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DEVELOPMENT AND BIOAVAILABILITY/BIOEQUIVALENCE OF A FIXED-
DOSE COMBINATION ANTIRETROVIRAL RECONSTITUTABLE SUSPENSION
INTENDED FOR PEDIATRICS

A Dissertation

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In partial fulfillment of the requirements for

the degree of Doctor of Philosophy

By

Fredrick Y. Esseku

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DEVELOPMENT AND BIOAVAILABILITY/BIOEQUIVALENCE OF A FIXED-DOSE COMBINATION ANTIRETROVIRAL RECONSTITUTABLE SUSPENSION INTENDED FOR PEDIATRICS

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ABSTRACT

DEVELOPMENT AND BIOAVAILABILITY/BIOEQUIVALENCE OF A FIXED-DOSE COMBINATION ANTIRETROVIRAL RECONSTITUTABLE SUSPENSION INTENDED FOR PEDIATRICS

By

Fredrick Y. Esseku

May 2011

Dissertation supervised by Moji Christianah Adeyeye, Ph.D.

This dissertation was designed to develop and assess bioavailability/ bioequivalence of age-appropriate fixed-dose combination granules of lamivudine/zidovudine/nevirapine as reconstitutable suspension for pediatrics for use in pediatric patients. The granules were developed via roller compaction process to improve granule flow characteristics, minimize segregation of the multi-component active pharmaceutical ingredients, and avoid introducing moisture into the formulation and thereby improve the stability of the product. Optimization of the roller compaction process for the roll pressure and the ratio of the horizontal feed screw speed to the roll speed showed that these parameters had minimal effect on granule quality. Subsequently, a replicated 3^2 factorial design was utilized to optimize the levels of the suspending agent

(Avicel RC 591) and the anticaking agent (Aerosil 200). Modeling of the formulation viscosity through multiple regression analysis showed that the viscosity was a quadratic function of the concentration of Avicel RC 591. An assessment of the stability of the granules for reconstitution under International Conference on Harmonization stability conditions at 40 ^{o}C/75 %RH and 30 ^{o}C/65 %RH indicated the granule formulation to be stable with an estimated shelf-life of not less than 6 months. Similarly, the evaluation of the chemical stability of the reconstituted suspension at 30 ^{o}C/65 %RH showed the suspension to be stable for several weeks.

A clinical batch of the granules for reconstitution manufactured under Good Manufacturing Practices was tested for bioavailability and bioequivalence in a clinical study. A randomized single dose two-way complete crossover design in 24 healthy adult cohorts was performed, and the plasma samples from the subjects were analyzed by HPLC with UV detection. Non-compartmental pharmacokinetic analysis was performed to obtain the C_{max}, AUC_{o-t}, and AUC_{o-inf} utilizing WinNonlin software. Analysis of variance of the formulation, period, sequence, and subject effects established the absence of any significant effects due to these parameters. Application of the two one-sided statistical tests using the Anderson-Hauck method showed that the 90 % confidence interval for the ratio of test/reference for various pharmacokinetic parameters was within 82.6 – 124.5 % bioequivalence limits for all the three drugs. This confirms that the fixed-dose combination granule for reconstitution was bioequivalent to the simultaneously administered single-entity reference products.

DEDICATION

To the Esseku Family

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

3TC – Lamivudine

AIDS – Acquired immunodeficiency syndrome

ANOVA – Analysis of variance

API – Active pharmaceutical ingredient

ARV – Antiretroviral

AUC_{0-t} – Area under the curve, time zero to time t

$AUC_{0-\infty}$ – Area under the curve, time zero to infinite time

AZT – Zidovudine

BE – Bioequivalence

BCS – Biopharmaceutics classification system

BUTH – Bowen University Teaching Hospital

C_{max} – Maximum plasma concentration

CoF – Goodness of fit

CRF – Case report form

CV – Coefficient of variation

ConiS – Coni-snap capsule

DB – Double blind capsule

DSC – Differential scanning calorimetry

FTIR – Fourier transform infrared spectroscopy

FDA – Food and Drug Administration

FDC – Fixed-dose combination

GCP – Good clinical practice

GMP – Good manufacturing practice

HAART – Highly active antiretroviral therapy

HCL – Hydrochloric acid

HPLC – High performance liquid chromatography

HIV – Human immunodeficiency virus

ICH – International Conference on Harmonization

IEC – Institutional Ethics Board

IND – Investigational new drug

IR – Infrared

IRB – Institutional Review Board

IST – Isothermal stress testing

MP – Methylparaben

NAFDAC – National Agency for Food and Drug Administration and Control

NCA – Non-compartmental analysis

Nev – Nevirapine

NNRTI – Non-nucleoside reverse transcriptase inhibitor

NRTI – Nucleoside reverse transcriptase inhibitor

PK – Pharmacokinetics

PP – Propylparaben

PXRD – Powder X-ray diffraction

t_{max} – Time to attain maximum plasma concentration

USP – United States Pharmacopoeia

WHO – World Health Organization

Chapter 1. Introduction

1.1. Statement of the Problem

The development of pediatric formulations is normally not a high priority during the development of new chemical entities into dosage forms for clinical use. This has resulted in a situation where there are several per oral formulations of drugs that can be used by the adult population for managing specific disease conditions without any corresponding age-appropriate formulations for pediatric patients. As a result of this, in some clinical settings, the adult formulations are extemporaneously reconstituted for administration to infants. This may take the form of breaking adult tablets or capsules and administering suitable fractions to the sick children. In addition, the solid oral dosage forms may be crushed and mixed with foods or dispersed in water and administered to children. This may result in inaccurate dosing manifested as over-dosing leading to severe toxicity. In contrast, children may be under-dosed when they do not receive the required amount of drug to effect the treatment. In the management of infectious diseases such as HIV/AIDS, malaria, and pneumonia, this could lead to worsening patient symptoms or the development of resistance by the pathogens. Age-appropriate pediatric formulations should be stable, palatable and be in a form that is convenient for administration to children. With a view to amend the Federal Food, Drug, and Cosmetic Act to improve the safety and efficacy of pharmaceuticals for children, the United States congress enacted the "Best Pharmaceuticals for Children Act". Although the act has encouraged pharmaceutical manufacturers to develop more pediatric formulations, there

still remain a disproportionate number of adult formulations without the corresponding pediatric formulations.

In the field on antiretroviral (ARV) therapy, several formulations are available for treating HIV/AIDS in adults without the age-appropriate formulations for pediatric patients. Effective treatment of HIV/AIDS requires highly active antiretroviral therapy (HAART). HAART consists of a combination of two or more drugs from at least two antiretroviral drug classes. The combination therapy provides adequate viral suppression in addition to providing mutational barrier to resistance, compared to monotherapy. Due to the advantages demonstrated by combination therapy, there has been a drive to formulate fixed-dose combination (FDC) therapies of drugs commonly co-administered together. The FDCs reduce the number and size of different formulations administered, reduce treatment cost, and improve compliance and treatment outcome. Furthermore, the Food and Drug Administration (FDA) recommends the development of FDCs where the evidence for safety and efficacy of the combined usage of the drugs in patients has been accumulated.

Lamivudine and zidovudine are nucleoside reverse transcriptase inhibitors (NRTI) which have been co-administered with nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) as combination therapy in HAART with demonstrated efficacy and safety. However, to date no per oral liquid formulation has been developed for use by children.

1.2. Hypothesis and Objectives

It is hypothesized that a fixed-dose combination of lamivudine, zidovudine and nevirapine developed as granules for reconstitution will be stable under storage conditions recommended by the International Conference on Harmonization (ICH). Further, it is envisaged that the FDC developed will be bioequivalent to the simultaneously administered single-entity oral liquid reference products.

Following from the hypothesis, the objectives for this dissertation are

1. To develop a fixed-dose combination of lamivudine, zidovudine and nevirapine, formulated as granules for reconstitution, using dry granulation roller compaction technique. The granules for reconstitution formulation will be optimized and characterized.

2. To evaluate the stability of the optimized formulation under accelerated and long-term stability storage conditions.

3. To conduct a pilot bioequivalence study between generic and reference products of lamivudine 150 mg/zidovudine 300 mg tablets. This will be done in order to determine the suitability of the international clinical site for further study.

4. To perform bioavailability/bioequivalence (Phase I) studies on the developed granules for reconstitution in comparison to a combination of Epivir® (lamivudine) oral solution, Retrovir® (zidovudine) syrup and Viramune® (nevirapine) suspension as reference products.

If successful, the fixed-dose combination formulation developed could be employed for the treatment of HIV/AIDS in treatment naïve children in resource poor settings. This could facilitate patient compliance with concomitant improved therapeutic outcome.

1.3 Literature Review

1.3.1 Antiretroviral Therapy

1.3.1.1 Global AIDS

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV). There are approximately a total of 33.4 million people living with HIV worldwide.[1] Globally, the HIV epidemic seems to have stabilized around this number in the last couple of years. This is due to increased education about the epidemic and the implementation of intervention policies to prevent, test, treat and care for those affected by the disease. The World Health Organization (WHO) in recent times recommended that "all adults and adolescents, including pregnant women, with HIV infection and a CD4 count of or below 350 cells/mm^3 should be started on antiretroviral therapy, regardless of whether or not they have clinical symptoms".[1] This has caused an apparent increase in the number of people living with HIV/AIDS who require treatment. In December 2009, it was estimated that antiretroviral therapy coverage among children less than 15 years of age was 28%.[1] The majority of these children live in resource poor settings in sub-Saharan Africa. Previous pharmacokinetic studies by Ellis et al in Zambian and Malawi children on FDC adult ARV tablets demonstrated that some

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children do not achieve optimal concentration of nevirapine in plasma.[2] Subsequent to this, Kashuba and Swanstrom also demonstrated lower lamivudine exposures and subtherapeutic nevirapine level in Malawian children receiving scored triple combination FDC ARV tablets.[3] There is thus a need to provide age-appropriate formulations of antiretroviral drugs to enhance access to treatment, particularly for use in infants and young children. The United States President's Emergency Plan for AIDS Relief (PEPFAR), is providing funding for the scale-up of HIV/AIDS services worldwide to the tune of $5.5 billion annually.[1, 4] This fund could be accessed for providing antiretroviral therapy for resource poor settings.

1.3.1.2 Life Cycle of HIV

The life cycle of HIV occurs in the body of the infected host. The envelope protein gp160 regulates the tropism[5] of HIV-1 and gp41 controls fusion of the virus lipid bi-layer with that of the host cell. The virus binds to CD4 receptors on macrophages and lymphocytes, and also binds to CCR5 or CXCR4 co-receptors on macrophages and T-lymphocytes (Figure 1.1). Upon fusion of the virus with the host cell, viral RNA is released into the host cytoplasm and undergoes replication to form RNA-DNA duplex. This duplex is short lived as viral RNA is degraded by RNase H to allow the formation of full length double stranded DNA copy of the virus, a process catalyzed by reverse transcriptase. The DNA of the virus is transported into the nucleus where it is integrated into host chromosome by viral integrase in a random location. Viral RNA and structural proteins are formed in the cytoplasm. The structural proteins assemble around viral RNA

to form a nucleocapsid. Reverse transcriptase enzyme, other proteins, and cell membrane are assembled and bud to form a new enveloped HIV particle.

The currently available drugs for treating HIV infection target potential sites for disruption of the life cycle to inhibit replication including 1. site of attachment to receptors and fusion with host cell, 2. reverse transcription, 3. integration, 4. transcription, 5. translation and 6. release of the viral particle (Fig 1.1). The antiretroviral agents co-administered in HAART are selected from these drug classes.
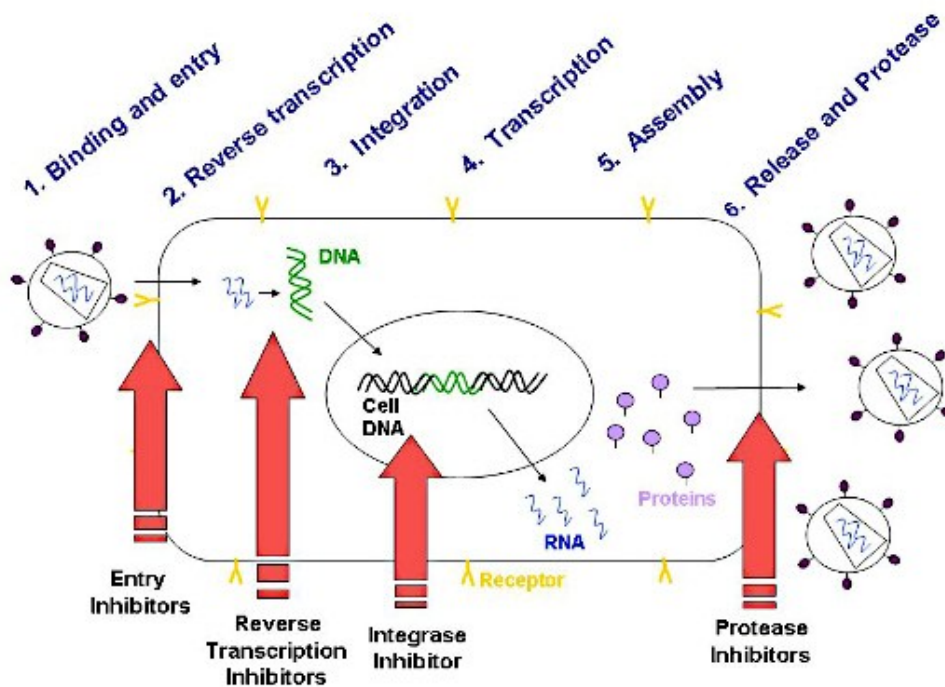
Fig 1.1 The life cycle of HIV.[6]

1.3.1.3 Rationale for Selecting the Antiretroviral Drug Combination

There are about 25 antiretroviral compounds licensed by the FDA for the treatment of HIV/AIDS. These approved drugs belong to the following classes: CCR-5 co-receptor antagonist, fusion inhibitors, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase strand transfer inhibitors and protease inhibitors (PI).[7] The use of antiretrovirals in pediatric patients is a rapidly evolving science, with frequent changes in the composition of the main first-line and second-line antiretroviral therapy. The backbone of antiretroviral regimen in treatment naïve children is 2 NRTIs plus 1 NNRTI or 2 NRTIs plus 1 protease inhibitor.[8, 9] The combination of AZT plus 3TC plus Nev (2 NRTIs +1 NNRTI) has been extensively used in adults and children and can be administered to children below 3 years of age.[10-13]

In vitro studies of the three-drug combination in peripheral blood mononuclear cells (PBMC) utilizing isobologram and the median-effect equation has been used to calculate the combination index for AZT/3TC/Nev.[14] The combination index method developed by Chou and Talalay, based on the median-effect principle of the law of mass-action, may be used to quantitate synergistic, antagonistic and additive effect for a drug combination.[15] Combination indices of <1, 1 and >1, indicate synergism, additive effect, and antagonism respectively. The combination index of percent of HIV-1 inhibition by AZT/3TC/Nev was less than 1, indicating synergistic activity.[14] Moreover, transmitted HIV drug resistance is low which makes these older drugs still effective in the treatment naïve children:[1] in children, HIV/AIDS infection is primarily due to mother-to-child

transmission. Furthermore, the Pediatric Antiretroviral Working Group (PAWG) of World Health Organization has recommended the combination regimen of AZT plus 3TC plus Nev [60 + 30 + 50 mg] as one of several ideal dosing strengths for priority antiretroviral products.[16] The combination regimen of AZT plus 3TC plus Nev [60 + 30 + 50 mg] was thus selected for development into the granules for reconstitution. It is expected that as pharmacokinetic studies of newer antiretroviral drugs are performed in children, the availability of clinical data will facilitate the formulation of new pediatric FDCs. See page 158 for further discussion.

1.3.1.4 Biopharmaceutics of the Antiretroviral Drugs

Lamivudine **A** (Fig. 1.2) is the (-)-enantiomer of 2'3'-dideoxy-3'-thiocytidine (3TC), a cytosine analog acting as a nucleoside reverse transcriptase inhibitor. The 2R cis(-)-enantiomer of 3TC is more potent and less toxic than the racemic mixture and it is the active pharmaceutical ingredient in drug products. Following oral administration, it is rapidly absorbed with an oral bioavailability of 86% (Table 1.1) and has a low plasma protein binding affinity. The phosphorylated (-)-enantiomer is more resistant to cleavage from nascent RNA/DNA duplexes by cellular 3'-5' exonucleases than the (+)-enantiomer and may explain the higher potency.[5] Upon entering cells by passive diffusion, 3TC is phosphorylated by deoxycytidine kinase to the monophosphate and subsequently by deoxycytidine monophosphate kinase and nucleoside diphosphate kinase to form lamivudine 5'-triphosphate. The intracellular half-life of lamivudine 5'-triphosphate is 12 to 18 hours. When the active anabolite is incorporated into HIV DNA, it acts as a

competitive inhibitor of reverse transcriptase to cause chain termination. Lamivudine is eliminated primarily as the unchanged drug in the urine. Lamivudine shows low affinity for human DNA polymerases, and thus has low toxicity to humans. Severe side effects associated with 3TC use are lactic acidosis and severe hepatomegaly.
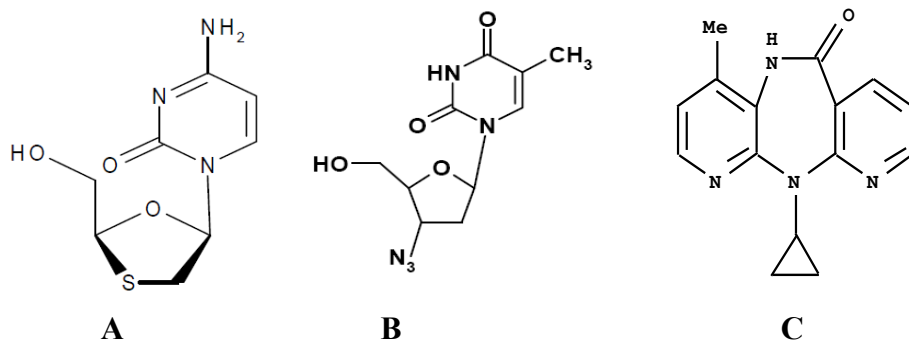
Fig.1.2 Chemical Structures of Lamivudine (A), Zidovudine (B) and Nevirapine (C).

Zidovudine **B** is 3-azido-2,3-dideoxythymidine (AZT), a thymidine analog. Intracellular zidovudine is phosphorylated by thymidine kinase to zidovudine 5'-monophosphate, which in turn is phosphorylated by thymidylate kinase to the diphosphate and by nucleoside diphosphate kinase to zidovudine 5'-triphosphate, the active form with intracellular half of 3 to 4 hours. Because the triphosphate lacks a 3'-hydroxyl group, incorporation by reverse transcriptase into nascent DNA terminates the proviral DNA chain. Zidovudine undergoes rapid first pass metabolism to form 5'-glucuronyl zidovudine and bioavailability is about 64% with plasma protein binding of approximately 25 %. The absorption is slowed by the presence of food, but there is no alteration in the area under the curve (AUC).[17] The penetration of zidovudine into the cerebrospinal fluid is a slow process and reaches an AUC of about 75% of the plasma

level.[18] Zidovudine is mainly excreted in the urine as 14 – 20 % of the unchanged drug and 74% of the glucuronide metabolite: less than 1% of an administered dose is internalized in cells as the active triphosphate.[19] Side effects associated with zidovudine use are neutropenia, anemia, myopathy, lactic acidosis and hepatomegaly.

Table 1.1. Pharmacokinetic parameters in adults

Parameter	Lam	Zid	Nev
Oral bioavailability (%)	86±16	64±10	>90
App. vol. distribution (L/kg)	1.3±0.4	1.6±0.6	1.21±0.09
Plasma protein binding (%)	<36	<38	60
Elimination half-life (hr)	5 – 7	0.5 – 3	25 – 30
Food effect	no	no	no

The chemical name of nevirapine **C** is 11-cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one. Nevirapine, an NNRTI is a non-competitive inhibitor that binds to an allosteric site on the HIV-1 to induce a conformational change at the active site and disrupt catalytic activity. Nevirapine is active against HIV-1 but not HIV-2 and resistance to the drug develops rapidly because its site of action is not essential for enzymatic action. It is rapidly absorbed; oral bioavailability is 90 % with no food effect. Nevirapine induces cytochrome CYP3A4 enzyme, a member of the cytochrome P450 mixed-function oxidase system, and a major enzyme in oxidation of xenobiotics in the body.[5] The autoinduction of CYP3A4 enzyme in turn results in induction of nevirapine metabolism thus reducing elimination half-life from 45 hours to

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25-30 hours after two weeks. It is eliminated primarily by oxidative metabolism by cytochrome CYP3A4 and CYP2B6 enzymes.[20] Less than 3% of parent drug is eliminated unchanged in the urine. In animal studies, the hydroxylated metabolites formed were 2-, 3-, 8-, and 12-hydroxynevirapine.[21] 4-carboxynevirapine, formed by secondary oxidation of 12-hydroxynevirapine, was a major urinary metabolite.

Nevirapine has been linked with hypersensitivity reactions which present clinically as severe and life threatening idiosyncratic skin rash. This dose-dependent rash is more common in females than males. Shenton *et al.* have observed similar characteristic rashes in rats and humans suggesting a common mechanistic pathway.[22-24] Immunohistochemistry analysis showed macrophage infiltration and ICAM-1 expression in the ears of treated rats as early as day 7 of treatment.[25] Investigations are underway to determine whether nevirapine or its reactive quinone methide metabolite (Fig 1.3) is responsible for the idiosyncrasy.[25, 26] The quinone methide is reactive because breaking of the double bond at C9 and formation of the double in the pyridine ring restores aromaticity and stability to the structure.
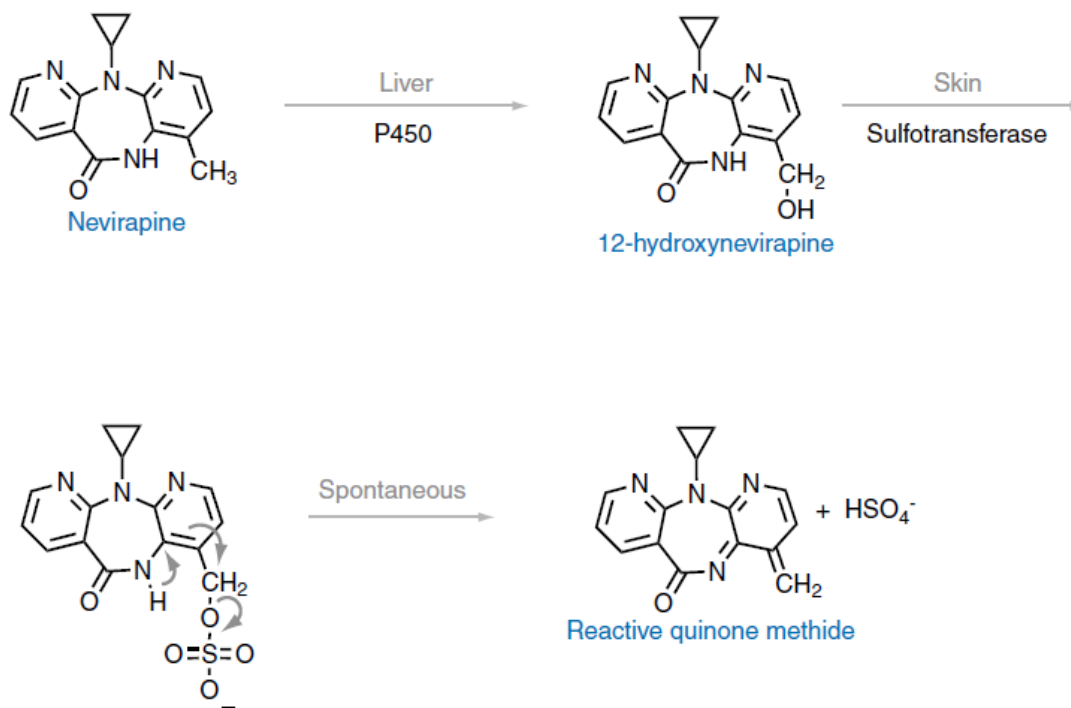
Fig 1.3 Postulated mechanism for formation of reactive nevirapine metabolite implicated in rash formation due to hypersensitivity reaction.[26]

In humans, the rash may develop 2-3 weeks after initiating nevirapine therapy. Further, CD4$^+$ T cells are essential to the development of the rash, and their depletion confers partial protective status to the patient. The low CD4$^+$ T cells protective effect is of interest in HIV/AIDS patients who typically present with depleted CD4$^+$ T cells levels. Pruritus may accompany the skin rash. Nevirapine may also cause hepatotoxicity when administered under normal prescribing conditions. Even though these severe reactions are rare, there is a need to exercise extreme caution in initiating therapy with nevirapine. Due to the hypersensitivity reaction, nevirapine therapy is initiated by a lead-in dose daily for

two weeks to trigger the development of tolerance in patients and thus minimize the risk of the idiosyncratic rash.

1.3.1.5 Fixed-Dose Combination Formulation

The high pill burden, frequency of administration, and co-administration of other medications for opportunistic infections do not encourage drug intake by people living with HIV/AIDS. For instance, lamivudine solution, zidovudine syrup and nevirapine suspension are given to children with HIV, and this makes the volume of the doses administered large. There are no fixed-dose combinations available for pediatrics. Fixed-dose combination (FDC) therapy may enhance the therapeutic outcome of antiretroviral therapy through a combination of factors. The FDC reduces the number and size of different formulations administered with a resultant reduction in the cost of the drugs administered. In addition, FDCs reduce the propensity for drug-excipient incompatibility and thereby enhance compliance and success of therapy. The FDA released a Guidance document on fixed dose combination and co-packaged products to promote the development of FDCs.[27] A major provision of this document was the waiver to conduct clinical studies for safety and efficacy for the FDCs since the clinical evidence for the safety and efficacy of the specific combination is available prior to the FDC formulation development. However, there is a requirement to perform bioequivalence studies to determine the bioavailability of the FDC product.

Fixed-dose combinations have some limitations due to the fixed ratio of the drugs. FDCs cannot be used for individualized dosing. For instance, where individuals or sub-populations show higher or lower nevirapine metabolism due to varying CYP3A4 or

CYP2B6 levels, the dose of nevirapine cannot be adjusted. Consequently, prior to start of FDC, patients are usually given two-weeks of nevirapine lead-in dose to minimize the risk of developing hypersensitivity reaction, but the FDC product cannot be used for that purpose. Correcting for the dose of nevirapine for the lead-in period may result in under-dosing of the other drugs in the combination.

1.3.2 Granules for Reconstitution into Suspension

The goal of the project is to formulate a fixed-dose combination granule for reconstitution into suspension which contains lamivudine/zidovudine/nevirapine 30 mg/60 mg/50 mg per 5 mL. The rationale for the choice of dosage form is to facilitate the titration of doses to meet individual pediatric patient requirements. Unlike oral solid dosage forms, the dose of oral liquid dosage forms can be tailored to meet the needs of patients, particularly children. Granule for reconstitution into suspension form is a type of oral liquid dosage form containing a coarse dispersion of the API in aqueous medium. The particle size range of the APIs is greater than 0.5 μm.[28] This formulation may be employed when the API has poor solubility or stability in water. Some desirable properties of a reconstitutable suspension are a fast and simple reconstitution, slow sedimentation rate to ensure uniform dose dispensing, an appropriate viscosity for easy pouring and redispersion and a flocculated system to ensure ease of redispersion. The suspending agent used should be hydrated quickly by hand shaking to ensure rapid reconstitution.[29, 30] The granules for reconstitution could be made by roller compaction process to prevent the segregation of the drugs from the excipients. In addition, the roller

compaction process will avoid the introduction of moisture into the product: moisture could compromise the stability of the formulation.

1.3.2.1 Physicochemical Properties of the Drugs

The physicochemical properties of the drug substances essential to formulation are solubility, dissolution, partition coefficient and pKa. The varied physicochemical properties (Table 1.2) could pose challenges in a fixed dose combination product.

Table 1.2 Physicochemical properties of the drug substances[31]

Parameter	Lamivudine	Zidovudine	Nevirapine
Empirical formula	$C_8H_{11}N_3O_3S$	$C_{10}H_{13}N_5O_4$	$C_{15}H_{14}N_4O$
CAS Number	134678-17-4	30516-87-1	129618-40-2
Molecular weight	229.26	267.2	266.3
Melting temp (^{o}C)	178	122	247
Aqueous Solubility (mg/mL)	70	20	0.1
pKa	4.3	9.68	2.8
Log P	0.06	0.09	2.05

Lamivudine and zidovudine are biopharmaceutics classification system (BCS) Class 1 compounds with high solubility and high permeability whereas nevirapine is a BCS class II compound with low solubility but high permeability.[31] Zidovudine which exists as anhydrous crystals has a solubility of 20 mg/mL. This solubility exceeds the 12 mg/mL of drug present in the proposed formulation. Therefore zidovudine will be

completely dissolved in solution in the reconstituted suspension. Lamivudine anhydrous, hemihydrate and 0.2 hydrate have been reported in the literature. The 0.2 hydrate has one mole of water per five moles of lamivudine.[32-34] The proposed dose of 6 mg/mL of lamivudine is far below the solubility (70 mg/mL) of the anhydrous and hydrated forms of the drug[33] and thus the drug will be in solution in the reconstituted product. However, there is a need to characterize and control the physical form of the drug in the granules for reconstitution.[35]

Nevirapine presents as anhydrous and pseudo-polymorphic hemihydrate forms. The two forms have different solubility, dissolution rates and stability.[36, 37] The anhydrous nevirapine has higher solubility in water than the hemihydrate. Marketed nevirapine (Viramune®) tablets contain the anhydrous crystals while nevirapine (Viramune®) suspension contains the hemihydrate crystals. Angel *et al.* reported that the when the anhydrous form was formulated as aqueous suspension, it was converted into the micrometer-sized hemihydrate crystals. The degree of conversion and the rate of growth of the hemihydrate crystals were dependent on the temperature and time.[38] Interestingly, the hemihydrate crystals could not be detected by optical microscopy or Fourier transform infrared (FT-IR) spectroscopy but by low voltage scanning electron microscopy (LVSEM) as a surface catalyzed nucleation. Due to the lower solubility of the hemihydrate crystals, the dissolution rate of the suspension was negatively impacted. Further, crystal growth in a suspension promotes sedimentation and may result in caking to form a non-redispersible sediment.[39] This is an unacceptable phenomenon as it leads to an untoward physical appearance. However, formulating the anhydrous nevirapine as granules for reconstitution significantly reduces the contact time in aqueous medium and

the probability of conversion to the hydrate. In addition, the presence of polyhydric alcohols (polyols) in the medium could adsorb onto the surfaces of crystals to prevent nucleation and the conversion of the crystal into hydrates.[40, 41] Polyols commonly used as excipients in suspensions such as xylitol, mannitol and sorbitol have been shown to possess the ability to inhibit recrystallization. Furthermore, the presence of polymers which adsorb to the crystal surface by specific interaction with the functional groups can inhibit crystal growth. For example, Ziller and Rupprecht successfully inhibited the growth of acetaminophen suspension by incorporating a small quantity of polyvinylpyrrolidone (PVP).[39] Thus, formulation strategies could be adopted to eliminate or minimize recrystallization and crystal growth.

Compounds are generally more chemically stable in water as dispersed solid particles than when dissolved. The high solubility of lamivudine and zidovudine could lead to a faster rate of degradation in solution since all the three drugs are susceptible to hydrolysis. By and large, these challenges could adversely impact the safety, efficacy, performance and stability of the drug product.

1.3.2.2 Drug Substance and Excipient Interaction

In multi-component systems such as fixed dose combination (FDC) drug formulations, the presence of drug substances and several excipients within the same formulation enhances contact among the different components and increase the propensity for interactions, which could lead to incompatibility. Primarily, scientific evaluation of the chemistry and physical properties of formulation components provides the most efficient tool in selecting compatible formulation components to prevent

incompatibility. In addition, drug-drug and drug-excipient compatibility testing, another tool for selecting compatible formulation components can reveal potential problems which otherwise may not be predicted during initial physicochemical evaluation.[42, 43] For example, the presence of unknown residues (impurities) in drug substances and excipients may cause interactions that may be detected by compatibility testing but otherwise couldn't have been predicted by basic structure-related scientific evaluation. Consequently, formulation development requires systematic solid-state or liquid-state characterization during preformulation to allow specifications to be set for the formulation components in line with International Conference on Harmonization (ICH) tripartite guideline Q6A "Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances".[35] The goal is to ensure the safety, efficacy, performance, and stability of the drug product.

Drug-drug, drug-excipient, or excipient-excipient interactions could impact material properties including hydration, dehydration, crystal growth, dispersibility, dissolution, powder flow, physical appearance of the dosage form and chemical degradation.[44, 45] Consequently, the drug substance and excipients are characterized for melting temperature, solubility, pKa, particle size, particle morphology, polymorphism, and hydration state.[46, 47]

Residual moisture can compromise stability of the formulation by facilitating molecular mobility and reactivity. However, in the solid state, the bipyramidal lamivudine anhydrous did not transform into acicular lamivudine hydrate when exposed up to 95 % relative humidity.[33] A similar observation has been made for nevirapine anhydrous in the solid state. The temperature and time for the drug-excipient study are

also critical as they influence the rate and extent of reactivity of the components. In addition, roller compaction and milling processes could induce dehydration or polymorphic phase conversions. This could mean that when hygroscopic excipients mixed with these drugs absorb atmospheric moisture, the minimal water absorbed may not cause the drugs to become hydrates.

