assembled in a long chain that includes several proteins (reverse transcriptase, protease, intergrase). Before these enzymes become functional, they must be cut from the longer polypeptide chain. Viral protease cuts the long chain into its individual enzyme components which then facilitate the production of new viruses.

Step 8

Protease and reverse transcriptase inhibitors

Inhibitors of this viral protease can be used to fight HIV infection. By blocking the ability of the protease to cleave the viral polypeptide into functional enzymes, protease inhibitors interfere with continued infection.

Mutations enable HIV to avoid treatments that involve only one drug, so there is growing use of multiple-drug therapies in which both a protease inhibitor AND a reverse transcriptase inhibitor are combined.

Step 9

Assembly & Budding

Finally, viral RNA and associated proteins are packaged and released from the lymphocyte surface, taking with them a swatch of lymphocyte membrane containing surface proteins. These proteins will then bind to the receptors on other immune cells facilitating continued infection.

Budding viruses are often exactly like the original particle that initially infected the host. In the case of HIV, however, the resulting viruses exhibit a range of variations which makes treatment difficult.

Scheme 1.1: Simplification of HIV life cycle at a cellular level (Cells alive, 2005).

1.5 TRANSMISSION OF THE HIV VIRUS

HIV can be transmitted by:

- Unprotected sexual activity (semen);
- Mother-to-child transmission (breast milk);
- Intravenous (IV) drug use (via sharing contaminated needles and/or syringes);
- Transfusion of infected blood or blood clotting factors;

- Use of contaminated needles, syringes, or surgical equipment;
- Occupational exposure (needle-stick injuries) (Elizabeth Glaser Pediatric AIDS Foundation, 2007).

HIV cannot be transmitted by:

- Hugging;
- Casual kissing;
- Saliva, tears or sweat;
- Touching;
- Sharing a home;
- Touching a toilet seat, telephone, or doorknob;
- Eating or playing together;
- Mosquitoes or other insects (Elizabeth Glaser Pediatric AIDS Foundation, 2007).

1.6 SYMPTOMS OF HIV IN CHILDREN

HIV children get sick frequently and severely. They suffer from the normal infections that any other child would get but because of their weakened immune system, these infections are more frequent, severe and much more difficult to treat.

Examples of common infections include infections of the ears, sinuses, lungs (pneumonia), blood (sepsis), urinary tract, bladder, intestines, and skin, as well as fluid around the brain (meningitis).

If the immune system is weakened beyond a certain point, children may also get infected with germs that would not cause disease in children with normal immune systems, or they may get sicker and have more extensive illness. They may develop opportunistic infections such as: Pneumocystis carinii pneumonia (PCP), Candida (thrush), Herpes simplex (HSV), Mycobacterium avium complex (MAC), Cryptosporidium, Cytomegalvirus, Cryptococcus, Toxoplasmosis, Herpes zoster, or chicken pox (which is much worse in children with HIV) (Elizabeth Glaser Pediatric AIDS Foundation, 2007).

1.7 TREATMENT FOR HIV

1.7.1 DEFINITION OF A FIXED-DOSE COMBINATION

A fixed dose combination is a combination of two or more actives in a fixed ratio of doses. This term is used generically to mean a particular combination of actives irrespective of the formulation or brand. It may be administered as single entity products given concurrently or as a finished pharmaceutical product (the WHO, 2004:13).

1.7.2 GOALS FOR ANTI-RETROVIRAL THERAPY

The primary goals of ARV therapy are to:

- Reduce HIV-related morbidity and mortality;
- Improve quality of life;
- Restore and preserve immunologic functions; and
- Maximally and durably suppress viral load (Department of Health and Human Services, 2004c:5).

The secondary goal is to decrease the incidence of HIV through:

- The increased uptake in voluntary testing and counseling with more people then knowing their status and practicing safer sex;
- The reduction of transmission in discordant couples; and
- Reducing the risks of HIV transmission from mother to child (Department of Health, 2004c:2).

Table 1.1: Criteria for ARV Initiation in Adults and Adolescents (Department of Health, 2004a:4).

ADULTS and ADOLESCENTS-including pregnant women

- CD4 ≤ 200 cells/mm^3 irrespective of stage;

Or

- WHO stage IV AIDS defining illness, irrespective of CD4 count;

And

- Patient expresses willingness and readiness to take ARVs adherently;
- Modified WHO stage 2 or 3 disease OR
- CD4 percentage < 20% in a child under 18 months old, irrespective of disease stage, OR
- CD4 percentage < 15% in a child over 18 months old, irrespective of disease stage (Department of Health, 2004a:17).

Strategies to improve adherence to ARV therapy

- Establish readiness to start therapy;
- Provide education on medication dosing
- Review potential side effects
- Anticipate and treat side effects
- Utilize educational aids including pictures, pillboxes, and calendars
- Engage family and friends;
- Simplify regimens, dosing, and food requirements;
- Utilize team approach with nurses, pharmacists, and peer counselors;
- Provide accessible, trusting health care team (Department of Health, 2004a:17).

1.7.3 ARVs: CLASSIFICATION AND MECHANISM OF ACTION

Table 1.2: Examples of ARV drugs currently on the market (Katzung, 2001:831-843).

Drug Class	Drug Name	Examples of Trade Names	Abbreviations
NRTI	• Zidovudine	Retrovir®	AZT/ ZDV
	• Didanosine	Videx®	ddI
	• Lamivudine	Epivir®	3TC
	• Zalcitabine	Hivid®	ddC
	• Stavudine	Zerit®	D4T
	• Abacavir	Ziagen®	ABC
	• Emtricitabine	Emtriva®	FTC
NNRTI	• Nevirapine	Viramune®	NVP
	• Delavirdine	Rescriptor®	DLV
	• Efavirenz	Sustiva®	EFV
PI	• Saquonavir	Invirase®	SQV
	• Ritonavir	Norvir®	RTV
	• Indinavir	Crixivan®	IDV
	• Nelfinavir	Viracept®	NFV
	• Amprenavir	Agenerase®	APV
FI	• Enfluvirtide	Fuzeon®	T20

1.7.3.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

The NRTIs cause viral termination by inhibiting the HIV-1 reverse transcriptase enzyme. It can also be incorporated into the growing viral DNA chain. Most have activity against HIV-1 as well as HIV-2. Cellular phosphorylation is required for activation (Katzung, 2001:831-838).

1.7.3.2 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The NNRTIs bind directly to a site on the viral reverse transcriptase enzyme which results in the blockade of RNA- and DNA-dependant DNA polymerase activities. It does not require phosphorylation to be active and it has specific activity against HIV-1 (Katzung, 2001:831-838).

1.7.3.3 Protease Inhibitors (PI)

PIs inhibit HIV-1 protease, an enzyme that cleaves viral precursor proteins and is critical to the production of mature infectious virions (Katzung, 2001:831-838).

1.7.3.4 Fusion Inhibitors (FI)

Enfuvirtide (DP-178 or T-20) and tifuvirtide (T-1249) are examples of fusion inhibitors. They are both peptides and are administered subcutaneously. They act by binding to gp41, thus preventing the binding of HR2 to the HR1 region and thereby blocking the fusion process. Conversely enfurvitide is inactive against HIV-1 group O viruses, HIV-2 and SIV (ANON: 2007).

1.7.4 ARVs: ADVERSE EFFECTS

Table 1.3: Important ARV adverse reactions (Reproduced from Department of Health, 2004b:38).

Anti-retrovirals	Adverse Effects
Abacavir	A potentially fatal hypertensive reaction occurs. Symptoms usually appear within 6 weeks of treatment initiation. The reaction is suspect if symptoms from 2 or more of the following groups are present: • Fever. • Maculopapular pruritic generalised rash. • Gastrointestinal symptoms. • Other symptoms (including pharyngitis, dysponea, cough, musculoskeletal disorders, malaise, fatigue, lymphadenopathy and paraesthesia). NB: never give abacavir to a child who has previously developed an abacavir hypersensitivity reaction.
Didanosine (ddI)	Pancreatitis, peripheral neuropathy, GIT effects (bloating, flatulence, nausea, diarrhea), lactic acidosis.
Efavirenz (EFV)	CNS disturbances (dysphoria, vivid dreams, distractedness, dizziness), GIT symptoms. Skin rash, *congenital abnormalies-Avoid*

	during 1st trimester of pregnancy.
Lamivudine (3TC)	Diarrhoea, pancreatitis, lactic acidosis.
Lopinavir/Ritonavir	GIT symptoms, lipid abnormalities (5%), lipodystrophic changes.
Nevirapine (NVP)	Skin rash (16%), nausea, vomiting, hepatitis (can be fatal).
Ritonavir	Bad taste, GIT symptoms, especially diarrhea. Raised liver enzymes, raised cholesterol and triglycerides, lipodystrophic changes.
Stavudine (d4T)	Peripheral neuropathy, hepatic steatosis, lactic acidosis, pancreatitis.
Zidovudine (AZT)	Bone marrow suppression (anaemia, neutropenia), GIT symptoms, myopathy, lactic acidosis. Cardiomiopathy in children.

1.7.5 AVAILABLE TREATMENT REGIMES AND DOSAGE FORMS

Table 1.4: Treatment Regimes and Dosage forms (World Health Organization, 2006:82-116).

1. Nucleoside Reverse Transcriptase

LAMIVUDINE		
FORMULATIONS	**DOSE**	**COMMENTS**
Oral solution: 10 mg/ml. Tablet: 150 mg.	Target dose: 4 mg/kg/dose bd (twice daily) to a maximum of 150 mg bd. Dose at < 30 days: 2 mg/kg/dose bd. Dose at ≥ 30 days: 4 mg/kg/dose bd. Dose at > 50 kg: 150 mg bd.	• Well tolerated. • No food restrictions. • Also active against hepatitis B. • The tablets can be crushed and the contents mixed with a small amount of food or water and immediately taken.
STAVUDINE (d4T)		
FORMULATIONS	**DOSE**	**COMMENTS**
Oral solution: 1 mg/ml. Capsules: 15 mg, 20 mg, 30 mg, 40 mg.	Target dose: 1 mg/kg/dose. Dose at < 30 kg: 1 mg/kg/dose bd. Dose at > 30 kg: 30 mg/dose bd. Adults > 60 kg: currently 40	General: • Well tolerated. • Do not use stavudine with zidovudine due to antagonistic effect.

	mg bd recommended;(using 30 mg dosing leads to delay or reduction of toxicity, although limited data on efficacy are available).	<u>Oral solution:</u> • Palatable and well tolerated but requires refrigeration after reconstitution. • Powder for oral solution should be protected from excessive moisture and stored in tightly closed containers at 25°C (permitted range: 15°C-30°C). • After re-constitution, needs refrigeration and storage in original container; discard any unused portion after 30 days. • Must be well shaken prior to each use. <u>Capsules:</u> • Can be opened and mixed with small amount of food or water (stable in solution for 24 hours if kept refrigerated).
ZIDOVUDINE		

FORMULATION	DOSE	COMMENTS
Syrup: 10 mg/ml. Capsules: 100 mg and 250 mg. Tablet: 300 mg.	Target dose for infants > 6 weeks old: Oral 180-240 mg/m^2 per dose given bd. Maximum dose 300 mg/dose given bd. Adult dose 250-300 mg/dose given bd. MTCT prevention dose: *Oral:* 4 mg/kg every 12 hours starting within 12 hours after birth and continuing up to 1-6 weeks of age, depending on national recommendations. *Intravenous:* 1.5 mg/kg infused over 30 minutes, every 6 hours until oral dosing is possible.	General: • Do not use stavudine with zidovudine due to an antagonistic effect. • No food restrictions. • Use with caution with anemia due to potential for bone marrow suppression. Syrup (oral solution): • Preferred in children < 8 kg since accurate dosing with capsules is not practical in smaller children. • Is stable at room temperature but needs storage in glass jars and is light-sensitive. Capsules: • May be opened and dispersed in water or on to a small amount of food and immediately ingested. • Storage at 15°C to 25°C. Tablets:

		• Storage at 15°C to 25°C. • 300 mg tablets are often not scored; may be cut in half with a tablet splitter in a pharmacy. • Tablets may be crushed and combined with a small amount of food and water immediately ingested.

ABACAVIR (ABC)		
FORMULATION	**DOSE**	**COMMENTS**
Oral solution: 20 mg/ml. Tablet: 300 mg.	Target dose < 16 years or < 37.5 kg: 8 mg/kg/dose bd. Maximum dose > 16 years or ≥ 37.5 kg: 300 mg/dose bd.	General: • Parents must be warned about potential hypersensitivity reaction. • ABC should be stopped permanently if hypersensitivity reaction occurs. • No food restrictions. Tablets: • Can be crushed and contents mixed with small amount water or food and immediately ingested.

		• Storage at room temperature (20°C to 25°C). Oral solution: • Storage at room temperature (20°C to 25°C); may be refrigerated.
DIDANOSINE (ddI)		
FORMULATIONS	**DOSE**	**COMMENTS**
Oral solution from pediatric powder/water: 10 mg/ml (in many countries must be made up with additional antacid). Chewable tablets: 25 mg, 50 mg, 100 mg, 150 mg, 200 mg. Enteric-coated beadlets in capsules: 125 mg, 200 mg, 250 mg, 400 mg (designed for once daily dosing; preferred but still not widely available).	Dose < 3 months: 50 mg/m^2 /dose bd. Dose at 3 months to < 13 years: 90-120 mg/m^2 /dose bd. Maximum dose, ≥ 13 years or > 60 kg: 200 mg/dose bd or 400 mg once daily. Once-daily dosing of chewable tablets is authorised in United Kingdom for children over the age of 6 years.	General: • ddI is degraded rapidly unless given as enteric formulation or combined with buffering agents or antacids. • In children this effect may be less marked and ddI may not have to be administered on an empty stomach. Oral suspension: • Is not easy to use and should be avoided if possible. • Should be kept

		refrigerated; stable for 30 days; must be well shaken. Tablets: • At least two tablets must be used at any one time for adequate buffering. • ddI tablets should be chewed, crushed or dispersed in water or clear juice before they are taken. • They should not be allowed to swallow whole. Enteric-coated beadlets in capsules: • Can be opened and sprinkled on a small amount of food.

2. Non-Nucleoside Reverse Transcriptase Inhibitors

EFAVIRENZ (EFV)		
FORMULATION	**DOSE**	**COMMENTS**
Syrup: 30 mg/ml. Capsules: 50 mg, 100 mg,	Target dosing: 19.5 mg/kg/day (syrup) or 15	General:

200 mg. Tablets: 600 mg.	mg/kg/day (capsule/tablet). Weight greater than 40 kg, 600 mg once daily.	• Storage at 25°C. • Insufficient data on dosing for children < 3 years old. • EFV can be given with food, especially high-fat meal, absorption is increased by an average of 50% as a result of a high-fat meal. • EFV is best given at bedtime in order to reduce CNS side-effects, especially during first two weeks. Capsules: • May be opened and added to a small amount of food or liquid; they have a very peppery taste but can be mixed with foods to disguise the taste.
NEVIRAPINE (NVP)		
FORMULATION	**DOSE**	**COMMENTS**
Oral suspension: 10 mg/ml.	Target dose for maintenance: 160-200 mg/m^2 to a maximum dose of 200 mg	General: • Parents must be warned

Tablet: 200 mg.	taken bd. Special considerations on dosing: a) <u>Induction dose:</u> once daily for 14 days. b) <u>Maintenance dose</u>: target dose is 160-200 mg/m^2/dose given twice daily adjusted for more aggressive dosing in younger ages. c) For children 14-24.9 kg the suggested dose is 1 tablet a.m. and ½ tablet p.m. Due to the prolonged half-life of nevirapine, the fluctuation in drug exposure associated with this dosing schedule is acceptable. d) If a mild rash occurs during the first 14 days of induction dosing, continue once daily dosing and only escalate dose once the rash has subsided and the dose is well tolerated. If a severe rash occurs (especially if accompanied by	about a potential severe, life-threatening rash during the 14-day lead-in period. The once-daily induction dose is used to reduce the frequency of rash. • NVP should be permanently discontinued and not restarted in children who develop severe rash. • Drug administration: avoid nevirapine if rifampicin is co-administered. • Can be given without regard to food. • Storage at 25°C. <u>Oral suspension:</u> • Must be shaken well. <u>Tablets:</u> • Are scored and can be divided into two equal parts to give a 100 mg dose; can be crushed and combined with a small amount of food or water and immediately administered.

	fever, blistering or mucosal ulcerations), discontinue drug. Dosing for MTCT prevention: 2 mg/kg/dose within 72 hours of birth once only. If the maternal dose of Nevirapine was given less than 2 hours before delivery, then administer 2 mg/kg/dose to the infant immediately after birth and repeat within 24-72 hours of first dose. If the infant weight is not available, administer 0.6 ml oral suspension.	

3. Protease Inhibitors

SAQUINAVIR (SQV)		
FORMULATION	**DOSE**	**COMMENTS**
Capsules: Hard gel capsules(hgc): 200 mg. Tablets: 500 mg.	hgc studies reported using 33 mg/kg three times a day.	General: • Should not be taken as sole protease inhibitor. • Should be taken with food as it enhances

		absorption; it is suggested that it be taken within two hours after a meal. Storage: • hgc do not need refrigeration. Pharmacokinetic data: • Safety and effectiveness not yet well established in younger children. • Not licensed for use in children under 16 years of age or less than 25 kg.

NELFINAVIR (NFV)		
FORMULATION	**DOSE**	**COMMENTS**
Powder for oral suspension: 50 mg per 1.25 ml scoop (200 mg per level teaspoon of 5 ml). Tablet: 250 mg, 625 mg.	< 10 kg: dose listed is targeted to achieve a dose of ~75 mg/kg/dose bd. ≥ 10 kg to 19.9 kg: dose listed is targeted to achieve a dose of ~60 mg/kg/dose bd. ≥ 20 kg: maximum recommended dose of 1250 mg/dose bd.	General: • Powder and tablets can be stored at room temperature. • Must be taken with food to improve absorption. • Drug interactions (less than ritonavir-containing protease inhibitors).

		• Because of difficulties with powder the use of crushed tablets is preferred (even for infants) if the appropriate dose can be given. Tablets: • May be halved, or crushed and dispersed in water or on to a small amount of food and immediately ingested. Pharmacokinetic data: • Available for all ages. • However, there is extensive pharmacokinetic variability in infants, with a requirement for very high doses in infants < 1 year of age.

LOPINAVIR/RITONAVIR (LPV/r) (Co formulation)		
FORMULATION	**DOSE**	**COMMENTS**
Oral solution: 80 mg/ml lopinavir plus 20 mg/ml ritonavir. Capsules: 133.3 mg	Lopinavir target doses: 5-7.9 kg: 16 mg/kg/dose bd.8-9.9 kg: 14	General: • Should be taken with food.

lopinavir plus 33.3 mg ritonavir. Tablets: 200 mg Lopinavir + 50 mg ritonavir.	mg/kg/dose bd. 10-13.9 kg: 12 mg/kg/dose bd. 14-39.9 kg: 10 mg/kg/dose bd. Equivalent to 300 mg/m^2. Ritonavir target doses: 7-15 kg: 3 mg/kg/dose bd..15-40 kg: 2.5 mg/kg/dose bd. Equivalent to 75 mg/m^2. Maximum dose: 400 mg lopinavir + 100 mg ritonavir taken bd.	• Preferably, oral solution and capsules should be refrigerated; however, can be stored at room temperature up to 25°C for two months; at > 25°C drug degrades more rapidly. • There are many drug-to-drug interactions because RTV inhibits cytochrome P450. Oral solutions: • Low volume but bitter taste. Capsules: • Large. • Should not be crushed or opened; must be swallowed whole. Tablets: • Do not have food restrictions although bioavailability is increased when administered with food. • Cannot be split.

RITONAVIR (RTV)		
FORMULATION	**DOSE**	**COMMENTS**
Soft gelatin capsules: 100 mg. Liquid: 600 mg ritonavir per 7.5 ml (80 mg/ml). Co-formulated with lopinavir.	Target dose treatment: < 2 years: not established. ≥ 2 to 16 years: 400 mg/m^2 bd by mouth up to a maximum 600 mg bd. • Started at 250 mg/m^2 and increased at intervals of 2 to 3 days by 50 mg/m^2 bd to reduce side effects. As a booster to Lopinavir: Ritonavir target doses: 7-15 kg: 3 mg/kg bd. 15-40 kg: 2.5 mg/kg bd.	General: • Used in low doses to increase effect of some protease inhibitors. • Liquid must be kept at 20°C to 25°C and in original bottle. • Liquid is foul-tasting and excipient contains 43% alcohol. • Soft gel capsules contain 12% alcohol excipient. • Should be taken with food. • Liquid may be taken alone or mixed with milk or food but should not be mixed with other fluids, including water. • Many drug-to-drug

		interactions because RTV inhibits cytochrome P450.

4. Fixed-dose combinations

ZIDOVUDINE (AZT) PLUS LAMIVUDINE (3TC)		
FORMULATION	**DOSE**	**COMMENTS**
Oral solution: not available. Tablet: AZT (300 mg) plus 3TC (150 mg).	Target dose: Zidovudine – 180-240 mg/m^2/dose bd. Lamivudine – 4 mg/kg/dose bd. Maximum dose: 1 tablet/dose bd.	General: • See comments under individual components. Tablets: • Storage between 2°C and 30°C. • No food restrictions. • Can be crushed and contents mixed with a small amount of water or food and immediately taken.

STAVUDINE (d4T) PLUS LAMIVUDINE (3TC)		
FORMULATION	**DOSE**	**COMMENTS**
Oral solution: Stavudine 10 mg + lamivudine 40 mg/5 ml. Tablets: d4T (30 mg) plus 3TC (150 mg) *or* d4T (40 mg) plus 3TC (150 mg).	Target dose: Stavudine - 1 mg/kg/dose bd. Lamivudine - 4 mg/kg/dose bd. Maximum dose: 1 tablet/dose bd.	General: • See comments under individual drug components. Tablets: • Preferably, should not be split unless scored.
ZIDOVUDINE (AZT) PLUS LAMIVUDINE (3TC) PLUS ABACAVIR (ABC)		
FORMULATION	**DOSE**	**COMMENTS**
Oral solution: not available. Tablet: AZT (300 mg) plus 3TC (150 mg) plus ABC (300 mg).	Target dose: Zidovudine – 180-240 mg/m^2/dose bd. Lamivudine – 4 mg/kg/dose bd. Abacavir – 8 mg/kg/dose bd. Maximum dose: 1 tablet/dose bd.	General: • See comments under individual drug components. • Parents must be warned about potential hypersensitivity reaction. • ABC should be stopped permanently if hypersensitivity reaction occurs.

STAVUDINE (d4T) PLUS LAMIVUDINE (3TC) PLUS NEVIRAPINE (NVP)		
FORMULATION	**DOSE**	**COMMENTS**
Tablet: d4T (30 mg) plus 3TC (150 mg) plus NVP (200 mg); *or* d4T (40 mg) plus 3TC (150 mg) plus NVP (200 mg). As of October 2006 not yet WHO prequalified: Tablet: 5 mg stavudine/ 20 mg lamivudine/ 35 mg nevirapine (baby). Tablet: 5 mg stavudine/ 40 mg lamivudine/ 70 mg nevirapine (junior). Suspension: Stavudine 10 mg/ 5 ml + lamivudine 40 mg + nevirapine 70 mg.	Maximum dose: One 30 mg d4T based tablet bd.	General: • Contains fixed dose of NVP, therefore cannot be used for nevirapine induction as nevirapine dose escalation required. • See comments under individual drug components. Tablets: • Preferably, should not be split unless scored.

1.8 CONCLUSION

The treatment regimes and dosage forms mentioned in this chapter give us an indication as to why patient compliance in ARV patients are so low and why there are so many patients that are drug resistant. Different dosage forms play an important part in patient adherence. For example, PI's only have the tablets and the capsules available and because the dosage is so high, patients would have to swallow a large number of tablets or capsules and this automatically decreases patient compliance and is impossible as a dosage form for babies.

A lot of the dosages are measured with body surface area and those patients that are in poor resource settings will not be able to take the proper measurement which may lead to wrong dosages and as a result drug resistance or decreased patient compliance.

As can be seen in the above table, very few fixed dose combinations exist in the form of an oral solution or suspension. A fixed dose combination containing lamivudine, stavudine and nevirapine in the form of an oral solution may address the above mentioned problems of patient compliance as it may reduce the number of daily doses significantly. In addition, it should be possible to calculate the liquid dose according to body weight, thus preventing suboptimal dosaging. It was therefore decided to combine the ARV's lamivudine, stavudine and nevirapine in an oral solution by using Pheroid™ technology (see Chapter 3).

CHAPTER 2

PHYSICO-CHEMICAL PROPERTIES OF LAMIVUDINE, STAVUDINE AND NEVIRAPINE

2.1 INTRODUCTION

As already mentioned in chapter 1, combination antiretroviral therapy has proven to be the most effective approach in treating HIV positive patients. This combination therapy leaves us with a major patient compliance problem for children and babies.

According to the WHO should paediatric formulations of those fixed dose combinations already available for adults, be produced:

- Stavudine/Lamivudine/Nevirapine;
- Zidovudine/Lamivudine/Nevirapine;
- Zidovudine/Lamivudine/Abacavir (WHO:2004:4).

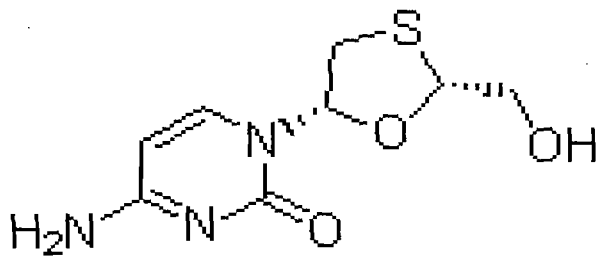
The combination that we suggest is generally the first line regimen, stavudine/lamivudine/nevirapine, used in South Africa and world wide. The reason we have chosen this regimen is due to the availability of the methods of analysis as well as the low toxicity.

We have not chosen the regimen containing zidovudine due to one of the toxicities, which is cardiomyopathy in children, since our focus is formulation of paediatric ARV's. The adverse effects of the various individual drugs can be viewed in table 1.3.

2.2 LAMIVUDINE (3TC)

2.2.1 CHEMICAL PROPERTIES

2.2.1.1 Structural formula and chemical name



Chemblink (2007).

3'-thia-2',3'-dideoxycytidine (The Merck Index, 2006:927).

2.2.1.2 Empirical formula

$C_8H_{11}N_3O_3S$ (The Merck Index, 2006:927).

2.2.1.3 Molecular weight

229.26 (The Merck Index, 2006:927).

2.2.1.4 Appearance

White to off-white crystalline powder (The Merck Index, 2006:927).

2.2.1.5 Melting point

160-162°C (The Merck Index, 2006:927).

2.2.1.6 Solubility

The solubility in water at 20°C is approximately 70 mg/ml (GlaxoSmithKline, 2001:1), which is about 120 times more than required by the BCS Class 1 (High Solubility-High Permeability) (FDA Guidance, August 2000: 2) for a 150 mg oral dose.

According to Jozwiakowski *et al.* (1996:193), lamivudine has a pKa of 4.3 and exists primarily in the un-ionized form when dissolved in distilled water (pH of 1% solution is 6.9). It is light insensitive and stable at a broad temperature range in both the solid state and in aqueous solutions.

Table 2.1: Equilibrium solubilities ((mg/ml) (standard deviation)) of lamivudine vs. solvent and temperature (Jozwiakowski *et al.*, 1996:193).

Solvent	5.0°C	15.0°C	25.0°C	35.0°C	45.0°C
			Form 1		
Water	37.8(1.8)	52.8(0.8)	84.9(1.4)	149.6(3.9)	360.0(9.2)
Methanol	18.7(0.9)	22.8(1.0)	28.3(1.1)	35.8(1.9)	48.8(0.8)
			Form 11		
Ethanol	7.0(0.1)	8.8(0.2)	11.4(0.3)	15.2(0.6)	19.6(0.5)
n-Propanol	4.1(0.04)	5.1(0.1)	7.2(0.1)	9.4(0.2)	13.3(0.4)
2-Propanol	2.7(0.03)	3.8(0.1)	4.9(0.2)	6.4(0.2)	8.0(0.0)
n-Butanol	2.9(0.3)	3.7(0.3)	5.4(0.5)	6.3(0.1)	8.8(0.3)
Sec-Butanol	2.1(0.04)	3.0(0.04)	4.0(0.1)	5.6(0.2)	7.8(0.3)
Ethyl acetate	0.019(0.005)	0.037(0.004)	0.0570(0.005)	0.086(0.008)	0.128(0.006)
Acetone	0.57(0.01)	0.74(0.02)	0.94(0.04)	1.52(0.02)	2.58(0.24)
Acetonitrile	0.47(0.02)	0.64(0.03)	0.91(0.01)	1.45(0.04)	2.15(0.06)

2.2.2 PHARMACOLOGICAL ACTION

Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It needs to be phosphorylated to its triphosphate form before it is active. 3TC-triphosphate inhibits DNA polymerase (Wikipedia, 2006).

2.2.3 PAEDIATRIC DOSE AND ADULT DOSE

Paediatric dose:

Oral: 3 months-12 years, 4 mg/kg (maximum 150 mg) twice daily.

Neonates < 30 days old: 2 mg/kg twice daily (Gibbon, C.J., 2003:313).

2.2.4 STABILITY

Lamivudine is known to be quite stable in solution and in biological fluids. Nguyen *et al.* (1995:1671-1682) studied factors affecting the chemical stability and preservative efficacy of lamivudine oral formulations. Five factors, each at three levels, were investigated in an optimisation study: pH (4.5, 5.5 and 7.5), sucrose, propylene glycol, glycerine and EDTA. All the solutions contained a constant concentration of lamivudine (1 mg/ml), parabens and artificial flavours. The formulations were tested after storage in sealed ampoules for 3 months at 40°C. The pH was found to be the factor with the least effect on the chemical stability of lamivudine (and the parabens). Results are presented in an ANOVA Table for Quadratic Model Fit (Table 2.2), which indicates that the degradation of lamivudine (after 3 months at 40°C) varies from about 0% to 12% with maximum lamivudine stability calculated for a sugar concentration of 23-27% w/v and a pH of 6.5-6.7. Lamivudine was least stable at pH 4.5.

Morris and Selinger (1994:255-264) studied the stability of lamivudine in urine samples for 5 hours at 58°C and found about 4.5% degradation after storage of samples for 4 days. Hoetelmans *et al.* (1998:387-394) and Harker *et al.* (1994:227-232) reported similar results on stability for lamivudine in human plasma and serum samples.

Table 2.2: ANOVA table for the quadratic model fit to the lamivudine chemical stability data for 3 months at 40°C (Nguyen *et al.*, 1995:1671-1682).

Source	Degrees of Freedom (df)	F-Ratio	Signif.	Coefficient	Std. Error
Constant	1			-0.009278	0.004766
*pH	1	194.40	0.0000	0.044843	0.003072
*Sucrose	1	1.79	0.1942	-0.003091	0.003072
*EDTA	1	1.93	0.1785	-0.004263	0.003072
*pH^2	1	35.03	0.0000	-0.051950	0.008778
*$Sucrose^2$	1	5.92	0.0232	-0.021358	0.008778
$EDTA^2$	1	5.47	0.0284	0.018090	0.007735
Residual	23				

R^2 =0.9214, R^2 (adjusted) =0.9009

* or ^{2} Indicate factors are transformed to the -1, 1 scale as opposed to their original units.

2.2.5 BIOAVAILABILITY

Lamivudine is rapidly absorbed with a bioavailability of between 80% to 88% in adults and adolescents and from 66% to 68% in children. Food delays the peak serum concentration; however, there is no significant difference in bioavailability when lamivudine is taken with food. Time to peak concentration is approximately 0.5 to 2 hours after a single 100 mg dose; with food, it increases to approximately 3.2 hours; with fasting, it is about 1 hour. Lamivudine crosses the blood-brain barrier and is distributed into the cerebrospinal fluid (CSF) to a limited extent. In children, CSF concentrations have ranged from 10% to 17% of the

corresponding non-steady-state serum concentration (Department of Health and Human Services, 2005:2).

2.2.6 ELIMINATION

Metabolism is a minor route of elimination. The majority of lamivudine is eliminated unchanged in urine by active organic cationic secretion. In most single-dose studies in infected patients, the mean elimination half-life ranged from 5 to 7 hours. Oral clearance and elimination half-life were independent of dose and body weight over an oral dosing range of 0.25 mg/kg to 10 mg/kg. The intracellular half-life lamivudine triphosphate is 11 to 15 hours; while the serum half-life of lamivudine is about 2.6 hours in adults and 1.7 to 2 hours in children. The renal clearance of lamivudine is greater than the glomerula filtration rate, implying active secretion into renal tubules (Department of Health and Human Services, 2005:2).

2.2.7 BIOTRANSFORMATION

Trans-sulfoxide is the only known metabolite of lamivudine; serum concentrations of this metabolite have not been determined (USP DI, 1999:1819).

2.2.8 DRUG AND FOOD INTERACTIONS

- Lamivudine and zalcitabine may inhibit the intracellular phosphorylation of one another. Consequently, lamivudine should not be co-administered with zalcitabine.
- When co-administered with sulfamethoxazole / trimethoprim, lamivudine exposure was increased by 44% and decreased by 30% respectively.
- Concurrent administration of lamivudine and drugs associated with pancreatitis (e.g., alcohol, didanosine, intravenous pentamidine, sulfonamides, and zalcitabine) or with drugs associated with neuropathy (dapsone, didanosine, isoniazid, stavudine, and zalcitabine) should be avoided or used with caution.
- Administration of lamivudine with indinavir and zidovudine resulted in a 6% decrease in AUC of lamivudine, no change in the AUC of indinavir, and a 36% increase in AUC of zidovudine (Department of Health and Human Services, 2005:2).

2.2.9 CONTRA-INDICATIONS

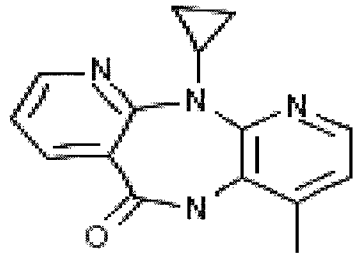
- Patients with a known hypersensitivity to any of the components of the products.
- Lactic acidosis and severe hepatomegaly with steatosis, including fatal cases, have been reported with the use of nucleoside analogues alone or in combination, including lamivudine and other antiretrovirals.
- In paediatric patients with a history of prior anti-retroviral nucleoside exposure, a history of pancreatitis, or other significant risk factors for the development of pancreatitis, lamivudine should be used with caution.
- Risk-benefit should be considered in patients with HIV and/or renal function impairment (Department of Health and Human Services, 2005:2).

2.3 NEVIRAPINE (NVP)

2.3.1 CHEMICAL PROPERTIES

2.3.1.1 Structural formula and chemical name

1-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one



Drug Bank (2007).

2.3.1.2 Empirical formula

$C_{15}H_{14}N_4O$ (The Merck Index, 2006:1123).

2.3.1.3 Molecular weight

266.30 (The Merck Index, 2006:1123)

2.3.1.4 Appearance

White to off-white crystalline powder (The Merck Index, 2006:1123).

2.3.1.5 Melting point

247-249°C (The Merck Index, 2006:1123).

2.3.1.6 Solubility

0.1 mg/ml at neutral pH; highly soluble at pH<3 (The merck Index, 2006:1123)

The active substance can exist in one polymorphic and one pseudo polymorphic form, i.e. an anhydrous form and the hemi hydrate. The anhydrous form was selected for the development of the oral tablet due to its higher intrinsic aqueous solubility (90 µg/ml at 25°C). The less soluble hemi hydrate is used in oral suspensions. Nevirapine is a weak base showing increased solubility at acidic pH values (Viramune: Scientific discussion, 2000:2).

Nevirapine is highly lipophyllic. It is only slightly soluble in water (0.1 mg/ml), forming a clear colourless solution, and is relatively insoluble in non-polar media. It is a weak base because of two pyridine nitrogen's. The measured ionisation constants are pKa = 2.8; pKa = -0.4. The first and second ionisation constants were determined spectrophotometrically (UV) and by NMR, respectively (Nevirapine Chemistry, 2006). According to The Merck Index (1996:6571) it is "highly soluble" at pH <3.

For the 200 mg oral dose nevirapine to be regarded as highly soluble, the solubility of the drug substance over the pH range 1.0-7.5 should be at least 200 mg per 250 ml (FDA Guidance for Industry, August 2000:2), i.e. 0.8 mg/ml.

According to the FDA's Guidance for Industry (August 2000) nevirapine can be classified as a class 2 drug substance (Low Solubility-High Permeability).

2.3.2 PHARMACOLOGICAL ACTION

The NNRTI's bind directly to a site on the viral reverse transcriptase that is near to but distinct from the binding site of the NRTI's. The NNRTI's have specific activity against HIV-1 and unlike the NRTI's, they neither compete with nucleoside triphosphates nor require phosphorylation to become active. RNA- and DNA-dependent DNA polymerase activities are blocked due to binding of the NNRTI drug to the enzyme's active site. The K103N mutation as well as the less critical Y181C/I mutation are generally associated with

resistance to an NNRTI. The rapid emergence of resistance prohibits monotherapy with any of the NNRTIs during treatment of HIV infections (Katzung, 2001:838).

The Department of Health and Human Services (2006:7) reports that nevirapine binds directly to heterodimeric HIV-1 RT and appears to inhibit RT activity by disrupting the catalytic site of the enzyme.

2.3.3 PAEDIATRIC DOSE AND ADULT DOSE

Paediatric Dosage:

According to dose finding studies the choice of a 7 mg/kg twice a day dose would result in a slight increase above the levels reported in adults. This was considered acceptable taking into account that the higher dose will help to assure adequate plasma concentrations to combat the higher viral load in children than in adult patients.

The final recommended doses for the different ages are therefore the following:

- Patients from 2 months to 8 years, 4 mg/kg once daily for 2 weeks followed by 7 mg/kg twice a day.
- Patients from 8 years to 16 years: 4 mg/kg once daily followed by 4 mg/kg twice a day (Viramune: Scientific discussion, 2000:12).

2.3.4 STABILITY

The anhydrous form was shown to be non-hygroscopic, and conversion to the hemi hydrate form did not occur even in samples exposed to 92% relative humidity for 24 months at 30 °C (Viramune: Scientific discussion, 2000:2).

At another website (Nevirapine Chemistry, 2006) the following is reported for nevirapine:

> *Nevirapine is a very stable compound. At pH 3 and 11 file half life values were determined to be approximately 1200 and 700 days, respectively. No decomposition of drug has been noted in stability studies of bulk drug for up to five years. No special storage requirements are required. An expiration period of 30 months has been granted for file nevirapine 200 mg tablets packaged in plastic bottles, and a period of*

24 months for file 20 mg tablet packaged in blister packs and stored between 15-30 °C.

Table 2.3: Stress conditions of nevirapine at ambient temperature for 30 days (Chan Li *et al.*, 2000:249).

Stress conditions	Nevirapine remaining (%)
1 N HCl	30
1 N NaOH	82
3% H_2O_2	72
Light (600 foot candles)	100

2.3.5 BIOAVAILABILITY

Nevirapine is rapidly absorbed following oral administration. Bioavailability is > 90%. Peak plasma concentrations of 2 mg/ml occur within 4 hours after dosing. At a therapeutic dose of 200 mg twice daily, mean steady-state C_{max} and C_{min} of nevirapine in plasma were 5.7 mg/ml and 3.7 mg/ml respectively. The area under the curve is 109 µg.h/ml (Viramune: Scientific Discussion, 2000:2).

2.3.6 ELIMINATION

It was reported by Department of Health and Human Services (2006:7) that in a pharmacokinetic study, approximately 81% of a radiolabeled dose was recovered in the urine, with greater than 80% of that made up of glucuronide conjugates of hydroxylated metabolites. Approximately 10% of a radiolabeled dose was recovered in the faeces. Less than 5% of the recovered radiolabeled dose was made up of the parent compound; therefore, renal excretion plays a minor role in elimination of the parent compound. In

children, nevirapine elimination accelerates during the first years of life, reaching a maximum at around 2 years of age, followed by a gradual decline during the rest of childhood.

2.3.7 BIOTRANSFORMATION

Nevirapine is extensively metabolised by the CYP3A P450 isoform to hydroxylated metabolites and then excreted, primarily in the urine (Katzung, 2000:836).

In humans, it is extensively metabolised via the oxidative cytochrome P450 pathway to several hydroxylated metabolites which are subsequently glucuronised. *In vitro* studies with human liver microsomes suggested that the metabolic pathway was primarily mediated by cytochrome P450 isozymes from the CYP3A family and to a lesser degree CYP2B6 (Viramune: Scientific discussion, 2000:8).

2.3.8 DRUG AND FOOD INTERACTIONS

According to Department of Health and Human Services (2006:7) the following interactions may occur:

Lower plasma concentrations may occur with drugs that are extensively metabolised by CYP3A if nevirapine is administered concurrently due to the fact that nevirapine is metabolised by and induces the activity of CYP3A isoenzymes.

When nevirapine is administered together with hormonal contraceptives then lower blood plasma levels occur, thus additional precautions should be taken in addition to hormonal contraceptives when nevirapine is administered to women with child bearing potential.

Caution is required when administering nevirapine with some protease inhibitors because plasma concentrations of the protease inhibitors may be reduced to subtherapeutic concentrations due to nevirapine-induced hepatic metabolism.

The use of nevirapine together with ketoconazole results in decreased plasma concentrations of ketoconazole and increased levels of nevirapine, therefore it is not recommended.

Nevirapine may decrease plasma concentrations of methadone by increasing its hepatic metabolism. Narcotic withdrawal syndrome has been reported in patients treated with nevirapine and methadone concurrently.

The administration of nevirapine together with rifampin and rifabutin result in subtherapeutic plasma levels of nevirapine because rifampin and rifabutin accelerate the metabolism of NNRTI's and it also results in increased levels of rifampin and rifabutin because nevirapine retards the metabolism of these drugs.

Concurrent use of St. John's Wort and nevirapine is expected to substantially decrease nevirapine concentrations and may result in suboptimal levels of nevirapine, loss of virologic response, and development of nevirapine resistance; thus concurrent use is not recommended.

2.3.9 CONTRA-INDICATIONS

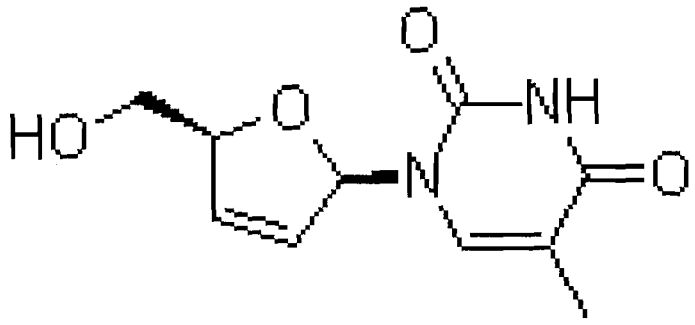
Patients with a hypersensitivity to nevirapine or any of the components should not use nevirapine.

Risk-benefit should be considered in patients with renal function impairment, as nevirapine metabolites are extensively eliminated by the kidneys (Department of Health and Human Services, 2006:7).

2.4 STAVUDINE

2.4.1 CHEMICAL PROPERTIES

2.4.1.1 Structural formula and chemical name



2' ,3' –Didehydro-3'-deoxythymidine

(Chemblink, 2007).

2.4.1.2 Empirical formula

$C_{10}H_{12}N_2O_4$ (The Merck Index, 2006:1510).

2.4.1.3 Molecular weight

224.21 (The Merck Index, 2006:1510).

2.4.1.4 Appearance

White crystalline powder (The Merck Index, 2006:1510).

2.4.1.5 Melting point

160-162°C (The Merck Index, 2006:1510).

2.4.1.6 Solubility

Solubility in water at 25°C is in the order of 90 mg/ml (Ghandi *et al.*, 2000:233), which is about 500 times more than required by the BCS Class 1 (FDA Guidance, August 2000: 2) for the stavudine 40 mg capsules. The effect of solubility of stavudine in an aqueous medium is not reported in literature. However, it is not expected that pH, in the range 1 to 7.5, will reduce the solubility to less than 0.16 mg per ml, which is the minimum for stavudine (40 mg capsules) to be regarded as "highly soluble."

It is reported by the Department of Health and Human Services (2004b:7) that the solubility of stavudine is about 83 mg/ml in water and 30 mg/ml in propylene glycol at 23°C. The n-octanol/water partition coefficient of stavudine at 23°C is 0.144.

2.4.2 PHARMACOLOGICAL ACTION

According to Katzung (2001:838) the NRTI's act by competitive inhibition of HIV-1 reverse transcriptase and can also be incorporated into the growing viral DNA chain and cause termination. Each requires intracytoplasmic activation as a result of phosphorylation by cellular enzymes to the triphosphate form. Most have activity against HIV-2 as well as HIV-1.

The specific mechanism of action of stavudine according to the Department of Health and Human Services (2004b:7) is that stavudine is a prodrug, thus it is phosphorylated by cellular kinases to the active metabolite, stavudine triphosphate. This active metabolite causes inhibition of HIV replication by two mechanisms. The first mechanism entails inhibition of HIV reverse trancriptase (RT) by competition of stavudine triphosphate with the natural substrate deoxythymidine triphosphate. Because stavudine lacks the 3'-OH group, its incorporation into viral DNA causes termination of DNA chain elongation. The other mechanism of action is reduction in the synthesis of mitochondrial DNA because stavudine triphosphate inhibits cellular DNA polymerase beta and gamma. A concentration of 0.009 mg/ml of stavudine is required to inhibit HIV replication by 50% in vitro.

2.4.3 PAEDIATRIC DOSE AND ADULT DOSE

Paediatric dose:

- Oral, > 3 months and ≤ 30 kg: 1 mg/kg 12 hourly.

- 30 kg and over, as for adults, which is 30 mg 12 hourly (Gibbon, C.J., 2003:313).

2.4.4 STABILITY

Kawaguchi *et al.* (1989:1944-1945) studied the stability of stavudine in an aqueous medium. The chemical stability ($T_{1/2}$) of stavudine at 37 °C at various pH conditions is shown in Table 2.4.

Table 2.4: Chemical stability of stavudine in an aqueous medium [from Kawaguchi *et al.*, (1944: 1945)].

pH	$T_{1/2}$ (Hours)
1.0	26
3.0	92
5.0	105
7.0	102
9.0	97

From the above data (Table 2.4), it can be seen that stavudine shows the highest stability between pH 5 & 7. Sarasa *et al.* (2000:187-188) reported that stavudine is degraded about 5% in human plasma samples over 24 hours at 25 °C.

Kawaguchi *et al.* (1944:1945) reported that the degradation of stavudine takes place through hydrolysis of the glycosyl-thymine bond under all pH conditions studied by them, i.e. acid and basic medium.

A study was done to describe the degradation of stavudine under different stress conditions (hydrolysis, oxidation, photolysis and thermal stress). The drug was found to hydrolyse in acidic, neutral and alkaline conditions and also under oxidative stress. The major degradation product formed under various conditions was thymine (Dunge *et al.*, 2005:1).

Stability studies have been conducted on 3 batches of stavudine stored at 25°C/60%RH (24 months) and 40°C/75%RH (6 months). The data revealed no significant change and a re-test period of 24 months has been allowed for stavudine when stored at/below 25°C in a well closed container (WHOPAR part 6).

Stability studies have been conducted on 3 batches of stavudine 40 mg capsules stored at 25°C/60%RH (12 months) and 40°C/75%RH (6 months). Stavudine showed some degradation though statistically the trend was acceptable for allowing a tentative shelf life of 2 years for the product when stored at/below 30°C (WHOPAR Part 6).

2.4.5 BIOAVAILABILITY

Following oral administration, stavudine is rapidly absorbed, with peak plasma concentrations occurring within 1 hour after dosing (WHOPAR Part 6).

2.4.6 ELIMINATION

Forty (40) % of overall clearance of stavudine is through renal elimination and is independent of the route of administration. About 50% undergoes nonrenal elimination. Although the exact metabolic fate is unknown, stavudine may be cleaved to thymine, and the subsequent degradation and/or utilisation of thymine may account for the unrecovered stavudine. There is no clarity on whether stavudine is removed by hemodialysis or peritoneal dialysis. There is an indication of active tubular secretion in addition to glomerular secretion. It is important to remember that the dosage of stavudine should be modified in patients with reduced creatinine clearance and those patients receiving maintenance hemodialysis because oral clearance of stavudine decreases and the terminal elimination half life increase as creatinine clearance decreases (Department of Health and Human Services, 2004b:7).

2.4.7 BIOTRANSFORMATION

Stavudine is phosphorylated intracellularly to stavudine triphosphate, the active substrate for HIV-reverse transcriptase (USP DI, 1999).

2.4.8 DRUG AND FOOD INTERACTIONS

According to the Department of Health and Human Services (2004b:5), the following drugs could cause peripheral neuropathy and should be used with caution with stavudine:

- Chloramphenicol
- Cisplatin
- Dapsone
- Didanosine
- Ethambutol
- Ethionamide
- Hydralazine
- Isoniazid
- Lithium
- Metronidazole
- Nitrofurantoin
- Phenytoin
- Vincristine
- Zalcitabine

Didanosine or hydroxyurea may increase the risk of potentially fatal hepatotoxicity or pancreatitis if taken concurrently with stavudine.

Concomitant use of stavudine and zidovudine is not recommended due to possible competitive inhibition of the intracellular phosphorylation of stavudine. *In vitro* studies detected an antagonistic antiviral effect between stavudine and zidovudine at a molar ratio of 20 to 1, respectively. Concurrent use is not recommended until *in vivo* studies demonstrate that these medications are not antagonistic in their anti-HIV activity (Department of Health and Human Services, 2004b:5).

2.4.9 CONTRA-INDICATIONS

Stavudine should not be taken by patients who have a hypersensitivity to this drug or any of its components. During pregnancy the combination of stavudine and didanosine should only be used if the benefits outweigh the risks. Fatal lactic acidosis has been reported in pregnant women treated with a combination of stavudine and didanosine with other antiretroviral agents. Lactic acidosis and severe hepatomegaly with steatosis have been reported (Department of Health and Human Services, 2004b:7).

2.5 CONCLUSION

As can be seen in this chapter, the characteristics of the three chosen ARV's show us that in theory they are compatible. This literature study showed that lamivudine stable is in the pH range of 6.5 – 6.7; stavudine between 5 and 7; and nevirapine from 3 to 11. Therefore, any Pheroid™ formulation, containing all three these ARV's, would need to have a pH between 6 and 7 to ensure stability of all the actives, but especially that of lamivudine.

In the following chapter the formulation of the Pheroid™ technology will be discussed.

CHAPTER 3

THE PHEROID™ TECHNOLOGY

3.1 Introduction

The Pheroid™ technology is based on what was previously called Emzaloid™ technology (Grobler *et al.*, 2007:3). "The Pheroid™ system is a stable structure within a system compromising of a unique submicron emulsion type formulation that can be manipulated in terms of morphology, structure, size and function. The Pheroid™ consists mainly of plant and essential fatty acids" (Grobler, 2004:4). It is able to enhance the absorption and/or efficacy of various categories of active ingredients and other compounds. In comparison with other systems, the Pheroid™ technology has been shown to result in major improvements in characteristics such as, the control of size, charge, and the hydrophilic-lipophilic nature of therapies (Grobler *et al.*, 2007:3).

3.2 Characteristics of the Pheroid™ system

The Pheroid™ delivery system is a colloidal system that contains unique and stable lipid-based submicron- and micron-sized structures, called Pheroids™, uniformly distributed in a dispersion medium that may be adapted to the indication. The purpose of the use of colloidal systems as carriers of drugs is to enhance the efficacy of the administered compounds whilst reducing the unwanted side-effects. Due to the different colloidal systems, such as simple colloids, multiple colloids and network colloids, various types of Pheroid™ structures can be formulated (Grobler *et al.*, 2007:4-5).

The Pheroids™ consist primarily of ethylated and pegylated poly unsaturated fatty acids, including the omega-3 and -6 fatty acids but excluding arachidonic acid (Grobler *et al.*, 2004:6). The Pheroid™ contains one unique component, namely nitrous oxide (N_2O) which has at least three different functions, namely, it contributes to the miscibility of the fatty acids, the self-assembly process of the Pheroids™ and the stability of the formed Pheroids™ (Grobler *et al.*, 2007:10).

3.3 The advantages of the Pheroid™ system

- Increased delivery of active compounds;
- Decreased time to onset of action;
- Reduction of minimal effective concentration;
- Increased therapeutic efficacy;
- Reduction in cytotoxicity;
- Penetration of most known barriers in the body and in the cells (e.g. keratinized tissue, intestinal lining, vascular system, fungi, bacteria, parasites);
- Ability to target treatment areas (e.g. Pheroid™-containing anti-virals have shown to have an impact on the viral load, perhaps by interfering with viral binding and/or budding);
- Lack of immunological response;
- Ability to transfer genes to cell nuclei;
- Reduction of drug resistance (Grobler, 2004:3).

3.4 The functions

The essential fatty acids that the Pheroid™ consists of are necessary for various cell functions. These fatty acids cannot be manufactured by human cells; they therefore need to be ingested. Some of the functions of this component of the Pheroid™ system are the following:

- Maintenance of membrane integrity of cells;
- Energy homeostasis;
- Modulation of the immune system through amongst others the prostaglandins/leukotrines;
- Some regulatory aspects of programmed cell death (Grobler, 2004:4).