Lamivudine, a primary amine, may undergo Maillard reaction. The Maillard reaction occurs between primary or secondary amines and the carbonyl groups on reducing carbohydrates.[48] The implication is that bulk sweeteners such as maltose which are reducing carbohydrates cannot be used to formulate lamivudine reconstitutable suspension. To date, no interactions have been reported between lamivudine, nevirapine and zidovudine in FDC formulations. Interestingly, acesulfame potassium, employed as sweetening agent in AZT/3TC/Nev tablets reacted with AZT to form 1-[5-hydroxymethyl-4-(5-methyl-2,3-dihydro-[1,2,3]triazole-1-yl)-tetrahydrofuran-2-yl]-5-methyl-1 H-pyrimidine-2,4-(1H,3H)dione.[49] Hence, drug-excipient interaction studies would be performed to determine the compatibility of the formulation components.

1.3.2.3 Solid State Characterization

Powder X-ray diffractometry, Fourier transform infrared spectroscopy, differential scanning calorimetry and thermogravimetry are some analytical techniques used for solid state characterization of drug-excipient interaction studies and stability testing of the formulation.[50-54] These analytical techniques are used to characterize starting materials in order to determine the polymorphic or hydration form as well as determine whether there are any solid state transformations due to processing. The

techniques are also used to rule out drug-excipient incompatibility or for excipients screening. The impact of the interaction on compatibility and stability could be evaluated by potency and dissolution testing using HPLC.

1.3.2.3.1 Powder X-ray Diffraction (PXRD)

The different crystal forms of the drugs have different degrees of solubility and this could result in varying dissolution rates with unpredictable bioavailability profiles. This requires the characterization of the drug substances and the drug product to be able to relate the dissolution and bioavailability to the physical form of the drug. Hence, the solid-state characterization techniques employed should be applicable to the analysis of the drugs in pure form and in the presence of other formulation components. That is, the method(s) employed should have the selectivity to identify the crystal form in a mixture and the sensitivity to detect varying levels of the drug. Powder X-ray diffractometry (PXRD) is a solid-state analytical technique that meets these requirements.

Powder X-ray diffractometry provides diffraction patterns unique to the crystal structure of the molecule.
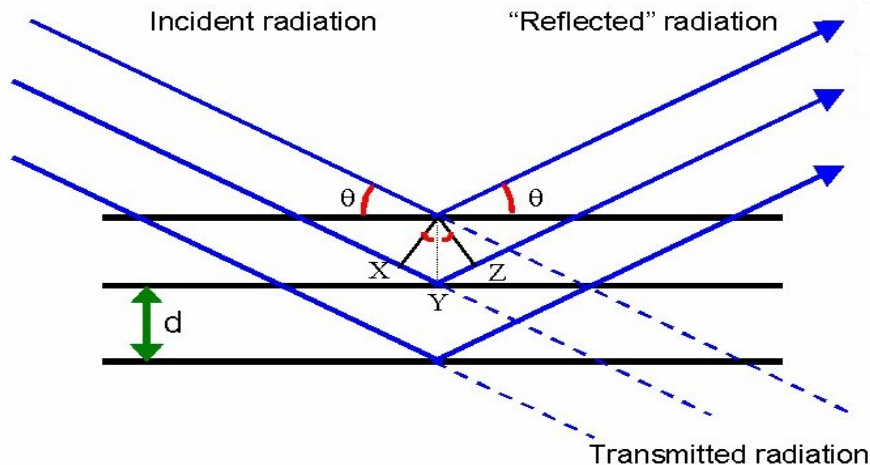
Fig 1.4 Diffraction of light by an optical grating

A crystal may be considered as being made of planes of unit cells (Fig 1.4) and the planes

diffract x-rays according to Bragg's law (Eq. 1.1) which is defined as

$$2d\sin\theta = n\lambda \qquad \text{(Eq. 1.1)}$$

where θ = Bragg angle

λ = wavelength

d = distance between adjacent planes

n = integer

When Bragg's law is fulfilled, the diffracted rays are in phase and there is constructive

interference. The size and shape of the unit cell determine the d-space while the position

of the various atoms in the cell and their atomic number determine the scattering power.

These properties define the powder diffraction pattern of a crystalline material.[55] Thus

peak position and peak intensity are used to characterize crystals. In a solid mixture, the

powder diffraction pattern of each crystalline phase is produced independently. This

implies that in a mixture of powders, the powder diffraction patterns of the individual

components will be summed up in the diffraction pattern obtained. The peak intensities

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and positions and any changes thereof may be used for substance identification, polymorphic phase identification and transformations, and to study interactions. For instance, PXRD may be used to analyze ribbons or granules produced by roller compaction to determine whether the mechanical stress applied by the rolls induced any phase changes.

1.3.2.3.2 Fourier Transform Infrared Spectroscopy

Molecular species may undergo transitions from one rotational or vibrational energy level to another by absorbing infrared (IR) radiation. Vibration motions are of two types: bending and stretching. Bending vibrations involve a change in the angle between two bonds whereas stretching vibrations involve changes in the interatomic distances along the axis of the bond between atoms.[56] In heteronuclear molecules, the vibrations or rotations results in fluctuating dipole moment. When the vibration frequency matches the frequency of the IR radiation, the IR radiation is absorbed and the amplitude of molecular vibration changes. Thus the molecule can absorb the IR radiation only if there is a net change in the dipole moment as it rotates or vibrates.[56] Hence, the IR absorption spectrum of a molecule is unique to the molecular structure and the environment in which it occurs, making it a useful tool for the identification of compounds.

The mid-IR spectral region of wavenumbers 4000 to 200 cm^{-1} is applied in Fourier-Transform infrared (FTIR) spectroscopy instruments in the identification of pharmaceutical compounds. The FTIR instruments provide (i) greater signal-to-noise ratio due to a large radiant power reaching the detector, (ii) high resolving power and wavelength reproducibility, and (iii) short data acquisition times since all signals reach

the detector simultaneously.[56] The IR spectrum consists of two major regions: the group frequency region and the fingerprint region. The group frequency region (3600 to 1250 cm^{-1}) represents the approximate frequency of the functional groups in a molecule. The fingerprint region (120 to 600 cm^{-1}) represents the general absorption maxima of the compound. By combining the data from the two regions, the general structure and functional groups in a compound can be identified. Frequently, the absorption spectrum of the unknown sample is compared to the spectra of the reference sample or a reference chart.

1.3.2.3.3   Differential Scanning Calorimetry (DSC)

Thermal analytical techniques provide yet another means of measuring the physical properties of pharmaceutical substances. Of particular interest in the analysis of small sample quantities is differential scanning calorimetry. Differential scanning calorimetry is the measurement of difference in energy input to a sample and a reference to keep them at the same temperature as a function of time and temperature. This may be used to obtain the melting temperature, glass transition temperature, and enthalpy of fusion or crystallization.[57] These parameters can contribute towards the determination of the drug substance's purity and the extent of solid-solid interaction.

At constant pressure, the energy exchanged between a system and its environment is the enthalpy change (dH) given by

$$\partial H = \partial U + P\partial V \qquad\qquad \text{(Eq. 1.2)}$$

For solids and liquids, the volume change is negligible and dV approximates to zero.

Thus $\qquad\qquad (\partial H) = (\partial U)p = (\partial q)p \qquad\qquad \text{(Eq. 1.3)}$

where *dU* is sum of internal energy of the system, *PdV* is change in amount of work and *dq* is heat flow. For temperature change from T_1 to T_2, the heat exchange entering or leaving the system is equal to enthalpy change:

$$\frac{dH}{dt} = C_p \frac{dT}{dt} + f(T,t)$$

(Eq. 1.4)

where H is enthalpy (J mol^{-1}), Cp is the heat capacity (JK^{-1} mol^{-1}), T is temperature (K) and t is time. Thus the total heat flow in the DSC is the sum of that related to the heat capacity and the kinetic response *f*(T,t) of the system.[56] The heat capacity is the amount of energy required to raise the temperature of a material by 1°C.[58] For a mixture of substances, the melting point of each substance and enthalpy of fusion are usually depressed due to the fact that melting process is a colligative property. Thus the enthalpy of fusion of the mixture may approximate the sum of the fusion enthalpies of the individual components. Some authors have used the differences between the experimental and predicted enthalpies of fusion (corrected for temperature) to describe the extent of interaction,[59] with the assumption that the observed differences are due to non-bonded interactions.

1.3.2.4 Preparation of Reconstitutable Suspension

Multiparticulate solids for reconstitution into oral suspension typically come as a finished formulation (powder or granules) requiring only the addition of water and agitation prior to dispensing. The solids may consists of the active pharmaceutical

ingredient(s); a suspending agent to keep the insoluble drug particles suspended; anticaking agent to prevent the granules from forming aggregates; preservatives to maintain the microbial integrity of the product; sweetening and flavoring agents to enhance the taste and flavor of the reconstituted product. The granules may be prepared by direct blending, wet granulation or dry granulation (roller compaction or slugging) processes depending on the bulk properties of the formulation components.

1.3.2.5 Dry Granulation Processing – Roller Compaction

Roller compaction is a dry granulation technology employed to improve flow characteristics, minimize segregation of components, increase particle size and bulk density of powders. The product contacting parts of the roller compactor are the feed hopper, feed screws (horizontal, vertical, tapered, angular), and the rolls. As the powder from the hopper is conveyed by the feed screw mechanism to the slip zone, the powder slips onto the roll surface (Fig. 1.5) where the material may experience deaeration and elastic deformation.[60] Rotation of the rolls transfers the material to the nip zone. In this region, the powder particles undergo rearrangement, densification and plastic deformation.[61] The material moves at the same speed as the rolls and as it passes through the region of maximum pressure between the rolls, it is compacted into a sheet called a ribbon or briquette. The nip angle is a function of both the powder properties such as compressibility and flow as well as the instrument parameters including feeding mechanism, roll dimensions and surface. As the compacted sheet enters the release zone, it may expand due to elastic recovery. The extent of elastic recovery is determined by the

material properties, deaeration, roll diameter and speed. Recent experiments to validate the Johanson[62] rolling theory of granular solids showed that the theory predicted well the effect of material properties on peak pressure and nip angle.[63] However, at higher roll speed, the model could not adequately predict due to air entrainment.

Milling of the sheets leads to the formation of more uniformly sized granules with improved flow properties.[64] Furthermore, the granules are composites of the individual powders constituting the powder blend and this minimizes or prevents segregation. Ribbons or the granules made from the ribbons may be characterized by porosity, particle size, tensile strength, density and solid fraction.[65, 66] Dry granulation by roller compaction is thus a useful process for making granules for reconstitution into suspension.
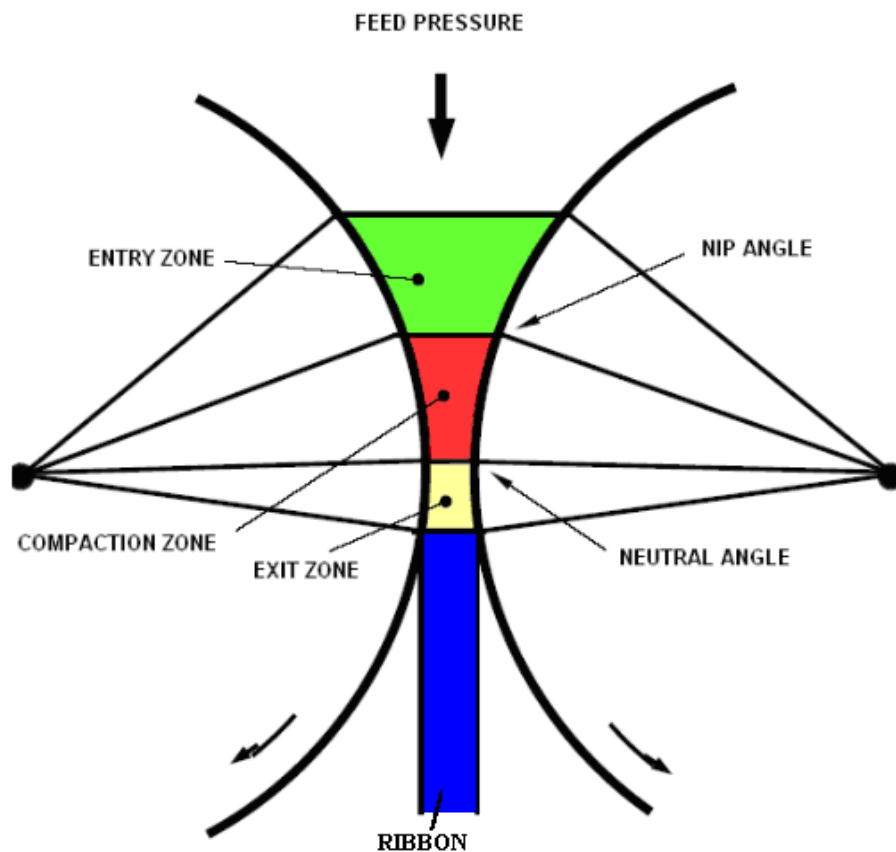
Fig 1.5 The zones in a roller compactor. Adapted from reference [67]

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The instrument parameters manipulated to produce variable ribbon properties are roll pressure, roll speed, feed screw speed, roll gap and dwell time. By modeling the friability of lactose ribbons formed under variable instrument parameters, it was established that the relative importance of these parameters in decreasing order was roll pressure, roll speed and horizontal screw speed.[68] Higher roll pressures produce dense compacts and larger granules due to stronger interparticle bonding during compaction. The roll speed is inversely related to the dwell time, the dwell time being the period required for particle rearrangement and bonding. By reducing the roll speed, the dwell time increases for stronger bonding to occur leading to stronger compacts. The roll gap determines the thickness of the compacted sheets. For a given roll pressure and speed, low feed rate forms thinner ribbons while high feed rates form thicker ribbons. Finally, the ratio of the feeding screw speed to roller speed determines the material throughput. The feeding screw transfers materials to the slip zone whereas the rollers transport the powder material from the slip zone to the nip zone. Therefore the ratio of roller speed to feed screw speed is critical in forming ribbons of uniform density. The flow of the granules and the distribution of the APIs in the granules may be assessed to establish whether the roller compaction process achieved the stated objectives.

1.3.2.6 Reconstitution of Granules for Coarse Suspension and Considerations

The final blend of the granules for reconstitution is usually dispersed in water to form a coarse suspension prior to dispensing to the patient. Dispersed particles in a

suspension, may experience diffusion via Brownian motion if the particle sizes are in the colloidal and molecular size ranges.

Brownian Motion: Brownian motion can affect the reconstitution and sedimentation rate of suspensions. Diffusion is dependent on Brownian motion, and diffusion coefficient has an inverse relationship with the radius of the particle. Thus, Brownian motion and convective currents can cause small particles (less than 0.5 μm) to remain dispersed continuously. The Einstein equation (Eq. 1.5) for translational Brownian motion [28] is given as

$$x = \sqrt{2Dt} \qquad \text{Eq. 1.5}$$

where x is the mean displacement in direction x in time t and D is the diffusion coefficient. The diffusion coefficient, D (cm^2/s) given by the Stokes-Einstein equation (Eq. 1.6) is a measure of the mobility of a molecule or particle in a liquid.[28]

$$D = \frac{RT}{6\pi\eta r N} \qquad \text{Eq. 1.6}$$

R is gas constant, T is absolute temperature (K), r is the radius of a spherical particle (cm), N is the Avogadro's number, and η is the viscosity of the medium (Poise). As the particle size becomes greater than 0.5 μm, as in coarse suspensions, Brownian motion is no longer able to keep the particles suspended and the particles sediment to obey Stokes' law (Eq. 1.7).

Sedimentation Velocity: Stokes' law[28] describes the velocity of sedimentation of spherical particles in dilute suspensions as

$$\upsilon = \frac{\partial^2 (\rho_s - \rho_o)g}{18\eta}$$

(Eq. 1.7)

υ = velocity (cm/sec)

∂ = diameter of particles (cm)

η = viscosity of dispersion medium (Poise)

ρ_s = density of dispersed phase (g/cm^3)

ρ_o = density of dispersion medium (g/cm^3)

g = acceleration due to gravity (cm/sec^2)

Stokes' law assumes that there are no interactions between the dispersed particles, there are no interactions between the dispersion medium and the dispersed particles, and the particles are spherical. By reducing the sedimentation velocity, the dispersed API particles can remain suspended long enough for uniform doses to be dispensed. Hence small particle size, high viscosity of dispersion medium and minimum difference in densities between the dispersed and dispersion phases reduce the sedimentation velocity. Most dispersed particles in pharmaceutical suspensions are anything but spherical. To account for the variable size and shapes of the API particles, the velocity may be adjusted by a porosity term.

Finely dispersed particles can associate through particle-particle interaction via Van der Waals forces to form loose aggregates with entrapped dispersion medium called flocs. The flocs settle quickly but can be redispersed easily due to the weak interactions holding the particles together. By adjusting the viscosity of the suspension, the sedimentation volume, redispersibility and flow of the suspension will be suitable for everyday manipulation by patients.

Sedimentation Volume: The sedimentation volume of a suspension is defined by the relationship

$$F = \frac{V_u}{V_o}$$

(Eq. 1.8)

where *Vu* is the volume of sediment and *Vo* is the initial volume of suspension. As F approximates 1, sedimentation is minimum and the product appears elegant. An accurate approach to assess uniform drug dispersion is to sample and analyze the top layer of the suspension for potency. In addition, the rheological properties, chemical stability of the drug substance, and acceptable taste, odor and color form essential components of formulation design and development.

Rheology of Suspensions: The rheological properties of a suspension can facilitate suspending the drug particles for a period of time and assure the measurement of an accurate dose. The Stokes' law provides a tool for manipulating the viscosity. Added to this, the viscosity could influence the dissolution mechanisms for suspensions by impacting mass transport phenomena such as shear at the particle surface, hydrodynamic and diffusion boundary layer thickness.[69] The viscosity is expected to remain constant throughout the shelf-life of the product as changes may lead to physical instability and inaccuracies in the dispensed dose. The effect of formulation components on viscosity may also be determined as an aid to excipient selection.

Rotational rheometer may be used to determine the rheological properties of non-Newtonian fluids such as suspensions. Accurate measurements of shear rate and shear stress of non-Newtonian fluids can be made on a cone and plate geometry (Fig. 1.6) of a

rotational rheometer. At shear rates where laminar flow exists, the layers of the fluid move in concentric circles. The rheometer measures the torque required to rotate the cone as it causes the layers of the fluid to move. The cone is attached to a calibrated spring and as it rotates in the fluid, the resistance of the fluid to the movement (viscosity) causes a deflection of the spring which is displayed on a digital display. The physical variables obtained by direct measurement are the torque **M** (dyne-cm), the angular velocity ω of the cone (rad/sec), the cone angle θ (degrees) and the radius **r** (cm) of the cone.[70]
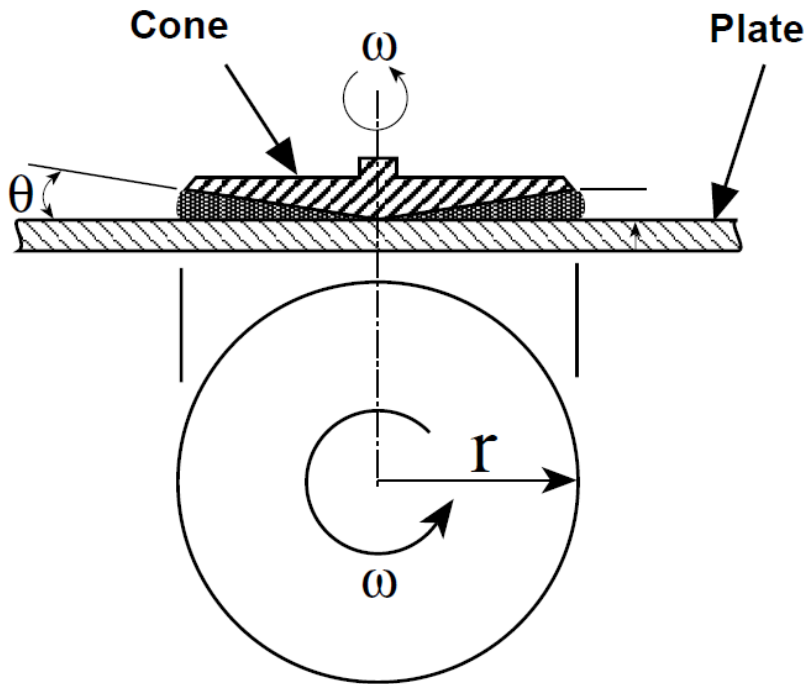
Fig 1.6 Geometry of a cone and plate rheometer.[70]

The relationship between shear rate (γ), shear stress (τ), viscosity (η) and the physical variables are as follows:

Shear rate (sec^{-1})　　　　　$\gamma = \dfrac{\omega}{\sin \theta}$　　　　　(Eq. 1.9)

Shear stress (dynes/cm^2) $\tau = \dfrac{M}{\left(\dfrac{2\pi r^3}{3}\right)}$ (Eq. 1.10)

Viscosity (poise) $\eta = \dfrac{\tau}{\gamma}$ (Eq. 1.11)

The viscosity of a system is dependent on temperature, pressure, shear rate, time, and history of the system. Most pharmaceutical suspensions intended for per oral administration are non-Newtonian exhibiting thixotropic flow behavior. Thixotropic systems display a reduction in viscosity with time when subjected to a constant shear rate.[71] When shear rate increasing at a constant rate is applied to a thixotropic system up to a point, and then reduced at the same rate, a plot of shear stress versus shear rate shows the downcurve displaced to the right of the upcurve (hysteresis) due to a decrease in viscosity (Fig. 1.7). The viscosity of the system decreases because the molecules in the system require time to reform the cohesive interactions which were disrupted due to shearing. Thixotropy is essential to pharmaceutical suspensions since the higher viscosity on storage could retard settling while the lower viscosity on agitation could facilitate pouring of a dose.
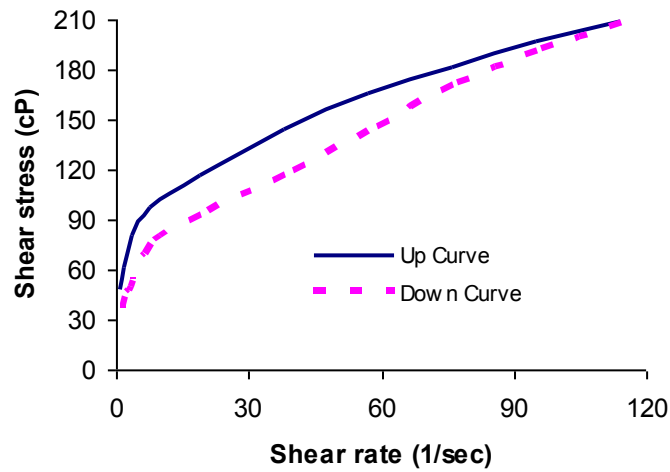
Fig. 1.7 Plot of shear rate vs. shear stress showing thixotropy.

Several models have been used to describe the flow properties of non-Newtonian systems. Two of these are the Casson and the Herschel-Bulkley models.[72, 73]

Casson model: $\quad\quad \sqrt{\tau} = \sqrt{\tau_o} + \sqrt{\eta \gamma}$ $\quad\quad$ (Eq. 1.12)

Herschel-Bulkley model: $\tau = \tau_o + k\gamma^n$ $\quad\quad$ (Eq. 1.13)

where γ = shear rate

$\quad$ τ = shear stress

$\quad$ η = viscosity

$\quad$ τ_o = yield stress

$\quad$ k = consistency index

$\quad$ n = flow index

The Casson model may be suitable for describing dispersions with high solid content. The yield stress and plastic viscosity obtained from the Casson model can be readily related to the desirable attributes of a suspension. The Herschel-Bulkley model may be used to describe the general flow of non-Newtonian liquids, particularly where the fluid exhibits a spectrum of rheological behavior under different conditions. The Herschel-Bulkley model defines the rheological properties of shear thinning systems as consistency index, **k>0** and the flow index, **0<n<1**; and **n > 1** corresponds to yield-dilatant behavior.[72, 73] The consistency index indicates flow behavior at low shear rate.

1.3.2.7 Chemical Stability of Granules and Suspensions

The chemical kinetics of drugs in a fixed-dose combination of granules for reconstitution into a suspension could be complex. The API in the granules (solid state) could have kinetic rates and pathways different from those of the drug in solution or the suspended drug particles. The mechanisms of degradation of drugs in pharmaceutical dosage forms usually follow zero-order, first-order or pseudo-first order kinetics. The linearized form of the zero-order rate equation (Eq. 1.14) is defined as

$$C_t = C_o - K_o t \qquad \text{(Eq. 1.14)}$$

where C_o and C_t are the drug concentrations at initial time and at time t. K_o is the zero-order rate constant, the quantity of drug degraded in a unit time. A plot of C_t vs. time yields a straight with the slope equal to K_o. The slope is negative since the concentration decreases with time. Shelf-life, the time for 10% of drug to degrade is

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$$t_{90} = \frac{0.1C_0}{K_o} \qquad \text{(Eq. 1.15)}$$

The reaction rate of zero-order reaction is independent of concentration but affected by other parameters such as pH.

In suspensions, as the dissolved drug degrades in solution, more of the suspended drug dissolves to replace the degraded amount thus keeping the amount of drug in solution constant. Under such conditions, the degradation kinetics appears to be independent of concentration of the drug and the reaction rate is described as apparent-zero-order kinetics.[74, 75] The zero-order rate (K_o) constant becomes equal to the product of the first-order rate constant (K) and the solubility of the drug (C_s) at the specific temperature (Eq. 1.16). When the drug reservoir is depleted, the suspension system behaves like a solution, following first-order kinetics.

$$C_t = C_o - (KC_s)t \qquad \text{(Eq. 1.16)}$$

For a first-order reaction, the reaction rate depends on the first power of concentration of one reactant. The linearized first-order reaction equation (Eq. 1.17) is

$$\ln C_t = \ln C_o - Kt \qquad \text{(Eq. 1.17)}$$

where K is the first-order rate constant. The shelf-life equation is

$$t_{90} = \frac{0.105}{K} \qquad \text{(Eq. 1.18)}$$

When the reaction rate depends on two reactants but one reactant is in excess and the apparent concentration does not change, the reaction is described as a pseudo-first-order reaction. The mathematical treatment is similar for a first-order reaction. The hydrolysis of drugs in aqueous solution is an example of pseudo-first order kinetics. This is due to the fact that in a solution, the solvent is in a large excess and thus the concentration of the solvent will not change significantly during the course of the reaction. The decomposition of drugs in solid dosage forms is frequently zero-order or first-order kinetics.

The ICH guidance document Q1A(R2) prescribes conditions for testing the stability of drug substances and drug products. The climatic conditions in Nigeria, the intended market, are hot and humid (zone IVA).[76, 77] Accelerated stability conditions are 40 ^{o}C / 75 % RH for 6 months and long-term stability conditions are 30 ^{o}C / 65 % RH for a minimum of 12 months.[78] The stability of reconstituted suspension may be evaluated for the duration of use,[75] while the powder is evaluated for the period stipulated for all dosage forms. Potency tests, dissolution tests and degradation products are usually assessed at well defined sampling time points during stability studies.

1.3.3 Bioavailability and Bioequivalence Studies

1.3.3.1 Bioavailability

The biological availability or bioavailability of a new per oral drug product is assessed to determine the release of the drug substance from the drug product and the absorption of the drug substance into the systemic circulation. The systemic exposure profile obtained is utilized to establish a therapeutic dosage regimen. Bioavailability is

defined in 21 CFR 320.1 as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action.[79] The bioavailability is obtained from the plasma drug concentration versus time profile. The pharmacokinetic (PK) parameters used to characterize bioavailability include (i) the area under the plasma concentration-time curve (AUC) which is a measure of the extent of drug absorption; (ii) the maximum plasma concentration (C_{max}) which is a measure of the rate of drug absorption; (iii) the time required to reach the maximum plasma concentration (t_{max}). Other PK parameters of interest are the elimination half-life and terminal disposition rate constant, which is often assumed to follow first order kinetics. The C_{max} and t_{max} are obtained directly from the plasma-concentration time profile.

Non-compartmental analysis: The non-compartmental (NCA) pharmacokinetic analysis is frequently used to derive the pharmacokinetic parameters in bioavailability studies. In NCA, the body is assumed to consist of a system within which there is an accessible pool for sampling and measuring drug concentration.[80] Measurement of the drug concentration in the accessible pool yields information for calculating the pharmacokinetic parameters. The NCA model presents several advantages for pharmacokinetic analysis. First, NCA requires fewer assumptions than that which is necessary with compartmental analysis. Secondly, numerous concentration points are obtained which reduce the uncertainty and make the measurements more robust.[81] Plotting this large number of point makes the AUC less prone to variability due to experimental errors.

The AUC from time zero to time t may be calculated by the trapezoidal rule (Eq. 1.19), while the AUC from time zero to infinity is obtained according to Eq. 1.20 where C_k is the last measurable concentration and λ is the terminal elimination rate constant.

$$AUC_{(0-t)} = \sum_{i=2}^{k} \left(\frac{C_{i-1} + C_i}{2} \right) (t_i - t_{i-1}) \qquad \text{(Eq. 1.19)}$$

$$AUC_{(0-\infty)} = AUC_{(0-t)} + C_k / \lambda \qquad \text{(Eq. 1.20)}$$

Relative bioavailability (f) is the ratio of the AUC of a drug from a test product to the AUC of the reference product in the same dosage form and given by the same route of administration.[82] When the doses of the test and reference products are the same,

$$f = \frac{AUC_{test}}{AUC_{reference}} \qquad \text{(Eq. 1.21)}$$

The relative bioavailability (Eq. 1.21) describes the fraction of the dose of the test product administered per orally that is absorbed and becomes systemically available for pharmacologic activity as compared to the reference product. When a generic (test) and an innovator (reference) show comparable AUC, the two products are accepted as bioequivalent.

1.3.3.2 Bioequivalence

Bioequivalence is defined in 21 CFR 320.1 as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of

drug action when administered at the same molar dose under similar conditions in an appropriately designed study.[79] Pharmaceutical alternative refers to products that are different dosage forms, different strengths or different crystal forms of the active moiety.[82] On the other hand, pharmaceutical equivalent refers to products containing the same active ingredient, in the same strength and dosage form and are intended for the same route of administration. The two bioequivalent products are fundamentally assumed to offer therapeutic equivalence in terms of formulation performance, efficacy and safety.[81]

The statistical criterion for bioequivalence as issued by the Food and Drug Administration requires that the 90 % confidence interval of the ratio of the average response for test to reference of AUC and C_{max} on a log-transformed scale should fall within 80 % to 125%.[79, 83] The log-transformation normalizes the distribution of the data: that is there is the assumption of log-normality for intersubject and intrasubject variabilities in the bioequivalence studies.[84] In other words, intrasubject variability is assumed to be the same for each subject and for each formulation. The log transformation further reduces the skewedness of the otherwise positively skewed data. The statistical test for the determination of the 90 % confidence interval is two one-sided t-tests. The two one-side t-test assesses whether (i) the test product is significantly less bioavailable compared to the reference listed product and (ii) the reference product is significantly less bioavailable compared to the test product. [81] Within the 80 % to 125% log-transformed interval, any observed differences between the test and reference products do not manifest in clinically significant effects. This is referred to as the 80/125 rule.
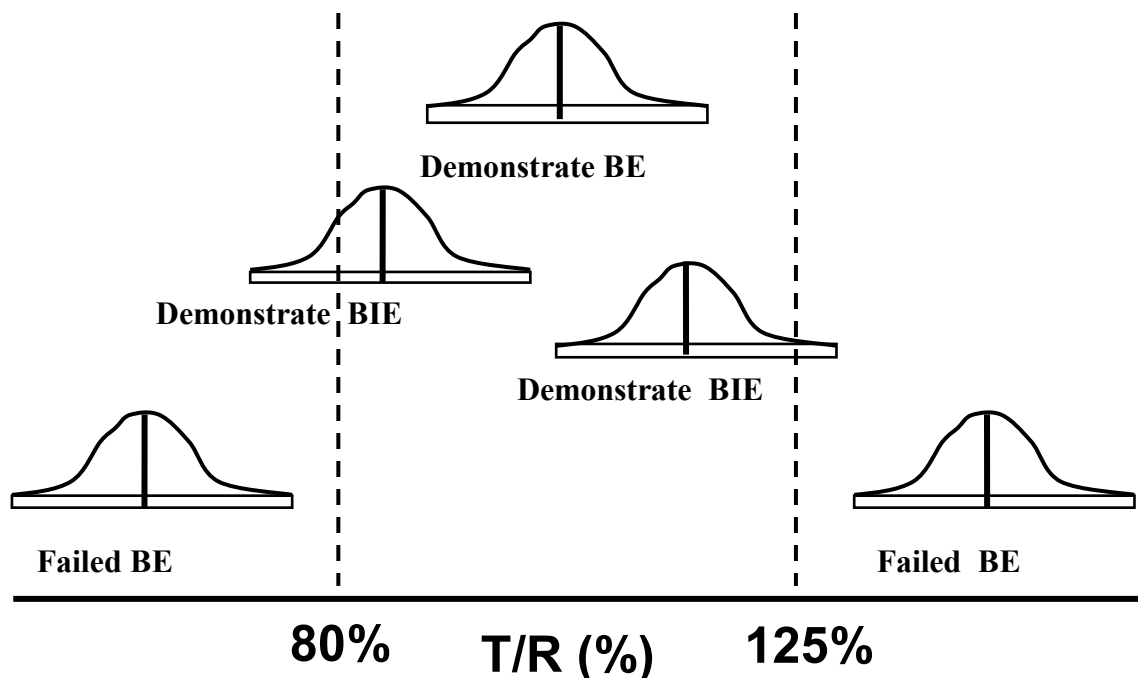
Fig. 1.8 Possible outcome of bioequivalence (BE) studies showing the 90 confidence intervals of log-transformed data (BIE – bioinequivalence, T – test, R – reference). Adapted from reference. [81]

The concept of bioinequivalence (Fig 1.8) is demonstrated when the ratio of the mean response for test/reference of AUC and C_{max} on a log-transformed scale lies within the 80 % to 125% band, but the range overlaps the confidence interval.[81, 84] The results of bioinequivalence may be due to poor performance of the formulation, excessive variability in product characteristics, sample size, or improper study design. Bioinequivalence may occur when the sample size is small, but the intrasubject variability is high resulting in a high total variance between the two study products.[85] In addition, if the plasma sampling intervals do not accurately capture the C_{max}, all the three pharmacokinetic parameters will be affected. The inability to capture the true C_{max} leads to decreased AUC. Finally, when the generic product shows a lot of variability in product performance attributes such as dissolution, it could lead to big differences or outliers

when the pharmacokinetic parameters for each individual is compared across products. When both the mean ratios and the range lie outside the confidence interval, the product is said to have failed bioequivalence. The outcome of the bioequivalence test determines whether the product may be approved for marketing and clinical use by the regulatory authorities.