3.5 Physical-chemical characteristics of the Pheroid™ system

Depending on the composition of the Pheroid™, they are metabolised in either the mitochondria or the peroxisomes of the cell, resulting in release of the active compound (Grobler *et al.*, 2007:24). The enhancement of the Pheroids™ is thought to be a result of the following mechanisms:

- Deposition of compound within organisms by the Pheroids™;
- Enhancer effect of individual fatty acids and N_2O upon barrier permeability and fluidity;
- Lipid concentration – the higher the free fatty acid concentration, the higher the delivery up to saturation point;
- Increased thermodynamic activity of active compounds due to less favourable environment for charged molecules;
- Increased partitioning into cells because of ion-pairing effects in a low dielectric medium and binding proteins in membranes (Grobler *et al.*, 2007:27).

Table 3.1: Similarities and differences of Pheroid™ and lipid-based delivery systems (reprinted with permission from Mrs. A. Grobler) (Grobler, 2004:6).

Pheroid™	Other delivery systems
Consists mainly of essential fatty acids.	Generally contain a proportion of substances foreign to the body, e.g. artificial polymers.
Studies have shown that it does not produce an immune response in man.	Some liposomal formulations have been shown to cause immune responses.
A variety of Pheroid™ types are formulated, depending on the composition and the method of manufacturing, e.g. bilayer membrane vesicle, a highly elastic or fluid bilayer vesicle with loose lipid packing, formation of small pro-Pheroids™, etc.	A variety of different liposome types have been described: single lamellar vesicles, multi-lamallar vesicles, nanosomes, etc.
The Pheroid™ can be manipulated in terms	Problems with the degree of repeatability of

of size, charge, lipid composition and membrane packing. The desired types can repeatably be obtained.	liposomal systems, liposomal types and sizes have been described.
A specific protein family in cell membranes is responsible for the binding and uptake of essential fatty acids. Thus, an affinity exists between the Pheroid™ and cell membranes since it is comprised of fatty acids.	Specific binding and uptake mechanisms by mammalian cells have not been described for other delivery systems.
To a certain extent, the Pheroid™ can be targeted at a sub-cellular level by the use of different combinations of fatty acids and/or other added molecules.	Since phospholipids are metabolised in the cell membrane, it is difficult to envisage how sub-cellular organelles can be easily targeted by this approach.
The Pheroid™ causes no cytotoxicity and assists with maintenance of cell membrane.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body.
Drug resistance was reduced or eliminated in all *in vitro* studies done.	Some delivery systems are prone to drug resistance or adverse immune responses.
The Pheroid™ is polyphilic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophylic or hydrophilic.
The Pheroid™ system passively targets the reticulo-endothelial system (RES).	Similarly, liposomal systems generally target RES.
The Pheroid™ reduces the volume of distribution and consequently the concentration at the target site is increased. An enhanced but narrow therapeutic index can be achieved, with a decrease in aspecific toxicity.	Similarly, liposomes encapsulating small molecule chemotherapeutic agents have been shown to reduce the volume of distribution and enhance the concentration of the active compound in tumors, resulting in a decrease of aspecific toxic effects.
Little leakage of the drug from the Pheroid™ before it reaches the target site has been observed.	Leaking of the drug in the plasma has been observed.

The Pheroid™, due to its composition is sterically stabilized without the disadvantages of increased size and decreased elasticity.	Delivery systems generally need to be stabilised.
The Pheroid™ contains no cholesterol, but however the interior volume is stably maintained.	Most lipid-based delivery systems contain phospholipids and cholesterol.
Pheroid™ enhances bioavailability by inhibiting the drug efflux mechanism.	Liposomal systems containing this feature have not been described.
The Pheroid™ enhances bioavailability of oral, topical and buccal administration of active compounds in all products tested so far. Absorption was similarly increased.	Some delivery systems have been shown to enhance absorption, whereas others decreased absorption.
The pharmacokinetics of active compounds changes during the entrapment process, resulting in a decrease in time needed to achieve maximum concentration levels.	Liposomes have similarly been shown to change the pharmacodynamics of active compounds.
Entrapment efficiency is high.	Due to charge and steric limitations of delivery systems, entrapment efficiencies may be problematic.
The loading capacity of the Pheroid™ is determined by the type of Pheroid™ formulated for a specific compound.	The interior or intra-membrane volume determines the loading capacity of most lipid-based delivery systems, it is therefore limited.
The Pheroid™ can be formulated as a pro-Pheroid™.	Liposomes can be formulated as pro-liposomes.
Micro-sponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other in the sponge spaces.	Combination treatments are problematic for most delivery systems.

Batch-to-batch reproducibility and stability have been proven	Large scale manufacturing of other delivery systems sometimes shows low batch-to-batch reproducibility and, in some instances, problems with size control.
The Pheroid™ showed *in vivo* stability during vaccine animal studies and in initial phase I volunteer trials.	Both product and *in vivo* chemical and physical instability are problematic for some lipid-based delivery systems.
Pheroid™ with entrapped small peptides and antibodies have been shown to interact with specific micro-domains on cells in culture. The purpose of the peptides and antibodies was drug targeting.	Antibody-containing liposomes for drug targeting have been described.

3.6 Conclusion

The Pheroid™ technology is a breakthrough in the pharmaceutical industry. It has therapeutic as well as preventative uses. During my study I have entrapped the anti-retrovirals in the Pheroid™ system and have studied the stability of the ARV's in the Pheroid™ system.

CHAPTER 4

VALIDATION, RESULTS AND DISCUSSION FOR THE ASSAY OF THE ACTIVES AND PRESERVATIVES

4.1 ORIGIN OF METHODS

This method was developed and validated under the guidance of Prof. J.L. du Preez at the Analytical Technology Laboratory (ATL) at the North-West University, Potchefstroom.

4.2 CHROMATOGRAPHIC CONDITIONS

Two separate methods were used. The one method was used to assay the actives and the preservatives together and the other method was used to assay the anti-oxidants. The assay, validation and results of the anti-oxidants is described in Chapter 5.

- **Analytical Instrument:** HP1100 series Agilent HPLC equipped with a pump, auto sampler, UV detector and Chemstation Rev. A.06.02 data acquisition and analysis software or equivalent.
- **Column:** Luna C 18(2) 250x4.6 mm (Phenomenex, Torrance, CA).
- **Mobile phase:** Acetonitrile (solvent B), water with 0.2% triethylamine, pH adjusted with phosphoric acid or ammonium hydroxide (solvent A).

Table 4.1: Gradient table.

Time(min)	Solvent A (%)	Solvent B(%)
0.00	12	88
1.50	12	88
8.00	65	35
10.00	65	35
12.00	65	35
12.10	12	88

- **Flow rate:** 1.0 ml/min.
- **Injection volume:** 20 µl.
- **Detection:** UV at 270 nm, 16 nm bandwidth.
- **Retention time:**

Lamivudine	± 4.2 minutes
Stavudine	± 5.3 minutes
Nevirapine	± 9.1 minutes
Methyl hydroxybenzoate (methyl paraben)	± 9.9 minutes
Propyl hydroxybenzoate (propyl paraben)	± 11.8 minutes

- **Run time:** 16 minutes

4.3 SAMPLE PREPARATION

FORMULATION A (Pheroid™):

1. Place a clean 100 ml volumetric flask on an analytical balance and tare.
2. Accurately weigh 5 ml of each of the samples at the indicated stability conditions (25°C+60%RH, 30°C+65%RH and 40°C+75%RH) of Pheroid™ formulation A into separate 100 ml volumetric flasks.
3. Add approximately 75 ml of methanol to each of the volumetric flasks, remove the stoppers and put the flasks in an ultrasonic bath containing luke warm water and sonicate for approximately 10-15 minutes.
4. Make sure that all of the pheroid formulation are dissolved. If solid particles are visible, shake the flask and sonicate it further until it is completely dissolved.
5. Allow the flasks to cool to room temperature and fill each flask to 100 ml with methanol.

6. Pipette 10 ml of this solution into a 50 ml volumetric flask and make up to volume with methanol. This is the final solution.

7. Filter the solutions through a 0.45 μm filter into auto sampler vials and analyse.

FORMULATION B (Pro-Pheroid™) :

STEP 1:

1. Place a clean dry 25 ml measuring cylinder on an analytical balance and tare.

2. Accurately weigh 25 ml of the pro-Pheroid™ solution.

3. Because we only need 10 ml of pro-Pheroid™ the following calculation must be done:

25 ml pro-Pheroid™ = A (the amount that 25 ml weighed)

therefore 10 ml = x

$x = \frac{10xA}{25\ ml}$

STEP 2:

1. Place a clean dry 50 ml volumetric flask on an analytical balance and tare.

2. Accurately weigh the amount that was calculated for x in the above calculation, of each of the samples at the stability conditions, 25°C+60%RH, 30°C+65%RH and 40°C+75%RH of the pro-Pheroid™ solution.

3. Add approximately 30 ml of 0.1 N HCl and put the flask in an ultrasonic bath containing lukewarm water and sonicate for approximately 1 hour.

4. If the particles have not yet dissolved then sonicate the solution further until there is a homogenous solution.

5. Allow the flask to cool to room temperature and fill each flask to 50 ml with 0.1 N HCl.

6. Use a 5 ml pipette and transfer 5 ml of the above solution into a 100 ml volumetric flask and make up to volume with methanol.

7. Thereafter pipette 10 ml of this second solution into a 50 ml volumetric flask and make up to volume with methanol; this is the final solution.

8. Filter the solutions through a 0.45 μm filter into auto sampler vials and analyse.

4.4 STANDARD PREPARATION

FORMULATION A & B:

1. Place clean dry weighing boats on an analytical balance and tare.

2. For Solution A, accurately weigh 100 mg of nevirapine, 100 mg of lamivudine and 100 mg of stavudine, each in its own weighing boat.

3. Place the three accurately weighed API's in a 100 ml volumetric flask.

4. Make up to volume with methanol.

5. Place a clean dry weighing boat on an analytical balance and tare.

6. For Solution B, accurately weigh 125 mg of methyl hydroxybenzoate and 5 mg of propyl hydroxybenzoate, each in its own weighing boat.

7. Add these substances into a 100 ml volumetric flask and make up to volume with methanol.

8. Then take 10 ml of Solution A and 10 ml of Solution B and add this to a 50 ml volumetric flask.

9. Make up to volume with methanol. This is the final Solution.

10. Filter the solutions into an auto sampler vial and analyse.

4.5 CALCULATIONS

FORMULATION A:

[Std] = mass of std (mg) /100 ml

= A x 10 ml

= B / 50 ml

= C (mg/ml) x 1000 ml

= D µg/ml

$$[\text{Sample}] = \frac{([\text{std}] \times \text{SAR})}{\text{STR}}$$

SAR=Sample peak areas

STR=Standard peak areas

FORMULATION B:

[Std] = see above calculation

$$[\text{Sample A}] = \frac{([\text{std}] \times \text{SAR})}{\text{STR}}$$

SAR=Sample peak areas

STR=Standard peak areas

We need to take into consideration the specific gravity, thus the following calculation must be done:

Actual [Sample] = [sample A] / SG

SG=Specific Gravity in wt/ml

4.6 VALIDATION TEST PROCEDURE AND ACCEPTANCE CRITERIA

4.6.1 SPECIFICITY

METHOD:

1. Prepare a sample from a placebo of the pheroid solution that does not contain the various actives which are being tested with this method.
2. Take 5 ml of the standard solution and individually dilute it with 5 ml of 0.1 N HCl, 1 M NaOH and 10 % H_2O_2 respectively.
3. Store the solution of the actives and the preservatives overnight at 40°C.
4. Store the solution containing the anti-oxidants overnight at room temperature.
5. Filter these samples into an auto sampler vial and analyse to determine whether any additional peaks were formed.

ACCEPTANCE CRITERIA:

- The placebo should not contain any peaks that will interfere with the determination of the actives.
- Extra peaks formed in the stressed samples should be discernable from those of the active components.

4.6.2 LINEARITY

METHOD:

1. Prepare a standard solution as described under standard preparation.
2. Inject variable volumes into the chromatograph to obtain standards from 75-125% of the expected sample concentration.

ACCEPTANCE CRITERIA:

- Linear regression analysis should yield a regression co-efficient (r squared) of ≥ 0.99.

4.6.3 ACCURACY

METHOD:

1. Spike amounts of active at concentrations of approximately 80%, 100% and 120% of the expected sample concentration.

2. Inject into the chromatograph in duplicate.

ACCEPTANCE CRITERIA:

- Recovery must be between 98-102%

4.6.4 PRECISION

4.6.4.1 INTRA DAY PRECISION (REPEATABILITY)

METHOD:

1. Measure approximately 3x8 ml (80%), 3x10 ml (100%) and 3x12 ml (120%) of the pheroid solution into 100 ml volumetric flasks and make up to volume with methanol.

2. Prepare a single standard at 100% of the expected sample concentration as described in the method.

3. Inject into the chromatograph in duplicate.

4.6.4.2 INTER DAY PRECISION

METHOD:

1. Analyse the same homogeneous sample in triplicate as described for intra-day precision (at 100% of the sample concentration) on two more occasions to determine the between-day variability of the method. On one occasion (day 3) a different analyst should perform the analysis on a different set of equipment.

ACCEPTANCE CRITERIA:

- % RSD calculated during repeatability studies must be less or equal than 2% (n=9).
- % RSD calculated during inter-day precision studies must be less or equal than 5% (n=9).

4.6.5 RUGGEDNESS

4.6.5.1 STABILITY OF THE SAMPLE SOLUTIONS

METHOD:

1. Prepare a sample as directed under sample preparation in paragraph 4.3.
2. Inject the sample into the chromatograph.
3. Leave the sample in the auto sampler tray and re-analyse over a period to determine the stability of the sample.

ACCEPTANCE CRITERIA:

- Sample solutions should not be used for a period longer than it takes to degrade by 2% and in the case of degradation, special precautions should be followed to compensate for the loss.

4.6.5.2 SYSTEM REPEATABILITY

METHOD:

1. Inject a sample six times consecutively in order to test the repeatability of the peak area as well as the retention time.

ACCEPTANCE CRITERIA:

- The peak and retention times should have an RSD of 2 % or less.

4.6.6 ROBUSTNESS

METHOD:

1. Make deliberate changes to the chromatographic conditions to determine the methods tolerance towards changes.

2. Change the flow rate, wavelength, injection volume, acetonitrile concentration and use a similar column from a different manufacturer.

ACCEPTANCE CRITERIA:

- The method should be able to tolerate a 5% variance in the chromatographic conditions.

4.6.7 SYSTEM AND METHOD PERFORMANCE CHARACTERISTICS (SYSTEM SUITABILITY)

METHOD:

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP tailing factor, capacity factor, and resolution between peaks and repeatability of multiple injections.

2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

ACCEPTANCE CRITERIA:

- The RSD should not exceed 2%.
- The column must have more than 3000 theoretical plates for all the peaks.
- The resolution between subsequent peaks must be greater than 2.

4.7 VALIDATION RESULTS

4.7.1 SPECIFICITY

Placebo and standard solutions were prepared as described in paragraph 4.4.

All samples were analysed at 270 nm.

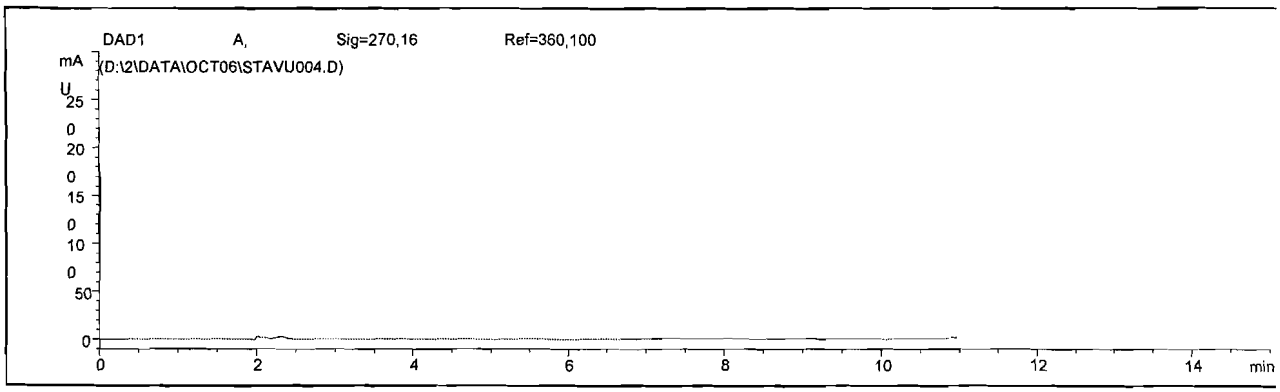


Figure 4.1: HPLC chromatogram of the placebo.

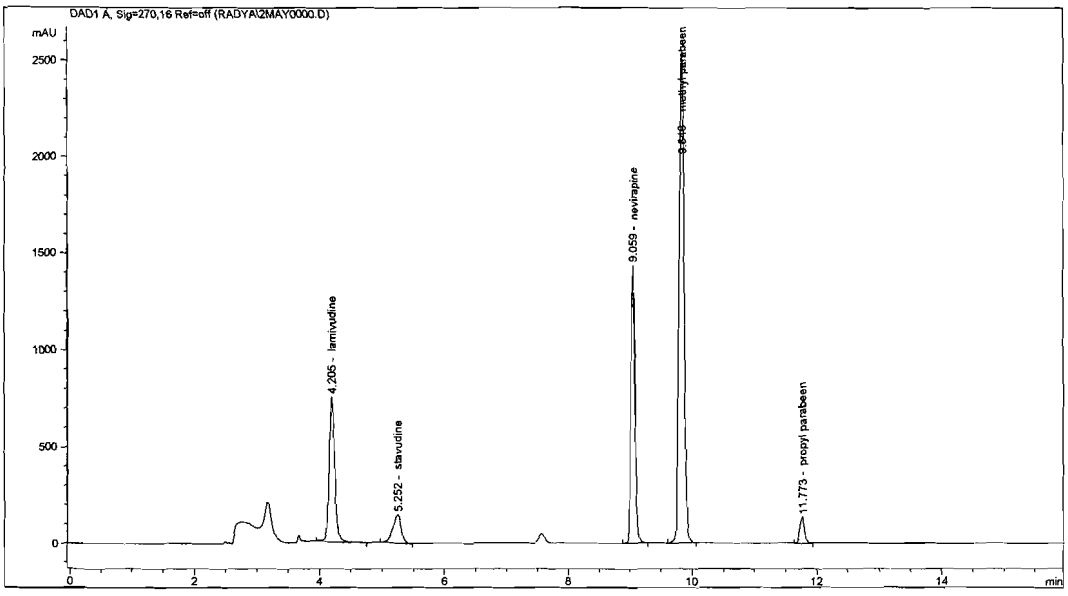


Figure 4.2: HPLC chromatogram of a standard solution.

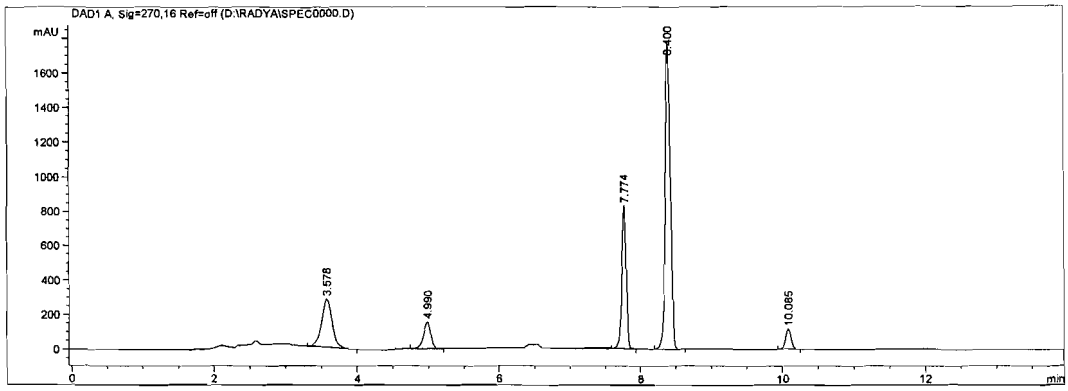


Figure 4.3: HPLC chromatogram of lamivudine, stavudine, nevirapine, methyl hydroxybenzoate and propyl hydroxybenzoate stressed in 0.1 N HCl at 40°C for 24 hours.

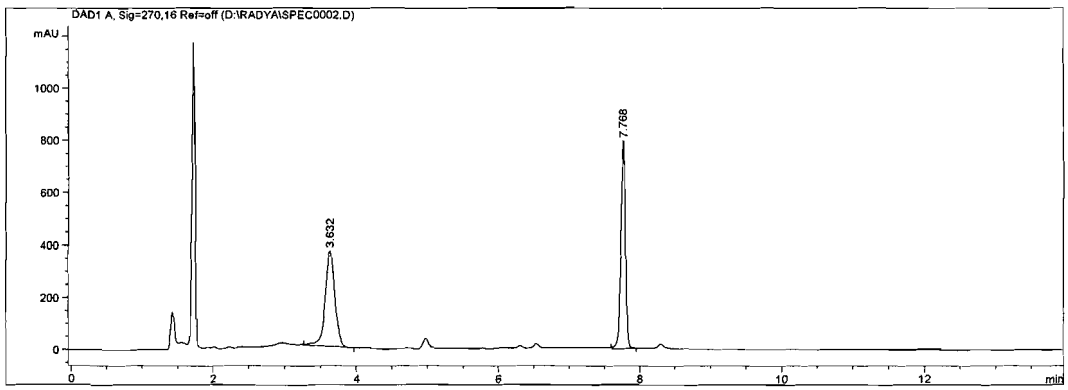


Figure 4.4: HPLC chromatogram of lamivudine, stavudine, nevirapine, methyl hydroxybenzoate and propyl hydroxybenzoate stressed in 1 N NaOH at 40°C for 24 hours.

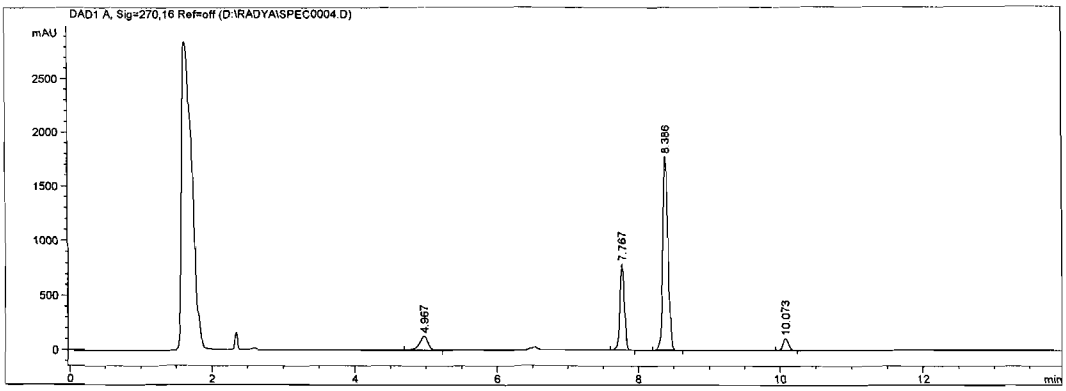


Figure 4.5: HPLC chromatogram of lamivudine, stavudine, nevirapine, methyl hydroxybenzoate and propyl hydroxybenzoate stressed in 10% H_2O_2 at 40°C for 24 hours.

4.7.1.1 CONCLUSION

In Figure 4.3 it can be seen that the peaks shifted and this could be because of the HCl. In Figure 4.4, the peaks of the preservatives degraded completely and this could have been due to the NaOH. In Figure 4.5, the H_2O_2 caused the lamivudine to degrade completely and it caused a certain amount of degradation in all the other substances as well.

4.7.2 LINEARITY

4.7.2.1 LAMIVUDINE

Table 4.3: Peak area and concentration for lamivudine.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
140.6	5604.1	5623.8	5614.0
160.7	6334.6	6310.4	6322.5
180.8	7000.8	7013.0	7006.9
200.9	7725.8	7693.3	7709.6

221.0	8370.6	8369.1	8369.9
241.1	9054.9	9059.1	9057.0

Table 4.4: Regression parameters for lamivudine.

REGRESSION PARAMETERS			
R squared	0.99997	**Lower 95%**	**Upper 95%**
Intercept	816.06714	904.54483	727.58946
Slope	34.21397	34.67020	33.75775

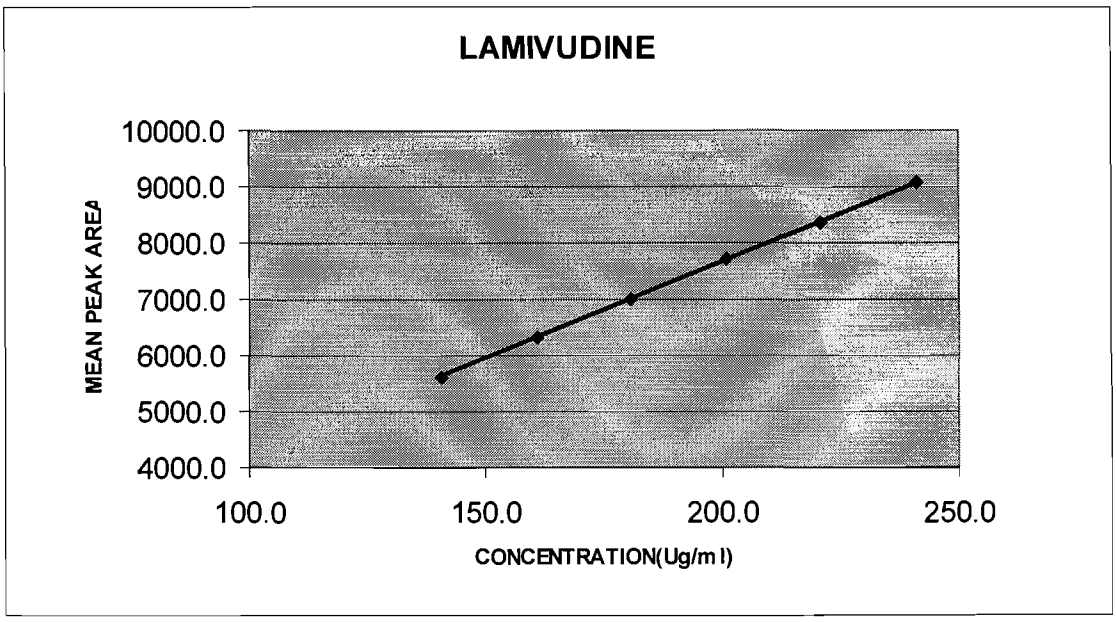


Figure 4.6: The linear regression curve for lamivudine.

4.7.2.2 STAVUDINE

Table 4.5: Peak area and concentration for stavudine.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
36.0	1557.2	1559.5	1558.3
41.2	1768.8	1765.4	1767.1
46.3	1988.4	1985.4	1986.9
51.5	2201.5	2201.3	2201.4
56.6	2396.8	2395.1	2395.95
61.8	2600.2	2596.9	2598.5

Table 4.6: Regression parameters for stavudine.

REGRESSION PARAMETERS			
R squared	0.9996	Lower 95%	Upper 95%
Intercept	102.72333	46.98089	158.46577
Slope	40.54217	39.41993	41.66441

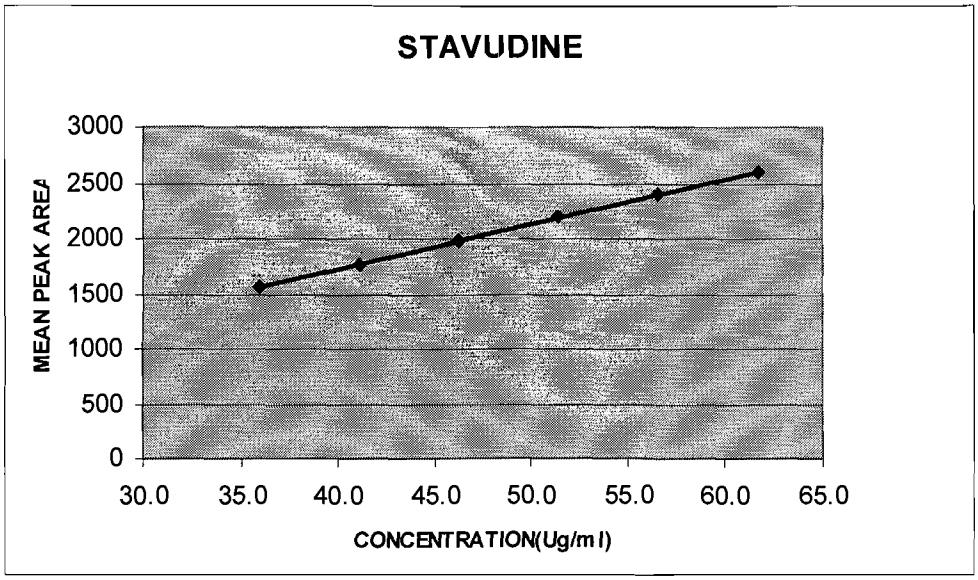


Figure 4.7: The linear regression curve for stavudine.

4.7.2.3 NEVIRAPINE

Table 4.7: Peak area and concentration for nevirapine.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
140.7	4344.6	4352.0	4348.3
160.8	4948.9	4956.8	4952.9
180.9	5589.9	5587.8	5588.9
201.0	6216.6	6212.8	6214.7
221.1	6799.0	6790.0	6794.5
241.2	7414.4	7399.9	7407.2

Table 4.8: Regression parameters for nevirapine.

REGRESSION PARAMETERS			
R squared	0.9998	Lower 95%	Upper 95%
Intercept	63.59238	-39.03268	166.21744
Slope	30.48034	29.95142	31.00925

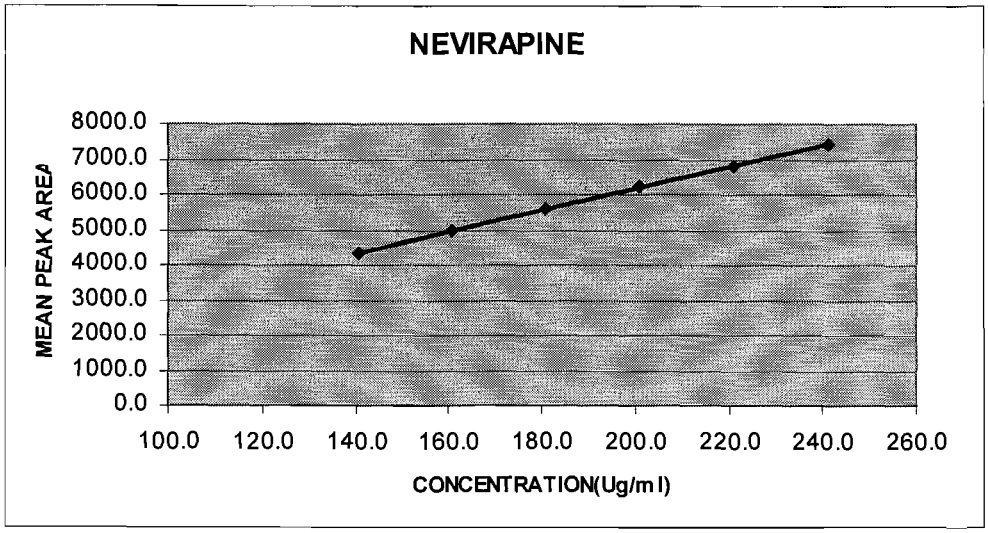


Figure 4.8: The linear regression curve for nevirapine.

4.8.2.4 METHYL HYDROXYBENZOATE

Table 4.9: Peak area and concentration for methyl hydroxybenzoate.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
176.7	12188.5	12206.0	12197.3
201.9	13839.5	13846.3	13842.9
227.2	15564.0	15591.0	15577.5
252.4	17171.8	17209.8	17190.8
277.6	18739.5	18647.8	18693.6
302.9	20312.3	20160.8	20236.5

Table 4.10: Regression parameters for methyl hydroxybenzoate.

REGRESSION PARAMETERS			
R squared	0.9993	**Lower 95%**	**Upper 95%**
Intercept	991.56071	440.14552	1542.97591
Slope	63.80094	61.53755	66.06433

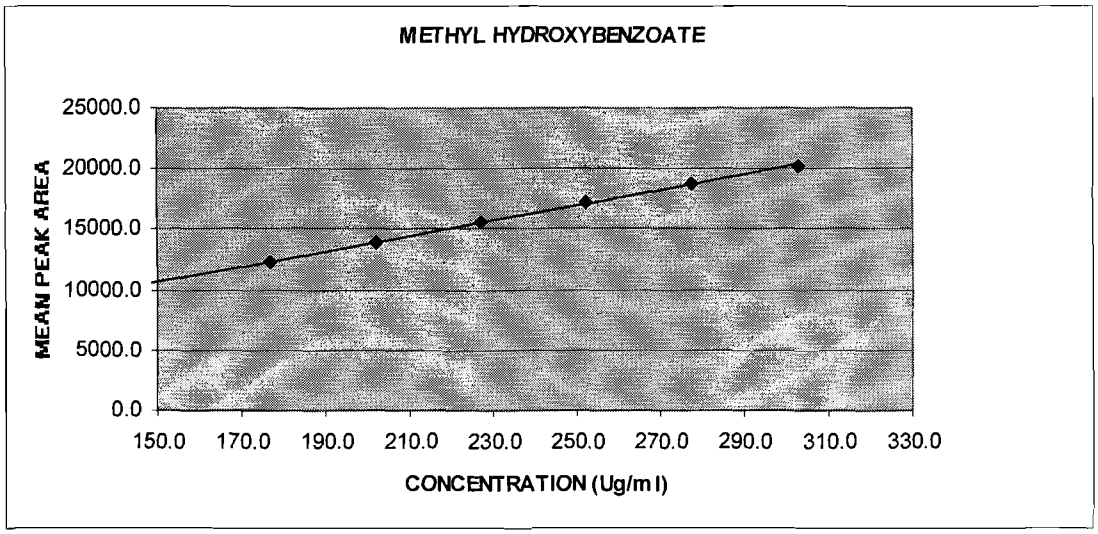


Figure 4.9: The linear regression curve for methyl hydroxybenzoate.

4.8.2.5 PROPYL HYDROXYBENZOATE

Table 4.11: Peak area and concentration for propyl hydroxybenzoate.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
7.9	484.0	516.7	500.4
9.1	541.4	538.1	539.8
10.2	611.6	629.9	620.8
11.3	686.9	703.3	695.1
12.5	763.0	752.3	757.7
13.6	820.6	825.2	822.9

Table 4.12: Regression parameters for propyl hydroxybenzoate.

REGRESSION PARAMETERS			
R squared	0.99461	Lower 95%	Upper 95%
Intercept	20.72333	-45.25569	86.70236
Slope	58.97707	52.94922	65.00492

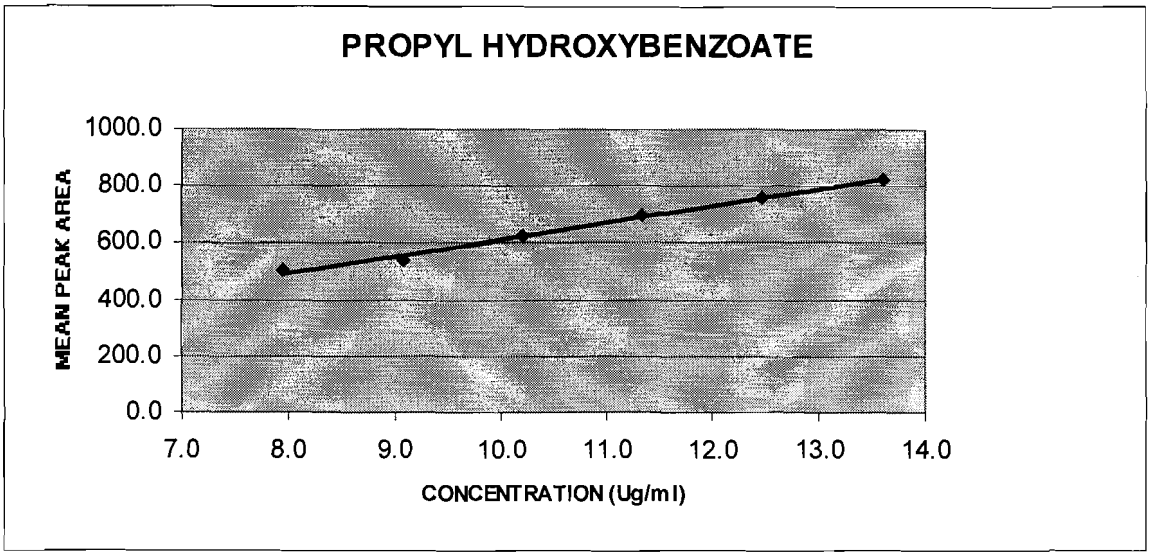


Figure 4.10: The linear regression curve for propyl hydroxybenzoate.

4.8.3 CONCLUSION

This method is linear over the concentration range of 140.6-241.1 µg/ml for lamivudine, 36.0-61.8 µg/ml for stavudine, 140.7-241.2 µg/ml for nevirapine, 176.7-302.9 µg/ml for methyl hydroxybenzoate and 7.9-13.6 µg/ml for propyl hydroxybenzoate. This method meets the criteria for acceptance and is therefore suitable for single point calibration.

4.8.4 ACCURACY AND PRECISION

4.8.4.1 LAMIVUDINE

Table 4.13: **Percentages lamivudine recovered.**

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
175.6	4929.0	4415.4	4672.2	172.4	98.2
175.6	4835.6	4886.0	4860.8	179.4	102.1
175.6	4853.2	4746.8	4800.0	177.1	100.9
219.5	5784.0	5842.2	5813.1	214.5	97.7
219.5	6106.1	6074.2	6090.2	224.7	102.4
219.5	5976.0	6029.0	6002.5	221.5	100.9
263.4	7325.5	7378.5	7352.0	271.3	103.0
263.4	7364.3	7242.4	7303.4	269.5	102.3
263.4	7387.6	7171.4	7279.5	268.6	102.0

Table 4.14: Confidence intervals for lamivudine.

Mean %	101.5
SD	1.78
% RSD	1.76

4.8.4.2 STAVUDINE

Table 4.15: Percentages stavudine recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
42.7	1453.4	1304.3	1378.9	42.9	100.4
42.7	1302.1	1398.1	1350.1	42.0	98.3
42.7	1397.9	1303.5	1350.7	42.0	98.3
53.3	1702.0	1707.4	1704.7	53.0	99.4
53.3	1797.5	1691.8	1744.7	54.2	101.7
53.3	1665.8	1738.9	1702.4	52.9	99.3
64.0	2051.3	2096.3	2073.8	64.5	100.7
64.0	2071.5	2030.4	2051.0	63.7	99.6
64.0	2009.6	2069.1	2039.3	63.4	99.0

Table 4.16: Confidence intervals for stavudine.

Mean %	99.63
SD	1.07
% RSD	1.07

4.8.4.3 NEVIRAPINE

Table 4.17: Percentages nevirapine recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
169.6	5343.1	5267.3	5305.2	166.1	97.9
169.6	5355.1	5362.5	5358.8	167.7	98.9
169.6	5346.2	5362.7	5354.5	167.6	98.8
212.0	6587.9	6610.5	6599.2	206.6	97.5
212.0	6758.3	6754.8	6756.6	211.5	99.8
212.0	6538.9	6644.4	6591.7	206.3	97.3
254.4	8041.8	8058.5	8050.2	252.0	99.1
254.4	8035.2	8077.4	8056.3	252.2	99.1
254.4	8028.9	8011.0	8020.0	251.1	98.7

Table 4.18: Confidence intervals for nevirapine.

Mean %	98.57
SD	0.77
% RSD	0.78

4.8.4.4 METHYL HYDROXYBENZOATE

Table 4.19: Percentages methyl hydroxybenzoate recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
205.0	14032.9	13718.8	13875.9	206.6	100.8
205.0	13944.1	13869.0	13906.6	207.0	1010.0
205.0	13970.3	14059.3	14014.8	208.6	101.8
256.3	16932.2	17085.5	17008.9	253.2	98.8
256.3	17491.7	17089.9	17290.8	257.4	100.4
256.3	16879.9	16916.4	16898.2	251.5	98.1
307.6	19730.6	19928.9	19829.8	295.2	96.0
307.6	19798.1	19874.2	19836.2	295.3	96.0
307.6	20468.9	19917.3	20193.1	300.6	97.7

Table 4.20: Confidence intervals for methyl hydroxybenzoate.

Mean %	98.95
SD	2.04
% RSD	2.06

4.8.4.5 PROPYL HYDROXYBENZOATE

Table 4.21: Percentages propyl hydroxybenzoate recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
10.0	598.2	589.8	594.0	9.8	98.1
10.0	600.4	600.1	600.3	9.9	99.1
10.0	598.3	599.5	598.9	9.9	98.9
12.5	738.1	740.1	739.1	12.2	97.6
12.5	766.5	759.6	763.1	12.6	100.8
12.5	725.8	743.7	734.8	12.1	97.1
15.0	899.7	901.9	900.8	14.9	99.2
15.0	900.2	903.6	901.9	14.9	99.3
15.0	897.9	896.9	897.4	14.8	98.8

Table 4.22: Confidence intervals for propyl hydroxybenzoate.

Mean %	98.88
SD	1.02
% RSD	1.03

4.8.5 CONCLUSION

The method yielded a mean recovery (accuracy) of 101.5 for lamivudine, 99.63 for stavudine, 98.57 for nevirapine, 98.95 for sodium methyl hydroxybenzoate and 98.77 for sodium propyl hydroxybenzoate.

The method yielded an intra-day precision of 1.76, 1.07, 0.78, 2.06 and 1.03 % RSD for lamivudine, stavudine, nevirapine, methyl hydroxybenzoate and propyl hydroxybenzoate respectively. These results are all acceptable.

4.8.6 PRECISION

4.8.6.1 INTER DAY PRECISION

4.8.6.1.1 LAMIVUDINE

Table 4.23: Inter-day precision for lamivudine

	Day 1	Day 2	Day 3	Between days
	97.7	97.2	98.0	
	102.4	100.5	100.6	
	100.9	99.5	99.8	
Mean	100.3	99.1	99.5	99.6

SD	1.96	1.37	1.08	1.60
RSD %	1.95	1.38	1.08	1.60

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	301.0	100.333	5.76333
Day 2	3.0	297.270	99.090	2.807
Day 3	3.0	298.503	99.501	1.741

ANNOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.407	2.0	1.204	0.350	0.718	5.143
Within Groups	20.623	6.0	3.437			
Total	23.030	8.0				

4.8.6.1.2 STAVUDINE

Table 4.24: Inter-day precision for stavudine

	Day 1	Day 2	Day 3	Between days
	99.4	98.5	100.0	
	101.7	98.6	100.3	
	99.3	100.2	99.7	
Mean	100.1	99.1	100.0	99.7
SD	1.13	0.79	0.25	0.93
RSD %	1.13	0.80	0.25	0.93

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	300.391	100.130	1.921
Day 2	3.0	297.307	99.1023	0.939
Day 3	3.0	300.016	100.005	0.094

ANNOVA

Source of	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Variation						
Between Groups	1.888	2.0	0.944	0.958	0.435	5.143
Within Groups	5.908	6.0	0.985			
Total	7.796	8.0				

4.8.6.1.3 NEVIRAPINE

Table 4.25: Inter-day precision for nevirapine

	Day 1	Day 2	Day 3	Between days
	97.5	98.2	100.9	
	99.8	99.7	101.6	
	97.3	99.5	100.1	
Mean	98.2	99.1	100.9	99.4
SD	1.12	0.70	0.61	1.4
RSD %	1.14	0.70	0.61	1.39

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	294.594	98.198	1.891
Day 2	3.0	297.422	99.140	0.725
Day 3	3.0	302.577	100.859	0.563

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.922	2.0	5.461	5.155	0.050	5.143
Within Groups	6.356	6.0	1.059			
Total	17.278	8.0				

4.8.6.1.4 METHYL HYDROXYBENZOATE

Table 4.26: Inter-day precision for methyl hydroxybenzoate

	Day 1	Day 2	Day 3	Between days
	98.8	101.4	100.8	
	100.4	101.0	99.6	
	98.1	101.8	100.0	
Mean	99.1	101.4	100.1	100.2
SD	0.96	0.31	0.50	1.14

RSD %	0.97	0.31	0.50	1.14

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	297.350	99.117	1.383
Day 2	3.0	304.206	101.402	0.148
Day 3	3.0	300.338	100.113	0.374

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.876	2.0	3.938	6.204	0.035	5.143
Within Groups	3.808	6.0	0.635			
Total	11.684	8.0				

4.8.6.1.5 PROPYL HYDROXYBENZOATE

Table 4.27: Inter-day precision for propyl hydroxybenzoate

	Day 1	Day 2	Day 3	Between days
	97.6	97.9	99.1	
	100.8	98.8	99.5	
	97.1	98.4	98.1	
Mean	98.5	98.3	98.9	98.6
SD	1.64	0.36	0.61	1.06
RSD %	1.67	0.37	0.61	1.07

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	295.514	98.505	4.053
Day 2	3.0	295.029	98.343	0.195
Day 3	3.0	296.677	98.892	0.553

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.478	2.0	0.239	0.149	0.864	5.143
Within Groups	9.603	6.0	1.604			
Total	10.081	8.0				

4.8.6.1.6 CONCLUSION

Inter-day precision was satisfactory with RSD's of less than 4%. The repeatability is still within acceptable limits, and the assay should perform well, even when executed by other personnel in a different laboratory.

4.8.7 SYSTEM REPEATABILITY

4.8.7.1 LAMIVUDINE

Table 4.28: System repeatability for lamivudine

Area	Retention time (minutes)
4492	4.205
4485	4.191
4513	4.193
4861	4.257
4943	4.271
4950	4.265
Mean	4707.3	4.23
SD	212.8	0.03
RSD %	4.5	0.8

4.8.7.2 STAVUDINE

Table 4.29: System repeatability for stavudine

Area	Retention time (minutes)
1281	5.252
1314	5.235
1314	5.240
1373	5.308
1474	5.324
1338	5.327
Mean	1349	5.281
SD	62.4	0.04
RSD %	4.6	0.7

4.8.7.3 NEVIRAPINE

Table 4.30: System repeatability for nevirapine

Area	Retention time (minutes)
6101	9.059
6116	9.057
6135	9.075
6213	9.056
6233	9.057
6236	9.047

Mean	6172.3	9.06
SD	56.3	0.008
RSD %	0.91	0.09

4.8.7.4 METHYL HYDROXYBENZOATE

Table 4.31: System repeatability for methyl hydroxybenzoate

Area	Retention time (minutes)
13879	9.849
14035	9.849
13978	9.895
13728	9.909
13677	9.907
13727	9.901

Mean	13837.3	9.88
SD	135.6	0.03
RSD %	0.98	0.27

4.8.7.5 PROPYL HYDROXYBENZOATE

Table 4.32: System repeatability for propyl hydroxybenzoate

Area	Retention time (minutes)
696	11.773
748	11.776
696	11.836
682	11.841
688	11.845
693	11.847
Mean	700.5	11.82
SD	21.8	0.03
RSD %	3.11	0.27

4.8.7.6 CONCLUSION

The system performance was satisfactory for lamivudine with RSD values of 4.5 % for peak area and 0.8 % for retention time respectively, for stavudine with RSD values of 4.6 % for peak area and 0.7 % for retention time respectively, for nevirapine with RSD values of 0.9 % for peak area and 0.09 % for retention time respectively, for sodium methyl hydroxybenzoate with RSD values of 0.98 % for peak area and 0.27 % for retention time respectively and for

sodium propyl hydroxybenzoate with RSD values of 3.1 % for peak area and 0.27 % for retention time respectively.

4.8.8 ROBUSTNESS

Various changes were made to the method. A change of ± 5 % was made to the flow rate, the wavelength, the gradient profile and the pH. Three different C-18 columns were also used. In spite of all these changes the method still worked well. The following columns were used:

- Phenomenex, Luna C-18, Torrance, CA
- Restek, ultra C-18, Bellfonte, Pensylvania (PA)
- Lycrospher, RP 18 EC, Darmstadt, Germany

4.8.9 CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Retention Times (minutes):

- Lamivudine ± 4.2 minutes
- Stavudine ± 5.3 minutes
- Nevirapine ± 9.1 minutes
- Methyl hydroxybenzoate ± 9.9 minutes
- Propyl hydroxybenzoate ± 11.8 minutes

Number of theoretical plates (N) plates/column (tangent method)

- Lamivudine 7852
- Stavudine 5710
- Nevirapine 91237

- Methyl hydroxybenzoate 67765
- Propyl hydroxybenzoate 73512

USP tailing factor (T):

- Lamivudine 0.982
- Stavudine 0.750
- Nevirapine 0.998
- Methyl hydroxybenzoate 0.931
- Propyl hydroxybenzoate 1.023

Capacity factor (k'):

- Larnivudine 1.627
- Stavudine 2.349
- Nevirapine 4.542
- Methyl hydroxybenzoate 5.051
- Propyl hydroxybenzoate 6.385

Resolution between peaks (R):

- Lamivudine – Stavudine 4.878
- Stavudine-Nevirapine 17.796
- Nevirapine-Methyl hydroxybenzoate 6.115
- Methyl hydroxybenzoate-propyl 13.219

4.8.10 SYSTEM SUITABIITY

- Make duplicate injections of standard solution.
- Calculate the relative standard deviation of the peak areas obtained.
- Calculate the number of theoretical plates for the paraben peaks.

The system is suitable to perform the analysis if the following criteria are met:

1. RSD of 2 injections not more than 2%
2. The column must have more than 3000 theoretical plates for all the peaks.
3. The resolution between subsequent peaks must be greater than 2.

4.9 SUMMARY

Table 4.33: Summary of the validation results for the simultaneous determination of lamivudine, stavudine, nevirapine, methyl hydroxybenzoate and propyl hydroxybenzoate in the Pheroid™ solution.

Tests	Results
Specificity	Does not comply
Linearity	Lamivudine r^2 =0.99997 Stavudine r^2 =0.9996 Nevirapine r^2=0.9998 Propyl hydroxybenzoate r^2=0.9993 Methyl hydroxybenzoate r^2=0.99461
Accuracy	Lamivudine 101.5% Stavudine 99.63% Nevirapine 98.57% Propyl hydroxybenzoate 98.95 % Methyl hydroxybenzoate 98.88%
Precision	Lamivudine 1.76% Stavudine 1.07% Nevirapine 0.78% Propyl hydroxybenzoate 2.06%

	Methyl hydroxybenzoate 1.03%
Robustness	Complies

4.10 CONCLUSION

The method performed well and should be suitable to analyse lamivudine, stavudine, nevirapine, sodium methyl hydroxybenzoate and propyl hydroxybenzoate simultaneously in the Pheroid™ or the pro-Pheroid™ solution for stability testing, quality control and batch release purposes.

The method can therefore be regarded as being stability indicating.

CHAPTER 5

VALIDATION PROCEDURE, RESULTS AND DISCUSSION FOR THE ASSAY OF THE ANTI-OXIDANTS

5.1 ORIGIN OF METHOD

The method was developed and validated under the guidance of Prof. J.L. du Preez at the Analytical Technology Laboratory (ATL) at the North-West University, Potchefstroom.

5.2 CHROMATOGRAPHIC CONDITIONS

The one method described in this chapter was developed to assay the anti-oxidants present in Formulations A and B (see Chapter 4). The three anti-oxidants used in the formulations under investigation are Butylated Hydroxytoluene (BHT), Butylated Hydroxyanisole (BHA) and Vitamin E.

- **Analytical Instrument:** HP1100 series HPLC equipped with a pump, auto sampler, UV detector and Chemstation Rev. A.06.02 data acquisition and analysis software or equivalent.
- **Column:** Luna C 18(2) 150x4.6 mm (Agilent).
- **Mobile phase:** 100% Acetonitrile.

Table 5.1: Gradient table.

Time	Solvent (%)
0.00	70.00
5.00	100.00
17.00	100.00
17.20	70.00

- **Flow rate:** 2.0 ml/min.
- **Injection volume:** 20 µl.
- **Detection:** UV at 210 nm, 16 nm bandwidth.
- **Retention time:**

BHT	± 1.37
BHA	± 4.06
Vitamin E	± 13.5

- **Run time:** 21 minutes.

5.3 SAMPLE PREPARATION

FORMULATION A:

1. Place a clean 100 ml volumetric flask on an analytical balance and tare.
2. Accurately weigh 5 ml of each of the samples at conditions, 25°C+60%RH, 30°C+65%RH and 40°C+75%RH of the Pheroid™ formulation into separate 100 ml volumetric flasks.
3. Add approximately 75 ml of methanol to each of the volumetric flasks, remove the stoppers and put the flasks in an ultrasonic bath containing luke warm water and sonicate for approximately 10-15 minutes.
4. Make sure that all the Pheroid™ are dissolved. If solid particles are visible, shake the flask and sonicate it further until it is completely dissolved.
5. Allow the flasks to cool to room temperature and fill each flask to 100 ml with methanol.
6. Filter the solutions through a 0.45 µm filter into auto sampler vials and analyse.