1.3.3.3 Sample Size

Another rule, the 80/20 rule, is employed to calculate the power for sample size determination prior to the bioequivalence study. The 80/20 rule requires that the number of subjects involved in the trial should be large enough to provide at least 80 % chance of detecting a 20 % difference in the average bioavailability. The sample size per sequence (n_e) is given by Eq. 1.22 where α is the level of significance, β is the probability of type II error and CV is the quotient of the square root of mean square error divided by the mean AUC of the reference product.[84] The CV may be obtained from published literature.

$$n_e \geq 2[t(\alpha/2, 2n-2) + t(\beta, 2n-2)]^2 [CV/20]^2 \qquad \text{(Eq. 1.22)}$$

A type I error occurs if truly non-equivalent products are declared to be bioequivalent (risk to patient) and type II error occurs if true equivalent products are declared as not bioequivalent (manufacturer's risk). Generally, small samples sizes (20 to 30 subjects) are used in bioequivalence studies which limit the power of the study if the intrasubject variance is large. [86]

1.3.3.4 Design of Bioequivalence Studies

For the *in vivo* measurement of the active moiety in bioequivalence studies, the

parallel design, the crossover design and the balanced incomplete block designs are used.

Parallel design: In this design, each group of subjects receives only one drug instead of

being dosed with both drugs at different periods. Parallel designs are useful when (i) the

drugs are very toxic such that it is unethical to expose the subjects to both products; (ii)

the study medications have very long elimination half-lives; and (iii) the study population

consists of very ill subjects. The variations observed in bioequivalence studies are due to

formulation, intersubject and intrasubject variabilities.[84] However, parallel designs are

unable to identify and separate out intrasubject and intersubject variabilities. In addition,

parallel designs require a larger number of subjects to provide valid statistical inference.

Hence parallel designs are not commonly used for bioequivalence studies.

Crossover designs: These are most commonly used in bioequivalence studies. In this

design, each group of subjects receives more than one drug product at different time

periods. Each group of subjects called a block receives a different sequence of drug

products. The assumptions in a crossover design are that the absorption, distribution,

biotransformation, and elimination in a subject are less variable from one time to the next

time of study, compared to the variability in the product performance. The crossover

design presents the following advantages: (i) Each subject serves as his control and

allows the use of a smaller number of subjects to provide precise statistical inference; (ii)

intrasubject comparison of formulations can be obtained while the intersubject variability

between formulations is eliminated; and (iii) provides the best unbiased estimate of

variance between the study products. However, crossover designs are not suitable for bioequivalence studies in which clinical outcome is the study endpoint or for drugs with very long elimination half-life since the long half-life will prolong the washout period. Extended washout period could cause the study subjects to drop out. To prevent the interference of drug carryover to subsequent treatment periods in crossover designs, which may bias any inferences drawn, a suitable washout period is built into the design.

1.3.3.5 Carryover Effects and Washout Period in Crossover Design

Carryover effect refers to residual concentration of drug in the plasma persisting after the end of a dosing period and into the subsequent dosing period. The washout period is the time between two treatment periods where no drug is administered and the residual concentration from the dose administered in the prior period does not carryover to the subsequent period. The washout period should be long enough for the effect of the drug to wear off.[83] In the case of immediate release oral suspension, this may be a few hours or days. A more stringent requirement is for the washout out period to be at least five-and-half times the elimination half-life of the drug.[82] This ensures that at least 95.5 % of the parent compound is eliminated. For products with active metabolites that have long half-life, the metabolites should be taken into account in establishing the washout period. To verify the absence of first order carryover effects, the biological fluid such as plasma collected before dosing should be analyzed for drug or metabolite content as appropriate. A practical limitation to this approach may be the sensitivity of the analytical method employed for analyzing the biological fluid. When carryover effects are

determined to be absent, the carryover term can be eliminated from the statistical model defining the pharmacokinetic parameter.

1.3.3.6 Blocking and Randomization

Subject characteristics including age, gender and weight could contribute to the variance observed in a bioequivalence study. Such variability or noise tends to confound the comparison between the test and reference formulations. Blocking out the noise due to these factors serves to control their effect on the response variables.[87] The principle of blocking matches the input variables pertaining to the subjects such that the effect of these variables is eliminated from the measured variables of primary interest. To form randomized blocks, the heterogeneous cohort (Fig. 1.9a) is grouped into homogeneous units or blocks (Fig. 1.9b) to represent a combination of gender, age, and weight. The treatments are assigned randomly to the blocks formed (Fig. 1.9c). It is desirable to form equal treatment group sizes with comparable group attributes to facilitate data analysis and interpretation.[84, 87] The randomization of the subjects in blocks ensures a balanced design. Randomized blocks may be analyzed statistically by the confidence interval approach or the analysis of variance approach.
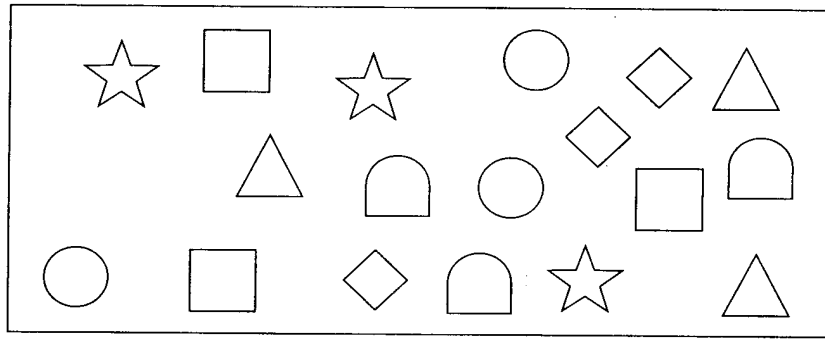
(a) Heterogeneous Experimental Units
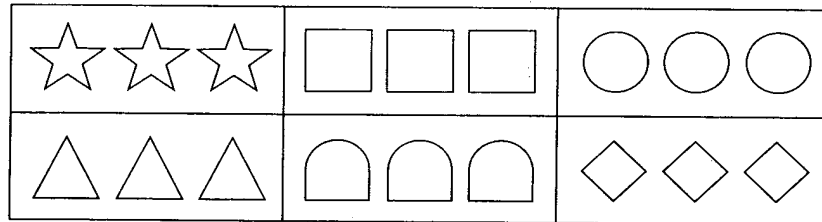
(b) Formation of Blocks
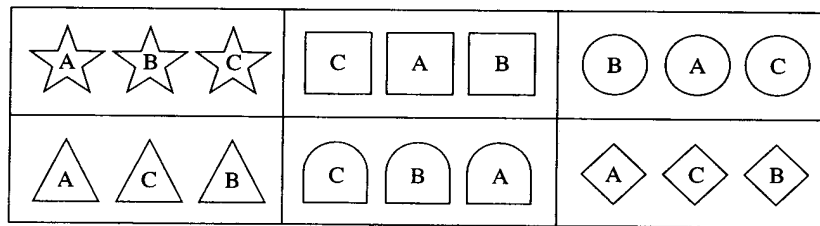
(c) Randomization of Treatments A, B, and C Within Each Block

Fig. 1.9 Steps in blocking and randomization [87]

Randomization and blinding are the two major design techniques for avoiding

possible bias in clinical studies. Randomization involves the probabilistic assignment of

subjects to treatment groups to remove the predictability of treatment assignment.[88] By

introducing the element of chance into the treatment assignment, there is less likelihood

of the subject characteristics influencing the outcome of the study. The stakeholders in a

clinical study are the subjects, investigators (physicians, nurses, pharmacists, laboratory

analysts, clinical coordinator) and the sponsor. These stakeholders can consciously or

unconsciously by commission or omission negatively bias the primary study endpoints.

45

To avoid this, treatments may be masked (blinded), if the dosage form lends itself to that. Blinding is performed to make the test and reference products look similar so that the subjects and the investigators are unable to differentiate between the test and reference (placebo) products. Comparing the different dosage forms, tablets are the easiest to blind. The techniques for blinding tablets include overencapsulation (Appendix 2), film coating, and manufacturing matching placebos. For liquid and powder products, blinding may require repackaging the formulations in identical packaging containers or applying identical labels. As part of the blinding process, the products should be adequately coded to facilitate unblinding, subsequent to the dosing and samples.

1.3.3.7 Statistical Methods for Average Bioequivalence

The data captured from the study should be evaluated for outliers before breaking the blind to ensure bias is not introduced into the analysis. The data exclusion criteria should be defined prior to the commencement of the study and justified. In a trial, all subjects enrolled and randomized into the planned treatment regimen groups are described as the intent to treat set.[88] However, often times, not all subjects in the intent to treat category follow the study protocol to the end and may drop out due to various reasons. The group of subjects who meet the entry criteria and follow the treatment protocol to completion form the protocol compliant set. The protocol compliant set typically receives the treatment, provides samples for measurements and does not violate the provisions of the protocol. Hence, full analysis set for the intent to treat subjects and per protocol analysis set for the protocol compliant subjects are compared to determine whether the inferences drawn from the two analysis sets are similar.[83, 88] Appropriate

statistical methods are applied to the two data sets. The confidence interval approach is considered an appropriate statistical method for assessing average bioequivalence. Added to this, the analysis of variance approach is applied to statistical inferences for the intersubject (carryover) effects and intrasubject (drug and period) effects.[84]

In a two-treatment two-sequence (2 X 2) randomized crossover design, the statistical model[84] for an observed pharmacokinetic response is given by

$$Y_{ijk} = \mu + S_{ik} + P_j + F_{jk} + Q_k + e_{ijk} \qquad \text{Eq. 1.23}$$

where Y_{ijk} = pharmacokinetic response of the ith subject in the kth sequence at the jth period

μ = overall mean

S_{ik} = random effect of the ith subject in kth sequence; i=1,2, ...n_k and k=1,2

P_j = fixed effect of the jth period; j=1,2

F_{jk} = direct fixed effect of formulation in the kth sequence which is administered at the jth period

Q_k = fixed effect of the sequence, k=1,2

e_{ijk} = the within subject random error in observing Y_{ijk}

The analysis of variance (ANOVA) of the formulation, period, sequence, and subject effects are determined prior to establishing bioequivalence (Table 1.3). The absence of any of these effects is necessary to show that the assessment of bioequivalence is purely due to the formulation differences between the two products. [86] The statistical

assumptions of the ANOVA are (i) randomization of the subjects to the sequences; (ii) the variance associated with the sequence and treatments should be homogeneous; (iii) the main effects model for subject, period, formulation, and sequence should be additive without any interactions; and (iv) the residuals of the model should be normally distributed and independent.[86]

Table 1.3. Analysis of variance table for 2 x 2 crossover design[84]

Source of variation	df	SS	MS=SS/df	E(MS)	F
Intersubjects					
Sequence	1	SS_{carry}	SS_{carry}	$\dfrac{2n_1 n_2}{n_1 + n_2}\left(C_T - C_R\right)^2 + 2\sigma_s^2 + \sigma_e^2$	$F_c = MS_{carry}/MS_{inter}$
Residuals	$n_1 + n_2 - 2$	SS_{inter}	$SS_{inter}/n_1 + n_2 - 2$	$2\sigma_s^2 + \sigma_e^2$	$F_v = MS_{inter}/MS_{intra}$
Intrasubjects					
Formulation	1	$SS_{formulation}$	$SS_{formulation}$	$\dfrac{2n_1 n_2}{n_1 + n_2}\left[\left(F_T - F_R\right) + \dfrac{C_R - C_T}{2}\right]^2 + \sigma_e^2$	$F_d = MS_{drug}/MS_{intra}$
Period	1	SS_{period}	SS_{period}	$\dfrac{2n_1 n_2}{n_1 + n_2}\left(P_1 - P_2\right) + \sigma_e^2$	$F_p = MS_{period}/MS_{intra}$
Residuals	$n_1 + n_2 - 2$	SS_{intra}	$SS_{intra}/n_1 + n_2 - 2$	σ_e^2	
Total	$2(n_1 + n_2) - 1$				

Footnotes: F_d is valid if $C_R = C_T$, n is the number of subjects in a treatment group, σ_e^2 is intrasubject variability, σ_s^2 is intersubject variability

1.3.3.8 Principles of Good Clinical Practice

Good Clinical Practice (GCP): It provides quality standards for ethical and scientific means of conducting clinical studies in human subjects. To ensure the safety, rights and well-being of human subjects, clinical trials should be performed according to the ethical principles emanating from the Helsinki Declaration.[89] In this regard, the study protocol should comply with all regulatory requirements including approval by a competent Institutional Review Board (IRB) or Ethics Committee (IEC). Generally, the benefits of a clinical trial should outweigh the associated risks.[90] The goals of clinical trial may be therapeutic or non-therapeutic trial such as pharmacokinetic or safety evaluation. The components of the ethical principles include subject recruitment, consent, and safety.

Recruitment of Subjects: The recruitment of subjects should consider the rights of study participants and steps should be taken to protect them from unnecessary risk. Subjects should be educated about their rights and responsibilities to ensure that both the subjects and the sponsor achieve the best possible outcome of the study. Further, subjects should not be coerced or induced by excessive monetary rewards or any other form of compensation to participate. This prevents a situation where the economically challenged find themselves exposed repeatedly to experimental medicines. The payments to the subjects should be prorated so that in case subjects withdraw before completing the study, the compensation will be commensurate to the individual participation. In addition, subjects recruited for a study should include individuals representing the demographics of the region and the disease being studied.[90, 91] This ensures the data generated from the

study will be relevant to the target population in which the product is intended to be used. However, the pediatric population represents a vulnerable subgroup and therefore initial evaluation of pediatric medicines could be performed in the adult population to obtain an indication of the safety parameters.[91]

Informed Consent: This should be obtained from all the study participants. The requirements of the study should be communicated to the participants in a language that they can understand, including the written informed consent document. The adult participants capable of sound decision making should provide consent by personally signing and dating the written informed consent form.[90] Participants should be made aware of their rights to decline to participate or to withdraw from the study at any time without any punitive consequences.

Risks: Efforts should be made to minimize the risk and distress that participants are subjected to. To be able to adequately appreciate the intricacies and the risks associated with the study, all the investigators conducting the study should have suitable training and skills to perform the assigned tasks and avoid hazards. Procedures and practices employed in the trials should be designed to minimize risk.[91] For instance, indwelling catheters could be used in place of repeated venipunctures for drawing blood samples, where applicable. The number of venipunctures, the volume of blood draws and the frequency of sample collection should be clearly stated at the onset of the study. Hence, a qualified physician should be enrolled in the study to be responsible for medical

decisions including the management of any adverse drug effects the subjects may experience.

Confidentiality: The records of all participants should be managed such that individual identities are not disclosed. This information should not be shared with the other volunteers engaged in the study. In addition, the study investigators should not be privy to this information, unless their assignments require them to know the information. Furthermore, the participant identifying information may not be associated with any of the research instruments or any publications made from the study. Thus, the privacy of the subjects is paramount.

Regulatory Approval: As part of GCP, an Investigational New Drug Application (INDA) should be submitted to the FDA for approval prior to the administration of any investigational products to humans. Although various products containing lamivudine, zidovudine, and nevirapine have been approved by the FDA, the granules for reconstitution is a "new product" without a prior marketing authorization and thus subject to INDA (21 CRF 312.40). To comply with this requirement, Form FDA 1571 should be completed and submitted to the FDA for approval. In addition, regulatory submission and approval is required by the National Agency for Food and Drug Administration and Control (NAFDAC), Nigeria, the country for the clinical site location.

1.3.3.9 Clinical Batch Manufacturing and Regulatory Considerations

In the clinical investigation of generic products, adequate safety and efficacy data from other existing clinical trials in humans can guide subsequent selection of the dose, the dosage form and the route of administration. To comply with the GCP standards[90], the test and reference products to be investigated in a clinical study should be manufactured, handled, and stored in accordance with Good Manufacturing Practice (GMP) as defined in the 21CFR 312. This responsibility rests with the sponsor of the study. Upon the successful development and complete characterization of the test product, the technology should be transferred to a GMP certified site for the manufacture, labeling and storage of the clinical batch. This implies that all the product components (active ingredients, excipients and packaging) should meet GMP standards. In view of the fact that a major cause of the failure of a generic product to achieve bioequivalence with the reference listed product is the variability in the test product, the need for complete characterization of the clinical batch cannot be overemphasized. Hence the manufacturing and the packaging batch records should be designed to capture the test procedures for product characterization. In addition, the certificate of analysis and the GMP certificate of compliance should be summarized in the investigator's brochure specific for the study. The investigator's brochure should also define the steps for reconciling the handling, use, and disposition of any unused products.

The GCP documentation requirements should meet both international and local regulatory requirements.[90] Some of the essential documents required for a clinical study are:

- Investigator's Brochure

- Protocol
- Case Report Form (CRF)
- Advertisement For Subject Recruitment
- Informed Consent Form
- Approval of Institutional Review Board (IRB)
- Regulatory Authority(ies) Authorization
- Medical/Laboratory/Technical Procedures /Tests
- Certificate(s) of Analysis of Investigational Product(s)
- Master Randomization List
- Monitoring Visit Reports

As previously discussed, the identity of the human subjects should not be disclosed in any clinical study reports. The subjects should report any events (such as accidents or illness unrelated to drugs used in the study or study protocol) or adverse drug reactions to the qualified physician engaged in the study for treatment and monitoring. Finally, the investigator should report any serious and unexpected adverse drug reactions to the IRB and the regulatory authorities.

Chapter 2. Development of Fixed-Dose Combination of Lamivudine, Zidovudine and Nevirapine, Formulated as Granules for Reconstitution

2.1 Introduction

Role of Physical-Chemical Properties: Oral liquid dosage forms facilitate the titration of doses to meet individual pediatric patient requirements. The choice between solution and suspension depends on factors such as the solubility of the drug, moisture content, crystal state of the actives and organoleptic properties.

Solubility: Lamivudine and zidovudine are soluble in water and could be formulated as solution. On the other hand, nevirapine has very low solubility and relating dose size to its solubility precludes nevirapine from being formulated as a solution. Formulating a suspension could overcome the low solubility challenge.

Moisture and Influence on Crystal State: The absence of water in dry powder formulations makes powders more stable than liquid formulations. Furthermore, dry powder formulation minimizes the probability of converting nevirapine anhydrous into nevirapine hemihydrate. Thus the formulation of granules for reconstitution into suspension could extend the shelf-life of the product. In addition, changes in the crystallinity of the API could be due to indirect incompatibility or to the introduction of moisture by one of the excipients. The objective of this step of the project was to

formulate a fixed-dose combination granule for reconstitution into suspension containing lamivudine/zidovudine/nevirapine 30 mg/60 mg/50 mg per 5 mL.

Segregation of the granule powder: The granules for reconstitution could be made by roller compaction process not just to avoid moisture but also to prevent the segregation of the drugs from the excipients. In a multi-drug formulation, segregation could be a real challenge and adequate steps should be taken during the manufacturing process to prevent segregation. By compacting the drugs into ribbons, dry granulation by roller compaction process lends itself to addressing this challenge. The probability of moisture causing unexpected interaction or reaction is also avoided by the roller compaction process.

Organoleptic properties: Drug-drug, drug-excipient, and excipient-excipient incompatibility [which could be established by isothermal stress testing (IST)] could affect the organoleptic properties such as color. Solid-state characterization and solution-state analysis may be used to assess the absence of interactions using techniques such as IST. Color changes in the mixture after IST or changes in the viscosity of the suspending agent could be used as indicators of incompatibility in the dosage form.

The taste of per oral pediatric formulations is critical as children are particularly averse to bitter taste. To enhance the taste, bulk sweeteners such as xylitol may be used. Xylitol is a non-cariogenic natural constituent of fruits and vegetables with sweetness equal to that of sucrose. Xylitol is a non-reactive crystalline material, stable to heat and air but moderately hygroscopic.[101] Compared to equivalent concentration of sucrose, it has a reduced tendency to cap-lock, has lower water activity and a higher osmotic pressure which enhances product microbial stability and freshness.[92] In addition, the

xylitol product literature states that "a 40 %w/w xylitol solution passes the BP preservative efficacy test".[92] Hence xylitol was selected as the bulk sweetener.

Xylitol has low specific surface area and exhibits brittle fragmentation.[93] Therefore, when xylitol is used as diluent in tablet formulations, it results in the formation of tablets with weak crushing strength.[94] The weak compaction properties of xylitol may be desirable in the formulation of granules for reconstitution. The roller compacted ribbons which have relatively weak strength and weaker granule strength would form granules that could be easily dispersed in water to form a suspension. Thus the compaction properties of xylitol make it a potential candidate for formulating granules for reconstitution by roller compaction.

In addition, roller compaction and milling processes can cause significant particle size reduction resulting in increased surface area and faster dissolution rate as described by the Noyes-Whitney[95] equation (Eq 2.1) below:

$$\frac{dM}{dt} = \frac{DS}{h}\left(C_s - C\right)$$
Eq. 2.1

dM/dt is the mass rate of dissolution, D is the diffusion coefficient of the solute in solution, S is the surface area of the particle, h is the thickness of the diffusion layer, Cs is the solubility of the solid, and C is the concentration of the dissolved drug in the bulk fluid at time t. The increased dissolution rate may be particularly beneficial to the dissolution of nevirapine, a BCS class II compound with low aqueous solubility.

High Intensity Sweeteners: High intensity sweeteners are often used in low amounts to supplement bulk sweeteners in formulations and minimize the physical size of the dose as well as enhance the flavor of the formulation. Sucralose, neotame, and saccharin are

some of the sweeteners in FDA approved products (Fig 2.1). However, the choice of high intensity sweetener will depend on compatibility with the other formulation components.

Preservatives: Considering antimicrobials for oral liquid formulations, the parabens have been shown to exhibit synergistic antimicrobial activity in a wide pH range. Hence the combination of methylparaben and propylparaben is frequently used in oral suspensions to maintain the microbial integrity of formulations.

Hydration Tendency of Suspending Agent: Furthermore, the granules for reconstitution into suspension are reconstituted by simple hand agitation prior to dispensing. This requires that the suspending agent selected should hydrate rapidly when water is added to be effective at suspending the dispersed drug particles. Avicel RC 591 has been shown to be an effective suspending agent in reconstitutable suspension.[96] Considering the tendency of one component, xylitol, to adsorb moisture, the incorporation of an anti-caking agent such as Aerosil 200 colloidal silicon dioxide to prevent the formation of lumps in the granules could be essential. Aerosil 200 has high porosity and preferentially absorbs moisture to keep the granules dry and free and flowing.

Sucralose   **Neotame**   **Saccharin**
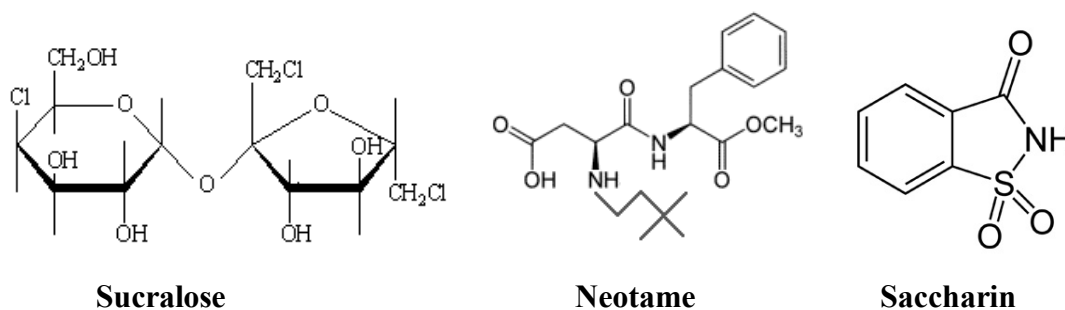
Fig. 2.1 Structures of high intensity sweeteners

Hence, it is hypothesized that fixed-dose combination of lamivudine, zidovudine and nevirapine formulated as granules for reconstitution can be developed using roller compaction process. Granulation by roller compaction process will eliminate the introduction of water into the formulation and thereby improve product stability. Furthermore, the formulated granules for reconstitution will be stable under ICH recommended stability testing conditions for tropical climate. The specific aims are (i) to develop FDC granules for reconstitution by roller compaction, and (ii) to characterize the formulation.

2.2 Experimental

2.2.1 Materials

Lamivudine, zidovudine and nevirapine reference standards (United States Pharmacopoeia, Rockville, MD); acetonitrile HPLC grade, ammonium acetate (Fisher Scientific, Hanover Park, IL); formulation components are listed in Table 2.1; all other reagents were of analytical grade.

2.2.2 Validation of Gradient HPLC method

2.2.2.1 Chromatographic Conditions for Gradient HPLC method

A gradient HPLC method was developed for the evaluation of the FDC granules for reconstitution. The chromatographic system consisted of Waters 2695 Separations module, 996 PDA detector, and Empower 2 chromatographic software (Waters Corporation, Milford, MA). The absorbance was monitored at 265 nm. The mobile phase was made of 50 mM ammonium acetate buffer pH 6.8 and acetonitrile. The mobile phase

was filtered and degassed before use. The gradient was from 5 to 55 % v/v acetonitrile over 26 min at a flow rate 1 ml/min. Column used was Luna-C18 5μm 250 x 4.6 mm column (Phenomenex, Torrance, CA).

2.2.2.2 Preparation of Standard Solutions

Solutions containing 10 mg/mL of the lamivudine, zidovudine, cytosine and thymine were prepared in Millipore water. 10 mg/mL stock solutions of nevirapine, methylparaben and propylparaben were prepared in acetonitrile/water 40 % v/v. Further dilutions were made using 30 % v/v acetonitrile/water solution. Quality control samples were similarly prepared.

2.2.2.3 Linearity and Range

To establish the linearity and the range, six concentrations in triplicate were used to construct the calibration curve. The range was calculated based on the estimated maximum amount of drug dissolved in 900 mL of dissolution medium. Linearity was examined over the following concentration ranges: lamivudine (20-80μg/mL), zidovudine (20-100μg/mL), nevirapine (20-80μg/mL), methylparaben (2-20μg/mL), propylparaben (0.8-10μg/mL), cytosine (0.8-10μg/mL) and thymine (0.8-10μg/mL). The six analyte solutions were mixed together in the same vials and analyzed. The data was fitted using the least sum of squares method.

2.2.2.4 Precision and Accuracy

Precision and accuracy were determined as 20%, 50% and 100% of the calibration curve range. Triplicate samples were analyzed at each level. Precision was computed as percent relative standard deviation (RSD) and accuracy was computed as percent relative recovery. Inter-day reproducibility was examined by repeating the procedure using independent samples on three separate days. The data was analyzed using two-way ANOVA.

Table 2.1. Components of Reconstitutable Granule Formulation

Ingredients	CAS Registry #	Manufacturer	Category	Percent w/w
Zidovudine USP	30516-87-1	Dayang Chemical	Active	2.58
Lamivudine USP	134678-17-4	Dayang Chemical	Active	1.29
Nevirapine anhydrous USP	129618-40-2	Dayang Chemical	Active	2.15
Avicel RC 591 (microcrystalline cellulose/ sodium carboxymethylcellulose)	9004-34-6 9004-32-4	FMC Biopolymer	Suspending agent	4.30
Sucralose NF	56038-13-2	Tate & Lyle	Sweetening agent	0.43
Xylitol NF	87-99-0	Roquette America Inc.	Sweetening agent	86.00
Aerosil 200 (Colloidal Silicon Dioxide USP)	7631-86-9	Degussa	Anticaking agent	1.98
Magnesium stearate USP	557-04-0	Spectrum Chemical	Lubricant	0.90
Methylparaben NF	99-76-3	Spectrum Chemical	Preservative	0.32
Propylparaben NF	94-13-3	Spectrum Chemical	Preservative	0.03

2.2.3 Formulation of Reconstitutable Granule Formulation

The components of the granules for reconstitution are listed in table 2.1. In view of the fact that the developed formulation will be tested in humans for bioavailability and bioequivalence, only excipients listed in the Food and Drug Administration's "Inactive Ingredients in Approved Products Database" were selected to avoid safety issues. The GMP batch for the clinical trials was manufactured at a GMP contract manufacturing site using GMP grades of the same formulation and packaging components. In addition, the formulation components were selected after preformulation studies using IST, solid state characterization techniques, and other screening methods described in the sections below.

2.2.3.1 Isothermal Stress Testing

Drug-drug and drug-excipient interactions were evaluated using isothermal stress testing (IST). Nevirapine, lamivudine and zidovudine powders were prepared singly and as mixtures in fixed ratios with 5 %w/w of water and stored in closed vials at 50°C for three weeks. [42] Similarly, identical dry blends were processed and stored at 50°C and at room temperature and used as controls. At the end of the storage period, the samples were visually inspected for organoleptic change. Powder x-ray diffraction (PXRD) characterization of the samples was performed to establish no changes in crystal form of the drugs. The validated HPLC-UV method was used to evaluate chemical changes.

2.2.3.2 Powder X-Ray Diffraction

Powder X-Ray Diffraction (PXRD) analysis was carried out to identify the polymorphic forms of the bulk drug substances. Isothermally stressed drug-drug mixtures

were also analyzed by PXRD. The powder samples were loaded unto the sample holder and tapped with the block gently to produce a smooth surface that flushes with the rim of the sample holder (back loading technique). The X'Pert Pro MPD diffracting system (PANalytical BV, Almelo, Holland) was used at the following settings: generator setting 40mA, 45 kV; anode Cu K-α1, K-α2, scan step size 0.017 degree 2θ, scan step time 75s, scan range from 3-50 degree 2θ.

2.2.3.3 Fourier Transform Infrared Spectroscopy

The Fourier Transform IR spectrometer was used to collect the spectra of the bulk APIs for identification purposes. Isothermally stressed drug-drug mixtures were also analyzed by FT-IR. NICOLET 380 FT-IR instrument and Omnic software (Thermo Electron Corporation, Madison, WI) were used for data collection. The scan range was 500-4000cm^{-1}, the data was averaged over 32 scans at a sensitivity of 50 and resolution of 4cm^{-1}.

2.2.3.4 Liquid State Characterization and Screening of Excipients

To choose between three high intensity sweeteners, (saccharin, neotame, and sucralose), 0.2% w/w of the sweeteners was added to different beakers containing 2% w/w Avicel RC591 (microcrystalline cellulose/sodium carboxymethylcellulose) in deionized water. The rheological properties of the suspension were measured on Brookfield LV DV-III Ultra rheometer using cone and plate geometry with spindle CP51 and electronic gap of 500 nm. The rheological properties were measured at a shear rate of

1 to 96 s^{-1}. The data was captured and analyzed using the Rheocalc software. The pH of the suspension was measured using a pH meter.

2.2.4 Manufacturing Process and Formulation Optimization

2.2.4.1 Manufacturing Process

Initial attempts to assess the compaction properties of binary mixtures of the three drugs and magnesium stearate formed weak ribbons that fragmented easily. The drugs constituted 99 % by weight fraction of the blend. Therefore, if the drugs had good compaction properties, the ribbons formed would have been strong. That the ribbons formed were weak was an indication that the drugs had poor compactibility. Unfortunately, the determination of the specific causes of the poor compactibility of the drugs was beyond the scope of this project. On the other hand, the binary mixture of xylitol and magnesium stearate formed relatively strong ribbons. Since xylitol constitutes greater than 80 % w/w of the final blend, the strength of the ribbons formed will be expected to parallel that of xylitol.

The granules were manufactured by screening through a 20 mesh screen, weighed quantities of lamivudine, zidovudine, nevirapine, xylitol, and magnesium stearate. The screened components were blended for 5 min utilizing a twin-shell V-blender (Patterson-Kelley Co. Stroudsburg, PA). The blend was roller compacted on a Fitzpatrick IR 220 chilsonator (The Fitzpatrick Co., Elmhurst, IL) followed by milling the ribbons on Fitz-mill communitor (The Fitzpatrick Co., Elmhurst, IL) at 1500 rpm screen size. The roller compaction parameters are described under the section for roller compaction optimization below. The granules produced were blended in a V-blender together with the other

screened formulation components for 5 min. This final blend was packaged into amber plastic bottles for storage before further characterization.

2.2.4.2 Optimization of Roller Compaction Parameters

Based on literature, the initial roll pressure ranged 145 to 2900 psi. Analysis of the ribbon strength on a beam bending test was used to reduce the limits of the roll pressure to 1300 to 1800 psi. The ratio of the horizontal feed screw speed to the roll speed was similarly selected. A 2 x 3 factorial design (Table 2.2) with two factors [roll pressure and the ratio of the horizontal feed screw speed to the roll speed] at three levels [low, medium, high] was used to optimize the roller compaction parameters. It is predicted that the roll pressure and the ratio of the horizontal feed screw speed to the roll speed could influence the break force of the ribbons formed.[68] The response variables were (a) the force required to break the ribbons, and (b) the fraction of granules less than 250 microns. Xylitol, the major component of the granules is brittle and thus the break force was more appropriate than indentation testing that is more suitable for testing the tensile strength of plastic deforming materials. The mean diameter of the suspending agent was 250 microns and therefore a similar mean particle diameter was applied to the granules made from the roller compacted ribbons. The experimental design and the statistical analysis of the response were performed using JMP statistical software, version 4 (SAS Institute Inc, Cary, NC).

Break Force: The three point bend rig method was used to measure the average force required to break the ribbons. The measurements were made on a TAXTplus texture

analyzer and Texture exponent 32 software was used for data acquisition (Texture Technologies Corp, Scarsdale, NY).

Particle Size Distribution: Dry sieve analysis was performed to determine the particle size distribution. The details of the method are described in section 2.2.5.1.

Table 2.2 Design of experiment for optimizing the roller compaction parameters. Vertical screw speed = 100 rpm, roller gap = 2 mm, RS = roll speed, HS = horizontal screw speed.

Run	Roll Pressure (psi)	HS/RS
A	1300	15/3
B	1300	15/4
C	1300	20/5
D	1550	15/3
E	1550	15/4
F	1550	20/5
G	1800	15/3
H	1800	15/4
K	1800	20/5

2.2.4.3 Suspension Formulation Optimization

A replicated 3^2 factorial design (Table 2.3) was utilized to optimize the levels of the suspending agent (Avicel RC 591) and the anticaking agent (Aerosil 200), each at three coded levels (-1, 0, 1). The anticaking agent inhibits settling and flocculation whereas the suspending agent influences the viscosity and the sedimentation rate of the reconstituted suspension. Aerosil 200 has also been shown to have effect on viscosity.

The hypothesis was that the suspending agent can suspend the drug particles long enough for a uniform dose to be measured. The response variables were (a) Viscosity: High at high level of Avicel RC 591 and (b) Sedimentation volume (F): F is closer to 1 at high level of Avicel RC 591. Least squares regression model was used to fit the viscosity data and the variance in the mean values were tested with ANOVA ($p < 0.05$), utilizing using JMP statistical software. It was also hypothesized that the presence of Aerosil 200 in the suspension could prevent the caking of the drug particles. The response variable was the absence of cakes in the suspension.

Table 2.3 Randomized 3^2 full factorial design matrix for formulation optimization. Avicel RC 591 [-1=1.0%, 0=2.0%, 1=3.0%], Aerosil 200 [-1=1.00%, 0=1.25%, 1=1.50%], all levels represent %w/v. The levels of the other formulation components were fixed as indicated in table 2.1.

Formulation #	Avicel RC 591	Aerosil
1	-1	-1
2	1	1
3	1	0
4	-1	1
5	0	-1
6	0	0
7	1	-1
8	0	1
9	-1	0

2.2.5 Characterization of Development Batch of Granules

Based on the results of the suspension optimization, the granules were manufactured and characterized for the relevant product attributes. The stability of both the granules and the reconstituted suspension including dissolution and potency are discussed under chapter 3.

Moisture Content: 5 g of sample was weighed and dried at 80 ^{o}C to constant weight. The loss in weight was computed as a percentage of the initial weight.

Density: A weighed quantity of granules was placed in a graduated cylinder and repeatedly tapped 200 times. The initial and final volumes of the powder in the cylinder were recorded. The bulk density, tapped density, and the compressibility index were calculated. This information was useful in selecting the bottle for packaging.

Particle Size Distribution: A quantity of granules was placed on a nest of standard USP sieves arranged in decreasing size down on Performer III sieve shaker (Cole-Parmer, Vernon Hills, IL). The sieves were agitated for 5 min and the weight of granules on each sieve determined. The geometric mean diameter and geometric standard deviation were computed.

Content Uniformity: The quantity (2.33 g) of granules equivalent to 5mL dose was weighed into a 200 mL flask. 100 mL of 40 % v/v acetonitrile/water solution was added

and sonicated for 30 min. 1 mL of this solution was diluted with 5 mL of 30 % v/v

acetonitrile/water. The solution was filtered through 0.45 micron nylon filter and

analyzed on HPLC in triplicate. The potency and the antimicrobial preservative content

were determined from the peak area response from the chromatogram. Six separate

measurements were made and the following computed:

(a) Amount of drug (mg)/ 5 ml

(b) Percent Potency (%) = Amount recovered x 100 % / Theoretical amount

(c) Acceptance Value $\left| M - \overline{X} \right| + ks$ Eq. 2.2

where $\overline{X}$ = mean content, M = reference value, k=acceptability constant, and s =standard

deviation. Measured volumes (5 mL) of the reference products (Epivir solution

(lamivudine), Retrovir syrup (zidovudine), and Viramune suspension (nevirapine) were

similarly analyzed.

Dissolution: Weighed sample (2.33 g) of granules was added to each of six dissolution

vessels containing deaerated 0.01N HCl at 37 $^\circ$C. The USP apparatus II (paddle method)

was run at 100 rpm for 60 minutes. Samples were collected at 0, 5, 10, 15, 30, 45 and 60

min, filtered through 0.45 micron nylon filter and analyzed on HPLC.

2.2.6 Clinical Batch Manufacturing and Characterization

Identification and Testing of Excipient: The excipients were identified using Fourier

Transform Infrared (FTIR) spectroscopy and chemical tests at a contract research

analytical testing facility. The FTIR spectra were collected using NICOLET 380 FT-IR

instrument and Omnic software (Thermo Electron Corporation, Madison, WI). The scan

range was 500-4000cm^{-1}, the data was averaged over 32 scans at a sensitivity of 50 and resolution of 4cm^{-1}.

Manufacturing and Packaging: To manufacture the clinical batch of granules for reconstitution under current Good Manufacturing Practices (cGMP), the lab developed technology was transferred to a GMP contract research manufacturing facility. The manufacturing and packaging batch records were transferred together with the product specifications. The batch was scaled-up to twice size of the laboratory batch. Following successful manufacturing of the demonstration batch, the GMP (clinical) batch was manufactured using similar parameters. The clinical batch was packaged into amber plastic bottles and appropriately labeled and stored. The batch was sampled and characterized for potency and dissolution. The batch was also evaluated for stability under long term-storage conditions.

2.3 Results and Discussions

2.3.1 HPLC method validation

The ICH guideline "Q2 (R1) Validation of Analytical Procedures: Text and Methodology" recommends validating analytical methods prior to employing the method for routine analysis. The HPLC method developed was validated according to this guidance document. The chromatogram (Fig. 2.2) shows well resolved peaks indicating the specificity of the method. The calibration curves were fitted by the least squares regression model. The intermediate precision was evaluated by means of two-way

analysis of variance (ANOVA). A p-value> 0.05 and Fcal < Fcrit indicates non

significant differences in the results between days or between levels.
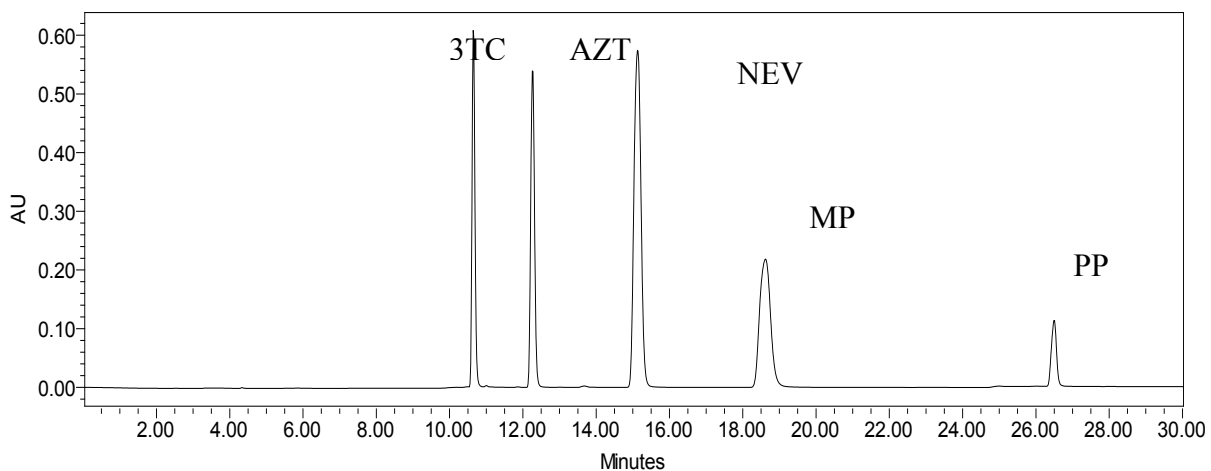
Fig 2.2 Chromatogram of granules for reconstitution showing resolved peaks for

lamivudine (3TC), zidovudine (AZT), nevirapine (NEV), methylparaben (MP), and

Propylparaben (PP).

All the analytes showed strong linear relationship between analyte concentration

and the peak area within the range evaluated (Table 2.4). The intraday accuracy for the

drugs ranged 99.15% to 100.12% while the precision ranged 0.17% to 1.32%. The

interday accuracy for the drugs was 99.02% to 100.20% with intermediate precision of

0.97% to 1.78%. The accuracy and precision for the preservatives were 99.10% to

103.46% and 1.49 to 3.35% respectively. The interday accuracy and intermediate

precision for the preservatives were 99.00% to 102.87% and 3.03% to 4.19%

respectively. The lower precision and accuracy of the preservatives compared to the

drugs was probably due to the lower concentration range tested. The ANOVA showed

that there were no significant differences ($p>0.05$ and Fcal < Fcrit) in accuracy or precision between the different concentrations examined within day or between days. Thus the validated HPLC method had the level of accuracy and precision required for analysis of the formulation samples.[97]

Table 2.4 HPLC-UV method validation. ANOVA (p > 0.05 and Fcal< Fcrit, for all samples)

Parameter	Lamivudine	Zidovudine	Nevirapine	Methylparaben	Propylparaben
Linear range (µg/mL)	20-80	20-100	20-80	2-20	0.8-10
R^2	0.9958±0.0050	0.9954±0.0032	0.9992±0.0014	0.9941±0.0037	0.9903±0.0064
Intraday					
Accuracy (%)	100.12	99.15	100.01	103.46	99.10
Repeatability (%)	0.17	0.77	1.32	3.35	1.49
Interday					
Accuracy (%)	99.02	99.81	100.20	102.87	99.00
Lower 95% CI	98.36	99.14	99.83	101.7	97.51
Upper 95% CI	99.69	100.47	100.56	104.05	100.48
Stdev	*1.76*	*1.76*	0.97	3.12	4.14
Intermediate precision (%)	1.78	1.76	0.97	3.03	4.19

2.3.2 Isothermal Stress Testing

Drug-drug interactions: Previous studies showed that lamivudine anhydrous was converted to the hydrate form in the presence of zidovudine and water at elevated temperature, although that did not affect the chemical stability of the drugs.[98] This was consistent with the fact that commercially available fixed-dose combination tablets containing lamivudine and zidovudine are stable under ICH stability storage conditions. This underscores the fact that the process for manufacturing the granules for reconstitution should be devoid of the addition of water to the product. HPLC analysis (Table 2.5) and solid state characterization using PXRD showed that there were no interactions between lamivudine and nevirapine or zidovudine and nevirapine.

Table 2.5 Recoveries of drugs from isothermally stressed samples. The physical blends of the drugs were stored under different conditions for 3 weeks.

		Drug Recovery (SD) %		
Sample #	Condition (3 weeks storage)	3TC	AZT	Nev
1	Room Temp	99.04 (0.20)	101.28(0.15)	94.70 (0.16)
2	50 $^{\circ}$C	96.24 (0.32)	100.11(0.31)	91.32 (0.27)
3	50 $^{\circ}$C/5% water	101.51(0.75)	98.87 (0.35)	97.75 (0.18)

Drug-excipient interactions: Wet drug-sucralose mixtures stored at 50°C showed brown discoloration but, samples stored at 40 $^{\circ}$C/75 % RH for three weeks did not produce any discoloration. Neotame-drug samples showed pale yellow discoloration at both temperatures. However, the recoveries of the drugs from drug-sucralose or drug-neotame mixtures were not affected. Stressed individual samples of neotame and sucralose showed similar discoloration. Neotame undergoes hydrolysis of the methyl ester moiety to form de-esterified neotame.[99] Sucralose (Fig. 2.1) also undergoes hydrolysis to form 4-chloro-4-deoxy-galactose (4-CG) and 1,6-dicholoro-1,6-dideoxyfructose (1,6-DCF).[100] Sucralose is listed as a component of a powder for oral suspension product in the Food and Drug Administration's "Inactive Ingredients in Approved Products Database", which indicates that the excipient is stable for use in the granules for reconstitution. Similarly, as at the end of January 2011, neotame was listed in the same database as a component of an oral spray. Saccharin, the other sweetener evaluated did not produce color changes in the samples. Thus all three high intensity sweeteners could potentially be used to formulate the granules for reconstitution. Furthermore, there were no observed color changes or significant degradation (less than 10%) of drugs recovered from the stressed mixtures of the drugs and the other formulation components (Table 2.6).

Table 2.6. Recoveries of 3TC/AZT/Nev mixture stressed with individual formulation components containing 5 % moisture stored at 50 $^{\circ}$C for 3 weeks

	Drug Recovery (SD) %		
Excipient	**3TC**	**AZT**	**Nev**
Avicel RC591	97.82 (0.18)	97.52 (0.46)	92.35 (1.21)
Xylitol	102.87(0.14)	102.61 (0.09)	94.69 (0.11)
Sucralose	101.00 (0.19)	100.63 (0.18)	95.15 (0.16)
Aerosil 200	99.59 (0.51)	98.77 (0.05)	94.96 (0.18)
Methylparaben	97.76 (0.30)	96.42 (0.29)	90.98 (0.28)
Propylparaben	97.95 (0.47)	98.62 (0.56)	90.35 (0.13)
Magnesium Stearate	98.63 (1.01)	97.98 (0.12)	99.63 (0.21)

2.3.3 Liquid State Characterization and Screening of Excipients

ANOVA of mean pH and viscosities of the suspensions containing high intensity sweeteners and Avicel RC 591 were significantly different, each with p-value<0.0001. Suspensions containing saccharin had acidic pH < 3 (Table 2.7), and Avicel RC 591, a water-dispersible hydrocolloid was flocculated reducing the apparent viscosity to approximately 1 cP at a shear rate of 57 s^{-1}. This shear rate is similar to that encountered in pharmaceutical suspension during pouring.[72] Saccharin, pKa of 1.6 produces protons in aqueous solution that react with the negatively charged carboxymethylcellulose anion leading to precipitation. The suspension formulated with both saccharin and Avicel RC 591 cannot suspend the drug for a uniform dose to be dispensed. Contrary to Avicel RC

591 technical literature that suggests the suspending agent to be stable at pH 3.5-11, the viscosifying property was markedly reduced below pH 5. Suspensions containing neotame (pH = 6.02) had no negative effect on the viscosity (Fig 2.3). Neotame has a pKa of 3.01 and the concentration of protons produced in the aqueous suspension was not high enough to precipitate the carboxymethylcellulose anion. On the other hand, sucralose is nonionic and had no effect on carboxymethylcellulose anion and thus no negative effect on the viscosity of the suspension (Fig 2.3). The pH of suspension containing sucralose was 6.08. Sucralose (Fig. 2.1) lacks UV/VIS absorption due to the absence of chromophores in the molecule. This deficiency is an advantage in HPLC-UV analysis, since it does not interfere with the API peaks. On the other hand, neotame has chromophores with UV/VIS absorption and could interfere with the selectivity of the HPLC-UV analysis for a multicomponent formulation sample. Hence, sucralose was selected instead of neotame for further studies.

Table 2.7 The pH of Avicel RC 591 and high intensity sweetener in deionized water

Sample	pH
Avicel RC 591	6.12
Neotame + Avicel RC 591	6.02
Sucralose + Avicel RC 591	6.08
Saccharin + Avicel RC 591	2.44
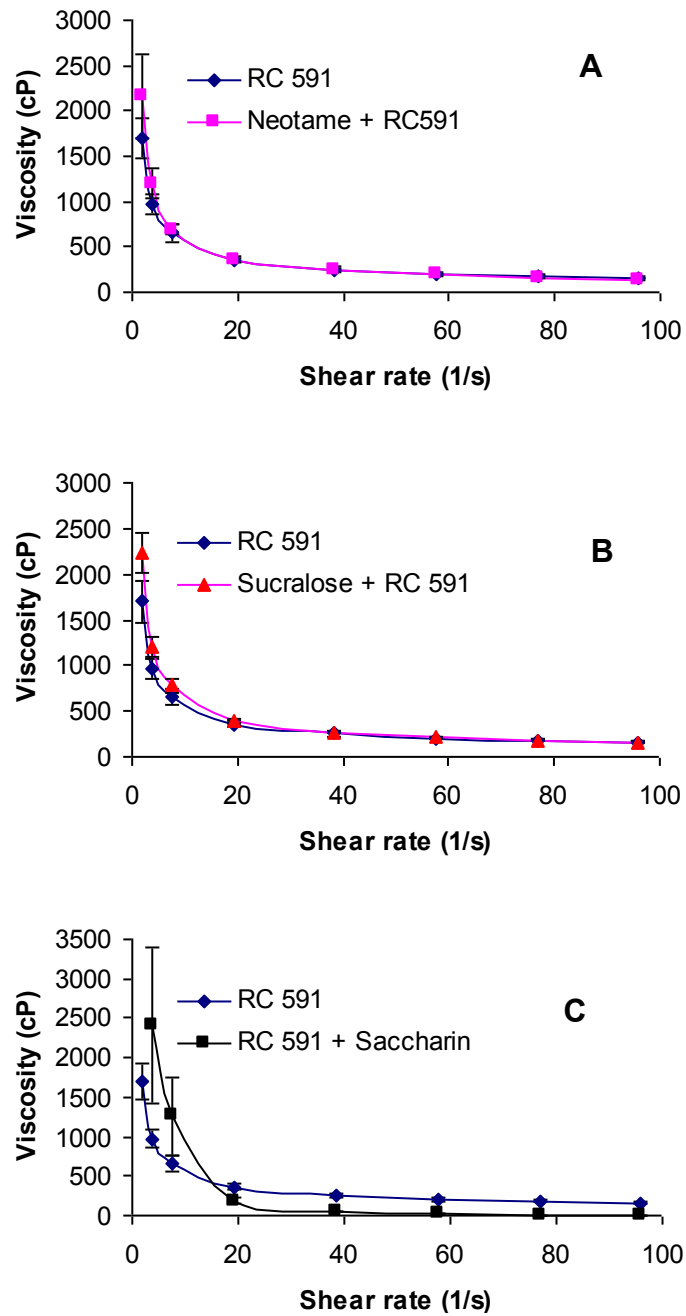
Fig 2.3 Viscosity of Avicel RC591 alone and in the presence of Neotame (A), Sucralose (B) and Saccharin (C).

2.3.4 Powder X-Ray Diffraction

Effect of Roller Compaction on Crystallinity of the Drugs: To determine whether the roller compaction process induced any polymorphic changes in the three drugs, the PXRD patterns before and after roller compaction were compared using granule samples. There were no differences in the diffraction patterns of lamivudine (Fig 2.4), zidovudine (Fig 2.5), and nevirapine (Fig 2.6) before and after roller compaction, indicating the absence of any polymorphic transformations. Further, samples of the roller compacted drugs stored at 40 ^{o}C/75 %RH for six months did not show any new peaks indicating the drugs were stable under the processing conditions.
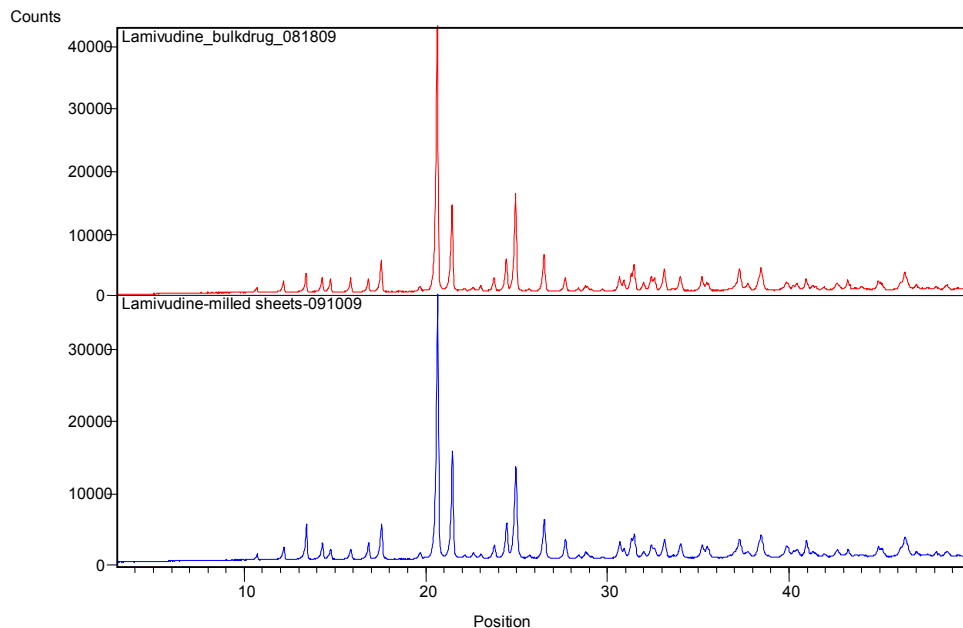
Fig 2.4 PXRD patterns of lamivudine powder before roller compaction (red) and granules after roller compaction (blue)

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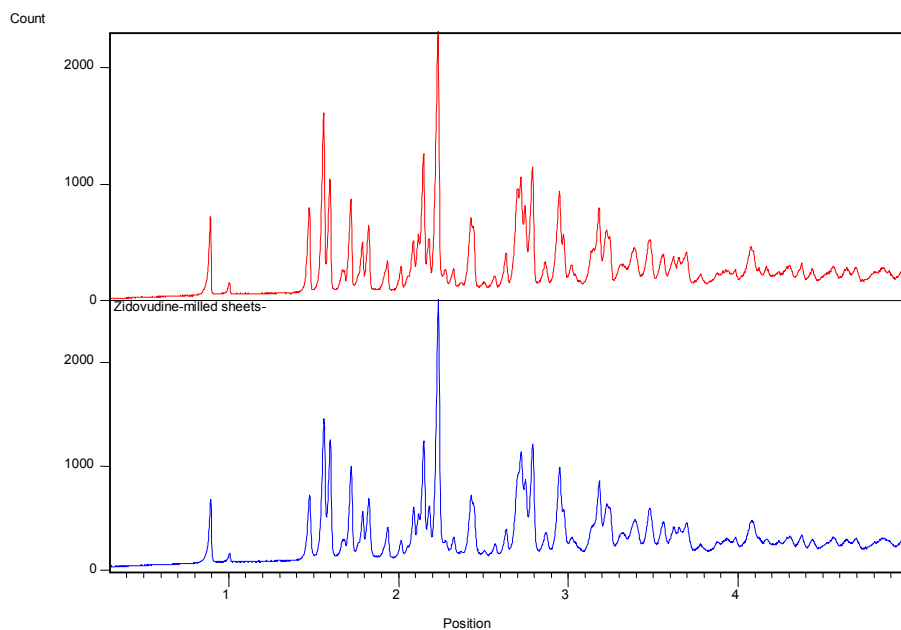
Fig 2.5 PXRD patterns of zidovudine powder before roller compaction (red) and granules after roller compaction (blue)
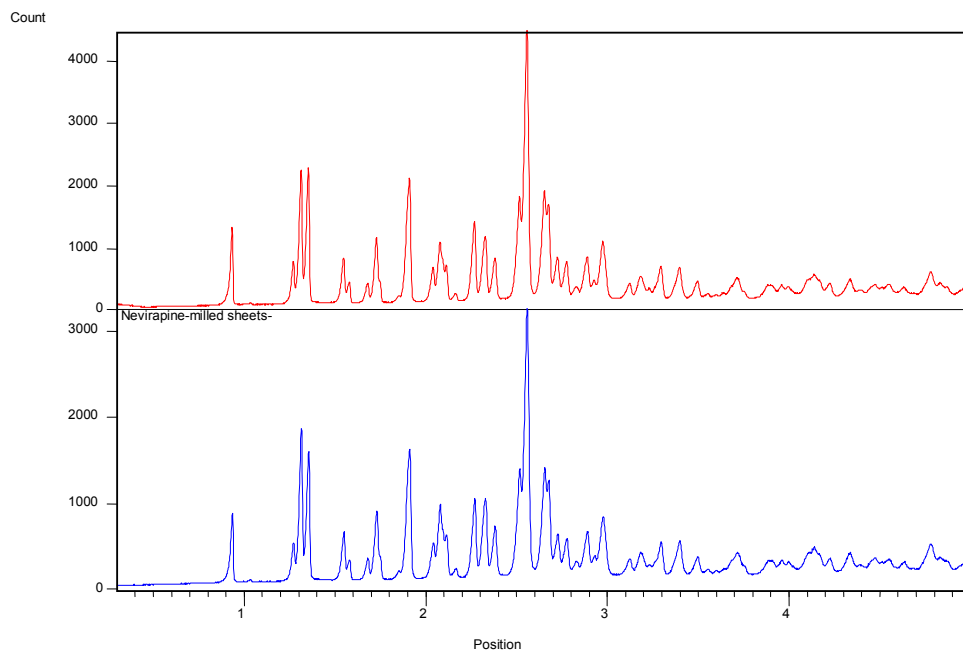
Fig 2.6 PXRD patterns of nevirapine powder before roller compaction (red) and granules after roller compaction (blue)

Crystallinity of Nevirapine and Lamivudine in Granules: In order to distinguish between the two crystal forms of nevirapine during stability studies on the granules, the powder X-ray diffraction (PXRD) patterns of nevirapine anhydrous and the recrystallized nevirapine hemihydrate were obtained and compared (Fig 2.7). The nevirapine hemihydrate had characteristic peaks at 5, 10.5, 11.5 and 14 deg 2θ which could potentially be used for distinguishing between the two crystal forms. Similarly, lamivudine 0.2 hydrate was also recrystallized and the PXRD pattern (Fig 2.8) compared with that of lamivudine anhydrous. The two forms of lamivudine have characteristic peaks suitable for identification of the two crystal forms.
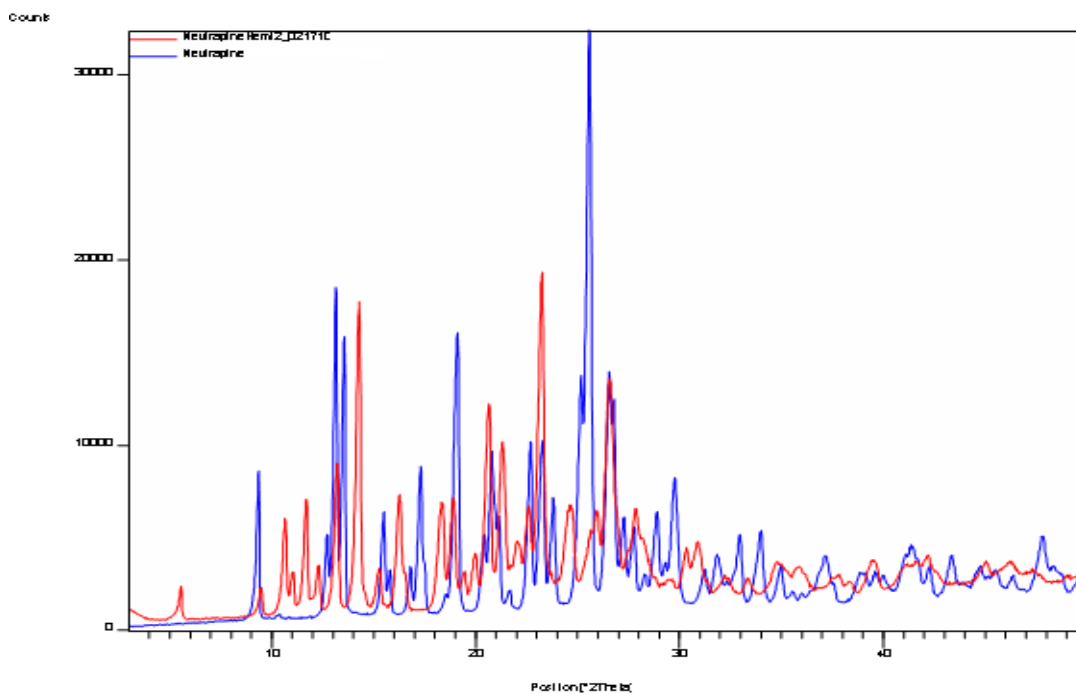
Fig. 2.7 PXRD patterns of nevirapine anhydrous (blue) and nevirapine hemihydrate (red) powder after recrystallization.
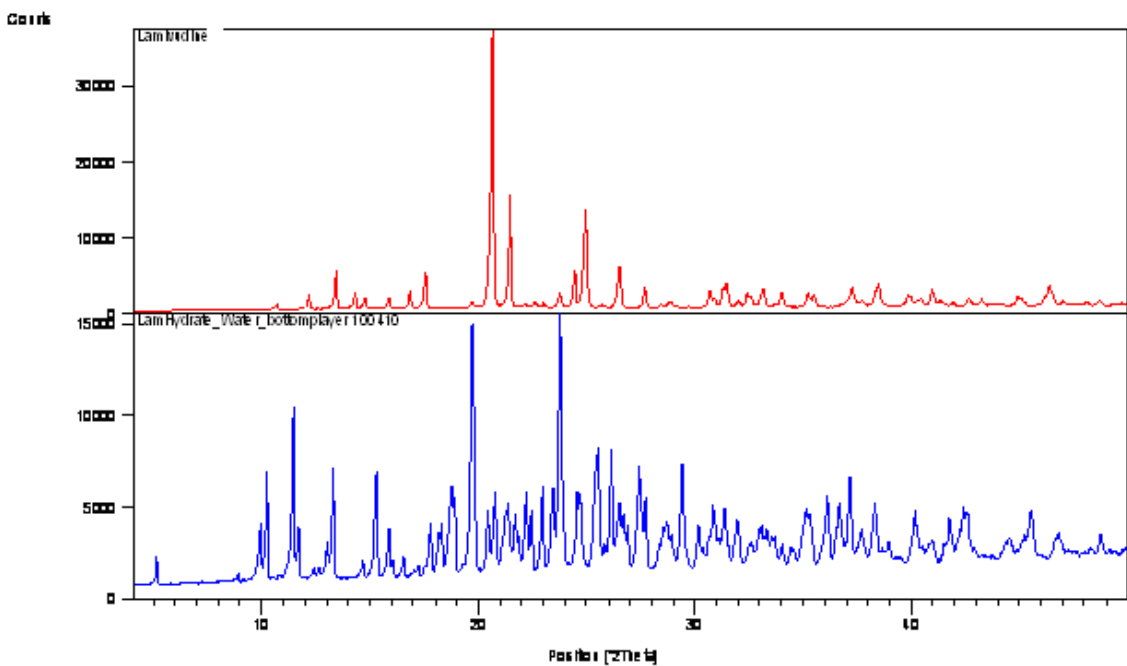
Fig. 2.8 PXRD patterns of lamivudine anhydrous (red) and lamivudine 0.2 hydrate (blue) powder after recrystallization.

Despite the characteristic peaks of the different crystal species of nevirapine, diffraction patterns obtained in the PXRD analysis could not be used to distinguish between granules containing nevirapine anhydrous or hemihydrate (Fig 2.9). This is due to the large mass fraction of xylitol in the blend compared to nevirapine. Xylitol suppressed the peaks of nevirapine in the diffraction pattern. The only different peaks that do not belong to xylitol are at 20.7^o and 21.5^o 2θ. These two peaks are common to lamivudine, zidovudine, nevirapine anhydrous and hemihydrate. Similarly, the characteristic peaks of lamivudine in the PXRD pattern were also suppressed by xylitol.
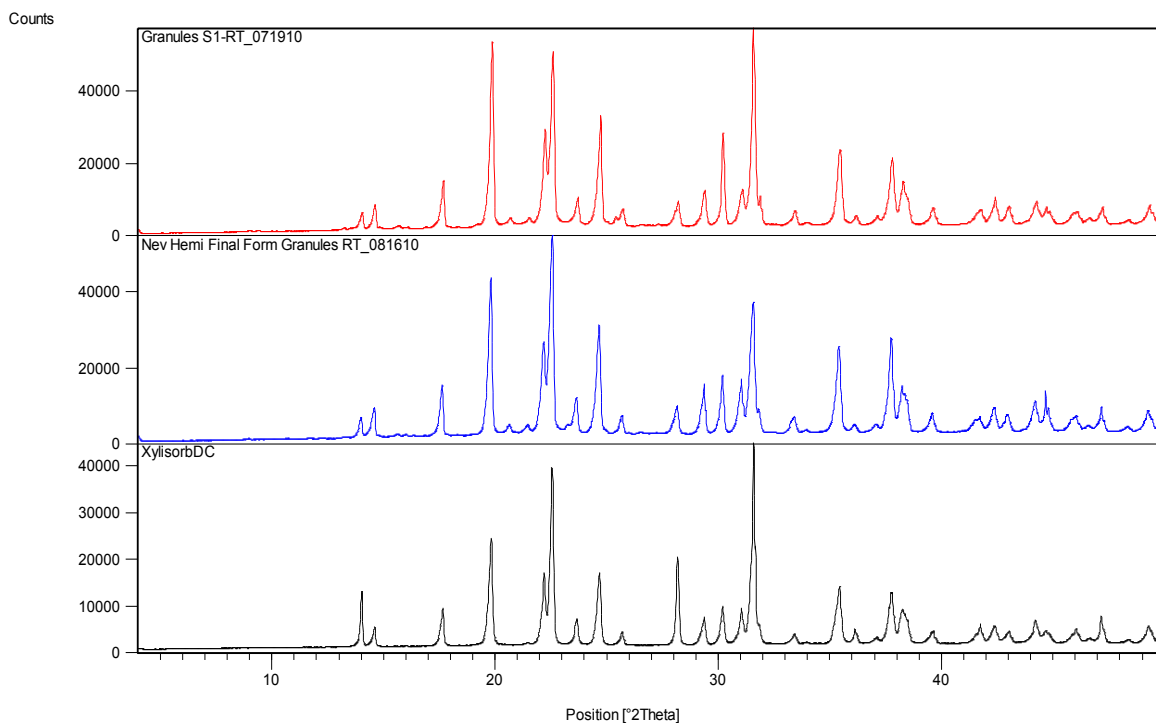
Fig 2.9 Diffraction patterns of pure xylitol (black), granules blend containing nevirapine hemihydrate (blue) and granules blend containing nevirapine anhydrous (red).