FORMULATION B:

STEP 1:

1. Place a clean dry 25 ml measuring cylinder on an analytical balance and tare.
2. Accurately weigh 25 ml of the pro-Pheroid™ solution.
3. Because we only need 10 ml of pro-Pheroids™ the following calculation must be done:

25 ml pro-Pheroid™ = A (the amount that 25 ml weighed)

therefore 10 ml = x

$x = \frac{10xA}{25\ ml}$

STEP 2:

1. Place a clean dry 50 ml volumetric flask on an analytical balance and tare.
2. Accurately weigh the amount that was calculated for x in the above calculation, of each of the samples at conditions, 25°C+60%RH, 30°C+65%RH and 40°C+75%RH of the pro-Pheroid™ solution.
3. Add approximately 30 ml of 0.1 N HCl and put the flask in an ultrasonic bath containing lukewarm water and sonicate for approximately 1 hour.
4. If the particles have not yet dissolved then sonicate the solution further until there is a homogenous solution.
5. Allow the flask to cool to room temperature and fill each flask to 50 ml with 0.1 N HCl.
6. Use a 5 ml pipette and transfer 5 ml of the above solution into a 50 ml volumetric flask and make up to volume with methanol. This is the final solution.
7. Filter the solutions through a 0.45 μm filter into auto sampler vials and analyse.

5.4 STANDARD PREPARATION

FORMULATION A:

1. Place a clean dry weighing boat on an analytical balance and tare.
2. Accurately weigh 10 mg of BHT, 10 mg of BHA and 20 mg of Vit E separately.
3. Transfer this together into a 100 ml volumetric flask and add approximately 50 ml of methanol. Place this in an ultra-sonic bath with luke warm water for about 20 minutes.
4. When all the particles have dissolved, make it up to volume with the methanol.
5. Filter the solutions into auto sampler vials and analyse.

FORMULATION B:

1. Place clean dry weighing boats on an analytical balance and tare.
2. For Solution A, accurately weigh 10 mg of BHT, 10 mg of BHA, each in its own weighing boat.
3. Place the two accurately weighed anti-oxidants in a 100 ml volumetric flask.
4. Make up to volume with methanol.
5. Place a clean dry 100 ml volumetric flask on an analytical balance and tare.
6. For Solution B, accurately weigh 20 mg of Vitamin E into the volumetric flask.
7. Add approximately 100 ml of distilled water and sonicate.
8. Then take 10 ml of Solution A and 10 ml of Solution B and add it to a 100 ml volumetric flask.
9. Make up to volume with methanol. This is the final solution.
10. Filter the solutions into an auto sampler vial and analyse.

5.5 CALCULATIONS

FORMULATION A:

[Std] =mass of std (mg) /100 ml

=A x 10 ml

=B / 50 ml

=C (mg/ml) x 1000 ml

=D µg/ml

$$[\text{Sample}] = \frac{([\text{std}] \times \text{SAR})}{\text{STR}}$$

SAR=Sample peak areas

STR=Standard peak areas

FORMULATION B:

[Std] = see above calculation

$$[\text{Sample A}] = \frac{([\text{std}] \times \text{SAR})}{\text{STR}}$$

SAR=Sample peak areas

STR=Standard peak areas

We need to take into consideration the specific gravity, thus the following calculation must be done:

Actual [Sample] =[sample A] / SG

SG=Specific Gravity in wt/ml

5.6 VALIDATION TEST PROCEDURE AND ACCEPTANCE CRITERIA

5.6.1 SPECIFICITY

METHOD:

1. Prepare a sample from a placebo of the Pheroid™ solution that does not contain the various actives which are being tested with this method.
2. Take 5 ml of the standard solution and individually dilute it with 5 ml of 0.1 N HCl, 1 M NaOH and 10 % H_2O_2 respectively.
3. Store the solution of the actives and the preservatives overnight at 40°C.
4. Store the solution containing the anti-oxidants overnight at room temperature.
5. Filter these samples into an auto sampler vial and analyse to determine whether any additional peaks were formed.

ACCEPTANCE CRITERIA:

- The placebo should not contain any peaks that will interfere with the determination of the actives.
- Extra peaks formed in the stressed samples should be discernable from those of the active components.

5.6.2 LINEARITY

METHOD:

1. Prepare a standard solution as described under standard preparation.
2. Inject variable volumes into the chromatograph to obtain standards from 75-125% of the expected sample concentration.

ACCEPTANCE CRITERIA:

- Linear regression analysis should yield a regression co-efficient (r squared) of ≥ 0.99.

5.6.3 ACCURACY

METHOD:

1. Spike amounts of active at concentration of approximately 80%, 100% and 120% of the expected sample concentration.

2. Inject into the chromatograph in duplicate.

ACCEPTANCE CRITERIA:

- Recovery must be between 98-102%.

5.6.4 PRECISION

5.6.4.1 INTRA DAY PRECISION (REPEATABILITY)

METHOD:

1. Measure approximately 3x8 ml (80%), 3x10 ml (100%) and 3x12 ml (120%) of the Pheroid™ solution into 100 ml volumetric flasks and make up to volume with methanol.

2. Prepare a single standard at 100% of the expected sample concentration as described in the method.

3. Inject into the chromatograph in duplicate.

5.6.4.2 INTER DAY PRECISION

METHOD:

1. Analyse the same homogeneous sample in triplicate as described for intra-day precision (at 100% of the sample concentration) on two more occasions to determine the between-day variability of the method. On one occasion (day 3) a different analyst should perform the analysis on a different set of equipment.

ACCEPTANCE CRITERIA:

- % RSD calculated during repeatability studies must be less or equal than 2% (n=9).
- % RSD calculated during inter-day precision studies must be less or equal than 5% (n=9).

5.6.5 RUGGEDNESS

5.6.5.1 STABILITY OF THE SAMPLE SOLUTIONS

METHOD:

1. Prepare a sample as directed under sample preparation, paragraph 5.3
2. Inject the sample into the chromatograph.
3. Leave the sample in the auto sampler tray and re-analyse over a period to determine the stability of the sample.

ACCEPTANCE CRITERIA:

- Sample solutions should not be used for a period longer than it takes to degrade by 2% and in the case of degradation, special precautions should be followed to compensate for the loss.

5.6.5.2 SYSTEM REPEATABILITY

METHOD:

1. Inject a sample six times consecutively in order to test the repeatability of the peak area as well as the retention time.

ACCEPTANCE CRITERIA:

- The peak and retention times should have an RSD of 2 % or less.

5.6.6 ROBUSTNESS

METHOD:

1. Make deliberate changes to the chromatographic conditions to determine the methods tolerance towards changes.

2. Change the flow rate, wavelength, injection volume, acetonitrile concentration and use a similar column from a different manufacturer.

ACCEPTANCE CRITERIA:

- The method should be able to tolerate a 5% variance in the chromatographic conditions.

5.6.7 SYSTEM AND METHOD PERFORMANCE CHARACTERISTICS (SYSTEM SUITABILITY)

METHOD:

1. Calculate the chromatographic performance characteristics of the separation, like retention time, USP tailing factor, capacity factor, and resolution between peaks and repeatability of multiple injections.

2. Use the data obtained to set realistic performance limits that should be met before the analysis can be performed.

ACCEPTANCE CRITERIA:

- The RSD should not exceed 2%.
- The column must have more than 3000 theoretical plates for all the peaks.
- The resolution between subsequent peaks must be greater than 2.

5.7 VALIDATION RESULTS

5.7.1 SPECIFICITY

Placebo and standard solutions were prepared as described in paragraph 5.4.

All samples were analysed at 270 nm.

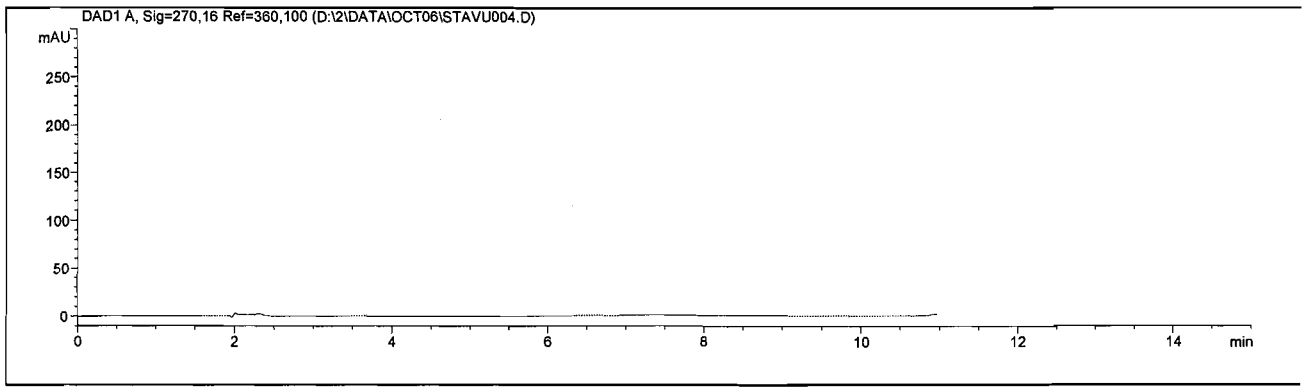


Figure 5.1: HPLC chromatograms of a placebo.

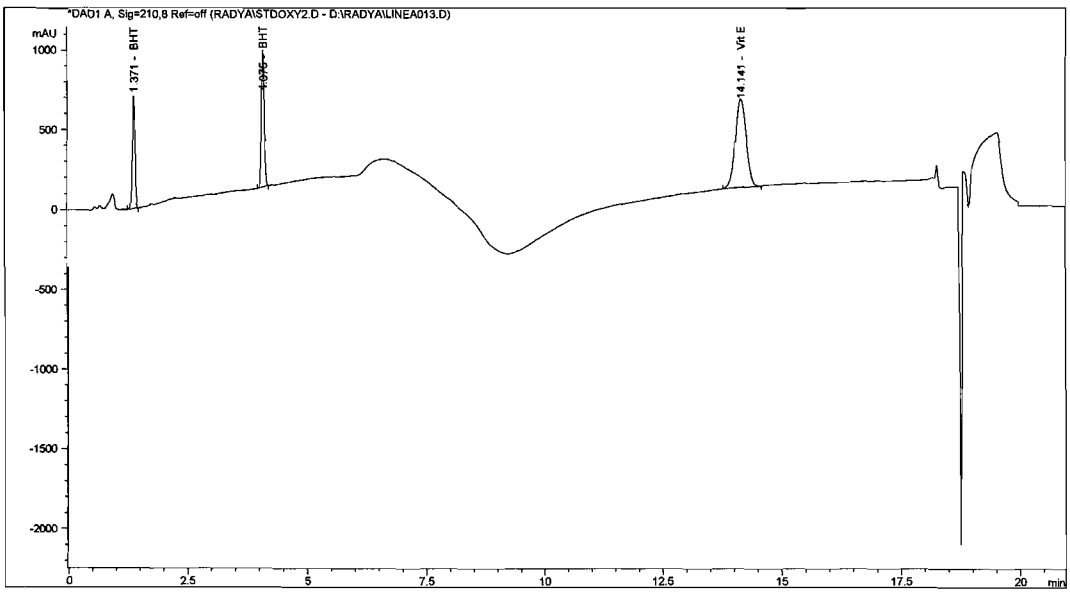


Figure 5.2: HPLC chromatograms of a standard containing BHT, BHA and Vitamin E.

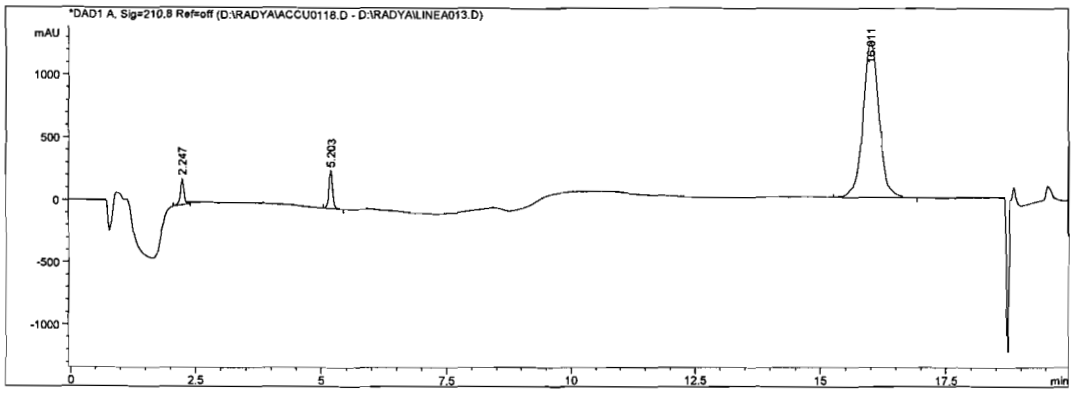


Figure 5.3: HPLC chromatogram of BHT, BHA and Vitamin E stressed in 0.1 N HCl at 40°C for 24 hours.

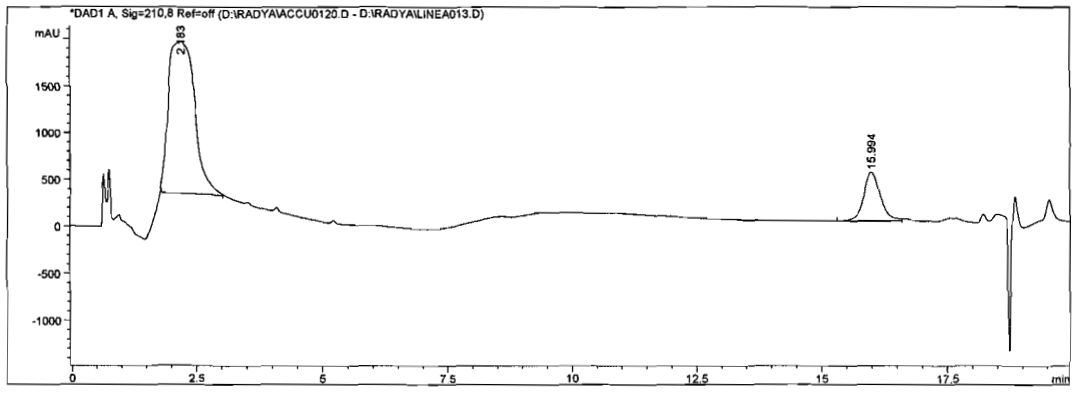


Figure 5.4: HPLC chromatogram of BHT, BHA and Vitamin E stressed in 1 N NaOH at 40°C for 24 hours.

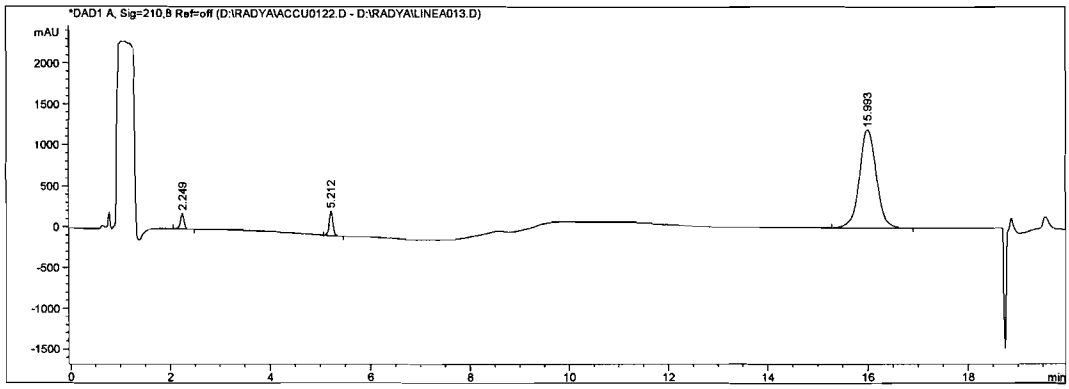


Figure 5.5: HPLC chromatogram of BHT, BHA and Vitamin E stressed in 10% H_2O_2 at 40°C for 24 hours.

5.7.1.1 CONCLUSION

In figure 5.4, it can be seen that only NaOH affects the anti-oxidants. The BHA and BHT degrades completely while the Vitamin E degrades to a certain amount. The anti-oxidants are however not affected by HCl or H_2O_2.

5.7.2 LINEARITY

5.7.2.1 BUTYLATED HYDROXYANISOLE

Table 5.3: Peak area and concentration for butylated hydroxyanisole.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
39.6	2132.6	2146.2	2139.4
45.2	2439.3	2504.0	2471.6

50.9	2770.1	2729.7	2749.9
56.5	2999.2	3015.2	3007.2
62.2	3279.1	3279.9	3279.5
37.8	3562.2	3602.2	3582.2

Table 5.4: Regression parameters for butylated hydroxyanisole.

REGRESSION PARAMETERS			
R squared	0.99863	**Lower 95%**	**Upper 95%**
Intercept	119.9033	45.42194	326.3714
Slope	32.27397	47.46132	52.61301

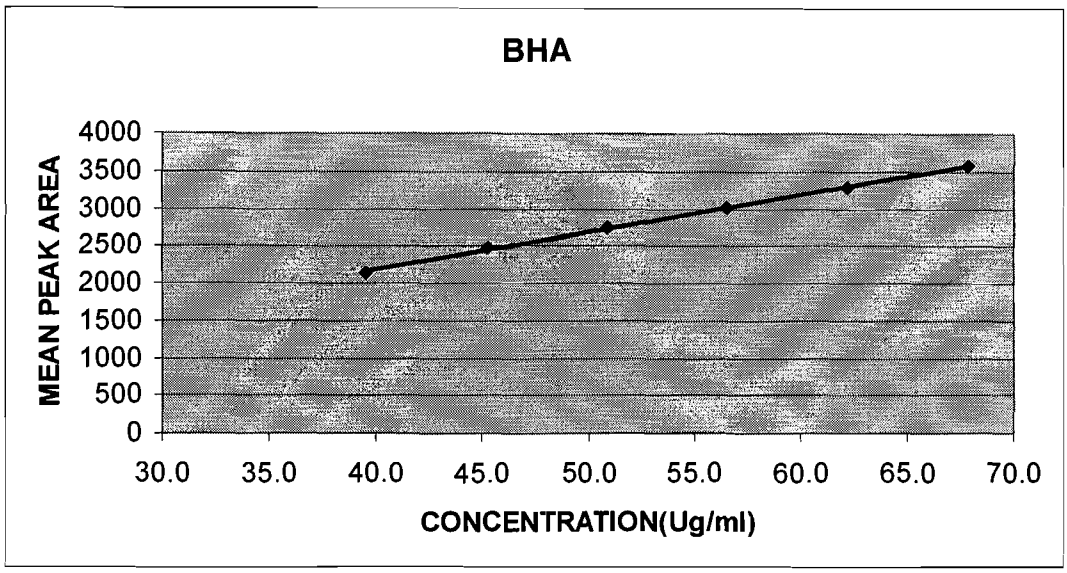


Figure 5.6: The linear regression curve for butylated hydroxyanisole.

5.7.2.2 BUTYLATED HYDROXYTOLUENE

Table 5.5: Peak area and concentration for butylated hydroxytoluene.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
41.0	1318.8	1424.9	1371.9
46.9	1477.0	1459.2	1468.1
52.7	1608.0	1596.7	1602.4
58.6	1810.7	1837.1	1823.9
64.5	1980.2	2005.9	1993.1

70.3	2160.8	2193.6	2177.2

Table 5.6: Regression parameters for butylated hydroxytoluene.

REGRESSION PARAMETERS			
R squared	0.98734	**Lower 95%**	**Upper 95%**
Intercept	491.59047	290.41476	692.76619
Slope	6.65503	5.60887	7.70119

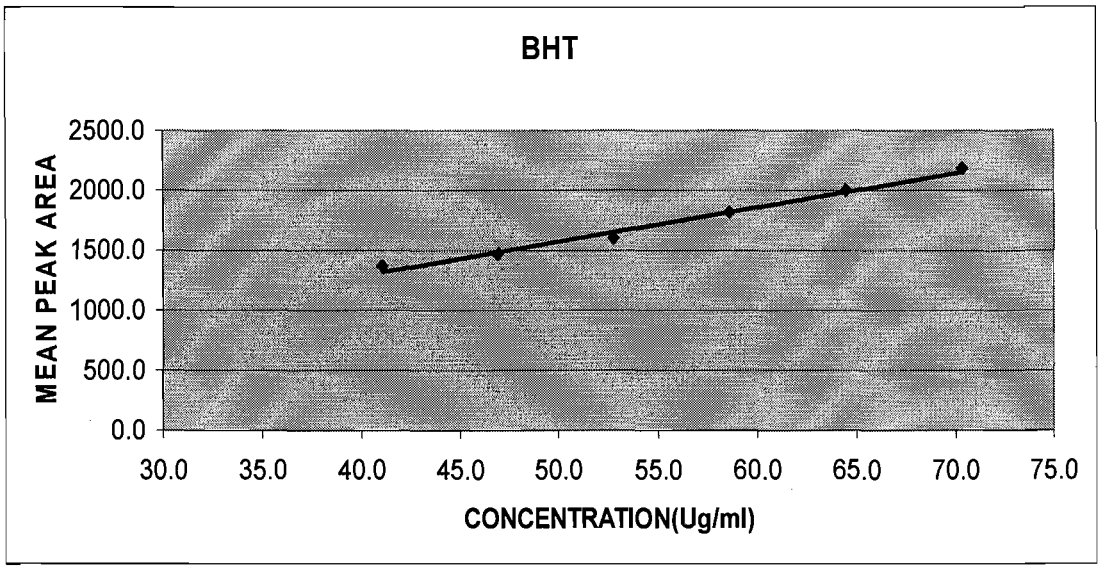


Figure 5.7: The linear regression curve for butylated hydroxytoluene.

5.7.2.3 VITAMIN E

Table 5.7: Peak area and concentration for vitamin E.

Concentration	Peak Area		
µg/ml	Area 1	Area 2	Mean
878.5	40026.8	39427.0	39726.9
1004.0	43093.1	42966.8	43029.9
1129.5	47213.4	48008.6	47611.0
1255.0	50922.9	51286.7	51104.8
1380.5	54392.2	53908.4	54150.3
1506.0	56878.7	56189.6	56534.1

Table 5.8: Regression parameters for vitamin E.

REGRESSION PARAMETERS			
R squared	0.99082	Lower 95%	Upper 95%
Intercept	15879.5514	11425.60488	20333.498
Slope	27.52216	23.84536	31.19897

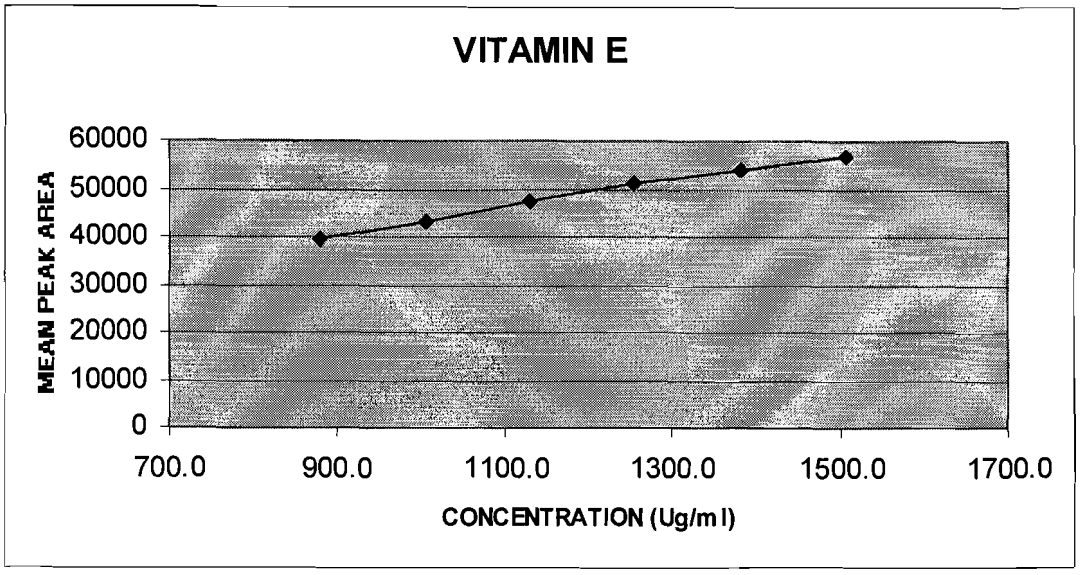


Figure 5.8: The linear regression curve for Vitamin E.

5.7.3 CONCLUSION

This method is linear over the concentration range of 39.6-67.8 µg/ml for butylated hydroxyanisole 41.0-70.3 µg/ml for butylated hydroxytoluene and 878.5-1506.0 µg/ml for Vitamin E. This method meets the criteria fro acceptance and is therefore suitable for single point calibration.

5.7.4 ACCURACY AND PRECISION

5.7.4.1 BUTYLATED HYDROXYANISOLE

Table 5.9: Percentages butylated hydroxyanisole recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
41.92	1487.2	1457.9	1472.55	41.9	100.0
41.92	1463.1	1442.9	1453.0	41.3	98.5

41.92	1468.5	1468.1	1468.3	41.8	99.7
52.4	1838.9	1809.5	1824.2	52.8	100.8
52.4	1862.5	1839.2	1850.85	53.6	102.4
52.4	1834.9	18451.1	1840.0	53.3	101.7
62.88	2173.7	2205.6	2189.65	64.1	102.0
62.88	2229.3	2218.8	2224.05	65.2	103.7
62.88	2239.4	2204.3	2221.85	65.1	103.6

Table 5.10: Confidence intervals for butylated hydroxyanisole.

Mean %	101.36
SD	1.66
% RSD	1.64

5.7.4.2 BUTYLATED HYDROXYTOLUENE

Table 5.11: Percentages butylated hydroxytoluene recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %
42.14	1030.8	1056.0	1043.4	42.1	99.8

42.14	1052.1	1049.0	1050.55	42.3	100.5
42.14	1064.5	1081.4	1072.95	43.2	102.6
52.68	1304.5	1278.4	1291.45	52.1	98.8
52.68	1331.3	1321.7	1326.5	53.5	101.5
52.68	1298.0	1312.0	1305.0	52.6	99.9
63.22	1553.1	1542.3	1547.7	62.4	98.7
63.22	1553.9	1548.5	1551.2	62.5	98.9
63.22	1567.5	1548.1	1557.8	62.8	99.3

Table 5.12: Confidence intervals for butylated hydroxytoluene.

Mean %	100.00
SD	1.26
% RSD	1.26

5.7.4.3 VITAMIN E

Table 5.13: Percentages vitamin E recovered.

Conc. Spiked µg/ml	Area 1	Area 2	Mean	Recovery µg/ml	Recovery %

943.5	31140.1	31170.5	311553.3	955.4	101.3
943.5	31399.2	31160.6	31279.9	959.2	101.7
943.5	31301.0	31423.5	31362.3	961.7	101.9
1179.4	37587.2	37549.7	37568.5	1152.0	97.7
1179.4	38014.8	37886.3	37950.6	1163.8	98.7
1179.4	37972.0	38178.2	38075.1	1167.6	99.0
1415.2	44099.9	46959.7	45529.8	1396.2	98.7
1415.2	47001.3	44342.5	45671.9	1400.5	99.0
1415.2	4144.4	44507.0	45974.7	1409.8	99.6

Table 5.14: Confidence intervals for vitamin E.