Dissolution of Different Species of Drugs: In view of the fact that PXRD could not distinguish between granules containing nevirapine anhydrous and hemihydrate, dissolution of the granules was evaluated as a means of distinguishing between the two crystal forms. The following test conditions were evaluated for the dissolution of the drugs: (a) 0.1 N HCl dissolution medium and USP apparatus II at 100 rpm could not discriminate between nevirapine anhydrous and the hemihydrate (Fig 2.10A). (b) When 0.01 N HCl dissolution medium and USP apparatus II at 50 rpm was employed, the granules formed a cone at the bottom of the vessel and the dissolution of the more soluble

drugs (lamivudine and zidovudine) was limited. (c) 0.01 N HCl dissolution medium and USP apparatus II at 100 rpm could discriminate between formulations containing nevirapine anhydrous and the hemihydrate (Fig 2.10B). Hence test conditions (c) were adopted for the analysis of the FDC granules. Moreover, lamivudine and zidovudine have high solubility and would be soluble in 0.01 N HCl dissolution medium.
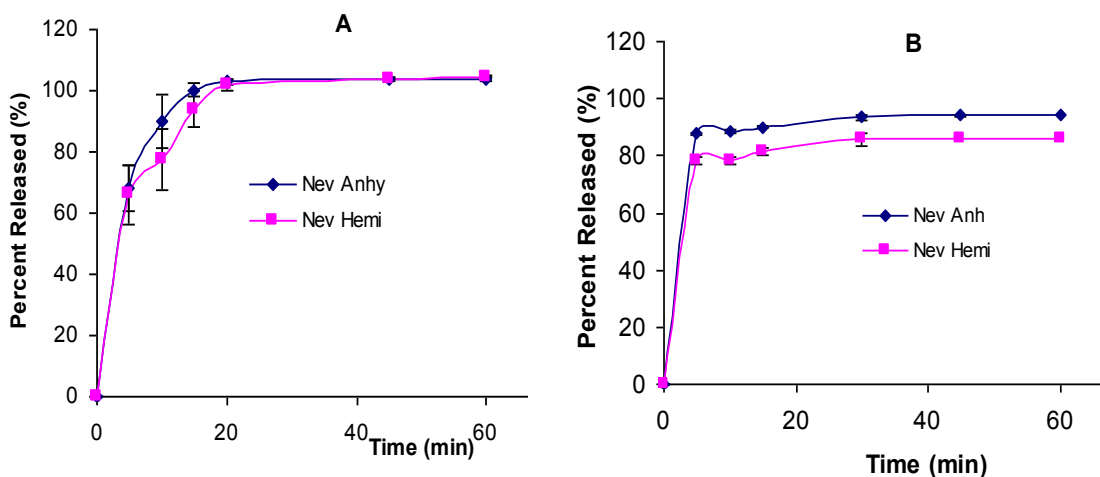
Fig 2.10 Dissolution of granule blend showing the profiles of nevirapine anhydrous and hemihydrate in (A) 0.1 N HCl dissolution medium and USP apparatus II at 100 rpm (B) 0.01 N HCl dissolution medium and USP apparatus II at 100 rpm.

2.3.5 Fourier Transform Infrared Spectra

The reflectance spectra of the bulk drugs and the USP reference standards of the drugs were obtained and compared. The overlay of the respective bulk and reference spectra of lamivudine (Fig 2.11), zidovudine (Fig 2.12) and nevirapine (Fig 2.13) did not reveal any differences confirming the identities of the active pharmaceutical ingredients. Analysis of isothermally stressed drug mixtures and physical mixtures of the similar

composition did not show any differences in the spectra indicating the absence of molecular interactions.
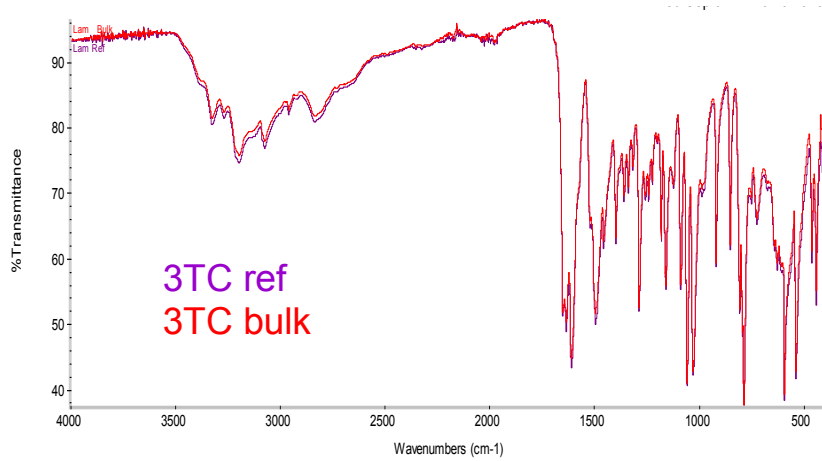
Fig 2.11 FT-IR spectra of lamivudine bulk and USP reference standard
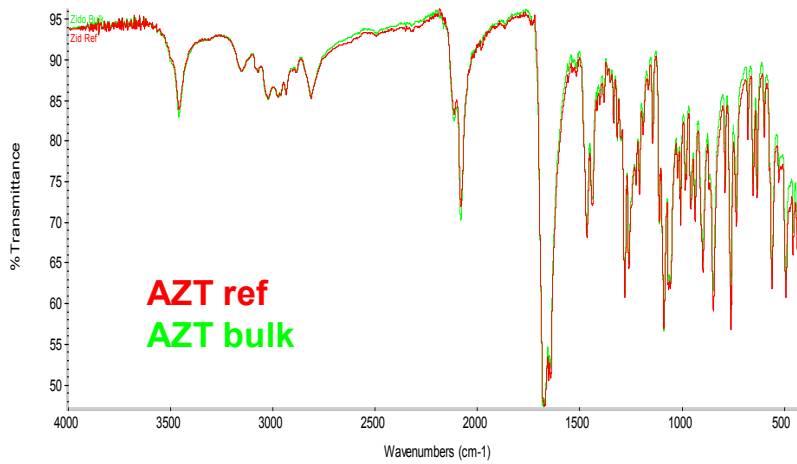
Fig 2.12 FT-IR spectra of zidovudine bulk and USP reference standard
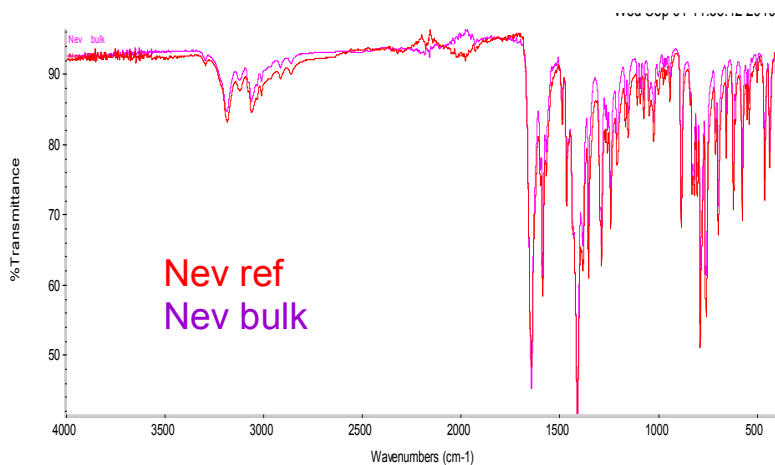
Fig 2.13 FT-IR spectra of nevirapine bulk and USP reference standard

2.3.6 Optimization of Roller Compaction Parameters

The average force required to break the ribbons ranged from 1182.51g to 2208.65g. The percent fraction of the granules made from the same ribbons of mean size less than 250 microns varied from 55.45 to 76.91 %. The results are summarized in Table 2.8. The data was subjected to multiple regression analysis using JMP software v4. The break force was fitted according to Eq. 2.2

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 \qquad \qquad \text{Eq. 2.2}$$

where X_1 = roll pressure and X_2 = speed ratio.

The R^2 was 0.3163 and the ANOVA p-value = 0.7710 indicated that the model could not adequately describe the observed data. Furthermore, from the effects table, the p-values were all greater than 0.5, an indication that the roll pressure, the speed ratio, and the interaction between the two terms could not adequately account for the variance in the observed data. Thus there was a weak relationship between the input variables and the

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break force. This could be due to the fact that the mechanical properties of the blend components (dependent on the large amount of xylitol) have a strong influence on the ribbon quality as explained below. However, this blend component was kept constant and was not included in the model (Eq 2.2). In addition, the same screen size was used to mill all the different ribbons. Since the screen size determines the particle size of the granules, the properties of the granules such as Carr's compressibility index which are related to the size of the granules were similar. Thus there was an absence of trends between average break force, fraction less than 250 microns and the compressibility of the granules. In particular, the hardness of the ribbons did not correlate with the particle size of the granules.

Xylitol, the major component of the blend consolidates by brittle fragmentation. The low specific surface area of xylitol minimizes the surface area available for interparticle bonding and this contributed to the ribbons which were not very hard.[93, 94] However, that property could be used to advantage in this formulation since hard granules for reconstitution could slow down the granule reconstitution process. Hence the quality of the granules obtained after milling the ribbons was suitable for the intended use.

Table 2.8 Optimization of the roller compaction parameters. RS = roll speed, HS = horizontal screw speed.

	Parameters				
	Roll Pressure		Ave. Break	% Fraction less	Ave Compressibility*
Run	(psi)	HS/RS	Force** (g)	250 microns	(%)
A	1300	15/5	1682 (24.1)	55.45	27.13 (1.53)
B	1300	15/4	1182 (23.9)	75.86	27.85 (0.46)
C	1300	20/5	1895 (23.6)	76.91	27.37 (0.86)
D	1500	15/5	1529 (27.1)	73.61	28.11 (0.83)
E	1500	15/4	2038 (44.1)	75.96	28.33 (0.00)
F	**1500**	**20/5**	**2098 (25.3)**	**75.27**	**26.92 (0.85)**
G	1800	15/5	2208 (22.1)	74.32	28.65 (0.82)
H	1800	15/4	1778 (29.3)	76.19	28.11 (0.83)
K	1800	20/5	1829 (39.0)	73.06	28.11 (0.83)

** % RSD, * SD

The Carr's compressibility index is an indication of the flowability of a powder, with lower numbers representing better flow properties. USP <1174> on powder flow defines Carr's compressibility index of 21 – 25 % as passable and 26 – 31 % as poor. The Carr's index obtained ranged from 26.92 – 28.65 % (n=3). It is expected that by adding Aerosil 200, a glidant, to the final granule blend, the flow will improve from poor to passable or better. Run F formed strong ribbons, had the best flow property, and greater than 75 % of the granules was less than 250 microns. Therefore, the parameters for run F (roll pressure of 1500 psi and ratio of the horizontal screw speed to the roll speed of 20/5)

were selected as the optimum roller compaction parameters. All the subsequent formulations were roller compacted using these parameters.

Initial Granule Formulation: After selecting sucralose as the sweetener of choice, the level of Aerosil 200 in the formulation was varied to select the concentration that will prevent lump formation in the granules. After four weeks storage of the granules at 40 ^{o}C/75 % RH, visual inspection of all the samples showed the absence of lumps and the granules were free flowing. This was an indication that all the levels of Aerosil 200 studied were effective at preventing the granules from lumping together. The colloidal sized Aerosil 200 formed films around the granule particles to prevent lump formation. The moisture uptake during that period was less than 0.4 %w/w. The minimal moisture uptake could be due to the fact the amber plastic bottles provided effective packaging barrier to water vapor. In particular, the packaging minimized the water uptake by the moderately hygroscopic xylitol.

2.3.7 Suspension Formulation Optimization

Suspension Sedimentation and Viscosity: Sedimentation was observed in samples containing the lowest levels of the independent variables together (-1, -1). That is, low Avicel RC591 and low Aerosil 200 concentrations were associated with sedimentation of the suspended drug particle. The formulations containing the other levels of Avicel RC591 and low Aerosil 200 did not show any sign of sedimentation. The observed pH of the formulations was independent of the concentration of the input variables.

The viscosity data was fitted by multiple regression analysis according to Eq. 2.3 using JMP software v4.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2 \qquad \text{Eq. 2.3}$$

where X_1 = concentration of Avicel RC591 and X_2 = concentration of Aerosil 200. The adequacy of the fitted model was checked by ANOVA and the variables which were not statistically significant were removed from the model. The square term of X_2 was not significant (p=0.0803) and was removed from the model. The final model (Eq. 2.4) was

$$Y = 681.84 + 436.045 X_1 - 39.90 X_2 - 52.90 X_1 X_2 - 120.69 X_1^2 \qquad \text{Eq. 2.4}$$

Table 2.9 Effect of optimization factors on viscosity modeled utilizing least squares regression model (r^2= 0.9654, n= 27).

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
X_1	1	1	3422434.4	588.5315	<.0001
X_2	1	1	28662.6	4.9289	0.0370
$X_1 * X_2$	1	1	33579.9	5.7745	0.0251
$X_1 * X_1$	1	1	87394.0	15.0285	0.0008

Both Avicel RC591 and Aerosil had significant effects (p<0.05) on viscosity of the suspension. Also the interaction between Aerosil 200 and Avicel RC 591 and the

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square term of Avicel RC591 had significant effects on viscosity (Table 2.9). However, the viscosity of the samples containing high levels of both components (1,1) was too high (> 1000 cP) to be utilized as an oral suspension. The residual plot (Fig. 2.14A) for viscosity did not show any pattern indicating the model assumptions were not violated. The surface plot (Fig. 2.14B) showed a curvature typical of a quadratic function. That is, the relationship between the concentration of Avicel RC 591 and the viscosity of the suspension was not linear. The increase in viscosity was a quadratic function of the Avicel RC 591 concentration.
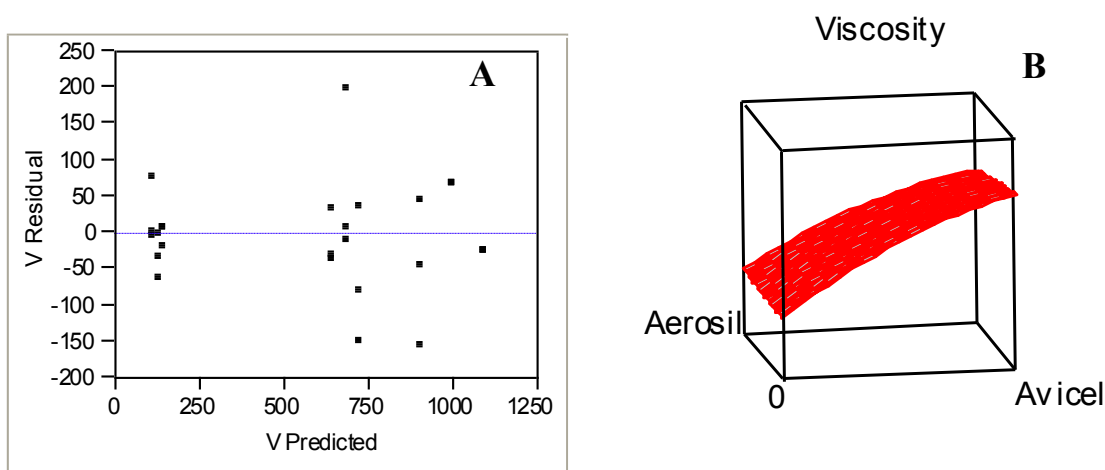
Fig. 2.14 The residual plot (A) of viscosity and the surface plot (B) showing effect of Avicel RC 591 (X1) and Aerosil 200 (X2) on viscosity.

Due to the fact low levels of Avicel RC591 and Aerosil 200 resulted in particle sedimentation and high levels of Avicel RC591 resulted in suspensions with excessive viscosity, the mid range concentration of these components were selected as the optimum concentrations of the two ingredients. Hence, 2% w/v of Avicel RC591 and 1 %w/v of Aerosil 200 were employed in the subsequent formulations.

2.3.8 Characterization of Granules (Development Batches)

Three development batches (1, 2, and 3) of the optimized formulation (Table 2.1) were manufactured, packaged and characterized. The particle size distribution, moisture content, flowability (indicated by Carr's index), content uniformity and dissolution were characterized.

Particle Size Distribution: The size distribution of the final granule blend for the three batches was determined by sieve shaking method. The mean particle diameter, d50, was 150 μm for all the three batches. The percent fraction less than 250 microns for batches S1, S2, and S3 were 80.00%, 76.91%, and 77.99% respectively. The fine particle size facilitates both reconstituting with water and suspending the drug particles since sedimentation rate is inversely related to particle size, according to Stokes' law.

Moisture Content: The initial moisture content of the granules was 0.3 %w/w, 0.3 %w/w and 0.2 %w/w for batches 1, 2 and 3. The low moisture content was important to maintain the granules in a free flowing state.

Flowability or Carr Compressibility Index: The compressibility indices for batches S1, S2, and S3 were 24.99±0.65, 24.76±0.98%, and 25.46±0.65% respectively. The compressibility indices were passable, and the flow of the final granule blend was better than the granules obtained after milling the roller compacted ribbons. The improved flow

was probably due to the addition of Aerosil, a glidant, which serves to reduce interparticle friction between the granules and thus improved flowability.

Content Uniformity of Granules: The mean content of the drugs ranged from 101.84±0.03% to 105.78±2.33% while the mean content of the preservatives ranged from 96.55±3.39% to 102.95±1.38% (Table 2.10).   The acceptance values for the analytes were calculated using Eq. 2.1. The USP33 <905> on uniformity of dosage units states that the maximum allowed acceptance value L1 (maximum range for deviation), for solid and liquid dosage forms of 10 dosage units is 15%. The maximum acceptance values calculated for the analytes was less than 10% (Table 2.10) indicating the five analytes passed the acceptance criteria for the uniformity of content. Thus the granules for reconstitution meet the specification for content uniformity. The clinical batch of granules had similar potency for lamivudine (97.49±0.46 %), zidovudine (101.55±0.56 %), and nevirapine (100.82±0.54 %).

Table 2.10 Content uniformity of granules.

Analyte	Batch S1		Batch S2		Batch S3		Acceptance value
	Ave (%)	SD	Ave (%)	SD	Ave (%)	SD	(%)
Lamivudine	102.85	1.35	102.45	0.56	104.28	0.21	9.87
Zidovudine	102.03	1.00	104.00	1.24	101.12	0.21	6.40
Nevirapine	103.12	1.72	101.31	3.33	101.84	0.03	7.79
Methylparaben	102.95	1.38	102.88	1.12	102.64	1.70	4.77
Propylparaben	102.81	0.82	101.17	1.53	96.55	3.39	6.19

Dissolution of Granules: Typical dissolution profiles of the three development batches performed using 0.01 N HCl dissolution medium and USP apparatus II at 100 rpm is represented in Fig 2.15. The drug release, corrected for potency, was rapid for all the three drugs. Lamivudine and zidovudine are BCS class I drugs that show rapid dissolution. For all the three batches, more than 80 % of lamivudine and zidovudine were released in 10 min which met the USP criterion of not less than 80 % dissolved in 30 min.

Similarly, more than 80 % of nevirapine was released within 10 min from all three batches which satisfied the USP criteria of not less than 75 % dissolved in 60 min. Nevirapine, a BCS class II drug showed rapid drug release than would be expected from this class of compounds. During the roller compaction, the pressure exerted by the rollers could have caused a reduction in the particle size distribution of nevirapine. In addition, milling the ribbons on the FitzMill would have further reduced the size distribution of the nevirapine particles. The reduction in particle size is inversely related to the surface area of the particles. The Noyes-Whitney equation (Eq. 2.1) shows that the mass rate of dissolution is directly related to the surface area of the particles. Thus as the size of the particles was reduced, the surface area increased resulting in increased dissolution rate. Furthermore, xylitol, a hydrophilic diluent was intimately mixed with nevirapine. Wetting of xylitol could have imparted faster wetting to the nevirapine particles to result in rapid dissolution. Finally, the dissolution profile of the clinical batch (discussed below) was similar to the profile of the development batch.
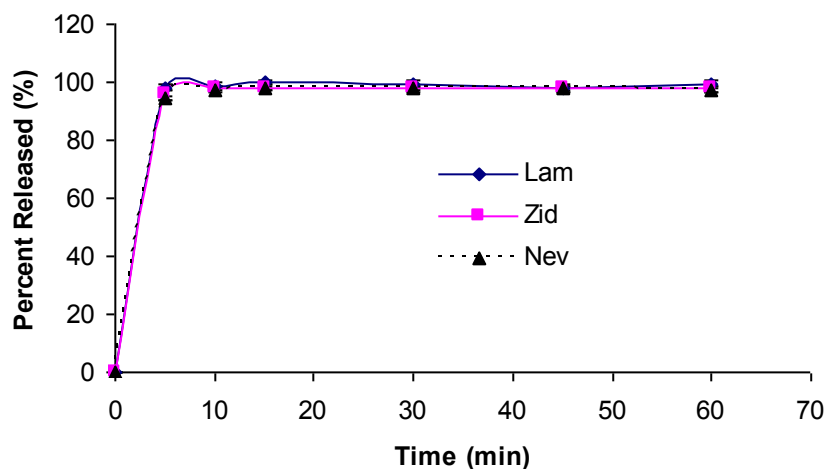
Fig. 2.15 Typical dissolution of development batch of granules in 0.01N HCl showing

profiles lamivudine (lam), zidovudine (zid), and nevirapine (nev).

Dissolution of Clinical Batch: The dissolution profile (corrected for potency) of the

clinical batch was determined under similar conditions. The profiles obtained for

lamivudine and zidovudine showed that more than 80 % was dissolved in 5 min (Fig

2.16), which satisfied the dissolution requirement of not less than 80 % dissolved in 30

min. In the case of nevirapine, the criterion is not less than 75 % should be dissolved in

60 min and the observed profile showed more than 80 % dissolved in 10 min, fulfilling

the USP requirement. Hence the dissolution profiles of the three drugs in the clinical

batch met the USP specifications and the batch was suitable for the intended purpose.
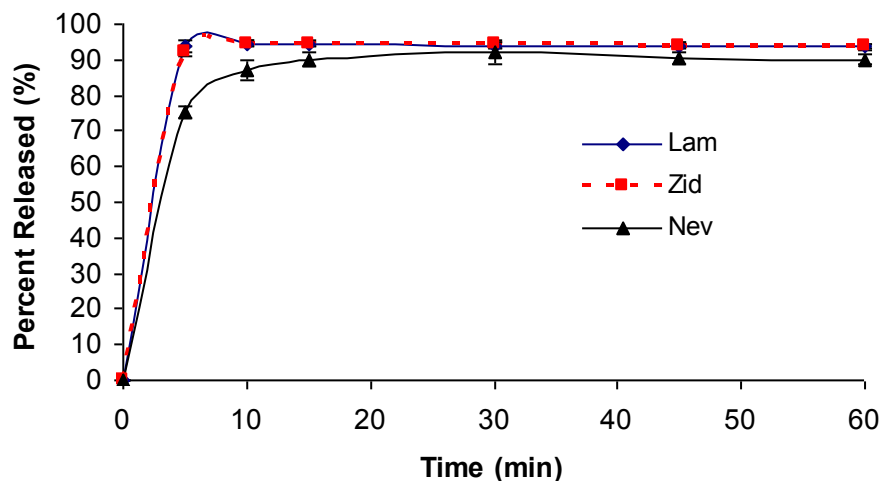
Fig 2.16 Dissolution of the clinical batch showing the profiles of lamivudine (lam), zidovudine (zid) and nevirapine (nev). The clinical batch contains nevirapine anhydrous.

2.4 Conclusions

The gradient high performance liquid chromatography method was validated for the drugs and the preservatives in the formulation. The method was suitable for the analysis of the fixed-dose combination granules and suspension without any interference from the formulation matrix.

There were no drug-drug interactions among the drugs and there were no drug-excipient interactions. Sucralose, the high intensity sweetener was preferred to neotame due to the lack of chromophores in the molecule and thus non-interference in HPLC-UV analysis. Furthermore, sucralose was stable and compatible with the drug and suspending agent; therefore it could be used as high-intensity sweetener in oral powders for reconstitution into suspension.

The roller compaction process was suitable for producing granules of uniform potency as indicated by the results of content uniformity. Powder X-ray diffraction analysis showed that the roller compaction process did not induce any polymorphic transformations in the drugs. The roller compaction process parameters were optimized for ribbon strength, particle size distribution and granule flow. In addition, the formulation optimization revealed that the Avicel RC591 and Aerosil 200 were important determinants of the viscosity of the suspension and the sedimentation rate.

The drugs and preservatives met the acceptance criteria for potency, and the granules had the desirable product characteristics. Dissolution conditions of 0.01N HCL medium and USP apparatus II at 100 rpm were identified to be discriminatory between granule formulations containing nevirapine anhydrous or nevirapine hemihydrate. The percent drug released for lamivudine, zidovudine, and nevirapine from all the three development batches and the clinical batch met the USP specifications for dissolution. The development batches and the GMP batch had the requisite potency and dissolution profiles.

Chapter 3. Evaluation of the Stability of Fixed-Dose Combination Granules for Reconstitution Formulation and the Reconstituted Suspension

3.1 Introduction

Drug products are expected to maintain their potency throughout the period of storage and use (shelf-life). The stability of the formulation is critical since a loss in potency could lead to under dosing, associated with ineffective therapy. Furthermore, the formation of toxic degradation products could make the product toxic and unsafe for patient administration.[76] The International Conference on Harmonization (ICH), the World Health Organization (WHO), and other regulatory authorities recommend conditions for stability studies.[78] Generally, the product should be packaged in the final container which is intended for the market and tested under conditions similar to those which prevail in the potential region of use.   The climatic conditions in sub-Saharan Africa, especially Nigeria, the intended market, are hot and humid (zone IVA)[76, 77] and the recommended accelerated stability conditions are 40 $^{\circ}$C / 75 % RH for 6 months and long-term stability conditions are 30 $^{\circ}$C / 65 % RH for a minimum of 12 months.[78]

In addition to the strength and purity, the product should maintain its antimicrobial integrity. The content of antimicrobial preservatives in a formulation should be greater than the minimum inhibitory concentration (MIC). The recommended usage levels (above MIC) for methylparaben and propylparaben in per oral liquid

formulations are 0.015-0.2% w/v and 0.01-0.02 % w/v respectively.[101] Although the preservatives show synergistic activity within a wide pH range of 4-8, the activity reduces above this range. This stems from the fact the two preservatives have pKa of 8.4 and above pH 8, the fraction of the ionized phenolate anion is increased. Since charged ions penetrate membranes poorly, the activity of the parabens is reduced at pH above 8. In addition, at higher pH, parabens undergo hydrolysis to form p-hydroxybenzoic acid, which has no antimicrobial activity. Hence, it is essential to monitor the pH of the reconstituted suspension during stability studies to ensure that the preservatives maintain their activity.

The data obtained from stability studies under ICH guidelines for long-term storage conditions may be used to establish the shelf-life of the product. This is done by extrapolating the kinetics of the degradation obtained under the long-term storage conditions, based on the principles of chemical kinetics. For granules, the quality attributes to be evaluated include potency, dissolution, and antimicrobial preservative content. In the case of suspension, the quality attributes to be monitored are potency, pH, and viscosity. For a fixed-dose combination product, several analytes made up of the active pharmaceutical ingredients and the preservatives have to be monitored.[49, 102]

The objectives for the stability studies were (i) to determine the stability of the granules for reconstitution under accelerated and long-term storage conditions, and (ii) to determine the stability of the reconstituted suspension under long-term stability conditions.

3.2 Experimental

3.2.1 Stability of Granules for Reconstitution

Stability Conditions: The stability of the granules was determined by placing granules in amber plastic bottles in a VWR 9000L stability chamber (VWR International, Bridgeport, NJ) at 40 ^{o}C/75 %RH and LH-1.5 stability chamber (Associate Environmental Systems, Ayer, MA) at 30 ^{o}C/65 %RH for six months. The granules were sampled at monthly intervals for potency, moisture uptake, and dissolution tests.

Potency and Antimicrobial Preservative Content: 2.33 g of granules (equivalent to 5 mL dose) was weighed into a 200 mL flask. 100 mL of 40 % v/v acetonitrile/water solution was added and sonicated for 30 min. 1 mL of this solution was diluted with 5 mL of 30 % v/v acetonitrile/water. The solution was filtered through 0.45 micron nylon filter and analyzed by the validated HPLC in triplicate. The potency and the antimicrobial preservative content were determined from the peak area response of the chromatogram.

Moisture Uptake: The moisture uptake was determined by weighing the bottles containing the final granules for reconstitution at pre-determined times. The weight gain was calculated as the percent moisture uptake.

Dissolution: A weighed sample (2.33 g) of granules was added to each of six dissolution vessels containing deaerated 0.01N HCl at 37 ^{o}C. The USP apparatus II (paddle method) was run at 100 rpm for 60 minutes. Samples were collected at 0, 5, 10, 15, 30, 45 and 60 min, filtered through 0.45 micron nylon filter and analyzed on HPLC.

3.2.2 Stability of Reconstituted Suspension

Reconstituting the Suspension: Sufficient amount of water was added to 93.01g of granules to make 200 mL, and shaken to form a suspension. The constituted suspension was equivalent to 6mg/mL of lamivudine, 10mg/mL of nevirapine, and 12 mg/ml of zidovudine. The suspension was reconstituted in amber plastic bottles.

Stability Conditions: The stability of the reconstituted suspension was determined by placing the plastic amber bottles containing the suspension in the stability chamber at long-term storage conditions (30 $^{\circ}$C/65 %RH) for four weeks. The samples were stored at long-term storage conditions to simulate in-use conditions. Samples were withdrawn after adequate shaking of the bottles at intervals for potency, antimicrobial preservative content, pH, and viscosity tests.

Potency and Antimicrobial Preservative Content: A volume of 5 mL was weighed into a 200 mL flask. 100 mL of 40 % v/v acetonitrile/water solution was added and sonicated for 30 min. 1 mL of this solution was diluted with 5 mL of 30 % v/v acetonitrile/water. The solution was filtered through 0.45 micron nylon filter and analyzed on HPLC in triplicate.

pH, Sedimentation Fraction and Viscosity: The electrode of a pH meter was placed in the reconstituted suspension and pH reading recorded. Triplicate measurements were made,

the average calculated and compared with the range. The viscosity was measured at intervals according to the procedure described in section 2.2.3.5.

In addition, alternating freeze-thaw cycles, each cycle lasting 24 hours was performed for two weeks. The samples for freeze-thaw testing were stored in a refrigerator (4 $^{\circ}$C) or at room temperature (25 $^{\circ}$C). The viscosity, pH, and redispersion were studied to determine the effect of freeze-thaw cycle on the physical stability of the suspension.

3.3 Results and Discussion

3.3.1 Stability of Granules

3.3.1.1 Potency of Drugs from Development Batch of Granules

The potency of the granules was evaluated at accelerated stability conditions (40 $^{\circ}$C/75 %RH) and long-term stability conditions (30 $^{\circ}$C/65 %RH) for hot humid tropical climate. The potency of the drugs should be within the 90 to 110 % range during the shelf-life of the product according USP specifications. The potency was normalized to the intial drug content, and not the label claim. Also, the long-term stability at 30 $^{\circ}$C/65 %RH was studied for six months in view of experimental constraints.

It was observed that, the potencies of the three drugs were within this range during the six-month study period indicating that the granules for reconstitution were stable. After six months at 30 $^{\circ}$C/65 %RH (Fig 3.1A), the potencies of drugs varied between 98.10 – 99.78 % for lamivudine, 98.03 – 99.45 % for zidovudine, and 99.05 – 100.4% for nevirapine.
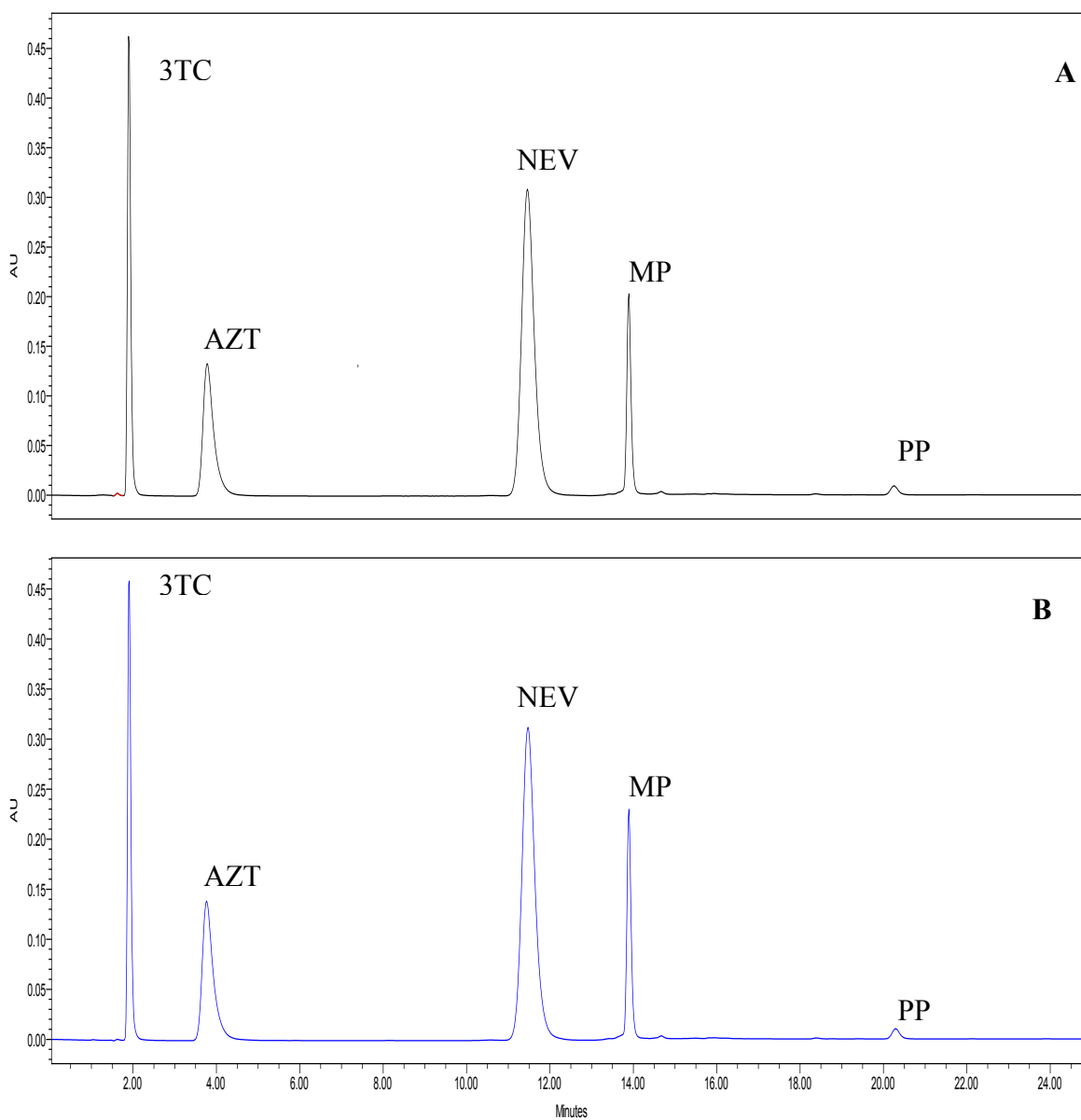
Fig 3.1 Chromatograms of stability samples of granules from the development batch (A) and the clinical batch (B) after storage at 30 $^\circ$C/ 665 %RH for 6 months. [3TC – lamivudine, AZT – zidovudine, NEV- nevirapine, MP – methylparaben, PP- propylparaben]

Similarly, the potencies of the preservatives were within 90 to 110 % range during the study period. After six months at 40 ^{o}C/75 %RH, the potency of propylparaben was greater than 91.13 % while the potency of methylparaben was greater than 93.92 %. At 30 ^{o}C/65 %RH, the percent recoveries for methylparaben and propylparaben ranged 97.60 – 99.15 % and 97.48 – 100.34 % respectively. These percent recoveries represent approximately 0.15 %w/v and 0.015% w/v of methylparaben and propylparaben respectively. The amounts recovered at 30 ^{o}C/65 %RH remained within the effective inhibitory concentration of 0.015-0.2 % w/v and 0.01-0.02 % w/v for methylparaben and propylparaben respectively.[101] Hence, the granules for reconstitution could be described as stable.

3.3.1.2 Potency of Drugs from Clinical Batch of Granules

The potency of the drugs in the granules from the clinical batch was assessed before and after 6 months storage at 30 ^{o}C /65 %RH (Fig. 3.1B). The potency of lamivudine before and after stability studies were 97.49±0.46 % and 99.56±0.81 % respectively (Table 3.1). The potency of zidovudine before and after stability studies were 101.55±0.56 % and 99.75±0.72% respectively. Similarly, the recoveries of nevirapine before and after stability test were 100.82±0.54 and 99.34±1.10% respectively. The differences in potency after stability testing for all three were negligible indicating the formulation was stable for at least six months. Therefore, the granules were stable and suitable for use in the clinical studies.

Table 3.1 Potency of the clinical batch before and after 6 months storage at 30 $^{\circ}$C /65 %RH

Compound	Recovery (%)	SD (%)
Initial Time		
Lamivudine	97.49	0.46
Zidovudine	101.55	0.56
Nevirapine	100.82	0.54
After 6 months at 30°C/65 % RH		
Lamivudine	99.56	0.81
Zidovudine	99.75	0.72
Nevirapine	99.34	1.10

3.3.1.3 Degradation Kinetics of Drugs from Development Batch of Granules

The principles of chemical kinetics were used to obtain the degradation rate constant. Although several researchers have assessed the stability of lamivudine, zidovudine and nevirapine combination in pharmaceutical dosage forms, the degradation kinetics are often not reported. However, the degradation kinetics of the three drugs in acids or bases follow pseudo-first order kinetics.[103-105] Hence first-order degradation kinetics were applied to all the drugs using the linearized first-order reaction equation (Eq. 3.1)

$$\ln A_t = \ln A_o - Kt \qquad \text{(Eq. 3.1)}$$

where K is the first-order rate constant and A_o and A_t refer to the initial amount of drug and the amount of drug remaining at time t respectively (Fig. 3.2).
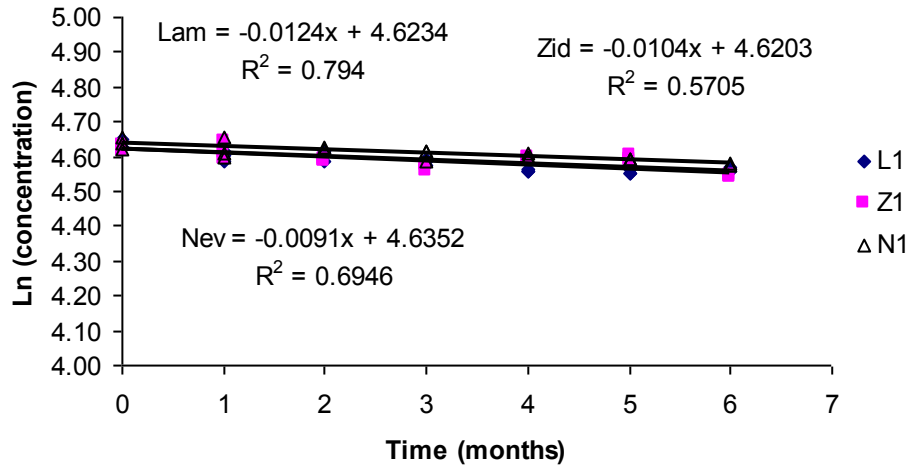
Fig 3.2A First-order degradation kinetics of development batch (1) of granules stored at 40 ^{o}C /75 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]
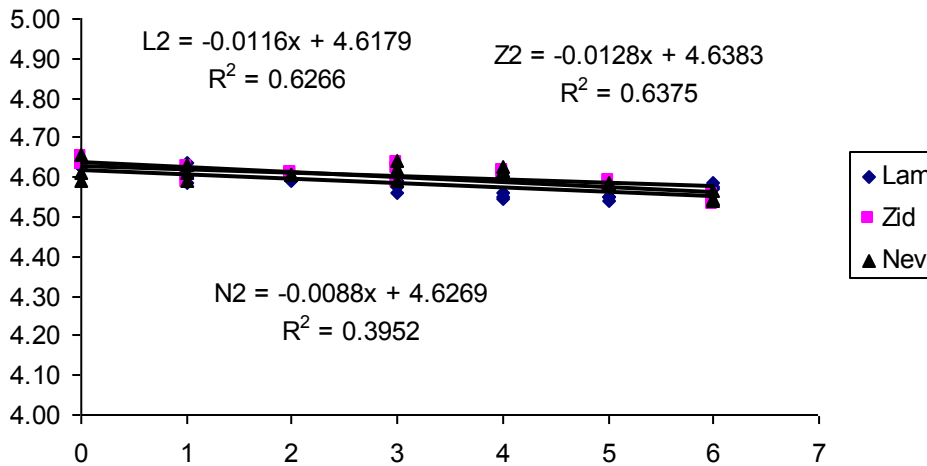
Fig 3.2B First-order degradation kinetics of development batch (2) of granules stored at 40 ^{o}C /75 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]

$y = -0.0121x + 4.6318$
$R^2 = 0.5322$

$Z = -0.0106x + 4.6176$
$R^2 = 0.6918$

$N = -0.0082x + 4.6159$
$R^2 = 0.5271$
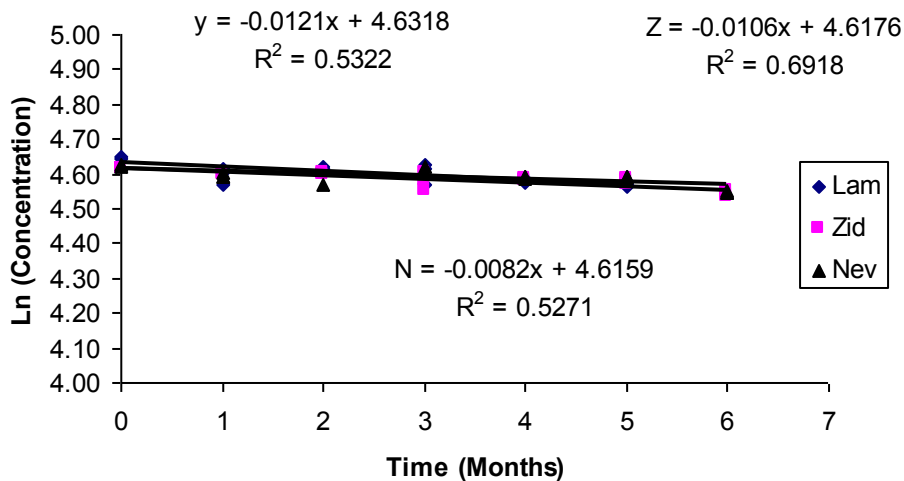
Fig 3.2C First-order degradation kinetics of development batch (3) of granules stored at 40 ^{o}C /75 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]

$L1 = -0.0032x + 4.6206$
$R^2 = 0.2461$

$Z1 = -0.0033x + 4.6183$
$R^2 = 0.1626$

$N1 = -0.0021x + 4.6139$
$R^2 = 0.0379$
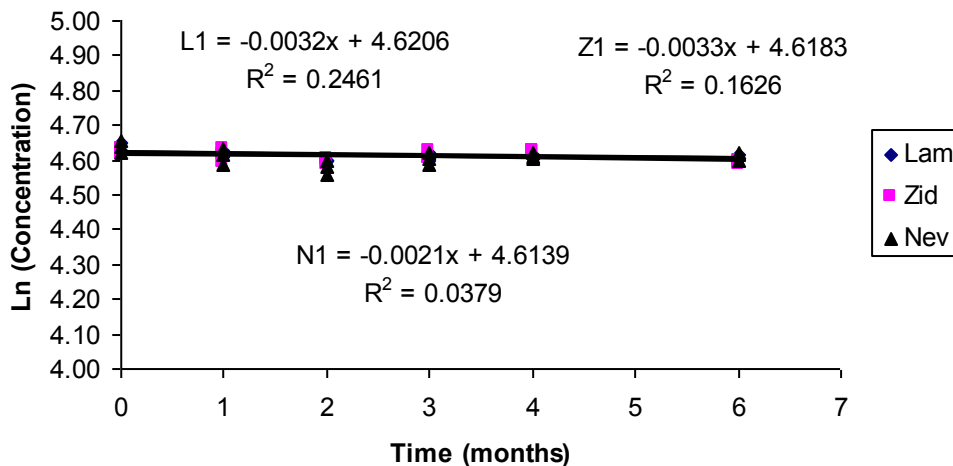
Fig 3.2D First-order degradation kinetics of development batch (1) of granules stored at 30 ^{o}C /65 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]
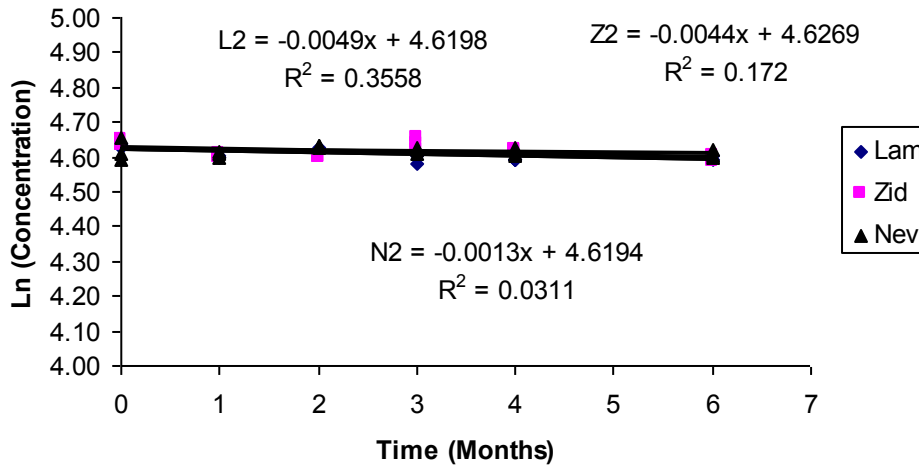
Fig 3.2E First-order degradation kinetics of development batch (2) of granules stored at 30 °C /65 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]
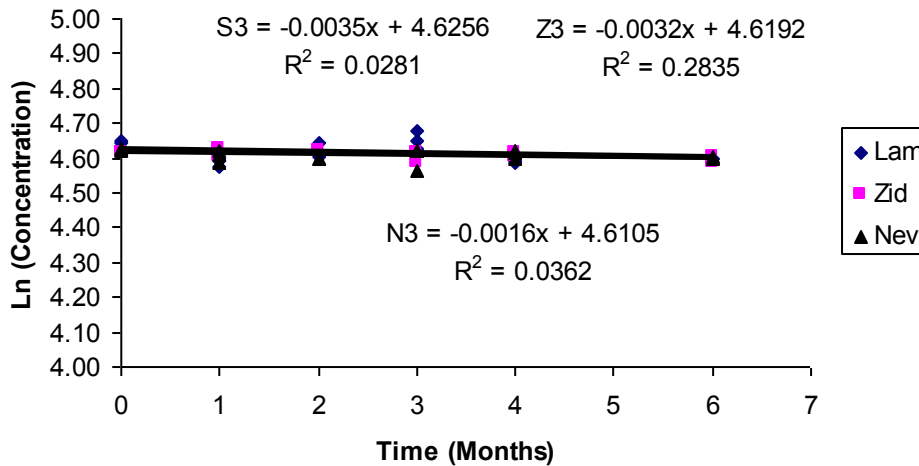
Fig 3.2F First-order degradation kinetics of development batch (3) of granules stored at 30 °C /65 %RH. Three determinations were made at each time point. [lamivudine (lam), zidovudine (zid), and nevirapine (nev)]

The degradation kinetics of the drugs in granules are summarized in Table 3.2. As expected, the degradation rate was faster at the accelerated stability conditions (40 $^{\circ}$C/75 %RH) compared to the degradation at the long-term stability conditions (30 $^{\circ}$C/65 %RH). This is due to the fact that reaction rate increases with temperature and moisture. This trend was common to all the three drugs studied during the six-month period.

Table 3.2 Degradation kinetics of the FDC granules for reconstitution obtained using first-order kinetics.

	Rate constant k (1/day)	
	40 $^{\circ}$C/75 %RH	30 $^{\circ}$C/65 %RH
Batch 1		
Lamivudine	0.0124±0.0030	0.0032±0.0030
Zidovudine	0.0104±0.0043	0.0033±0.0040
Nevirapine	0.0091±0.0029	0.0021±0.0056
Batch 2		
Lamivudine	0.0116±0.0043	0.0043±0.0037
Zidovudine	0.0128±0.0046	0.0044±0.0051
Nevirapine	0.0088±0.0052	0.0013±0.0039
Batch 3		
Lamivudine	0.0121±0.0062	0.0035±0.0138
Zidovudine	0.0106±0.0037	0.0032±0.0028
Nevirapine	0.0082±0.0043	0.0016±0.0045

During the development of the HPLC-UV method, the analysis of stressed solutions of the drugs showed peaks for the degradation products for nevirapine, lamivudine (cytosine and uracil) and zidovudine (thymine). During the stability studies of the granules in solid-state, peaks for the degradation products were not detected, although potency had reduced. Peak purity testing also revealed that all the peaks observed in the chromatograms were pure; that is, the peaks were due to single parent compounds. The degradation products might not have been detected due to the fact that volatile degradation products might have been formed, or the products formed were not detectable at the wavelengths used to monitor the chromatogram.

3.3.1.4 Moisture Uptake of Drugs from Development Batch of Granules

The moisture gained after six months ranged from 2.08±0.05% to 2.19±0.05% at 40 $^{\circ}$C/75 %RH and 0.53±0.31% to 0.79±0.41% at 30 $^{\circ}$C/65 %RH. High moisture content usually causes formation of lumps in the powder. The granules stored at 30 $^{\circ}$C/65 %RH were devoid of lumps after six months and were free flowing. In contrast, the samples stored at 40 $^{\circ}$C/75 %RH contained loose lumps, but were easily broken up by gentle agitation. The granules were packaged in amber plastic bottles to protect the product from light as well as to limit the adsorption of moisture. Since the moisture gained was low at both storage conditions, it can be inferred that the amber plastic packing bottle was effective at minimizing the entry of moisture into the product.

3.3.1.5 Dissolution of Drugs from Development Batch of Granules

The dissolution profiles of the products stored at 40 ^{o}C/75 %RH and 30 ^{o}C/65 %RH were assessed at specified intervals during the six months study period. The percent released was normalized to the initial potency of the drug. The maximum percent of lamivudine released from granules (Fig. 3.3) after six months at 40 ^{o}C/75 %RH was less than that for samples stored 30 ^{o}C/65 %RH due to the faster degradation at the higher temperature. However, more than 85 % of lamivudine was released within 10 min which met the USP criterion of not less than 80 % released in 30 min. The same trend was observed for all the three batches. Due to the rapid drug release, the f2 similarity factor was not calculated. In computing the f2 similarity factor, the regulatory guidelines recommend that dissolution points greater than 85% should not be used.

The maximum percent of zidovudine released from the granules (Fig. 3.4) after six months at 40 ^{o}C/75 %RH was also less than the drug released from the sample stored at 30 ^{o}C/65 %RH. This is validated by the greater drug degradation at 40 ^{o}C/75 %RH. More than 85 % of zidovudine was released within 10 min which also met the USP criterion of not less than 80 % released in 30 min. In the case of nevirapine, there were no differences in the dissolution profiles between samples stored at 40 ^{o}C/75 %RH and samples stored 30 ^{o}C/65 %RH (Fig. 3.5). This could be due to the fact less than 100 % of the drug was released from the granules. More than 80 % of nevirapine was released within 10 min which satisfied the USP criterion of not less than 75 % released in 60 min.

The quality and purity attributes of the granules evaluated during the stability studies remained within the acceptable limit. Hence, the granule for reconstitution will be expected to retain potency and dissolution profiles during the product shelf-life.
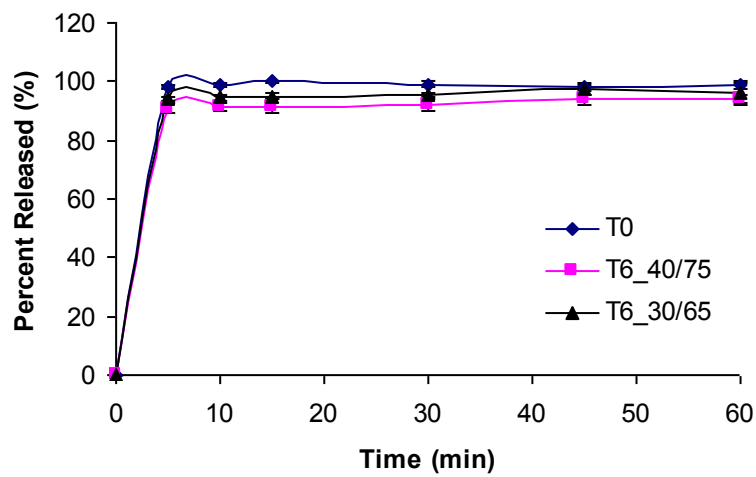
Fig. 3.3 Dissolution of development batch of granules (batch 1) showing the profiles of lamivudine at time zero (T0), after six months at 40 ^{o}C/75 %RH (T6_40/75) and 30 ^{o}C/65%RH (T6_30/65).
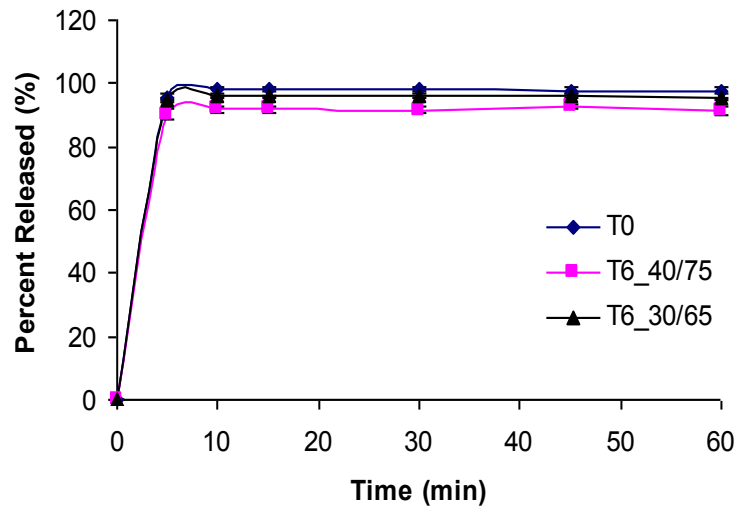
Fig. 3.4 Dissolution of development batch of granules (batch 2) showing the profiles of zidovudine at time zero (T0), after six months at 40 $^{\circ}$C/75 %RH (T6_40/75) and 30 $^{\circ}$C/65%RH (T6_30/65).
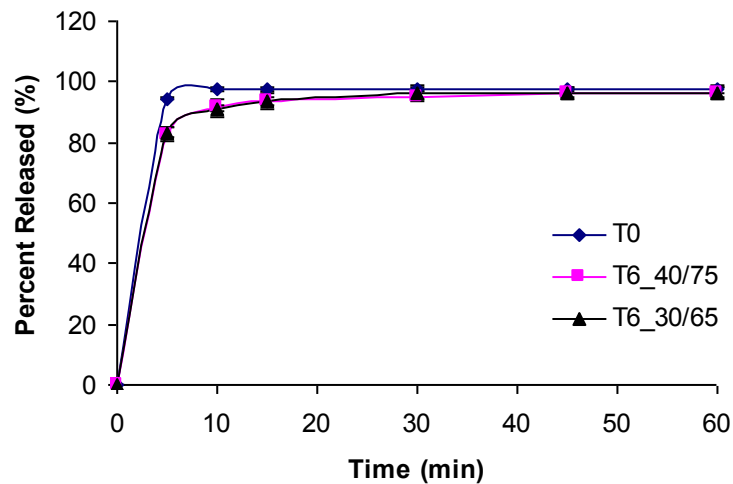
Fig. 3.5 Dissolution of development batch of granules (batch 3) showing the profiles of nevirapine at time zero (T0), after six months at 40 $^{\circ}$C/75 %RH (T6_40/75) and 30 $^{\circ}$C/65%RH (T6_30/65).

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3.3.1.6 Dissolution of Clinical Batch of Granules

The dissolution of the clinical batch of granules for reconstitution was evaluated after six months at 30 $^{\circ}$C/65%RH. For lamivudine and zidovudine, the time required for percent dissolved to reach 80% did not change upon stability testing (Table 3.3). In the case of nevirapine, the time required for 75 % to dissolve increased from 5 min to 15 min after stability testing. However, this slowing down of dissolution was still within the USP specification of not less than 75 % in 60 min. Hence the clinical batch showed acceptable dissolution profile after stability testing.

Table 3.3 Dissolution of the clinical batch showing the time taken for percent dissolved to meet USP specifications.

	Initial	After 6 months at 30 $^{\circ}$C/65%RH
	Time for 80 % of drug to dissolve (min)	
Lamivudine	5	5
Zidovudine	5	5
	Time for 75 % of drug to dissolve (min)	
Nevirapine	5	15

3.3.2 Stability of Reconstituted Suspension

3.3.2.1 Potency

The potency of the reconstituted suspension was evaluated for four weeks at 30 ^{o}C/65%RH. The recommendation is for reconstituted suspension to be used for a two-week period and then discarded. Hence the four-week study was adequate for assessing the stability of the suspension. The variance in the initial and final potencies was statistically tested utilizing analysis of variance (ANOVA) test (p-value < 0.05). The mean percent recoveries of the drugs from the suspension are summarized in Table 3.4. ANOVA of potency of lamivudine from the three batches at initial time and after four weeks showed no significant differences with p = 0.1466 and the Fcal (3.8949) less than Fcrit (9.5521). At four weeks, the potency of zidovudine for the three batches ranged from 97.84 % to 99.15%. ANOVA of the initial and final potencies across the three batches showed no significant differences between the batches with p = 0.5448 and Fcal was less than the Fcrit. Equally, the potency of nevirapine ranged from 98.11% to 98.32 % across the three batches. ANOVA of initial and final potency of nevirapine across the three batches did not show any significant difference. In addition, the potencies of the preservatives, methylparaben and propylparaben remained unchanged after four weeks. Thus, the potency of the drugs and the antimicrobial preservative content in the suspension remained unchanged after four weeks indicating that the formulation was chemically stable.

Table 3.4. Percent recoveries of drugs from reconstituted suspension at 30 °C/65 %RH for 4 weeks (within-use period). N=3, SD = standard deviation.

Time (weeks)	Batch 1 Ave (SD) %	Batch 2 Ave (SD) %	Batch 3 Ave (SD) %	ANOVA P-value	Fcal	Fcrit
Lamivudine						
0	104.44 (4.65)	102.75 (3.66)	98.80 (0.73)	0.1466	3.8949	9.5521
4	100.90 (1.57)	100.85 (1.97)	95.47 (1.13)			
Zidovudine						
0	102.06 (1.27)	99.62 (2.55)	100.68 (0.71)	0.5448	0.7488	9.5521
4	99.15 (1.52)	97.84 (1.91)	99.04 (0.67)			
Nevirapine						
0	100.67 (0.29)	100.10 (1.66)	100.17 (0.49)	0.9617	0.0395	9.5521
4	98.32 (0.20)	98.11 (1.29)	98.13 (1.42)			

3.3.2.2 pH and Sedimentation Fraction

The pH at time 0 and 4-weeks ranged from 7.01 to 7.06 among the three batches. ANOVA of the pH showed no significant differences (p = 0.2841) between the pH values at the initial and final time points. This indicated that the formulation was stable and there was an absence of reaction products that could alter the pH of the formulation. Furthermore, an absence of sedimentation was observed among the three batches: the sedimentation fraction was 1.

3.3.2.3 Rheology of the Reconstituted Suspension

Apparent Viscosity: The rheological properties of the reconstituted suspension were determined using a cone and plate Brookfield rheometer. The viscosity was measured at a shear rate of 0.76 to 114 s^{-1}. The apparent viscosity of the suspension was consistent within batches and across batches during long-term storage at 30 $^{\circ}$C/65 %RH for 4 weeks (Table 3.5). ANOVA test on the viscosity of the suspension showed no significant differences (p-value = 0.4464) between initial and final time points and across batches. In view of the fact the viscosity of the suspension is partly affected by pH, the lack of change of viscosity further confirms that pH has not changed, an indication that the formulation is stable.

The viscosity of the down-curve was displaced to the left of the up-curve, a phenomenon consistent with thixotropic behavior (Fig 3.6). When shear is applied to a system and the viscosity decreases with increasing shear, the system is described as shear- thinning. If on removal of the shear, the viscosity does not immediately return to original value, but recovers gradually over time to the original viscosity, the system is said to be thixotropic. When increasing shear rate was applied to the suspension and subsequently removed, the viscosity on decreasing the shear rate was lower (Fig 3.6) indicating that the system had not regained the original structure. Thus the suspension was thixotropic. Thixotropic behavior is of advantage in suspension formulations because upon agitation, the viscosity reduces to enhance flow (pourability) and continues to be so for a uniform dose to be dispensed. On standing, the system gradually returns to the original viscosity by reforming the interparticle interactions of the dispersed system.

Table 3.5 Apparent viscosity of suspension measured at a shear rate of 57 s^{-1}.

Time (weeks)	Average viscosity mPa.s (RSD)		
	Batch 1	Batch 2	Batch 3
0	262.0 (4.9)	282.7(3.2)	252.0 (9.4)
4	224.4 (13.4)	260.3 (10.2)	272.8 (9.1)
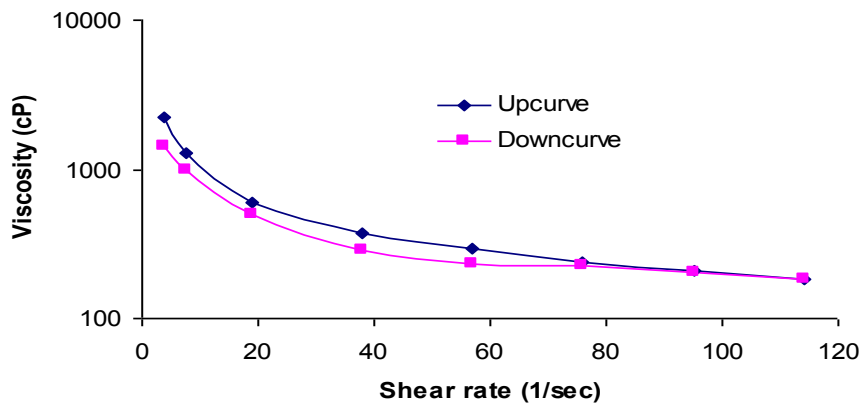
Fig 3.6 Rheogram of the suspension showing thixotropic behavior.

The Hershel Bulkley and the Casson models: The viscosity data was fitted to the Hershel Bulkley model (Fig. 3.7) and the Casson model (Fig. 3.8).[73] The goodness of model fit (CoF) for the Casson model ranged between 86.30 to 95.20 %, while the CoF of the Herschel Bulkley model was 85.20 to 99.00 %, indicating that both of the models could adequately describe the viscosity of the suspension. The Herschel Bulkley model defines the rheological properties[72] of shear thinning systems as consistency index, **k>0** and the flow index, **0<n<1** and for shear thickening systems, **k>0** and **n >1**. The observed consistency index was greater than zero and flow indices were consistent with this definition of shear thinning.
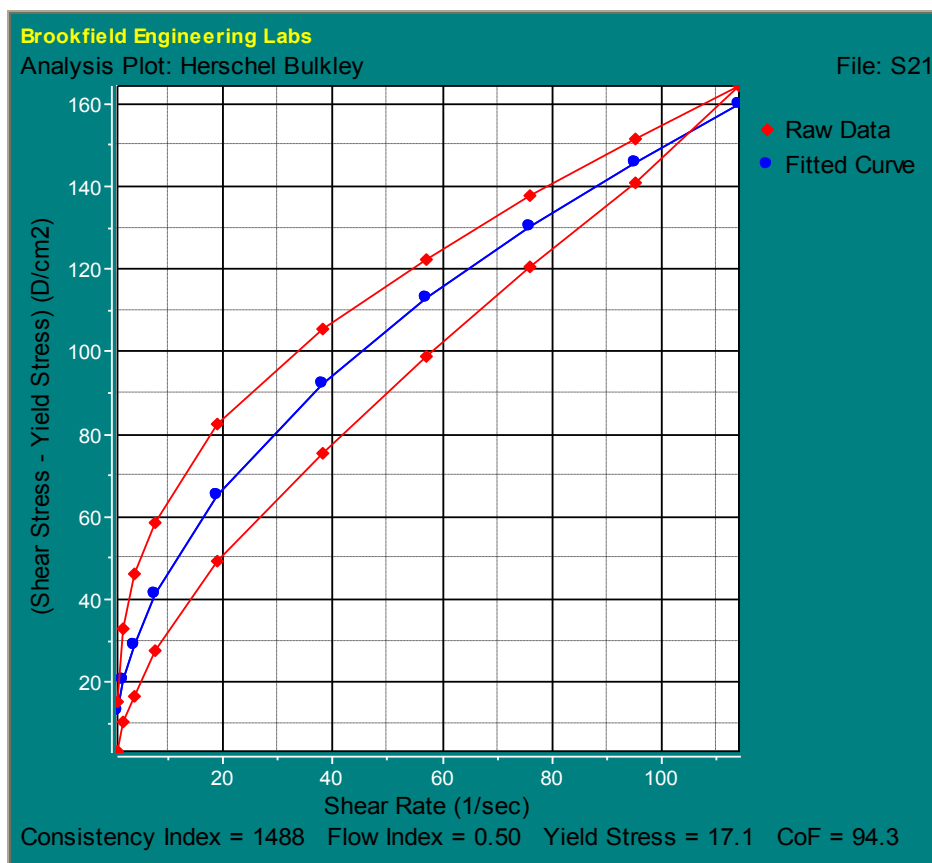
Fig. 3.7 A plot of the Herschel Bulkley model (blue line) and the raw viscosity data (red line).