Mean %	99.7
SD	1.4
% RSD	1.4

5.7.5 CONCLUSION

The method yielded a mean recovery of 101.36 for butylated hydroxyanisole, 100.0 for butylated hydroxytoluene and 99.7 for vitamin E.

5.7.6 PRECISION

5.7.6.1 INTER DAY PRECISION

5.7.6.1.1 BUTYLATED HYDROXYANISOLE

Table 5.15: Inter-day precision for butylated hydroxyanisole.

	Day 1	Day 2	Day 3	Between days
	100.8	101.5	99.6	
	102.4	99.1	100.8	
	101.7	102.2	99.4	
Mean	101.6	100.9	99.9	100.8
SD	0.65	1.34	0.63	1.17
RSD %	0.64	1.35	0.63	1.16

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	304.841	101.614	0.628
Day 2	3.0	302.821	100.940	2.798
Day 3	3.0	299.791	99.930	0.597

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.307	2.0	2.153	1.606	0.276	5.143
Within Groups	8.046	6.0	1.341			
Total	12.353	8.0				

5.7.6.1.2 BUTYLATED HYDROXYTOLUENE

Table 5.16: Inter-day precision for butylated hydroxytoluene.

	Day 1	Day 2	Day 3	Between days
	98.8	101.6	98.0	
	101.5	100.2	97.0	
	99.9	96.3	97.4	
Mean	100.1	99.4	97.5	98.97
SD	1.10	2.25	0.42	1.8
RSD %	1.10	2.26	0.43	1.8

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3.0	300.167	100.056	1.829
Day 2	3.0	298.121	99.374	7.595

Day 3	3.0	292.474	97.491	0.267

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.585	2.0	5.292	1.638	0.270	5.143
Within Groups	19.382	6.0	3.230			
Total	29.967	8.0				

5.7.6.1.3 VITAMIN E

Table 5.17: Inter-day precision for vitamin E.

	Day 1	Day 2	Day 3	Between days
	97.7	100.4	99.2	
	98.7	100.1	99.6	
	99.0	99.5	100.3	
Mean	98.45	99.98	99.68	99.37
SD	0.56	0.37	0.44	0.81
RSD %	0.57	0.37	0.45	0.81

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Day 1	3	295.3633	98.45444	0.471239828
Day 2	3	299.9514	99.98379	0.207833832

Day 3	3	299.0442	99.68139	0.296945874

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.935758	2	1.967879	6.048687415	0.0364424	5.143249382
Within Groups	1.952039	6	0.32534			
Total	5.887797	8				

5.7.6.1.4 CONCLUSION

Inter-day precision was satisfactory with RSD's of less than 4%. The repeatability is still within acceptable limits, and the assay should perform well, even when executed by other personnel in a different laboratory.

5.7.7 SYSTEM REPEATABILITY

5.7.7.1 Butylated hydroxytoluene

Table 5.18: System repeatability for BHT.

Area	Retention time (minutes)
2662	1.364
2642	1.365
2625	1.368
2640	1.366
2613	1.383
2625	1.383

Mean	2634.5	1.3715
SD	15.7	0.008
RSD %	0.60	0.60

5.7.7.2 Butylated hydroxyanisole (BHA)

Table 5.19: System repeatability for BHA.

Area	Retention time (minutes)
3516	4.046
3485	4.043
3491	4.048
3485	4.045
3428	4.076
3484	4.076

Mean	3481.5	4.06
SD	26.4	0.01
RSD %	0.75	0.36

5.7.7.3 Vitamin E

Table 5.20: System repeatability for Vitamin E.

Area	Retention time (minutes)
8830	13.507
8818	13.475
8753	13.464
8771	13.431
8775	13.552
8821	13.579
Mean	8794.7	13.5
SD	29.3	0.05
RSD %	0.33	0.38

5.7.7.4 CONCLUSION

The system performance was satisfactory for BHT with RSD values of 0.60 % for peak area and 0.60 % for retention time respectively, for BHA with RSD values of 0.75 % for peak area and 0.36 % for retention time respectively and for Vitamin E with RSD values of 0.33 % for peak area and 0.38 % for retention time respectively.

5.7.8 ROBUSTNESS

Various changes were made to the method. A change of ± 5 % was made to the flow rate, the wavelength, the gradient profile and the pH. Three different C-18 columns were also used. In spite of all these changes the method still worked well. The following columns were used:

- Agilent Luna C-18
- Restek ultra C-18, Bellfonte, Pensylvania (PA)
- Lycrospher RP 18 EC, Darmstadt, Germany

5.7.9 CHROMATOGRAPHIC PERFORMANCE PARAMETERS

Retention Times (minutes):

- BHT ± 1.37
- BHA ± 4.06
- Vitamin E ± 13.5

Number of theoretical plates (N) plates/column (tangent method)

- BHT 5647
- BHA 25945
- Vitamin E 12484

USP tailing factor (T):

- BHT 1.355
- BHA 0.982
- Vitamin E 1.017

Capacity factor (k'):

- BHT 0.988

- BHA 3.604
- Vitamin E 13.168

Resolution between peaks (R):

- BHT-BHA 23.761
- BHA-Vit. E 30.775

5.7.10 SYSTEM SUITABIITY

- Make duplicate injections of standard solution.
- Calculate the relative standard deviation of the peak areas obtained.
- Calculate the number of theoretical plates for the paraben peaks.

The system is suitable to perform the analysis if the following criteria are met:

1. RSD of 2 injections not more than 2%.
2. The column must have more than 3000 theoretical plates for all the peaks.
3. The resolution between subsequent peaks must be greater than 2.

5.8 SUMMARY

Table 5.2: Summary of the validation results for the simultaneous determination of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and vitamin E in the Pheroid ™solution.

Tests	Results
Specificity	Does not comply
Linearity	Vit E r^2 =0.99082

	BHA r^2 =0.99863 BHT r^2 =0.98734
Accuracy	Vit E =99.7% BHA =101.36% BHT= 100.0%
Precision	Vit E =1.4% RSD BHA= 1.64% RSD BHT= 1.26% RSD
Robustness	Complies

5.9 CONCLUSION

The method performed well and should be suitable to analyse BHT, BHA and Vitamin E simultaneously in the Pheroid™ or the pro-Pheroid™ solution for stability testing, quality control and batch release purposes.

The method can therefore be regarded as being stability indicating.

CHAPTER 6

STABILITY TESTING: AN ESSENTIAL ATTRIBUTE FOR DRUG PRODUCTS

6.1 INTRODUCTION

Everything that is formed by the human hands, from the exquisite Parthenon to the trivial milkshake, all are subject to loss in quality (decay). So too are pharmaceutical excipients no exception to this statement (Rhodes, C.T., 2007:2).

There is a vast difference in the rate at which drug products degrade. Some products, if properly stored and packaged, retain integrity for approximately ten years (although the maximum shelf life in many jurisdictions is five years). Other products such as radiopharmaceuticals must be used within a day or so (Rhodes, C.T., 2007:2).

6.2 REASONS FOR STABILITY TESTING

- Concern for the patient's well-being;
- Protection of the integrity as well as the reputation of the producer;
- To obey the law and fulfill the requirements as ruled by the regulatory agencies;
- To provide reference information that may be vital when formulating another product in the future (Rhodes, C.T., 2007:11).

6.3 ADVERSE EFFECTS THAT MAY OCCUR DUE TO INSTABILITY OF THE PRODUCTS

- 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;
- Modification of any factor of functional relevance (Rhodes, C.T., 2007:3).

6.4 MODES OF DEGRADATION

6.4.1 CHEMICAL

Oxidation, solvolysis, hydrolysis, etc. are all common forms of degradation (Rhodes, C.T., 2007:12).

6.4.2 PHYSICAL

Abrasion, impact, vibration and temperature fluctuations such as freezing, thawing or shearing are all factors that cause physical degradation. Tablet friability, tablet impact resistance, suspension redispersibility, or injection syringeability are all physical test methods that may be used; however they are still nonofficial and variable (Rhodes, C.T., 2007:12).

6.4.3 BIOLOGICAL AND MICROBIOLOGICAL

Across the globe, microbiological as well as non-microbiological organisms cause degradation of the pharmaceutical products and thus degradation (Rhodes, C.T., 2007:12).

6.5 STABILITY PROGRAMME

Two formulations were prepared for this study namely, Formulation A (Pheroid™) and Formulation B (pro-Pheroid™).

According to the ICH (International Conference and Harmonisation), these formulations were put on stability for three months on technical requirements for the registration of pharmaceuticals for human use (ICH Q6A, 1999).

6.5.1 STORAGE TEMPERATURES

The formulations were stored at three different temperatures and humidities according to ICH conditions. Controlled storage facilities were used during the stability period. The formulations were kept at the following conditions:

- 25°C + 60 % RH;
- 30°C + 65 % RH;
- 40°C + 70 % RH.

6.5.2 STABILITY TESTS CONDUCTED

All tests were done using calibrated and/ or validated test apparatuses, where applicable. The following tests were conducted:

- Visual assessment;
- pH;
- Viscosity;
- Actives and preservatives concentration assay;
- Anti-oxidants concentration assay;
- Microbial limit tests.

6.6 TESTS METHODS

To ensure the accuracy of the test results, all tests were carried out under Good Laboratory Practice (GLP) conditions.

6.6.1 VISUAL ASSESMENT

A visual assessment was carried out on each batch. The colour as well as the physical appearance was examined.

6.6.2 pH

The pH was measured using a Mettler Toledo 7 multi pH meter, with an in-lab routine pro-electrode. The pH meter was calibrated with buffer solutions of pH 4 and 7 each time before using it.

6.6.3 VISCOSITY

The viscosity of the test samples was measured using a Brookfield Programmable Model DV-II + Viscometer, together with the Helipath stand and a Labcon low temperature water bath. The temperature was set at 25°C and the SC4-18 spindle was used to determine the viscosity. The spindle rotated for 35 minutes at 2 minute intervals from 0.3, 0.6, 1.5, 3.0, 6.0, 12.0, 30.0, 60.0 and back again to 0.3 rpm. Seventeen readings were taken of each sample.

6.6.4 ASSAY: LAMIVUDINE, STAVUDINE, NEVIRAPINE AND PRESERVATIVES

The Hewlett Packard Agilent 1100 series HPLC instrument was used, together with the G1322A degasser, G1312A binary pump, and the G1329A ALS auto-sampler. Methanol was used as a solvent to enhance the stability of nevirapine, as it has a low solubility in water.

Standard preparation: Refer to chapter 4, paragraph 4.4;

Sample preparation: Refer to chapter 4, paragraph 4.3.

6.6.5 ASSAY: ANTI-OXIDANTS

The Hewlett Packard Agilent 1100 series HPLC instrument was used, together with the G1322A degasser, G1312A binary pump, and the G1329A ALS auto-sampler. Methanol was used as a solvent to enhance the stability of nevirapine, as it has a low solubility in water.

Standard preparation: Refer to chapter 5, paragraph 5.4;

Sample preparation: Refer to chapter 5, paragraph 5.3.

6.6.6 MICROBIAL LIMITS TEST

These tests were done according to the USP 29, (2006). The tests were done on each of the samples at conditions 25°C+60%RH, 30°C+65%RH and 40°C+75%RH as well as the initial batch of Formulation A and Formulation B.

The testing was subcontracted to Consulting Microbiological Laboratories in Johannesburg, South Africa (CML). Tests were conducted for the following micro organisms:

- *E. coli*
- *Staphylococcus aureus*
- *Salmonella*
- *Pseudomonas*
- Total plate count
- Yeast count
- Mould count

6.7 RESULTS AND DISCUSSION

6.7.1 VISUAL ASSESSMENT

6.7.1.1 RESULTS

Visual assessment was performed on the initial sample and thereafter on each of the samples of Formulation A as well as Formulation B. Results of the visual assessment tests are given in Table 6.1 and Table 6.2.

Table 6.1: Visual assessment results for Formulation A, which was the Pheroid™ formulation.

Visual Assessment

Initial	Storage Temperatures	Appearance after 3 months
Off-white, homogenous milky solution.	25°C + 60% RH	Off white to a light yellow milky solution.
	30°C + 65% RH	Slightly dark yellow milky solution.
	40°C + 75% RH	Bright yellow (Dark yellow) milky solution.

Stability testing was done over a period of 3 months and the appearance of formulation A remained the same throughout the 3 months of stability testing. Pictures of the appearances at the end of the three month study at 25°C + 60% RH, 30°C + 65% RH and 40°C + 75% RH are shown below (figures 6.1 – 6.3).

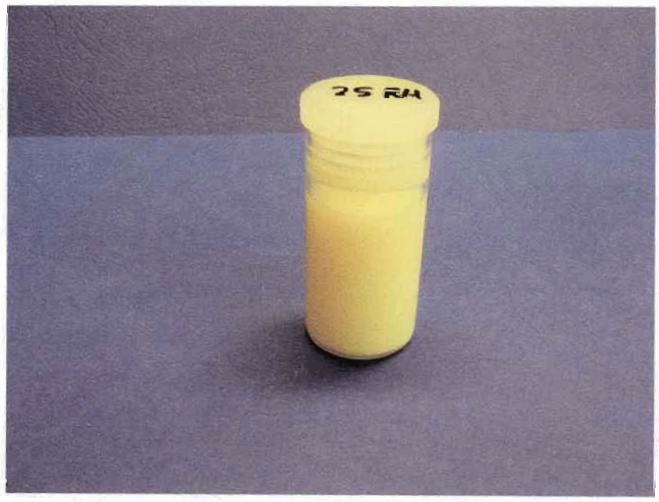


Figure 6.1: Appearance of Formulation A, 25°C + 60% RH after 3 months.

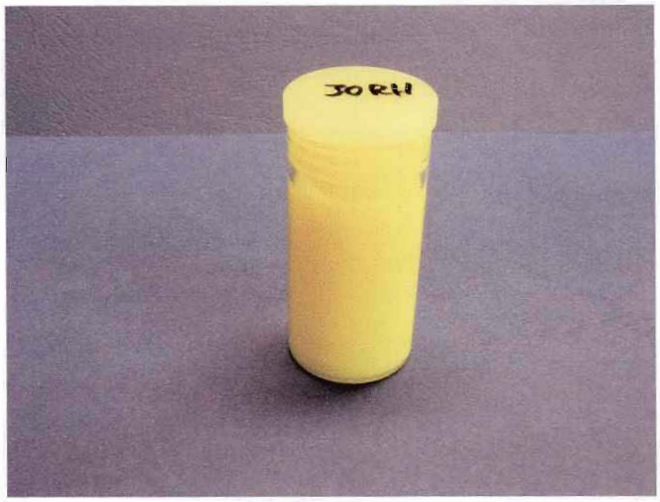


Figure 6.2: Appearance of Formulation A, 30°C + 65% RH after 3 months.

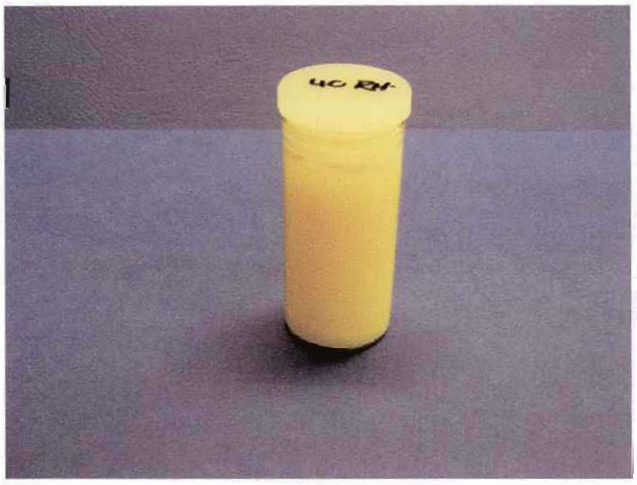


Figure 6.3: Appearance of Formulation A, 40°C + 75% RH after 3 months.

Table 6.2: Visual assessment results for Formulation B, which was the pro-Pheroid™ formulation.

Visual Assessment		
Initial	**Storage Temperatures**	**Appearance after 3 months**
Off white to a very light yellow, heterogeneous milky solution, with suspended particles present.	25°C + 60% RH	Light yellow milky solution with suspended particles.
	30°C + 65% RH	Slightly dark yellow milky solution with suspended particles.
	40°C + 75% RH	Bright yellow (dark yellow) milky solution with suspended particles.

Stability testing was done over a period of 3 months and the appearance of Formulation B remained the same throughout the 3 months of stability testing.

6.7.1.2 DISCUSSION

It was noticed that as the temperature and humidity was increased, the solutions became a darker yellow. This could be because of the degradation of stavudine in to its breakdown products. Another reason for the yellow colour could be because of the free radicals present in the formulation since one of the side effects of ARV's is free radical formation. Thus, to prevent the formation of free radicals, the amount of anti-oxidants needs to be increased or different anti-oxidants need to be added to the formulations.

6.7.2 pH

6.7.2.1 RESULTS

The pH results of the accelerated stability study are given below in Table 6.3.

Table 6.3: pH results for Formulation A, which is the Pheroid™ formulation, after accelerated stability testing.

pH				
Initial	**Storage conditions**	**First Month**	**Second Month**	**Third Month**
6.44	25°C + 60% RH	6.51	6.34	6.54
	30°C + 65% RH	6.56	6.46	6.45
	40°C + 75% RH	6.63	6.56	6.57

Literature study showed that lamivudine stable is in the pH range of 6.5 – 6.7; stavudine between 5 and 7; and nevirapine from 3 to 11. Therefore, any Pheroid™ formulation, containing all three these ARV's, would need to have a pH between 6 and 7 to ensure stability of all the actives, but especially that of lamivudine. From this results it seemed that the pH of the Pheroid™ formulation remained stable and within acceptable limits throughout the 3 month stability study.

6.7.2.2 DISCUSSION

The viscosity of Formulation B was not suitable for pH determinations. Formulation B needs to be diluted with 0.1 N HCl and this would have affected the pH readings. The results in table 6.3 show that there wasn't a change in the pH readings for Formulation A throughout the 3 months of stability testing, thus indicating that the pH was stable.

6.7.3 VISCOSITY

6.7.3.1 RESULTS

The viscosity results for Formulation A are given in the Table 6.4 below.

Table 6.4: Viscosity results (cp) of Formulation A during accelerated stability testing.

Viscosity				
Initial	**Storage conditions**	**First Month**	**Second Month**	**Third Month**
1.07	25°C + 60% RH	1.07	0.53	1.07
	30°C + 65% RH	1.07	0.53	1.07
	40°C + 75% RH	1.07	0.53	1.07

6.7.3.2 DISCUSSION

In Formulation A, the decrease in viscosity in month 2 is unexplainable and may be due to an instrumental error because the viscosity remains constant for the initial, first month and third month tests. Viscosity test on Formulation B was not done because it was too viscous.

6.7.4 MICROBIAL LIMIT TEST

6.7.4.1 RESULTS

The results for the microbial limit tests can be seen in Table 6.5 and Table 6.6.

Table 6.5: Microbial limit test for Formulation A during accelerated stability testing.

Test Organism	Initial	First Month	Second Month	Third Month
E. coli	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
S. aureus	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Detected/10 g **30°C / 65% RH:** Detected/10 g **40°C / 75% RH:** Detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
Salmonella	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
Pseudomo	Not		**25°C / 60% RH:**	**25°C / 60% RH:**

nas	detected/ 10 g		Not detected/10 g **30°C / 65% RH**: Not detected/10 g **40°C / 75% RH:** Not detected/10 g	Not detected/10 g **30°C / 65% RH**: Not detected/10 g **40°C / 75% RH:** Not detected/10 g
Yeast count	< 10 cfu/g		**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g
Mould count	< 10 cfu/g		**25°C / 60% RH:** 20 cfu/g **30°C / 65% RH:** 20 cfu/g **40°C / 75% RH:** 5 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g
Total plate count	< 10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** 5 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g

- Cfu/g/ml: Colony forming unit per gram or ml of sample tested.
- <10 cfu/g/ml: Not detected in 1 ml of the 1 in 10 dilution of sample.

Table 6.6: Microbial limit test for Formulation B during accelerated stability testing.

Test Organism	Initial	First Month	Second Month	Third Month
E. coli	Not detected/ 10 g	**25°C / 60% RH:** Detected/10 g **30°C / 65% RH:** Detected/10 g **40°C / 75% RH:** Detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
S. aureus	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
Salmonella	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:** Not detected/10 g
Pseudo-monas	Not detected/ 10 g	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:**	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:**	**25°C / 60% RH:** Not detected/10 g **30°C / 65% RH:** Not detected/10 g **40°C / 75% RH:**

		Not detected/10 g	Not detected/10 g	Not detected/10 g
Yeast count	< 10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g
Mould count	< 10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g
Total plate count	< 10 cfu/g	**25°C / 60% RH:** 20 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** <10 cfu/g	**25°C / 60% RH:** <10 cfu/g **30°C / 65% RH:** <10 cfu/g **40°C / 75% RH:** 5 cfu/g

- Cfu/g/ml: Colony forming unit per gram or ml of sample tested.
- <10 cfu/g/ml: Not detected in 1 ml of the 1 in 10 dilution of sample.

6.7.4.2 DISCUSSION

The *staphylococcus aureus* detected in Formulation A during the second month and the *E. coli* detected in Formulation B during the first month may be due to a problem during the

auto-claving procedure of the packaging containers. The storage bottles have to be sterile before using them. For all the other results, < 10 reflects the accuracy of the test procedure and for all practical purposes implies the absence of the organism indicated.

6.7.5 ACTIVES AND PRESERVATIVE CONCENTRATION ASSAY

6.7.5.1 RESULTS: FORMULATION A

Formulation A was subjected to an accelarated stability study over the period of three months. Active and preservative content was determined at monthly intervals – results are given in Table 6.7. Assay values given as the average of duplicate determinations from the same vial.

Table 6.7: Assay of actives and preservatives - results for Formulation A over 3 months of accelerated stability studies.

	Lamivudine	Stavudine	Nevirapine	Methyl-paraben	Propyl-paraben
Initial (%)	104.3	85.4	77.5	134.3	-
1st Month					
25°C / 60% RH	104.8	53.2	47.0	110.9	-
30°C / 65% RH	108.6	28.9	33.6	110.3	-
40°C / 75% RH	109.9	3.7	71.5	108.1	-
2nd Month					
25°C / 60% RH	108.0	33.6	13.30	130.0	-
30°C / 65% RH	104.5	-	14.3	123.9	-

40°C / 75% RH	102.2	-	34.7	125.2	-
3rd Month					
25°C / 60% RH	109.2	22.3	6.5	130.8	-
30°C / 65% RH	108.9	-	-	128.8	-
40°C / 75% RH	114.7	-	-	134.6	-

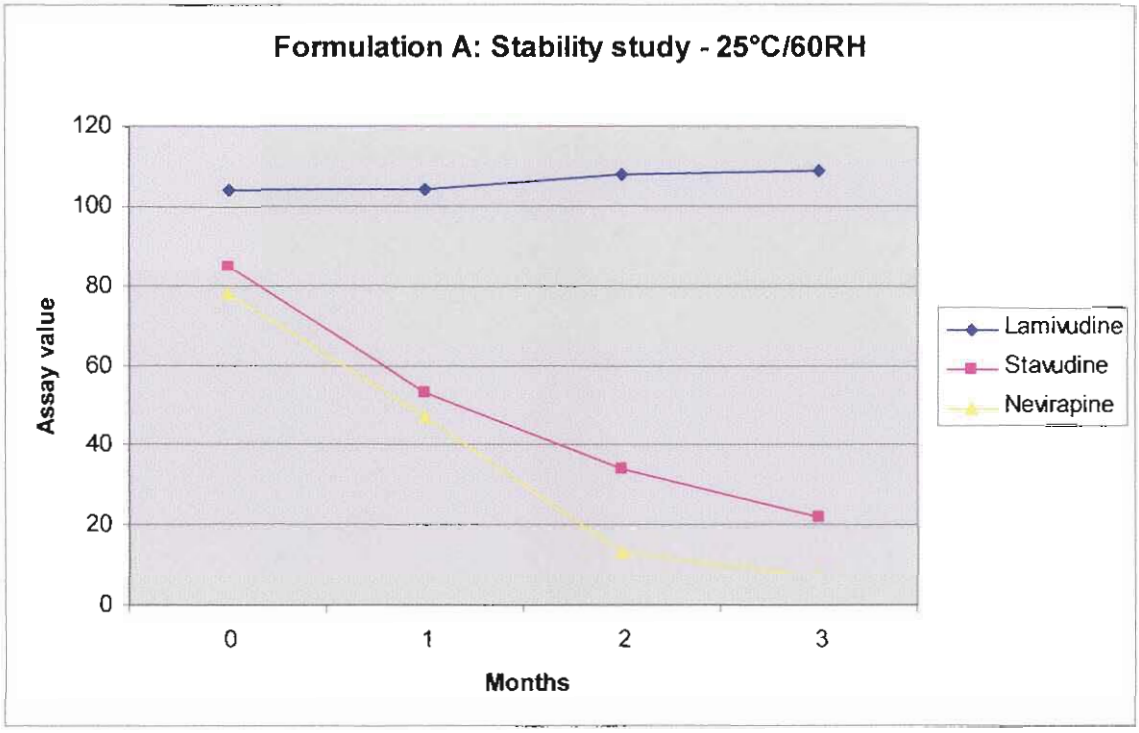


Figure 6.4: Stability study results for the three API's in Formulation A subjected to 25°C / 60% RH.