The flow index n, obtained from Herschel Bulkey model describes the rheological behavior of the suspension. When n < 1, the suspension exhibits shear thinning behavior and as n approaches 1, the suspension becomes more Newtonian. When aggregation occurs due to increased interparticle interaction, suspensions exhibit more shear thinning behavior as greater shear will be required to break the interparticle interactions. Subsequently, the value of the flow index, n decreases with increasing aggregation.[106] Therefore if aggregation of the suspended nevirapine particles occurs on storage, it would be expected that flow index n will decrease. However, comparing the flow index at the

initial and the final time points, there is a marginal increase in **n** for all three batches. This shows that particles did not aggregate on standing, further validating the absence of any apparent visible sedimentation. ANOVA of the mean flow index did not show any significant differences (p = 0.7482) between the flow indices at initial time point and after four weeks. In addition, the yield stress and consistency index are inversely related to the flow index.[106] Therefore as flow index increases, yield stress and consistency index decrease, corresponding to the trend observed in Table 3.6. The Casson model also showed a decrease in yield stress with time while plastic viscosity increased (Table 3.7). This could mean that if the reconstituted suspension is stored for a long period, the yield stress could decrease sufficiently to result in particle sedimentation. However for the purposes of reconstituting the suspension and using within two weeks, this observation should have no effect on the formulation and the ability to dispense a uniform dose.

Table 3.6. The Herschel Bulkley parameters for the suspension before and after

stability studies

Time	Consistency Index	Flow Index	Yield Stress	CoF
	Batch 1			
0	1713.3 (478.8)	0.44 (0.05)	42.7 (6.9)	91.4 (2.9)
4	1150.3 (560.2)	0.56 (0.09)	23.03 (9.7)	98.4 (1.0)
	Batch 2			
0	3326.5 (615.7)	0.33 (0.06)	40.7 (10.9)	80.2 (5.4)
4	1286.0 (176.0)	0.54 (0.03)	26.9 (13.5)	95.3 (1.0)
	Batch 3			
0	1938.0 (220.6)	0.35 (0.08)	31.3 (6.0)	87.2 (1.7)
4	2281.5 (33.23)	0.49 (0.11)	26.4 (8.0)	96.4 (1.2)
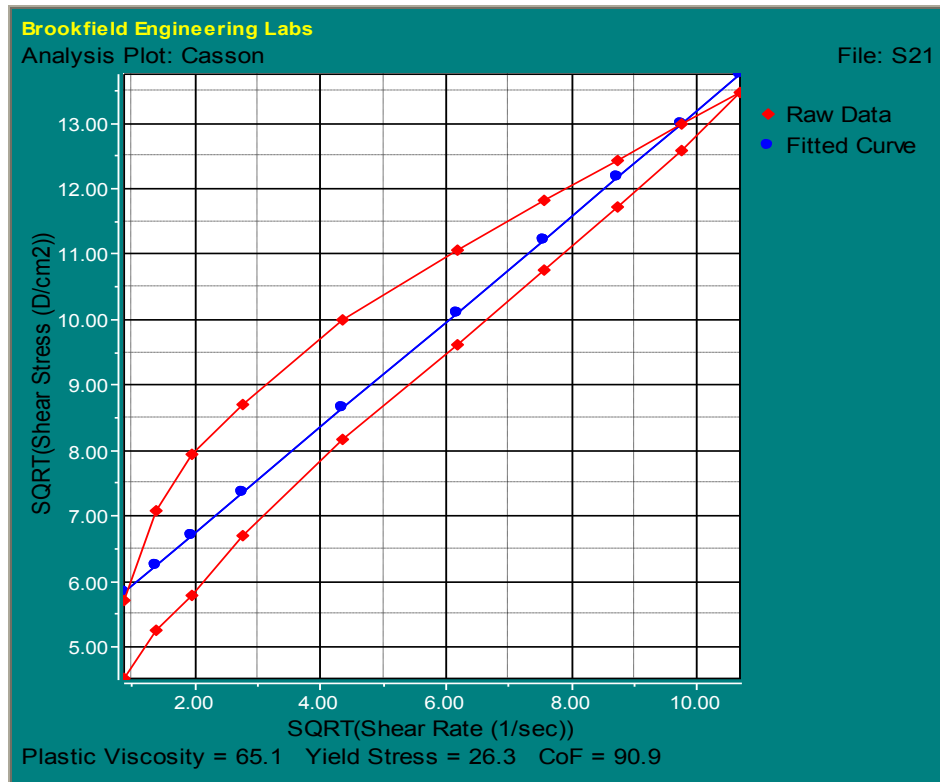
Fig. 3.8 A plot of the Casson model (blue line) and the raw viscosity data (red line).

Table 3.7. The Casson parameters for the suspension before and after stability studies

Time	Plastic Viscosity	Yield Stress	CoF
	Batch 1		
0	35.4 (2.4)	54.0 (2.5)	93.8 (1.4)
4	60.1 (10.5)	28.1 (4.5)	95.6 (0.3)
	Batch 2		
0	31.3 (6.0)	59.6 (4.7)	90.8 (0.3)
4	62.1 (6.0)	32.7(10.7)	92.9 (2.1)
	Batch 3		
0	30.1 (0.6)	54.4 (7.8)	93.1 (0.3)
4	57.2 (3.8)	38.7 (5.1)	94.7 (0.5)

Freeze-Thaw Stability: The reconstituted suspension was subjected to freeze-thaw cycling to determine the effect of temperature on the physical stability of the suspension. The apparent viscosity was monitored over the two week period and there were no significant changes in the apparent viscosity. Thus intermittently taking the suspension out of a refrigerator to dispense a dose will not negatively affect the physical stability of the suspension. Similarly, the pH of the suspension remained unchanged.

3.4 Conclusions

The stability of the development batches of granules for reconstitution was evaluated at accelerated and long-term storage conditions. Lamivudine, zidovudine, and nevirapine were stable in the formulation during the six month period showing minimal degradation. The dissolution profiles of the drugs before and after stability studies did not show any significant changes. The potency and dissolution profiles met the USP criteria. In addition, after the stability testing of the clinical batch at long-term stability conditions, the potency and dissolution attributes met the USP specifications.

The reconstituted suspension retained its potency throughout the study period. The pH and apparent viscosity of the suspension did not change with time. In addition, the Herschel Bulkley model adequately described the shear thinning parameters of the suspension. The flow index obtained from the Herschel Bulkley showed small increments with time while the yield stress and consistency index showed marginal reductions. To conclude, the granules for reconstitution and the reconstituted suspension exhibited both physical and chemical stability.

Chapter 4. Pilot Bioequivalence Study between Generic and Reference Products of Lamivudine 150 mg/Zidovudine 300 mg Tablets

4.1 Introduction

The design and conduct of clinical studies if not properly controlled can negatively affect the outcome of the study. For instance the local facilities available for housing the subjects could influence the decision to keep subject in-house over night prior to dosing as well the ability to take frequent samples within the first 24 hr of dosing. Further, the ability to estimate the local intrasubject variability could influence the number of subject enrolled in the study to avoid the unacceptable conclusion of bioinequivalence. To overcome these limitations, it is critical to perform a pilot study prior to the pivotal clinical study to evaluate some of these variable parameters. Therefore, the pilot bioavailability/bioequivalence clinical study was designed to assess the suitability of the proposed clinical site in Lagos, Nigeria for the study. The objective of the pilot study was to determine the pharmacokinetic bioequivalence between generic and reference fixed-dose combination products of lamivudine 150 mg/zidovudine 300 mg tablets.

Although more than 25 antiretroviral compounds have been approved for marketing and clinical use in the treatment of HIV AIDS[7] in adults, 14 compounds are approved for use in children and 13 of these are available in acceptable pediatric formulations.[9] Most of the newer protease inhibitor ARV class of drugs has not been

licensed by the FDA for use in children either due to safety concerns or a lack of clinical data on the use of the drugs in children. There is thus a limited number of age-appropriate pediatric formulations, particularly in resource limited settings. Therefore, scored fixed-dose combination ARV tablets for adults are frequently administered to children. The administration of the scored tablets to children could lead to sub therapeutic doses with an increase in the development of resistance.[2, 3, 107] Further, it may lead to lower adherence to medication in children.[9] Added to this, children often have higher viral loads compared to adults[9] and there is the need to ensure accurate dosing to suppress viral replication to reduce the viral RNA load in the blood. Hence, the second objective of this chapter of the research was to assess the *in vivo* quality of the FDC ARV adult tablets through a bioavailability and bioequivalence study. The in vitro quality of the tablets was evaluated in previous research by this laboratory.

4.2 Experimental

4.2.1 Materials

Ammonium acetate, potassium dihydrogen phosphate (KH_2PO_4), triethylamine (TEA), HPLC grade methanol and acetonitrile, heparinized BD vacutainer tubes, cryogenic vials (Fisher Scientific, Hanover Park, IL); Aprobarbital (Alltech, Deerfield, IL); Drug free human plasma (Plasmacare Inc., Pittsburgh, PA); 2ml micro-centrifuge tubes (VWR, West Chester, PA). Lamivudine 150 mg/zidovudine 300 mg test tablets (generic) and reference tablets (innovator) were obtained from the National Institute for Pharmaceutical Research and Development (NIPRD) and local drug stores in Nigeria.

4.2.2 Bioanalytical Assay

HPLC Chromatographic Condition: Chromatographic separation was performed on Shimazdu HPLC SCL-10A controller with SIL-10A auto injector linked with UV-VIS detector SPD-10A; Luna C_{18} 150mm × 4.6mm, 5μm column (Phenomenex, Torrance, CA); solvent system comprised of 25mM KH_2PO_4 (65%v/v) pH adjusted to 6.5 with triethylamine and methanol (35%v/v); injection volume was 50 µl; isocratic elution at a flow rate maintained at 1.0 ml/min; detection wavelength was 245nm using EZChrome^{TM} Software (Version 2.1).

Preparation of Standard Solutions: Solutions containing 1mg/mL of the lamivudine or zidovudine was prepared by dissolving the weighed samples in deionized water. Solvent for aprobarbital was methanol:water 50/50 %v/v. Further dilutions were made using 25 mM potassium dihydrogen phosphate buffer. The aprobarbital was used as internal standard.

Liquid-Liquid Extraction: A volume of 376 μL of thawed plasma was added to micro centrifuge tube followed by 24 μL of the internal standard (aprobarbital) and 576 μL of chilled acetonitrile. The tube was vortexed for 3 min and centrifuged at 11000 RPM for 10 min at 4 ^{o}C. 800 μL of the supernatant was evaporated under nitrogen at 37 ^{o}C. The dry residue was reconstituted with 800 μL of the buffer component of the mobile phase (25 mM KH_2PO_4) and analyzed by HPLC-UV method.

Calibration Curves: The appropriate volumes of the standard solutions of lamivudine, zidovudine, nevirapine and aprobarbital were added together in a micro-centrifuge tube containing 376 μL of drug free plasma and extracted using the extraction procedure above. Quality control (QC) samples at three levels were also prepared by spiking blank plasma with the working drug and internal standard solutions. The spiked samples were then extracted using liquid-liquid extraction method.

Validation: To establish the linearity and the range, replicate samples were used to construct a six-point calibration curve. The range was estimated based on the literature values for plasma analysis of the three drugs. Linearity was examined over the concentration range for lamivudine (50-2400ng/mL) and zidovudine (50-2000ng/mL). The data was fitted using the least sum of squares method. Precision and accuracy were determined by analyzing replicate samples at low, medium and high levels of the calibration curve. Precision was computed as percent relative standard deviation (RSD) and accuracy was computed as percent relative recovery. Inter-day reproducibility was examined by repeating the procedure using independent samples on three separate days. The data was analyzed using two-way ANOVA.

Short- and Long-term Stability: To monitor the stability of the extracted samples during running on the HPLC, spiked plasma samples at three different concentrations were kept at room temperature for 12, 24 and 48 hr. After the set time period, the plasma samples were subjected to liquid-liquid extraction procedure and the extracted drug samples were

analyzed using HPLV-UV method. Spiked plasma samples kept under the refrigeration condition – 20^0C for 30 days were assessed for long term stability studies.

4.2.3 Research Protocol Approval and Study Design

Institutional Review Board (IRB) protocol was submitted to the Duquesne University IRB for approval: the approved protocol was assigned the number 05/103 (See appendix 3). Similar submission was made to the University of Lagos, Nigeria, the clinical site for approval.

The criteria for enrolling subjects into the study were (a) Adult males determined to be healthy on the basis of a pre-trial physical examination, medical history and the results of blood biochemistry and hematology tests; (b) subject competent and willing to sign informed consent form voluntarily after being given all the detailed information about the study; and (c) willingness to be hospitalized for a 24-hour intensive sampling period. The subjects were excluded if (a) hypersensitive to study drugs; (b) drinking alcohol, smokers; (c) anemic or exhibited abnormal laboratory test, (d) if there were any clinically significant diseases or findings during the screening, medical history or physical examination that, in the opinion of the investigator, may interfere with the study; and (e) exposure to experimental or prescribed drugs within 30 days prior to the beginning of the study. Subjects who met the eligibility criteria were administered the consent form (see appendix 4).

Study Design: The study was designed as a single-dose, two-sequence two-treatment crossover design. The subjects were randomly assigned to the two arms of the study. The

two sequences were (a) test product during period one and reference product during period two or (b) reference product during period one and test product during period two.

Dosing: Following an overnight fast, 12 subjects were administered 150/300mg 3TC/AZT tablets with 240 ml of water. Volunteers were fed with regular diet after 4 hours of dosing. The test product was 150/300mg 3TC/AZT Duovir® tablets (Cipla India) and the innovator product was 150/300mg Combivir® tablets (GSK, UK). Nevirapine 200 mg tablets provided for simultaneous administration was inadvertently not given to the subjects.

Blood Sampling and Analysis: Blood samples were collected before dosing and at pre-determined intervals of 0.25, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4, 6, 8 hrs post dosing. All the samples were centrifuged and the plasma samples were kept frozen at - 80°C until assayed using a validated isocratic HPLC-UV method.

Evaluation of Pharmacokinetic Parameters and Statistical Analysis: The peak area ratio for the drug/internal standard was obtained and used to determine the drug concentration in the plasma. Non-compartmental pharmacokinetic analysis (NCA) was used to determine pharmacokinetic (PK) parameters for each subject. Area under curve from zero to the last measurable time point (AUC_{0-t}) and the area under curve from time zero to infinity ($AUC_{0-\infty}$) was computed by non-compartmental model with extravascular input using *WinNonlin Professional*® (version 5.2) software (Pharsight Corporation, Mountain View, CA). The maximum plasma concentration (C_{max}) and the time to attain the

maximum plasma concentration (t_{max}) were determined from the concentration-time profile curves. The AUCs and C_{max} were log transformed. The geometric mean ratios of test/reference of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ for 3TC and AZT were used to determine the average bioequivalence of the drug. The 90% confidence intervals (CI) for the ratios were determined. The Food and Drug Administration guidelines for average bioequivalence requires that the 90% CI for the ratio of mean C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of test to reference lies within the specified interval of 80-125%.

4.3 Results and Discussion

4.3.1 Validation of HPLC-UV Method

The chromatogram showed well resolved peaks indicating specificity of the method. The linear dynamic range for lamivudine was $50 - 2400$ ng/mL ($r^2 = 0.9980$) and the linear range for zidovudine was 50-2000 ng/mL ($r^2=0.9994$). The intra-day accuracy and precision for lamivudine were 100.04 % and 0.86% respectively. The inter-day accuracy and intermediate precision for lamivudine were 100.10 % and 0.65% respectively. In the case of zidovudine, the intra-day accuracy and precision were 98.57 % and 2.74% respectively. The inter-day accuracy and intermediate precision for zidovudine were 99.96 % and 1.39 % respectively. Two-way analysis of variance of the accuracy and precision within days and between days did not show any significant differences ($p > 0.05$ and Fcalculated < Fcritical) indicating that the method was suitable for the intended purpose.

4.3.2 Pharmacokinetic Analysis

The ratio of the peak area of the drug to the peak area of the internal standard (aprobarbital) was used to construct a calibration curve. The calibration curve was used to determine the concentration of the plasma drug concentration. Initial inspection of the data showed that the data for two subjects was very different from that from the rest of the cohort. The data for the two subjects was treated as outliers and eliminated from further analysis. Therefore, subsequent data analysis was performed for the remaining 10 subjects.

Non-compartmental pharmacokinetic analysis was performed to obtain the area under the curve from time zero to time t (AUC_{0-t}) and the area under the curve from time zero to time infinity ($AUC_{0-\infty}$). The linear-log trapezoidal rule was used for the calculation of AUC, assuming the drug disposition followed first-order kinetics. The maximum plasma concentration (C_{max}) and the time to achieve the maximum plasma concentration (t_{max}) were obtained from the graph. The t_{max} for lamivudine in the test and reference products was 0.93 ± 0.15 min and 0.75 ± 0.16 min respectively (Fig 4.1). Similarly, the t_{max} for zidovudine in the test and reference products was 0.73 ± 0.11 min and 0.63 ± 0.07 min respectively (Fig 4.2).
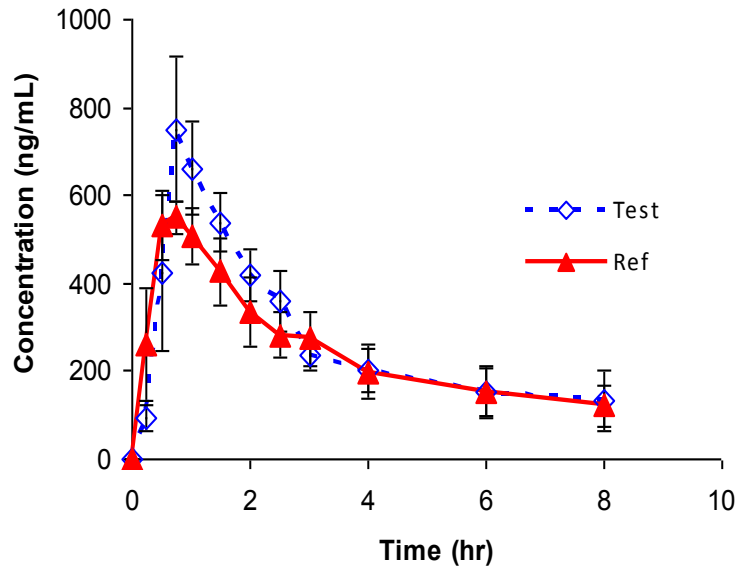
Fig 4.1 Plasma concentration-time profiles for lamivudine from the test and reference products. The data represents the mean (± standard error) for 10 subjects.
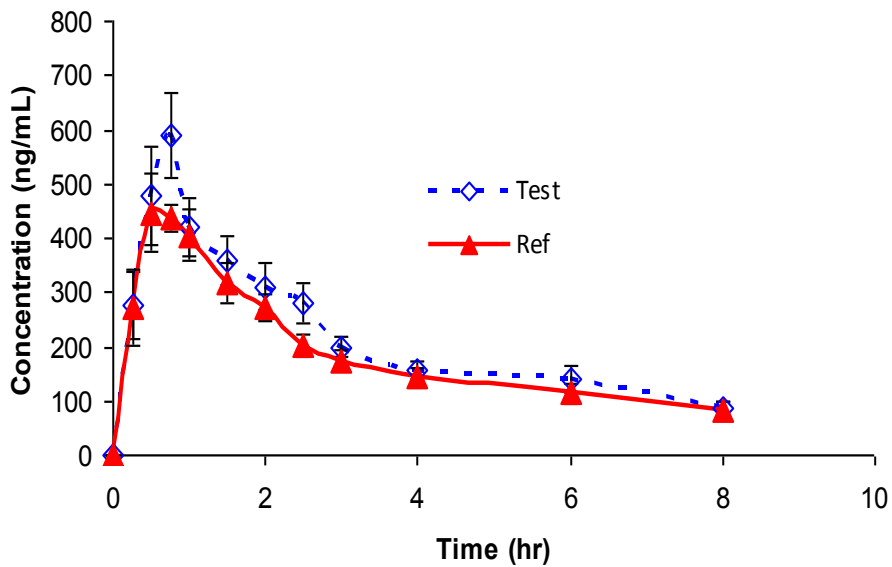
Fig 4.2 Plasma concentration-time profiles for zidovudine from the test and reference products. The data represents the mean (± standard error) for 10 subjects.

4.3.3 Statistical Analysis for Bioequivalence Assessment

Bioequivalence is characterized by three pharmacokinetic parameters: Cmax, AUC_{0-t} and $AUC_{0-\infty}$. Therefore these pharmacokinetic parameters were log transformed and the ratio of the test to reference PK parameters as well as the 90 % confidence interval determined. The analysis was done using WinNonlin 5.2. ANOVA test was performed to determine the effect of the model parameters. The model parameters were sequence, subject nested in sequence, period and formulation. ANOVA test revealed that the effect of subject nested in sequence was significant (p-value = 0.00552) for lamivudine (Table 4.1). This implies that for the two formulations to be bioequivalent, the distribution of the subject within the sequence will have to be taken into account. A probable cause of this is a carryover effect from the previous dose. However, the washout period was long enough to ensure complete elimination of lamivudine before the second dose. Besides, the plasma samples collected just before the second dosing did not contain any drug. A more plausible cause of this is a large intra-subject variability compared to the small sample of 10 subjects. In particular, the mean plasma concentration-time profiles for the test product (Fig 4.1 and 4.2) exhibited larger variability (larger standard error bars) compared to the reference product. This implies that the drug disposition in the subject during period 1 and period 2 were different.

Table 4.1 Analysis of variance of the model parameters for lamivudine

Hypothesis	DF	SS	MS	F_stat	P_value
Sequence	1	0.00825	0.00825	0.01718	0.89896
Subject (Sequence)	8	3.84175	0.48022	7.27077	0.00552
Formulation	1	0.14793	0.14793	2.23968	0.17288
Period	1	0.03466	0.03466	0.52479	0.48945
Error	8	0.52838	0.06605		

The FDA specification for bioequivalence requires that the ratio of the test to reference pharmacokinetic parameters should be within the 80 – 125 % limits of the 90 % confidence interval. For lamivudine, the confidence intervals for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ all extended across the lower limits (Table 4.2) of the bioequivalence limits indicating that lamivudine in the two formulations were bioinequivalent. In the case of zidovudine, the 90 % confidence interval for $AUC_{0-\infty}$ was 80.07 – 120.39 % which was within the bioequivalence limits (Table 4.3). However, the confidence intervals for the C_{max} and AUC_{0-t} extended across the lower limits of the bioequivalence boundary. Therefore, zidovudine in the test and reference products was bioinequivalent. Thus the test and innovator fixed-dose combination tablets of lamivudine/zidovudine were not bioequivalent. The observance of bioinequivalence could be due to the small sample size used in the pilot clinical study and a larger intra-subject variability. The larger variability, especially in lamivudine test product (Fig 4.1) produced an apparent higher

bioavailability of the test product. However, in vitro dissolution and potency tests on the tablets showed consistent results.[108]

Table 4.2. Geometric least square mean values of pharmacokinetic parameters, ratio, and 90 % confidence interval for lamivudine

Parameters	Test	Ref	Test/Ref	90 % Confidence Interval
Cmax (ng/mL)	721.57	792.92	91.00	73.52 – 112.64
AUC_{0-t} (ng*h/mL)	1688.53	1971.64	85.64	68.85 – 106.52
$AUC_{0-\infty}$ (ng*h/mL)	2092.72	2740.39	76.37	53.32 -109.37

Table 4.3. Geometric least square mean values of pharmacokinetic parameters, ratio, and 90 % confidence interval for zidovudine

Parameters	Test	Ref	Test/Ref	90 % Confidence Interval
Cmax (ng/mL)	516.71	662.36	78.01	63.48 – 95.87
AUC_{0-t} (ng*h/mL)	1420.52	1607.23	88.38	68.02 – 114.84
$AUC_{0-\infty}$ (ng*h/mL)	2002.39	2039.45	98.18	80.07 – 120.39

4.4 Conclusions

The validated HPLC-UV method had the requisite accuracy and precision for the analysis of the plasma samples. The test and reference fixed-dose combination lamivudine 150 mg/zidovudine 300 mg antiretroviral tablets were not bioequivalent. The

sample size for the pivotal bioequivalence studies should be increased to enhance the likelihood of a bioequivalence outcome. Finally, due to some administrative inconvenience and logistic problems, the clinical site in Lagos, Nigeria could not be used for the Phase I clinical study. Hence an alternate location should be identified for the Phase1 bioequivalence clinical trial. Lessons learned from this pilot study formed the basis for the development of protocols for the clinical study (chapter 5).

Chapter 5. Bioavailability/Bioequivalence Phase 1 Clinical Studies of Granules for Reconstitution

5.1 Introduction

The bioavailability and bioequivalence of a new formulation should be assessed to determine the rate and extent of drug release from the dosage form and the subsequent absorption into the plasma. The intricacies of bioequivalence clinical trials are underscored by the science-driven guidances that are established by the regulatory agencies. Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.[79] Two bioequivalent products are fundamentally assumed to offer therapeutic equivalence in terms of formulation performance, efficacy and safety.[81, 85] The statistical criterion for bioequivalence as issued by the Food and Drug Administration (FDA) requires that the 90 % confidence interval of the ratio of the average response for the test to the reference three-fold pharmacokinetic (PK) parameters on a log-transformed scale should fall within 80 % to 125 %.[79, 83] The statistical test for the determination of the 90 % confidence interval is the two one-sided t-test. The t-test assesses whether (i) the test product is clinically significantly less bioavailable compared to the reference listed product and (ii) the reference product is significantly less bioavailable compared to the test product.[81] The three-fold PK parameters that qualify

bioequivalence are (i) the maximum plasma concentration (C_{max}); (ii) the area under the plasma concentration-time curve from time zero to time t (AUC_{0-t}); and (iii) the area under the plasma concentration-time curve from time zero to time infinity ($AUC_{0-\infty}$). Other bioavailability PK parameters of interest are the time required to reach the maximum plasma concentration (t_{max}), the elimination half-life, and the terminal elimination rate constant.

To assure the safety, rights and well-being of human subjects, clinical trials are performed according to the Helsinki Declaration[89] on ethical principles in medical research as required by the local Institutional Review Board (IRB). Such principles include informed consent, subject confidentiality, adverse events reporting, and trained personnel should conduct the study. The regulatory guidances by the Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) are established to safeguard the rights and safety of subjects, and ensure Good Clinical Practice (GCP) or quality standards for ethical and scientific means of conducting clinical studies in human subjects. [79, 90] Hence, a clinical study protocol designed to follow ICH-GCP will be developed and submitted for approval. The goal is to test the hypothesis that lab-developed fixed-dose combination granules for reconstitution comprising lamivudine, zidovudine and nevirapine will be bioequivalent to the co-administered single entities of the reference products.

To test the hypothesis, the following specific aims were proposed.

Specific Aim 1: To develop a clinical research proposal and submit for approval from the Institutional Review Board. Approval from the other regulatory authorities with jurisdiction over the study will be obtained.

Specific Aim 2: To assess the bioavailability and bioequivalence of the granules for reconstitution in adult cohorts. The clinical batch will be used for the clinical study in adult cohorts at the Bowen University Teaching Hospital (BUTH) in Nigeria.

5.2 Experimental

Materials: Ammonium acetate, potassium dihydrogen phosphate (KH_2PO_4), triethylamine (TEA), HPLC grade methanol and acetonitrile, heparinized BD vacutainer tubes, cryogenic vials (Fisher Scientific, Hanover Park, IL); Aprobarbital (Alltech, Deerfield, IL); Drug free human plasma (Plasmacare Inc., Pittsburgh, PA); 2ml micro-centrifuge tubes (VWR, West Chester, PA).

5.2.1 Clinical Research Protocol

A clinical research proposal designed under Good Clinical Principles guidances and justifying the significance of the study was submitted to the following institutions for approval: (i) Institutional review Board – Duquesne University, Pittsburgh PA, protocol number 09-56.

(ii) Institutional review Board – Roosevelt University, Schaumburg, IL, protocol number 2010-73.

(iii) Ethics Board – Bowen University Teaching Hospital, Ogbomosho, Nigeria. The submission included (i) Transmittal form, (ii) Advertisement for subject recruitment, (iii) Informed consent; and (iv) National Institute of Health (NIH) training certificate for the conduct of studies in human subjects. See Appendices 5 and 6 for IRB approval letter and sample of the informed consent form.

In partnership and under Cooperative Research Agreement (CRDA) with a start-up company, Elim Pediatric Pharmaceuticals Inc, Investigational New Drug (IND) application was submitted to the FDA on FDA Form 1571. The rationale for the IND submission was that although all the three drugs being investigated in the study have been approved by the FDA for marketing, the granules for reconstitution is a new formulation without prior exposure in humans. Hence, an IND was required prior to testing. The IND consisted of (i) the protocols which had been approved by the IRB, (ii) investigator's brochure, (iii) chemistry, manufacturing, and control information (iv) pharmacology and toxicology information, and (v) previous human experience with the investigational drugs. Similar submission was made to the National Agency for Food and Drug Administration and Control (NAFDAC) in Nigeria.

Other research instruments developed were Case Report Form (CRF), Medical/Laboratory/Technical Procedures /Tests, Certificates of Analysis of Investigational Products, Master Randomization List, and Monitoring Visit Reports. Based on the lessons learned from the pilot study (chapter 4), several protocols were developed detailing every step. For instance, there were protocols for reconstituting the granules with water, product administration using oral syringe, centrifugation of blood and storage of plasma.

5.2.3 Bioavailability and Bioequivalence Clinical Trials

5.2.3.1 Study Design

The clinical phase was conducted as a joint project with Ms Anjali Joshi. The study protocol titled "Three-way Crossover Bioequivalence Study of Pediatric

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Formulations of Lamivudine/Zidovudine/Nevirapine" was designed and submitted to the regulatory authorities. The three-way crossover was designed such that it could be split into two two-way crossover designs. The products under investigation in the three arms of the study were (i) AZT/3TC/Nev 60/30/50 mg per 5 mL granules for reconstitution. (ii) AZT/3TC/Nev 60/30/50 mg per tablet for oral disintegration. (iii) Per-oral liquid formulations containing single entities of AZT, 3TC or Nev. The same drugs at the same doses were investigated; therefore teasing out the results into two two-way crossover design did not have a negative impact on the results. Hereafter, the clinical study will be referred to as two-way crossover study.

Two-way Crossover Study Design: The study was designed as an open label two-sequence two-period crossover design. The sequences were Test-Reference (TR) or Reference-Test (RT) and the dosing periods were I and II. Of the three drugs, nevirapine had the longest half-life of 45 hr for a single dose. Therefore a washout period of 14 days was included to account for over 7 half-lives. The 7 half-lives will ensure that at least 99% of the drug is eliminated before the dose is administered during period II.

5.2.3.2 Enrollment of Subjects

Announcement for Study: Advertisements announcing the study were posted at the Bowen University Teaching Hospital (BUTH) for two months prior to the commencement of the study to invite volunteers. The ICH guidance document E11 on

clinical investigation of medicinal products in the pediatric population suggests that for relative bioavailability comparison of pediatric formulations, adult subjects may be used.[91] The use of adults permits the initial assessment of pharmacokinetic parameters as well as the safety profile of the new formulation. Hence, healthy adult volunteers were invited to participate in the study.

Eligibility Criteria: The eligibility criteria included adults between 22 and 55 years; Healthy, determined on the basis of a pre-trial physical examination, medical history, electrocardiogram and the results of blood biochemistry and hematology tests; Subject is competent and willing to sign informed consent form voluntarily after being given all the detailed information about the study; Willing to be hospitalized for 24-hour intensive sampling period.

Exclusion Criteria: The exclusion criteria was hypersensitivity to study drugs; Abnormal laboratory biochemistry values; Smokers; Any clinically significant diseases or findings during the screening, medical history or physical examination that in the opinion of the investigator, may interfere with the study; Treatment with experimental drugs within 30 days prior to study entry.

Based on the above protocol, 44 adult cohorts were screened, and 11 subjects were disqualified from further continuation in the study. Twenty-four subjects were dosed and donated samples.

5.2.3.3 Dosing and Sample Collection

Following an overnight fast, the volunteers were dosed with either the reference drugs or test suspension. All test suspensions were freshly prepared and administered without being stored for dosing during the subsequent periods. The reference drug regimen consists of 12 mL of Epivir® (lamivudine) 10 mg/mL solution plus 24 ml of Retrovir® (zidovudine) 10 mg/mL syrup plus and 20 mL of Viramune® (nevirapine) of 10 mg/mL suspension. The test drug was 20 mL of reconstituted granules for suspension containing lamivudine 30mg/zidovudine 60 mg/nevirapine 50 mg per 5 mL of suspension. The doses were dispensed with oral syringes. The subjects were administered the doses together with 240 ml of water. The registered nurse in-charge of dosing checked the oral cavity of the volunteers to ensure the dose had been swallowed.

One venipuncture was made just before the dosing and an in-dwelling catheter inserted for blood sample collection during hospitalization for 24 hours. Fresh venipunctures were made for each ambulatory sample collection. The total number of venipunctures during the study period were 10 (5 venipunctures X 2 doses). 5 mL of blood samples were collected before dosing and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 12, 24, 48, 72, 96 hours after dosing. The total blood sample collection from each patient for the duration of the study was 150 mL (5 mL X 15 samples X 2 doses).

The blood samples collected in heparinized labeled tubes were centrifuged at room temperature for 15 minutes at 2700RPM and the plasma was transferred to labeled cryogenic vials and stored at -80 ^{o}C. The frozen plasma was shipped on dry ice from BUTH, the clinical site, to Duquesne University for HPLC-UV analysis.