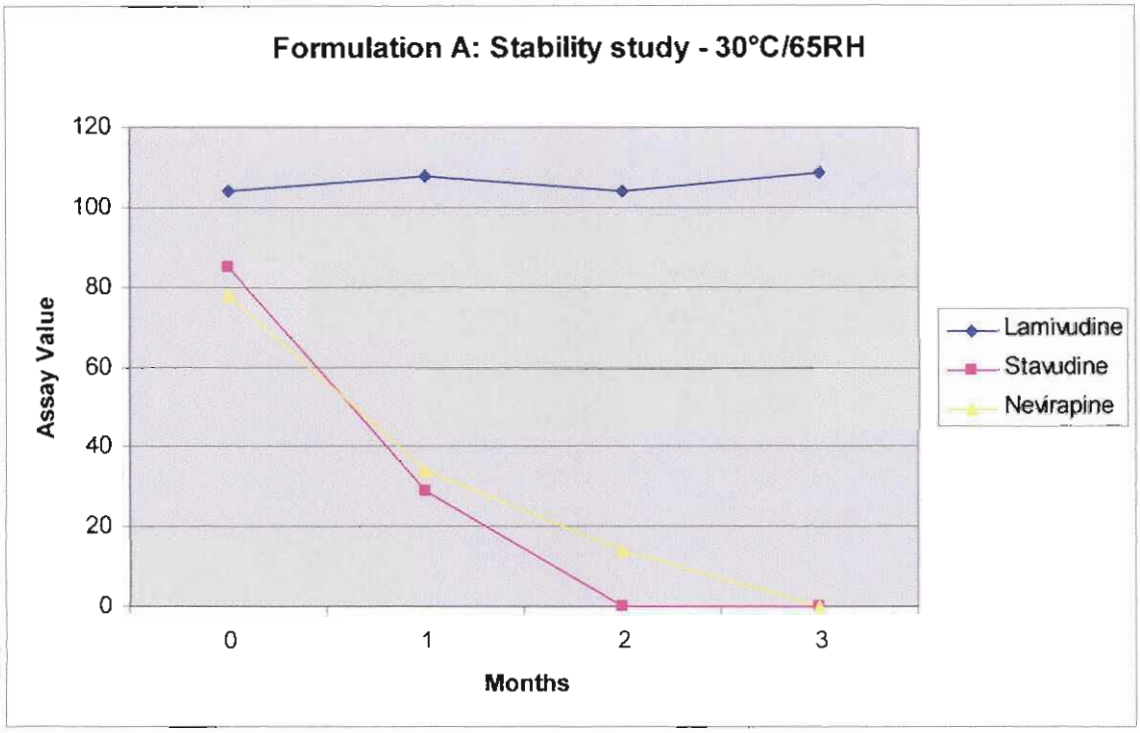


Figure 6.5: Stability study results for the three API's in Formulation A kept at 30°C / 65% RH.

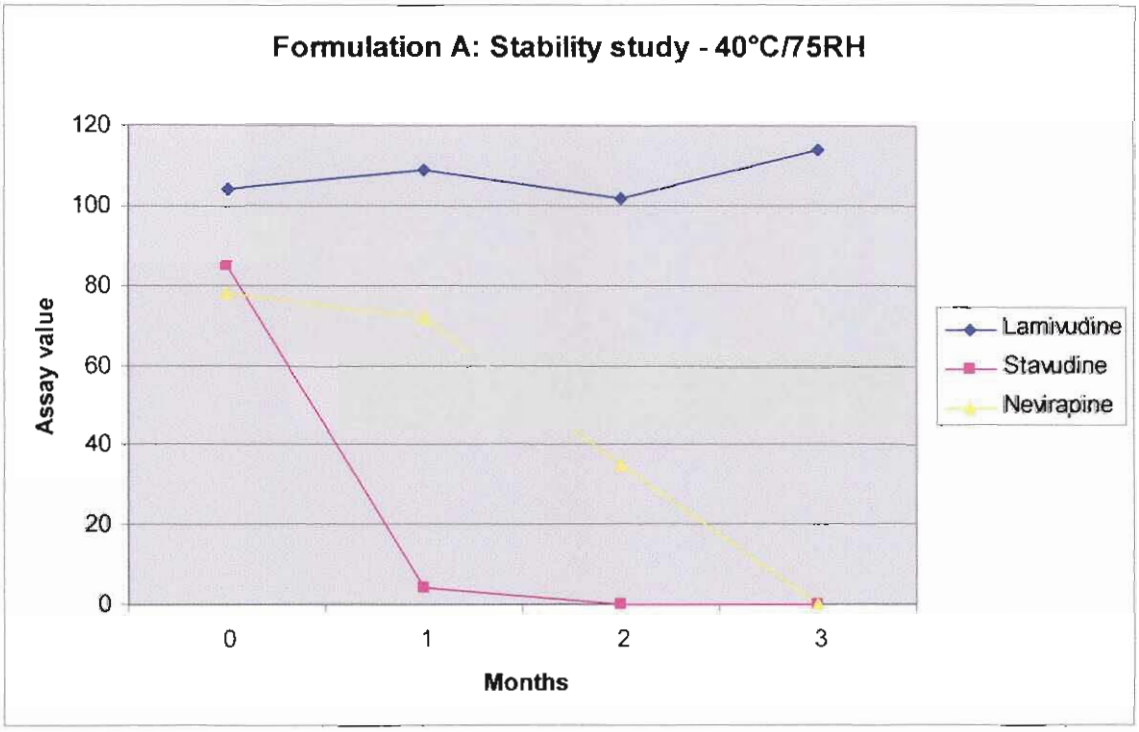


Figure 6.6: Stability study results for the three API's in Formulation A stored at 40°C / 75% RH.

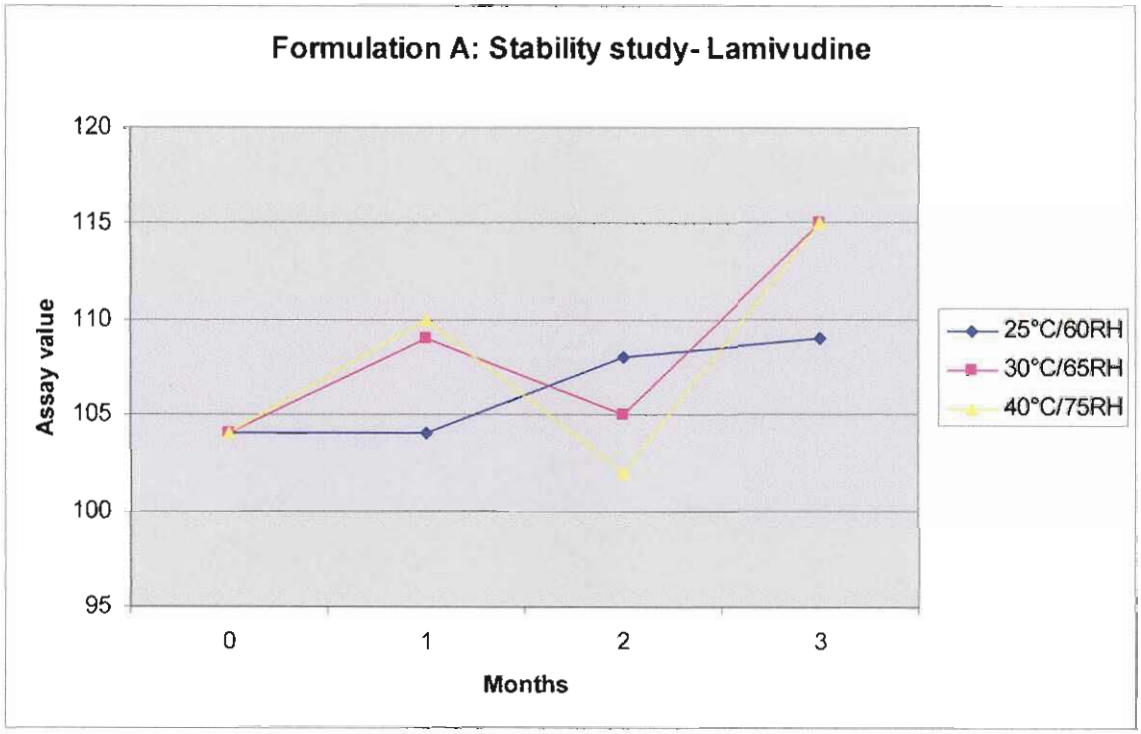


Figure 6.7: Stability of lamivudine in Formulation A over the three month period.

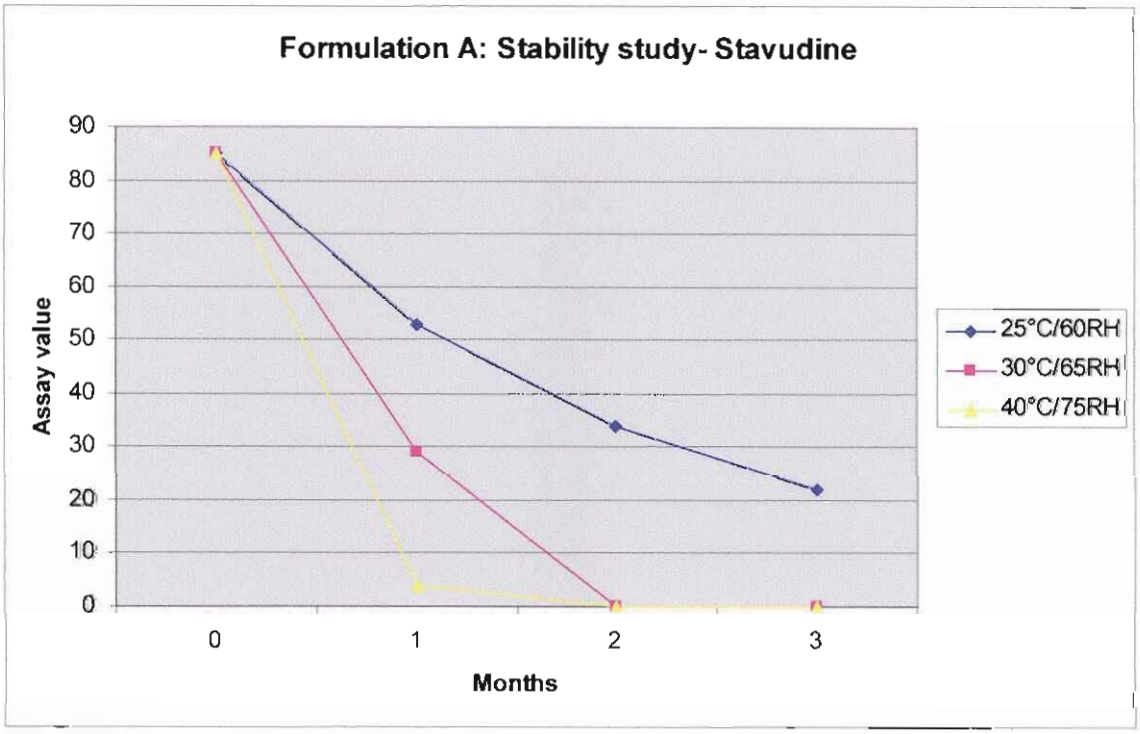


Figure 6.8: Stability of stavudine in Formulation A over the three month period.

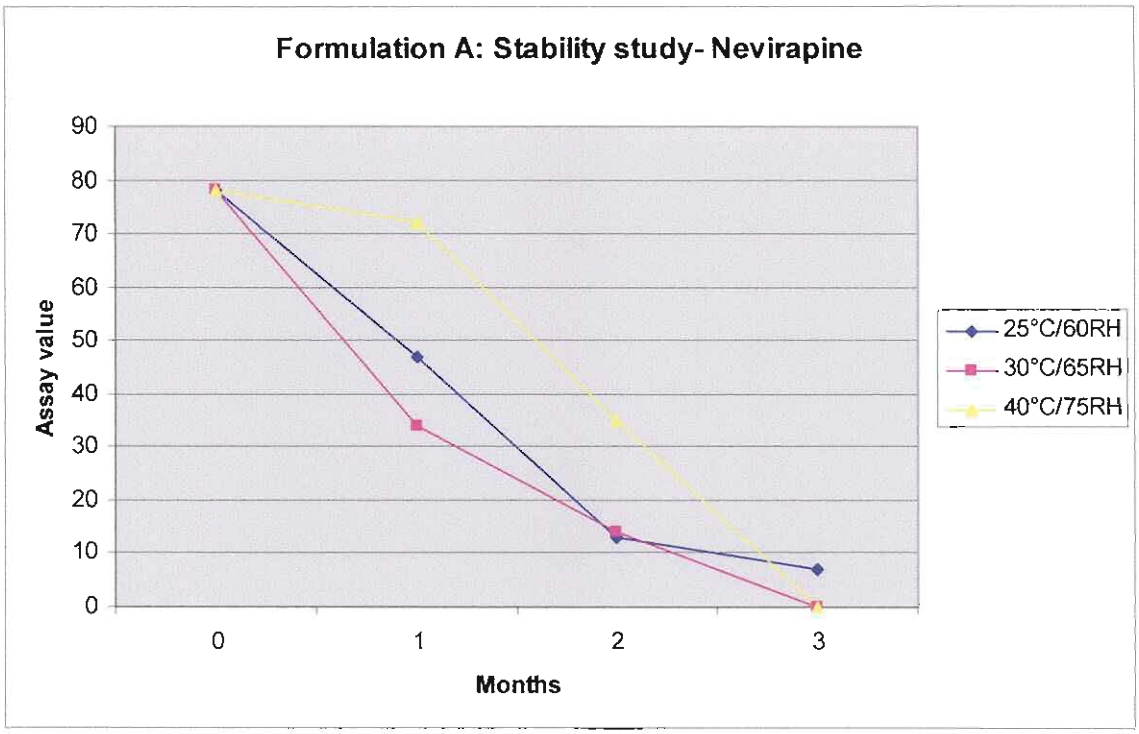


Figure 6.9: Stability of nevirapine in Formulation A over the three month period.

6.7.5.2 DISCUSSION

Lamivudine remained stable throughout the 3 months stability study (Figures 6.4 - 6.7). The initial assay of stavudine was not within specifications, indicating instability within the Pheroid™ formulation (Figures 6.4 and 6.8). During month 1 at 40°C / 75% RH the stavudine content declined to ± 3 % (Figure 6.8). During the second and third month at 30°C / 65% RH and 40°C / 75% RH there was a complete breakdown of the stavudine, thus indicating that as the temperature was increased, the degradation of stavudine increased (Figure 6.8). The nevirapine results were inconclusive. This might be due to a problem with the homogeneity of the formulation and/or because nevirapine is almost insoluble in aqueous solutions. Also, the stability of nevirapine in this system was not tested and it is possible that degradation of the nevirapine might be taking place. Methyl hydroxybenzoate remains stable throughout the duration of the study. The large percentage yield of the methyl hydroxybenzoate could be due to an initial weighing error. The concentration of the propyl hydroxybenzoate in the formulation was 0.2 µg/ml, thus the concentration was too small to be detected. The concentration of propyl hydroxybenzoate was supposed to be 2 µg/ml, but a calculation error was made by the Pheroid™ technician during upscaling of the production

process. The mistake was only discovered after the product had already been used in this study.

6.7.5.3 RESULTS: FORMULATION B

Formulation B was subjected to an accelerated stability study over the period of three months. Active and preservative content was determined at monthly intervals – results are given in Table 6.8. Assay values given as the average of duplicate determinations from the same vial.

Table 6.8: Assay of actives and preservatives - results for Formulation B over 3 months of accelerated stability studies.

	Lamivudine	Stavudine	Nevirapine	Methylparaben
Initial (%)	105.1	100.8	104.2	527.0
1st Month				
25°C / 60% RH	98.9	99.3	86.6	132.3
30°C / 65% RH	115.0	110.0	19.3	135.2
40°C / 75% RH	99.6	94.7	19.9	120.3
2nd Month				
25°C / 60% RH	129.4	115.9	50.7	145.3
30°C / 65% RH	119.3	111.7	24.35	133.3
40°C / 75% RH	111.55	103.2	58.3	135.6

3rd Month				
25°C / 60% RH	110.8	109.9	11.09	127.5
30°C / 65% RH	114.5	107.7	27.9	127.1
40°C / 75% RH	115.1	108.9	16.7	129.9

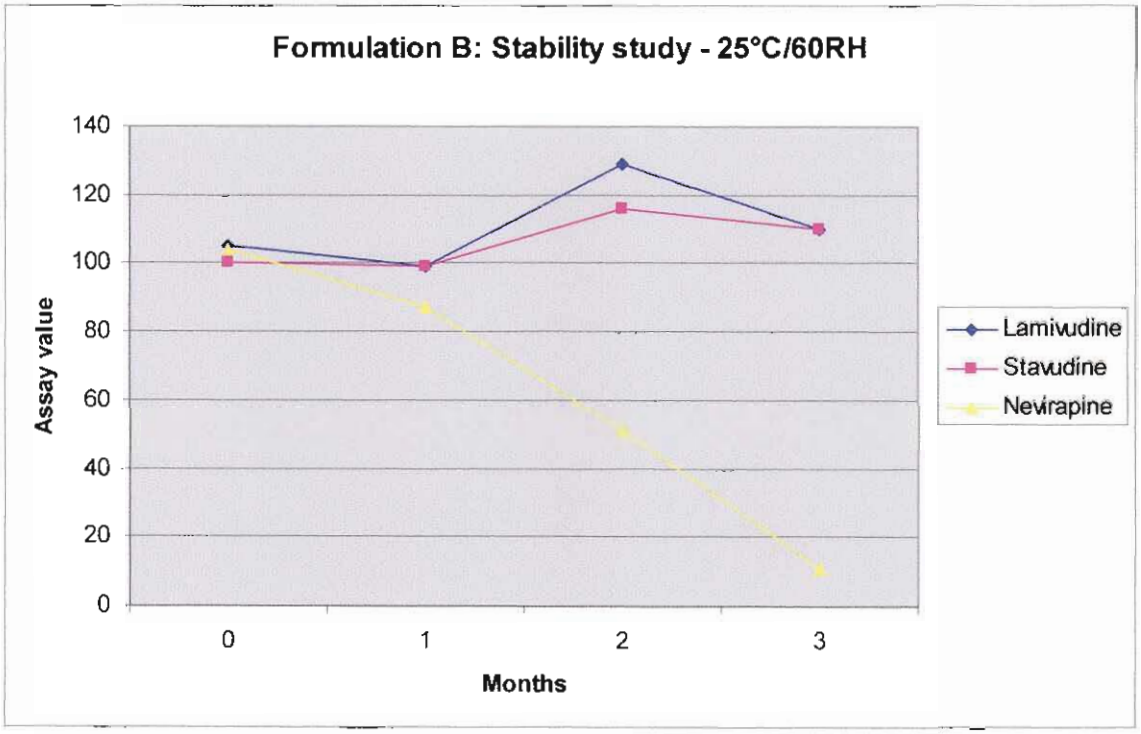


Figure 6.10: Stability study results for the three API's in Formulation B stored at 25°C / 60% RH.

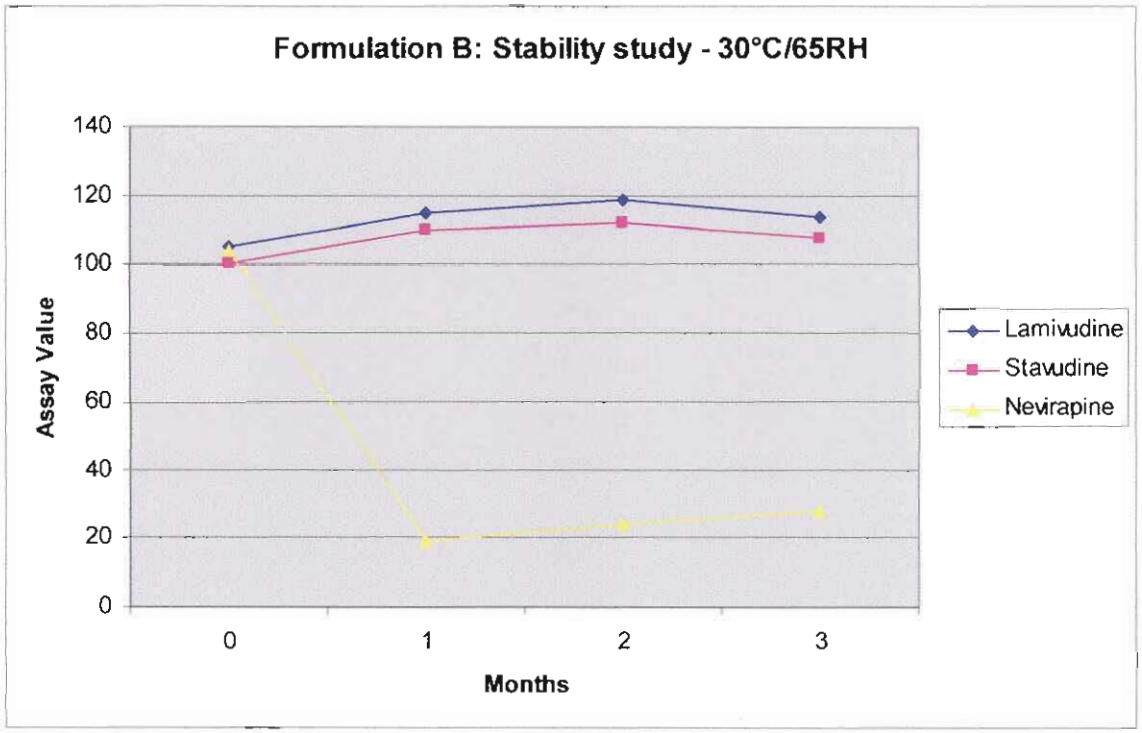


Figure 6.11: Stability study results for the three API's in Formulation B stored at 30°C / 65% RH.

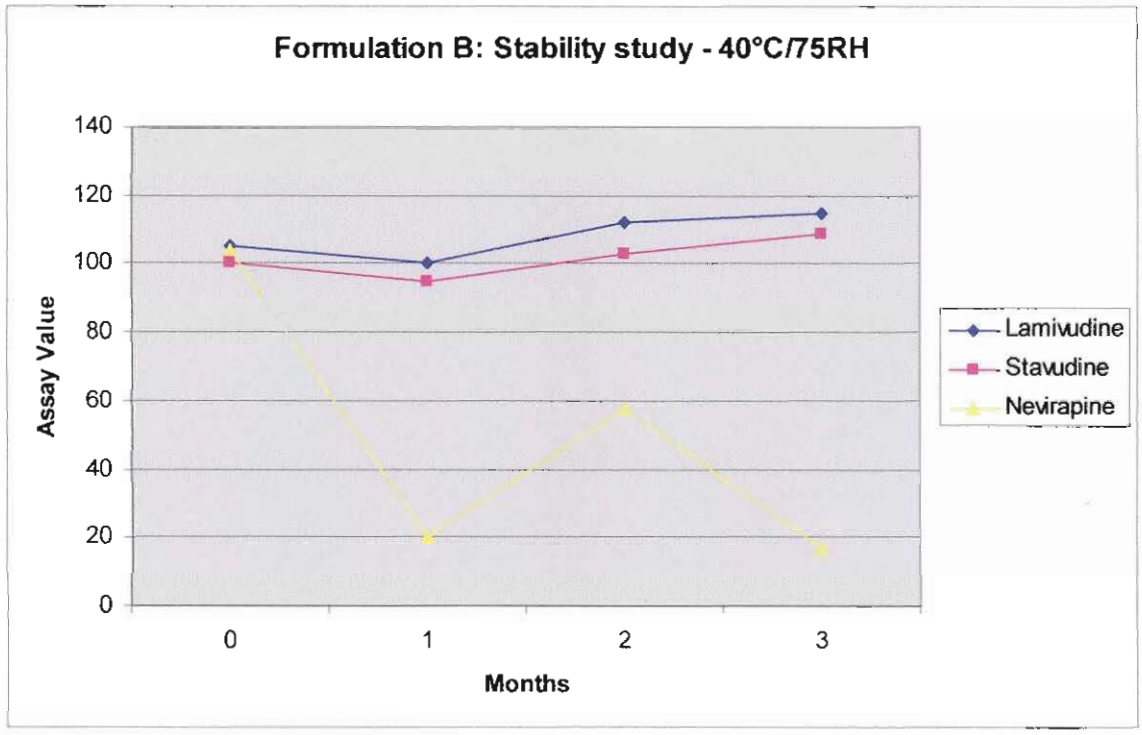


Figure 6.12: Stability study results for the three API's in Formulation B stored at 40°C / 75% RH.

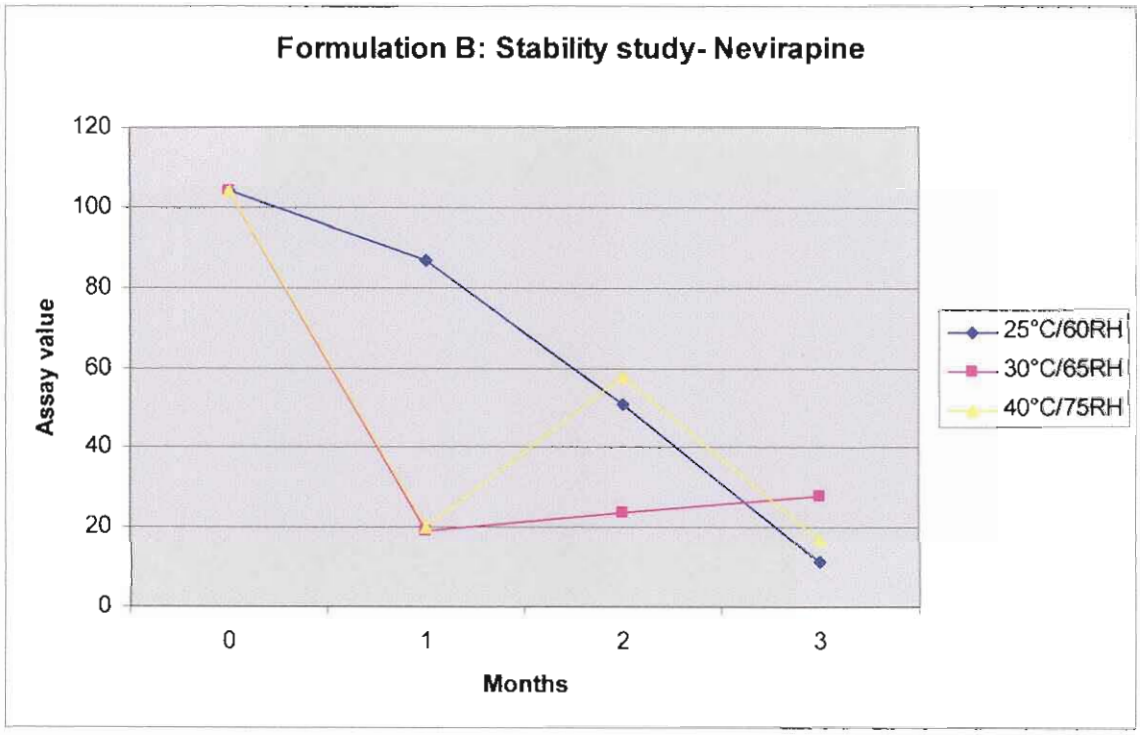


Figure 6.13: Stability of nevirapine in Formulation B over the three month period.

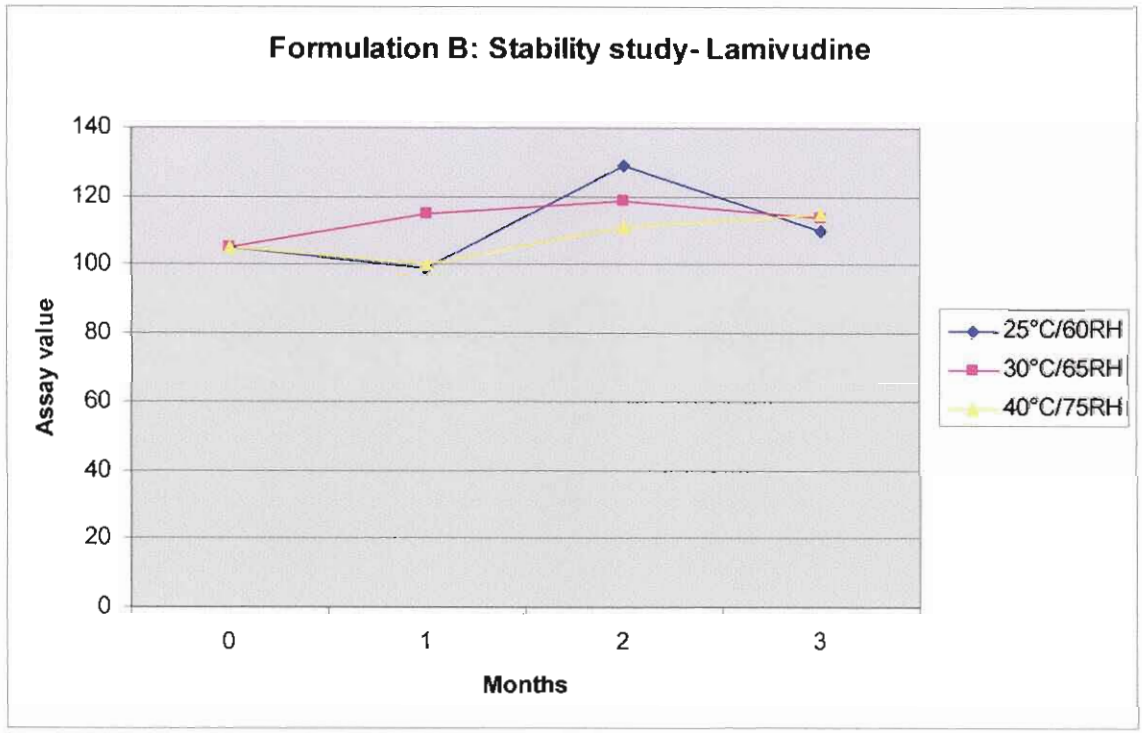


Figure 6.14: Stability of lamivudine in Formulation B over the three month period.

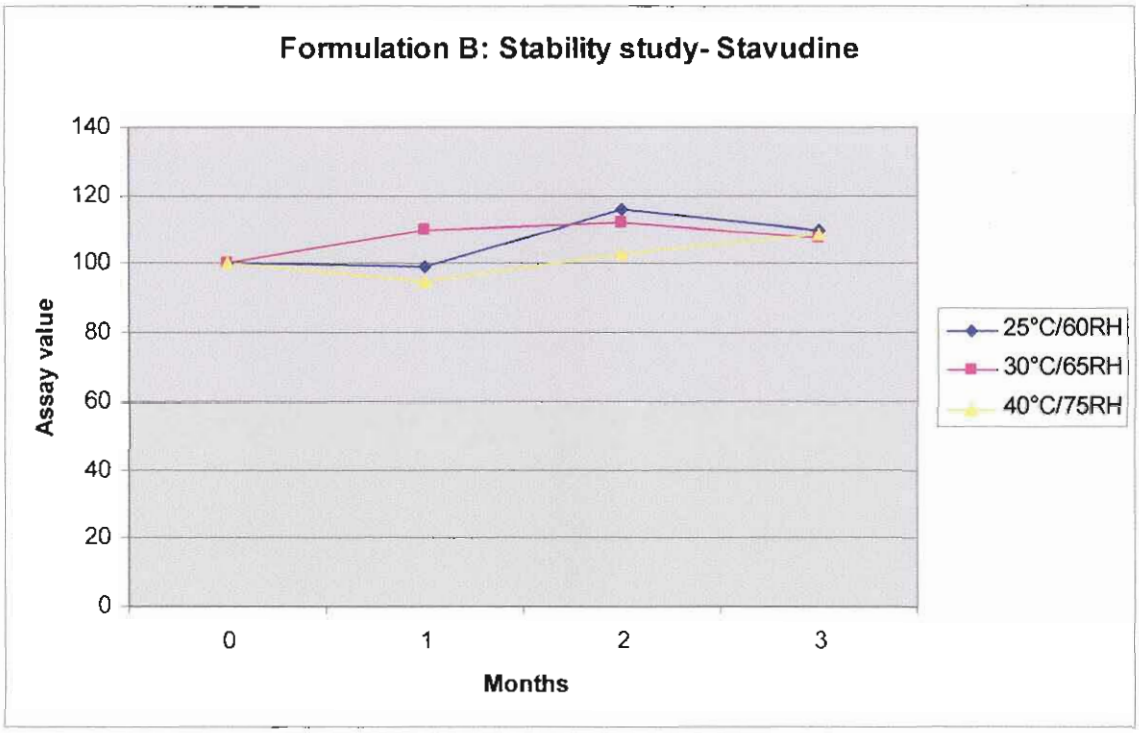


Figure 6.15: Stability of stavudine in Formulation B over the three month period.

6.7.5.4 DISCUSSION

Lamivudine once again remained stable throughout the duration of the study (Figure 6.14). The nevirapine results were again inconclusive (Figure 6.13). This might be due to a formulation problem and/or because nevirapine is also insoluble in the pro-Pheroid™ system. This was unexpected since the pro-Pheroid™ system does not contain an aqueous phase like Pheroids™ do. Possible degradation of the nevirapine in this system was never investigated. In contrast to Formulation A, stavudine remains stable in Formulation B (Figure 6.15).

The initial concentration of methyl hydroxybenzoate is a complete outlier. This could possibly be due to instrument malfunction and should be regarded as an experimental error. The results attained for month one, two and three remain stable, thus indicating that methyl hydroxybenzoate is a suitable preservative for the pro-Pheroid™ system. However, the large values could be due to an initial weighing error.

6.7.6 ANTI-OXIDANTS CONCENTRATION ASSAY

6.7.6.1 RESULTS: FORMULATION A

Formulation A was subjected to an accelerated stability study over the period of three months. Anti-oxidants was determined at monthly intervals – results are given in Table 6.9. Assay values given as the average of duplicate determinations from the same vial.

Table 6.9: Assay of anti-oxidants - results for Formulation A after 3 months of accelerated stability studies.

	BHT	BHA	Vit E
Initial	103.4	100.1	221.1
1st Month			
25°C / 60% RH	95.4	76.2	67.3
30°C / 65% RH	100.0	62.7	54.4
40°C / 75% RH	97.8	49.5	50.7
2nd Month			
25°C / 60% RH	100.8	66.1	57.0
30°C / 65% RH	94.4	63.3	52.4
40°C / 75% RH	100.5	60.0	49.7
3rd Month			
25°C / 60% RH	95.6	72.4	65.6
30°C / 65% RH	95.0	66.0	54.0

40°C / 75% RH	102.2	62.8	56.3

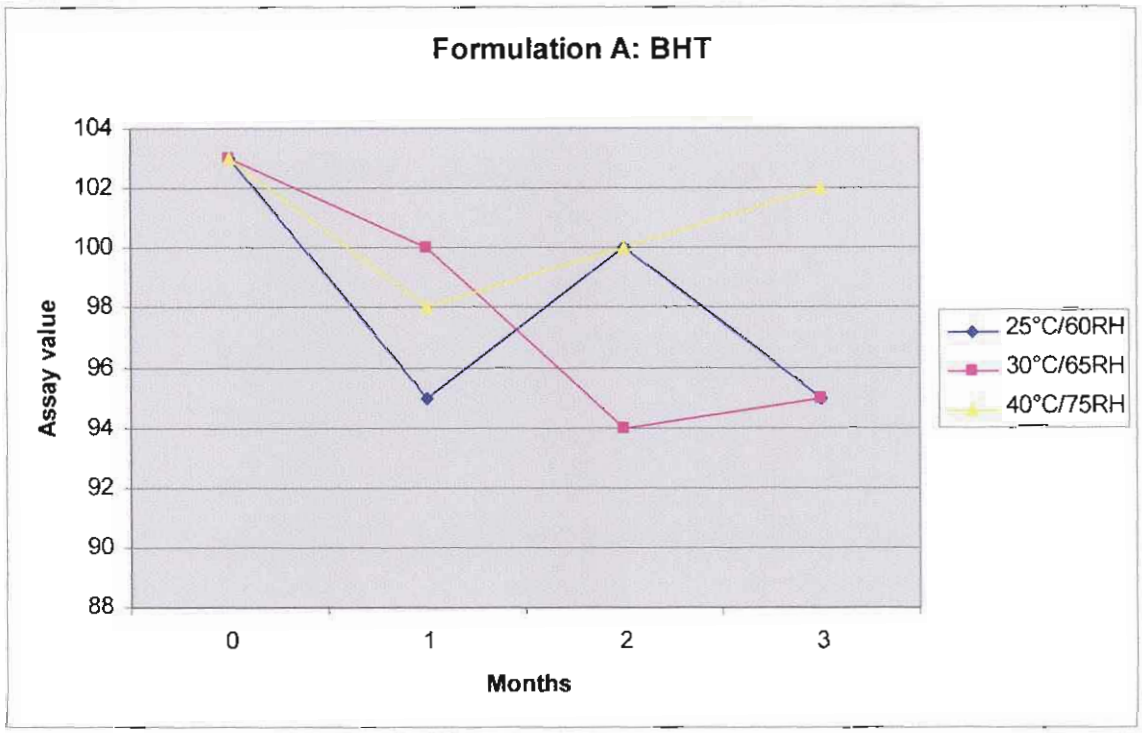


Figure 6.16: Stability of BHT in Formulation A over the three month stability period.

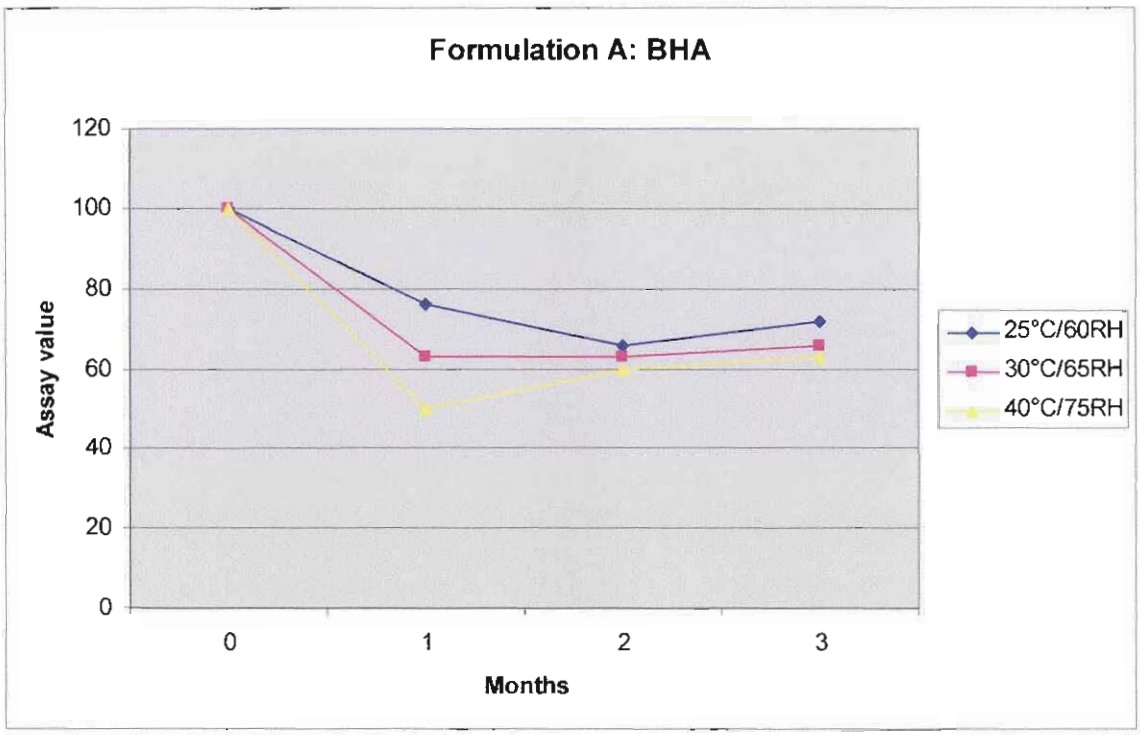


Figure 6.17: Stability of BHA in Formulation A over the three month stability period.

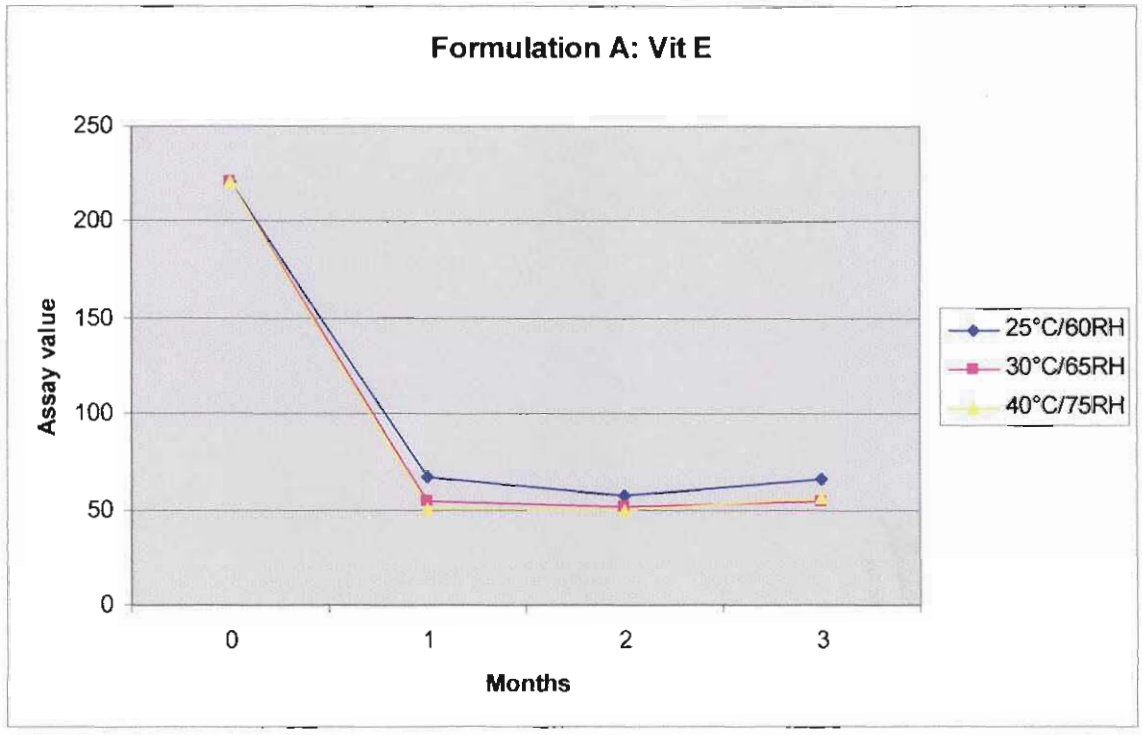


Figure 6.18: Stability of Vit E in Formulation A over the three month stability period.

6.7.6.2 RESULTS: FORMULATION B

Formulation A was subjected to an accelerated stability study over the period of three months. Anti-oxidants was determined at monthly intervals – results are given in Table 6.10. Assay values given as the average of duplicate determinations from the same vial.

Table 6.10: Assay of anti-oxidants - results for Formulation B after 3 months of accelerated stability studies.

	BHT	BHA	Vit E
Initial	103.1	89.3	105.7
1st Month			
25°C / 60% RH	114.0	86.0	92.5
30°C / 65% RH	132.0	98.0	94.0
40°C / 75% RH	128.6	97.3	91.0
2nd Month			
25°C / 60% RH	114.0	94.7	131.1
30°C / 65% RH	132.0	98.0	121.0
40°C / 75% RH	128.6	97.3	127.4
3rd Month			
25°C / 60% RH	123.7	109.3	103.6

30°C / 65% RH	122.6	98.7	98.0
40°C / 75% RH	131.3	110.7	104.9

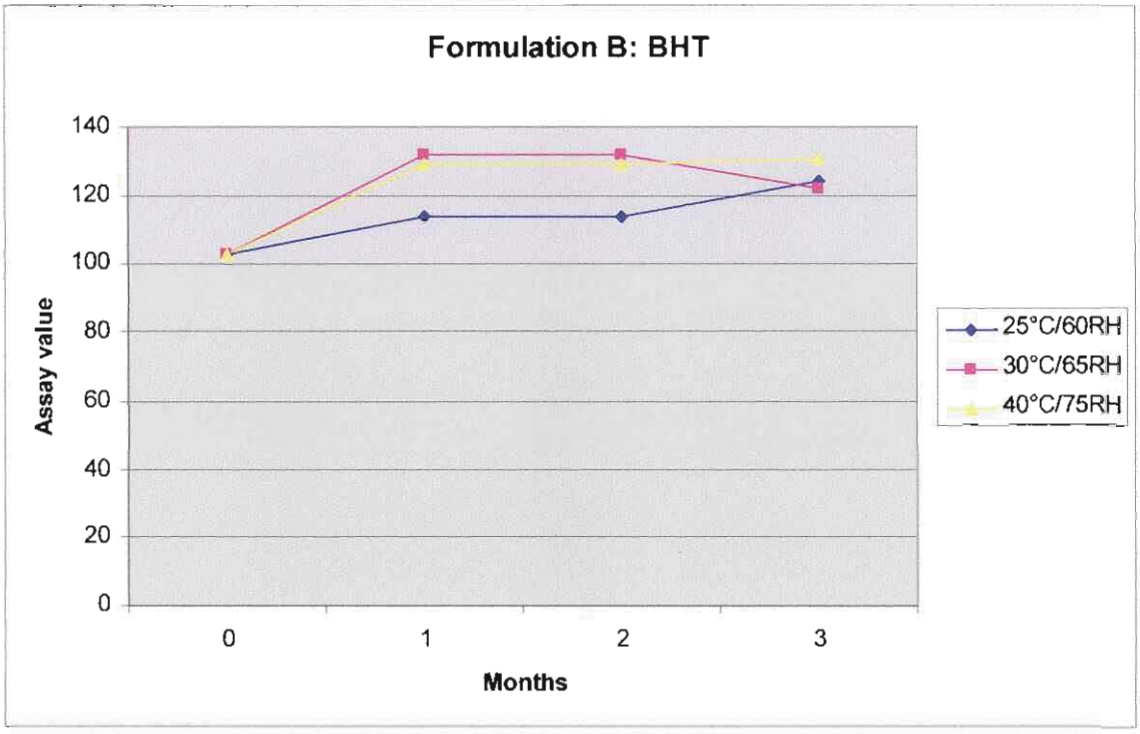


Figure 6.19: Stability of BHT in Formulation B over the three month stability period.

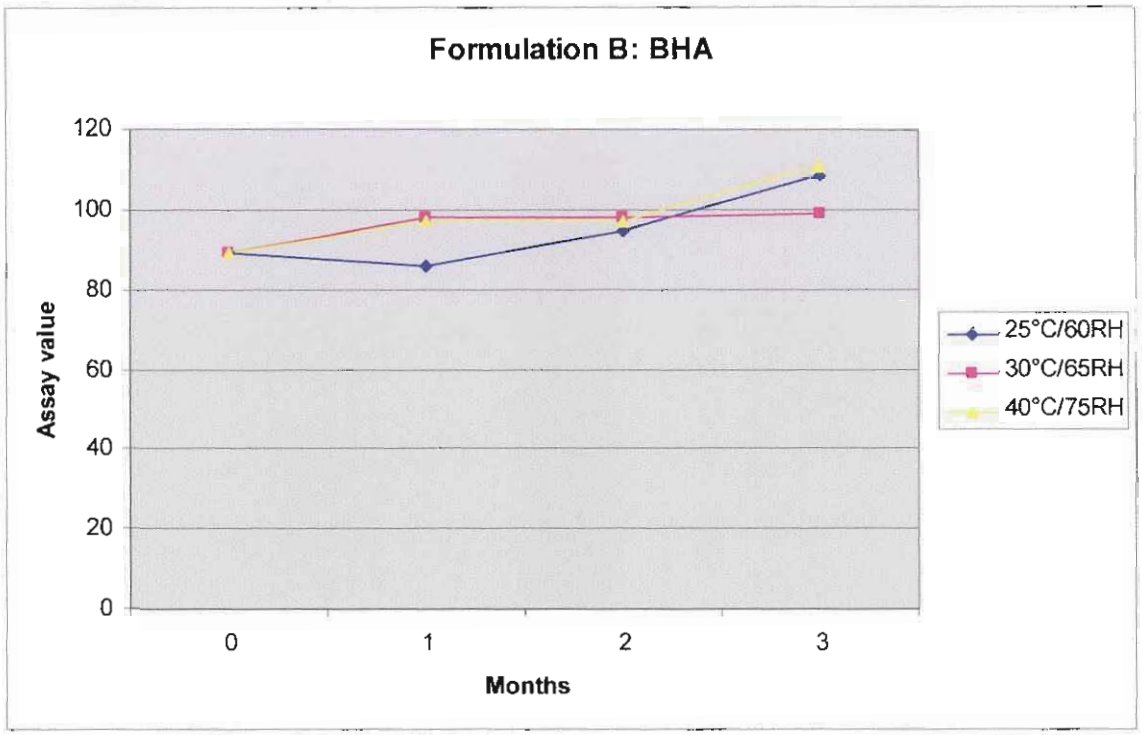


Figure 6.20: Stability of BHA in Formulation B over the three month stability period.

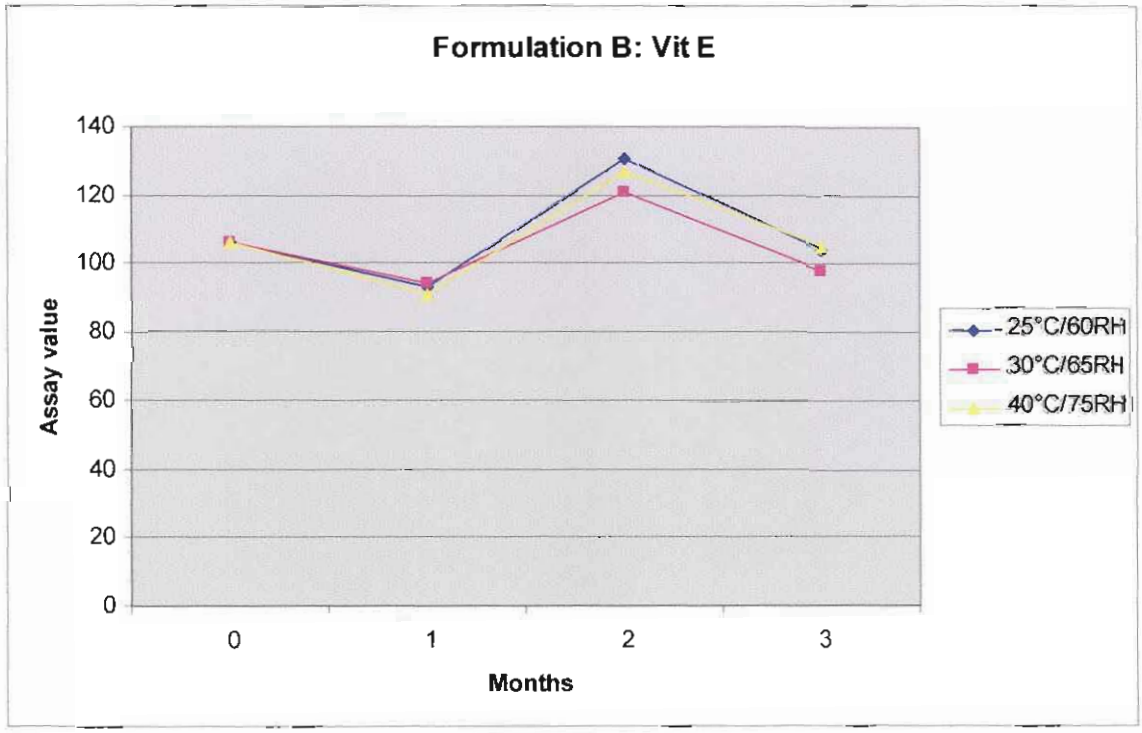


Figure 6.21: Stability of Vit E in Formulation B over the three month stability period.

6.7.6.2 DISCUSSION

Anti-oxidants work by consuming oxygen at a faster rate than the rate at which the drug substance reacts with oxygen, and in such cases they will protect the drug substance until they themselves are completely depleted (Rhodes, C.T., 2007:128).

The concentration of BHT remains stable for Formulation A and Formulation B. The concentration of BHA decreases with time in formulation A, but remains stable in Formulation B. The initial result for vitamin E is an outlier and could be due to instrument malfunction or an experimental error. The values for vitamin E also remain stable throughout the study.

These results indicate some problems with the chosen anti-oxidants. Either the anti-oxidants were not suitable to protect this formulation, or they were in some way incompatible with the delivery system. It is also possible that the assay method was not entirely suited for use with pheroid systems. This last option is backed by the inconsistent HPLC results obtained. It seems that an HPLC method specific to the Pheroid™ and pro-Pheroid™ systems should be developed and validated. The possible inclusion of alternative anti-oxidants such as TBHQ, sodium sulfite and ascorbic acid, should also be investigated.

6.7 FINAL CONCLUSION AND DISCUSSION

Formulation of Pheroid™ - and pro-Pheroid™ -based anti-retroviral preparations proved to be challenging, and in some cases not viable. Some API's, preservatives and anti-oxidants were incompatible with this delivery system.

HPLC was used to determine the concentrations of actives and excipients. The method used was validated for each compound, but not in the presence of the Pheroid™ delivery system. When the final product was analysed, assay results were highly variable, indicating the need to develop an HPLC method for Pheroid™ -based systems. Despite this, certain stability trends could still be observed.

Two formulations, A (Pheroid™-based) and B (pro-Pheroid™ -based), both containing lamivudine, stavudine and nevirapine, were subjected to accelerated stability testing. Assay results show the following:

- Lamivudine content remained stable, though not always within specification, in both the formulations throughout the three months of testing.

- Nevirapine's results varied dramatically for Formulation A, however the general trend seems to indicate a decline in concentration over time.

- It was thought that higher values would be obtained for nevirapine in Formulation B, which does not contain an aqueous phase. Results produced unexpectedly low values.

- In Formulation A, stavudine degrades completely in all but one sample. The sample kept at 25°C / 60% RH still contained a small amount of stavudine after three months.

- Stavudine content of Formulation B remained constant throughout stability testing. It can be concluded that stavudine is suitable for inclusion in Formulation B, but not Formulation A.

- Assay values for methyl hydroxybenzoate for both formulations remained stable throughout the three month period. Therefore, methyl hydroxybenzoate is a suitable preservative for these delivery systems.

An investigation is currently under way to find the most suitable anti-oxidant for use with the Pheroid™ delivery system.

This study has highlighted many of the problems and pitfalls that may be encountered when working with Pheroid™ and pro-Pheroid™ delivery systems. It has laid the groundwork for formulating three important ARV's into a convenient and effective dosage form. Much work remains to be done, both in formulation and in assay method development, before Pheroid™ -based multi-ARV dosage forms can undergo further testing.

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