5.2.3.4 Bioanalytical Assay

Preparation of Standard Solutions: Solutions containing 1mg/mL of the lamivudine or zidovudine was prepared by dissolving the weighed samples in deionized water. Solvent for nevirapine and aprobarbital (internal standard) was methanol:water 50/50 %v/v. Further dilutions were made using 25 mM potassium dihydrogen phosphate buffer. The aprobarbital was used as internal standard.

Liquid-Liquid Extraction: 200 μL of chilled acetonitrile was added to a micro-centrifuge tube. 376 μL of thawed plasma was added followed by 24 μL of the internal standard (aprobarbital) and 576 μL of chilled acetonitrile. The tube was vortexed for 3 min and centrifuged at 1200 g for 12 min at 4 ^{o}C. 800 μL of the supernatant was evaporated under nitrogen at 37 ^{o}C. The dry residue was reconstituted with 800 μL of the buffer component of the mobile phase (25 mM KH_2PO_4) and analyzed by HPLC-UV method.

Calibration Curves: The appropriate volumes of the standard solutions of lamivudine, zidovudine, nevirapine and aprobarbital were added together in a micro-centrifuge tube containing 376 μL of drug free plasma and extracted using the extraction procedure above.

Chromatographic Conditions: An isocratic HPLC method at a flow rate of 1 mL/min was used for the plasma analysis. The chromatographic system consisted of Waters 2695 Separations module, 996 PDA detector, and Empower 2 chromatographic software (Waters Corporation, Milford, MA). The chromatogram was monitored at 245 nm. The solvent system comprised of 25mM KH_2PO_4 (65%v/v) pH adjusted to 6.5 with

triethylamine and methanol (35%v/v) and Column used was Luna-C18 5µm 150 x 4.6 mm column (Phenomenex, Torrance, CA). The injection volume was 100 µL.

Validation: To establish the linearity and the range, replicate samples were used to construct a six-point calibration curve. The range was estimated based on the literature values for plasma analysis of the three drugs. Linearity was examined over the concentration range of lamivudine (100-2400ng/mL), zidovudine (100-2000ng/mL), and nevirapine (100-3000µg/mL). The data was fitted using the least sum of squares method. Precision and accuracy were determined by analyzing replicate samples at low, medium and high levels of the calibration curve. Precision was computed as percent relative standard deviation (RSD) and accuracy was computed as percent relative recovery. Inter-day reproducibility was examined by repeating the procedure using independent samples on three separate days. The data was analyzed using two-way ANOVA.

5.2.3.5 Pharmacokinetic and Statistical Analysis

The peak areas obtained from the chromatograms were used to construct plasma concentration time profiles. The pharmacokinetic parameters were the primary study endpoints and these were determined by non-compartmental analysis using WinNonlin software (Pharsight Corporation, Sunnyvale, CA). The maximum plasma concentration ($C_{max,}$) and the time to attain maximum concentration (t_{max}) were obtained directly from the graphs. The area under curve of plasma concentration-time curves (AUC_{o-t}, $AUC_{o-\infty}$) were obtained by applying trapezoidal rule. The bioequivalence comparison between the tests and reference drugs was performed using the two one-sided test. The FDA statistical

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criterion for bioequivalence requires that the 90 % confidence interval of the ratio of the average response for the test to the reference three-fold pharmacokinetic parameters (C_{max}, AUC_{0-t}, $AUC_{0-\infty}$) on a log-transformed scale should fall within 80 % to 125 %.[79, 83]

5.3 Results and Discussion

5.3.2 Bioanalytical Assay

The chromatogram obtained showed well resolved peaks of lamivudine, zidovudine, nevirapine and the internal standard (Fig 5.3). This indicated the specificity of the isocratic HPLC-UV method. However, there were two large peaks from the blank plasma, probably due to heparinized vacutainer tubes used to collect the blood to prevent clotting. The HPLC-UV method was validated for range, precision, and accuracy. The linear range for lamivudine was 100 – 2400 ng/mL with an R^2 value of 0.9959±0.0028 (Table 5.1). The linear range for zidovudine and nevirapine were established over the ranges of 100 – 2000 ng/mL and 100 – 3000 ng/ml respectively with the corresponding R^2 values of 0.9945±0.0020 and 0.9946±0.0044 respectively. The linear ranges examined covered the estimated ranges of the plasma concentrations of the three drugs.

The intraday accuracy ranged from 97.86±2.41% to 100.71±0.47% and the intraday precision ranged from 0.47 5 to 2.46%. The interday accuracy and intermediate precision were evaluated using two-way ANOVA to determine whether the recoveries from the different concentration levels or the different days were significantly different. The significance level α was set at 0.05. The interday accuracy and intermediate precision for lamivudine were 98.87±1.88% and 1.90% respectively. The p-value was 0.0931, and Fcal (4.5560) was less than Fcrit (6.9443) indicating that there were no

significant differences between the assay values on different days. The interday accuracy and intermediate precision for zidovudine were 98.38±1.61% and 1.63% respectively. The p-value was 0.6474, and Fcal (0.4856) was less than Fcrit (6.9443) indicating that there were no significant differences between the assay values on different days. Similarly, the interday accuracy and intermediate precision for nevirapine were 97.57±1.88% and 1.93% respectively. The p-value was 0.9271, and Fcal (0.0772) was less than Fcrit (6.9443) indicating that there were no significant differences between the assay values on different days. The validated method was stable and produced consistent results within different concentration levels and between days and thus was suitable for application to the analysis of the plasma. Analysis of plasma samples stored at room temperature (mimicking HPLC run conditions) and at -20 $^\circ$C (Chapter 4) showed that the drugs were stable in the plasma.

Table 5.1 Validation parameters for the isocratic HLPC-UV method for plasma analysis

Parameter	Lam	Zid	Nev
Range (ng/mL)	100 - 2400	100 - 2000	100 - 3000
R^2	0.9959 (0.0028)	0.9945 (0.0020)	0.9946 (0.0044)
Intraday			
Accuracy (%)	100.71 (0.47)	99.27 (1.81)	97.86 (2.41)
Precision (%)	0.47	1.82	2.46
Interday			
Accuracy (%)	98.87 (1.88)	98.38 (1.61)	97.57 (1.88)
Int. Precision (%)	1.90	1.63	1.93
LOD (ng/mL)	22.20	29.20	12.35
LOQ (ng/mL)	66.59	87.61	37.06
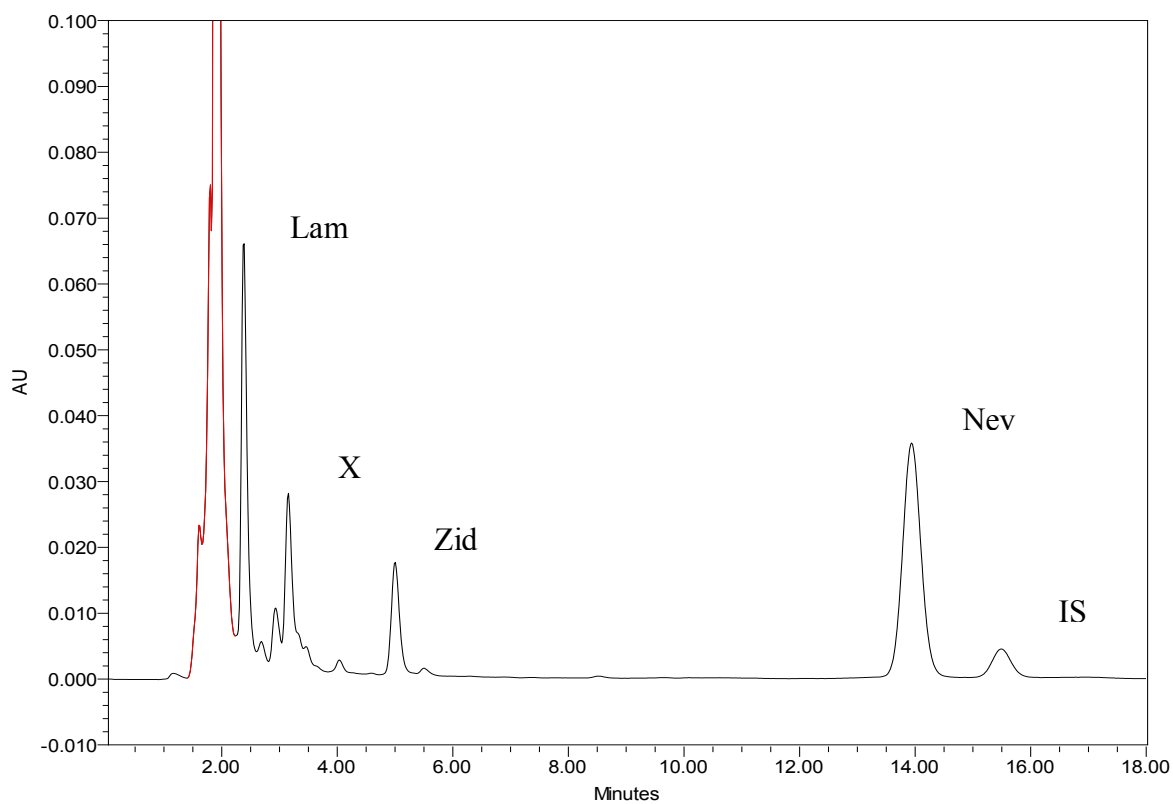
Fig 5.3 Chromatogram showing the peaks of a calibration curve standard. [Lamivudine (lam), zidovudine (zid), nevirapine (nev), aprobarbital (IS), and unknown (X)]
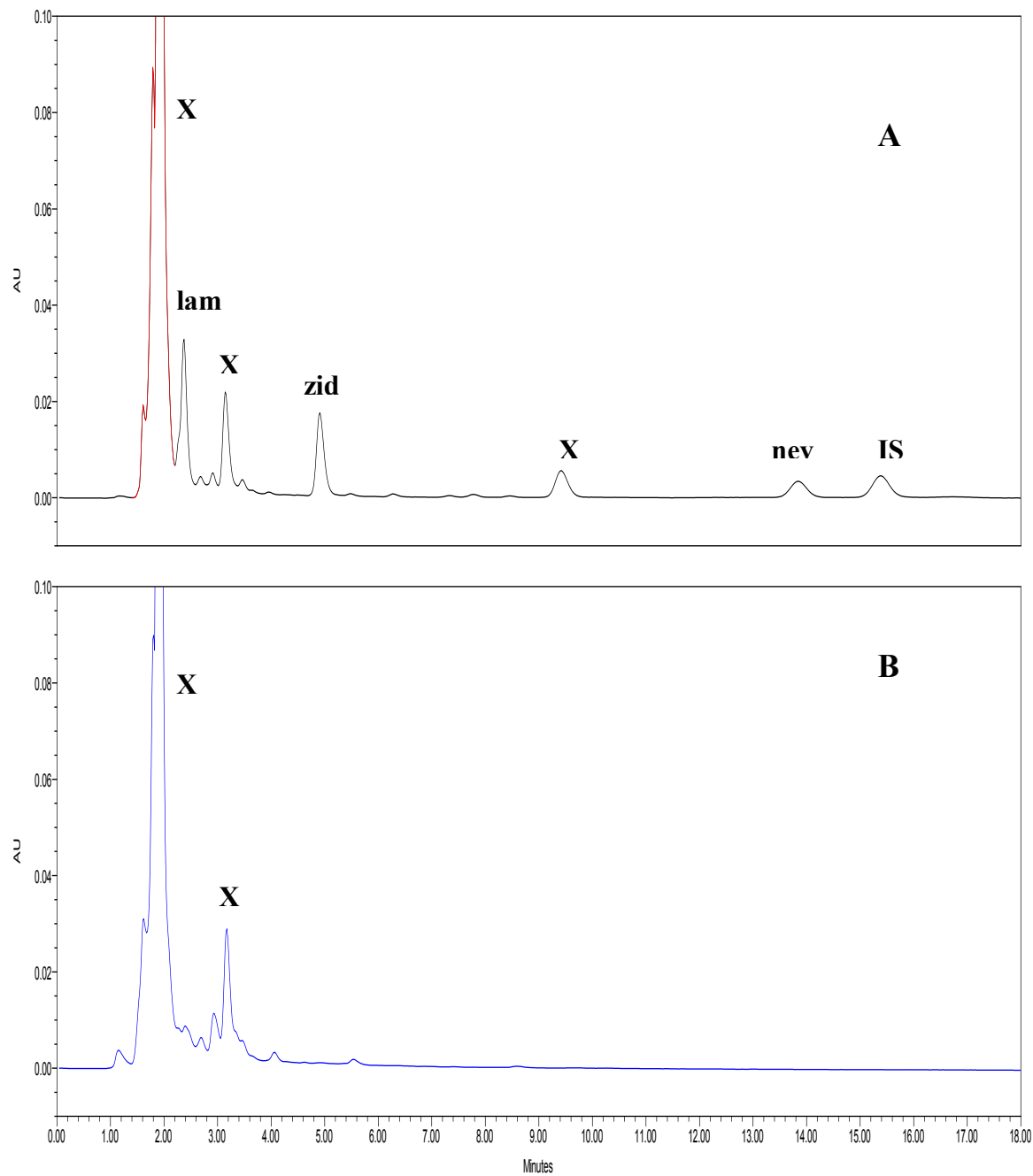
Fig 5.4 Chromatogram of (A) sample of plasma from a subject and (B) blank plasma.

[Lamivudine (lam), zidovudine (zid), nevirapine (nev), aprobarbital (IS), and unknown

(X)]

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5.3.3 Demographics and Safety Results

The study population was made up of 24 healthy adults: all the subjects were of black race. Forty-four adults aged 22-55 years were screened, 11 were disqualified based on the exclusion criteria. Of the eligible 33 cohort, 24 subjects were dosed and blood samples were collected from all the 24 subjects (Table 5.2). All the 24 subjects completed the study and did not miss any samples. Therefore all the 24 subjects were protocol compliant and data from these subjects was included in the pharmacokinetic and statistical analysis. There were no reports of severe adverse events by any of the study subjects indicating that the drugs were safe.

Table 5.2 Demographics of the study population.

Parameter	Group A	Group B
Males	12	6
Females	4	2
Weight (kg)	61.4	63.9
Age (yr)	31.7	33.5

5.3.4 Pharmacokinetic Analysis

The plasma samples were analyzed using the validated HPLC-UV method. The peak areas obtained from the chromatograms were converted to the ratio of the peak area of the drug to the peak area of the internal standard. The external calibration curves were then used to convert the peak area ratio into the concentration of the drugs. Thus plasma concentration time profiles were obtained. These data points were plotted as plasma

concentration-time curves using Phoenix WinNonlin 6.1 (Pharsight Corporation, Sunnyvale, CA). The non-compartmental pharmacokinetic analysis was performed using the log trapezoidal method to yield the area under the curve (AUC). The linear terminal portion of the curve was used to obtain the elimination rate constant and the elimination half-life. The maximum plasma concentration (C_{max}) and time to attain maximum plasma concentration (t_{max}) were read directly from the graphs. The mean plasma concentration profiles of the reference product and the test suspension were compared for lamivudine, zidovudine, and nevirapine.

Time to Maximum Plasma Concentration and Elimination Half-Life: The mean plasma concentration-time profiles for lamivudine in the test and reference products looked similar (Fig 5.5). The median (range) of t_{max} for lamivudine for test and reference products were the same 0.75 (0.5-1.5) hours. The elimination half-lives for the test and reference products were 5.14 h and 5.95 h respectively. With respect to zidovudine, the mean plasma concentration-time profiles for the test and reference products also looked similar (Fig 5.6). The median (range) of t_{max} for zidovudine for test and reference products were 0.75 (0.25-1.5) h and 0.5 (0.25-1.5) h respectively. The elimination half-life ($t_{1/2}$) was 3.01 h and 3.38 h for the test and reference products respectively. In the case of nevirapine, the mean plasma concentration-time profiles for the test and reference products also looked similar (Fig 5.7). The median (range) of t_{max} for nevirapine for test and reference products were 4.5 (0.75-12.0) h and 3.0 (1.5-6.0) h respectively. The $t_{1/2}$ was 58.4 h and 54.7 h for the test and reference products respectively. See the section on drug disposition below for the discussion.

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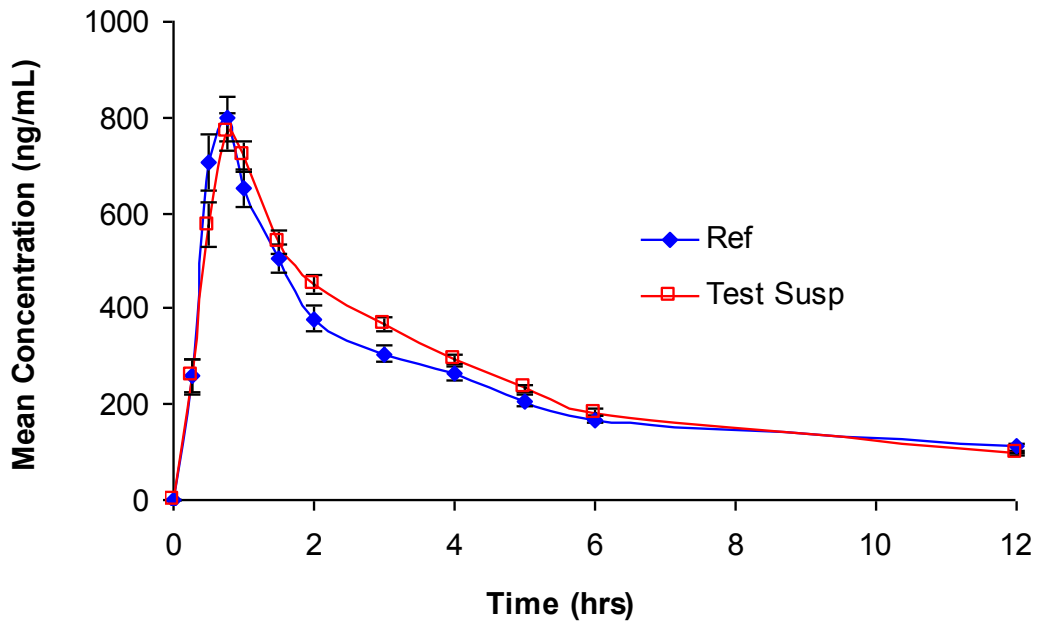
Fig 5.5 Mean (± standard error) plasma concentration-time profiles of lamivudine for the reference product (ref) and the test suspension (n=24).
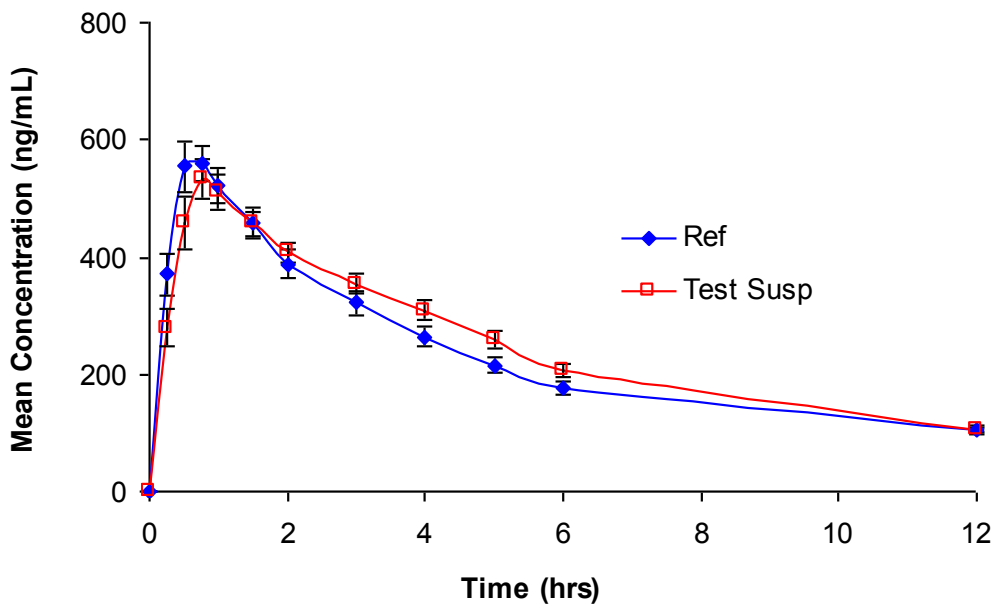
Fig 5.6 Mean (± standard error) plasma concentration-time profiles of zidovudine for the reference product (ref) and the test suspension (n=24).

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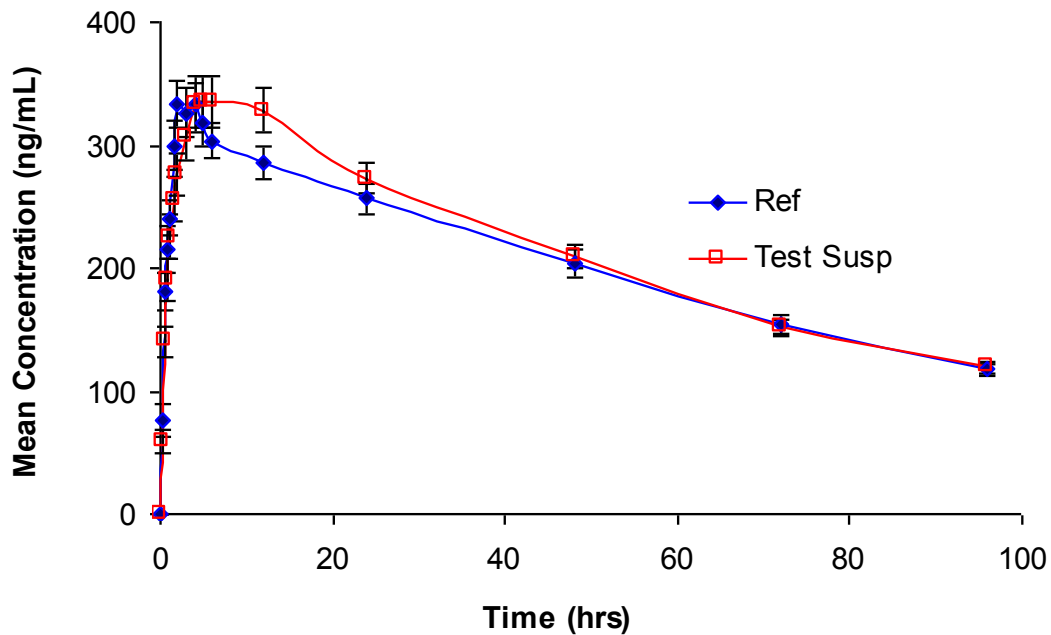
Fig 5.7 Mean (± standard error) plasma concentration-time profiles of nevirapine for the reference product (ref) and the test suspension (n=24).

Maximum Plasma Concentration and Area Under the Curve: The maximum plasma concentration (C_{max}) and the area under the curve (AUC) describe the exposure levels of the drugs in the blood. The C_{max} for lamivudine ranged 600.81-1146.99 ng/mL and 601.97-1304.99 ng/mL for the test and reference products respectively. The corresponding AUC_{0-t} for the test and reference lamivudine ranged 2322.56-3991.81 ng*h/mL and 1986.02-4045.76 ng*h/mL respectively. In the case of zidovudine, test and reference C_{max} ranged 411.65-856.41 ng/mL and 275.14-958.95 ng/mL respectively with the corresponding AUC_{0-t} 2040.03-3941.85 ng*h/mL and 1693.32-4121.39 ng*h/mL. With respect to nevirapine, the C_{max} for the test and reference product were 230.61-

704.53 ng/mL and 258.33-629.44 ng/mL respectively. The AUC_{0-t} ranged 13448.92-31218.68 ng*h/mL and 14260.02-29541.48 ng*h/mL for the test and reference nevirapine products respectively.

Drug Disposition and Special Patient Populations: The C_{max} for lamivudine and zidovudine were relatively lower compared to results from other studies due to the fact that the test dose administered (3TC=120 mg, AZT = 240 mg) was lower compared to the other doses studied elsewhere (3TC=150 mg, AZT = 300 mg).[13, 108, 108] The dose of the formulation was 3TC/AZT/Nev 30/60/50 mg/5mL, but this was scaled-up (3TC=120 mg/ AZT = 240 mg/ Nev = 200 mg) four times to the corresponding adult dose in order to increase the plasma concentration in adults and thereby increase the peak area detected by HPLC-UV method. Of the three drugs, nevirapine is the most toxic; therefore nevirapine dose was used as the upper limit. Since the ratio of the doses is fixed for the FDC granules (test product), the doses of lamivudine and zidovudine were lower than the typical adult doses.

The t_{max} and the elimination half-lives for the three drugs were comparable to data in the prescribing information for the innovator products[17, 109] or the results from other studies[13], although $t_{1/2}$ for nevirapine was slightly higher. The blood sampling was truncated at 96 h, but the half-life of single dose of nevirapine is 45 h.[109] The sampling was not extended long enough to capture up to three half-lives of nevirapine elimination. Thus the elimination rate constant captured for nevirapine could have been underestimated and this would have overestimated the half-life of nevirapine since half-life ($t_{1/2}$) is an inverse function (Eq 5.1) of the elimination rate constant (k).

$$t_{1/2} = \frac{0.693}{k} \qquad \text{Eq. 5.1}$$

Nevirapine Disposition: The pharmacokinetic parameters of nevirapine in children 3 months and older and adults are the same. Although the prescribing information for nevirapine (Viramune®)[109] indicates an absence of racial differences in the disposition of nevirapine, other studies state otherwise. Differences in nevirapine exposure levels in adults may be due to drug metabolism enzyme differences. Nevirapine is subject oxidative metabolism by CYP2B6 and CYP3A.[20] Cytochrome CYP2B6 516TT polymorphism has been shown to influence the plasma and intracellular concentrations and toxicity of NNRTIs (nevirapine and efavirenz) in HIV-infected patients.[110] Furthermore, Wyen et al demonstrated that CYP2B6 983T>C genotype leads to lower nevirapine metabolism in Black patients with an associated elevated plasma concentration.[20] The Black population has an increased variant allele frequency in this gene, which results in lower metabolic activity and higher nevirapine exposure. Similarly, higher nevirapine exposure level in Black children has also been reported.[107] However to facilitate accurate dosing by healthcare providers and caregivers, the manufacturers of nevirapine products, the World Health Organization, and other regulatory bodies do not emphasize this in dosing regimen. This has implications on the safety and toxicity in this sub-population. Hence there is a need for comparison of pharmacokinetic data from different Black populations (i.e. adults and children) to provide a basis for the development of pharmacogenomic database and prescribing information for nevirapine.

Drug Disposition in Paediatrics and HIV Patients: Lamivudine plus zidovudine plus nevirapine is recommended as a preferred first-line of antiretroviral therapy for treatment naïve infants (less than 12 months) and children 12 to 36 months.[1] Older treatment naïve children may also receive this combination. The dose of the FDC granules for reconstitution formulation was 3TC/AZT/Nev 30/60/50 mg/5mL. This ratio was recommended by the Paediatric Antiretroviral Working Group (PAWG) of World Health Organization recommended as an ideal dosing strength for priority antiretroviral product.[16, 111] This ratio based on body surface area (BSA) was developed by considering the clinical data from pediatric HIV/AIDS patients. This recommended dose was specifically for pediatric patients and does not require dose adjustment before administration to pediatric HIV/AIDS patients. In particular, the dose of nevirapine was increased, since the apparent clearance is higher in children due to a larger body surface area.[124,125] In this fixed-dose combination, the ratio of lamivudine to zidovudine to nevirapine is 30:60:50, whereas in adults, the ratio is 30:60:40 (150:300 mg:200 mg). This formulation is not recommended for adults since it will lead to nevirapine overexposure and toxicity. Hence, it can be concluded that this FDC granules for reconstitution is age-appropriate for pediatrics compared to the scored pediatric ARV tablets administered to children. The impact of under-dosing using adult FDC formulations cannot be over emphasized.

Drug-drug interactions: Nevirapine is principally metabolized by the liver via the cytochrome P450 isoenzymes CYP2B6 and CYP3A4, which it induces and is thus prone to numerous drug-drug interactions. Co-administration of drugs that are metabolized by

these enzyme systems may yield lower plasma concentrations. [109]   Drugs that induce these enzymes in turn can cause low plasma level of nevirapine. For instance, rifampin co-administered for tuberculosis, an opportunistic infection in HIV, may reduce the plasma concentration of nevirapine and reduce efficacy. On the other hand, enzyme inhibitors may cause increased plasma concentration of nevirapine and result in toxicity.

Lamivudine is excreted mainly unchanged in the urine and plasma levels are not affected by other drugs. However, zalcitabine may inhibit the intracellular phosphorylation of lamivudine into lamivudine triphosphate, and thus reduce efficacy.[17] In the case of zidovudine, stavudine and doxorubicin may antagonize the drug and reduce efficacy.[17] However, during this study, there were no reports of adverse events or drug-drug interactions since the subjects did not co- administer these drugs.

5.3.5 Statistical Analysis

Analysis of Variance: Analysis of variance test was performed on the C_{max}, AUC_{0-t}, $AUC_{0-\infty}$ to determine whether the model parameters had effect on the observed pharmacokinetic parameters. The ANOVA model (Eq. 5.2) tested was

$$Y_{ijk} = \mu + S_{i(k)} + P_j + F_{jk} + Q_k + e_{ijk} \qquad \text{Eq. 5.2}$$

where Y_{ijk} = pharmacokinetic response of the ith subject in the kth sequence at the jth period

μ = overall mean

P_j = fixed effect of the jth period; j=1, 2

Q_k = fixed effect of the sequence, k=1, 2

F_{jk} = fixed effect of formulation in the kth sequence which is administered in the jth period

S_{ik} = random effect of the ith subject nested in kth sequence

e_{ijk} = the within subject random error in observing Y_{ijk}

With the exception of subject with random variability, all the other parameters had fixed effects. The observed PK parameters were assumed normally distributed, independent, and the variance was homogeneous. The desirable result was that, all the model parameters will have no effect of the observed PK parameters. For the AUC_{0-t} for lamivudine, the ANOVA showed that the sequence (p-value= 0.6668), subject (p=0.8676), period (p=0.2289) and formulation (p=0.7303) were all not significant (Table 5.3). ANOVA of the C_{max} and $AUC_{0-\infty}$ for lamivudine revealed that all the parameters were not significant. Similar ANOVA for zidovudine and nevirapine showed that the model effects were not significant. It could be inferred that the observed differences in the PK parameters between test and reference products did not depend on the model parameters.

Table 5.3 ANOVA of the model parameters for AUC for lamivudine

Source	DF	SS	MS	F_stat	P_value
Sequence	1	0.0044	0.0044	0.1904	0.6668
Subject (Sequence)	22	0.5063	0.023	0.6168	0.8676
Formulation	1	0.0571	0.0571	1.5316	0.2289
Period	1	0.0045	0.0045	0.1219	0.7303
Error	22	0.8208	0.0373		

Two One-sided t-Tests of Bioequivalence: The bioequivalence between test and reference products were statistically tested using the two one-sided t-tests. The two one-sided tests hypotheses were

$$H_{01} : \mu_T - \mu_R \leq \theta_L \quad \text{vs.} \quad H_{a1} : \mu_T - \mu_R > \theta_L \qquad \text{Eq. 5.3}$$

and

$$H_{02} : \mu_T - \mu_R \geq \theta_U \quad \text{vs.} \quad H_{a2} : \mu_T - \mu_R < \theta_U \qquad \text{Eq. 5.4}$$

The first null hypothesis H_{01} (Eq. 5.3) was used to test whether the difference between the mean test (μ_T) and the reference (μ_R) PK parameter was less than the lower limit (θ_L) of the bioequivalence limit. In effect, H_{01} the tests if the test product was less bioavailable compared to the reference product. By rejecting H_{01}, it could be inferred that the difference between mean test and reference parameters was greater than the lower limit of the bioequivalence limit. The second null hypothesis H_{02} (Eq 5.4) tested whether the difference between the test and the reference PK parameter was greater than the upper

limit (θ_U) of the bioequivalence limit. This tests whether the test product was more

bioavailable compared to the reference product. By rejecting $H_{02,}$ then it can be inferred

that the difference between the test and the reference PK parameters is less than the upper

limit of the bioequivalence limit. In rejecting H_{01} and H_{02}, it is concluded the difference

between the test and the reference products is greater than the lower limit of the

bioequivalence limit, but less than the upper limit of the bioequivalence limit (Eq. 5.5),

hence the products are bioequivalent.

$$\theta_L < \mu_T - \mu_R < \theta_U \qquad \text{Eq. 5.5}$$

The Phoenix WinNonlin 6.1 software package uses the Anderson-Hauck test

statistic $(t_{AH})^{84}$ procedure to test the null hypotheses (Eq. 5.3 and Eq. 5.4) using Eq. 5.6

where n_1 and n_2 are the sample sizes of the test and reference products.

$$t_{AH} = \frac{\mu_T - \mu_R - (\theta_L + \theta_U)/2}{\sigma\sqrt{\dfrac{1}{n_1} + \dfrac{1}{n_2}}} \qquad \text{Eq. 5.6}$$

t_{AH} follows a non-central t-distribution with the non-centrality[84] parameter $\bar{\delta}$ is given by

$$\delta = \frac{\theta_L - \theta_U}{2\sigma\sqrt{\dfrac{1}{n_1} + \dfrac{1}{n_2}}} \qquad \text{Eq. 5.7}$$

The 90 % confidence interval at a significance level of 0.05 was used to establish the

bioequivalence intervals for the drugs. The lower and upper limits of the bioequivalence

limits are 80 – 125 %.[83] The C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for lamivudine, zidovudine, and nevirapine were transformed using natural log (ln), and the above steps used to calculate the bioequivalence interval for each parameter. Subsequently, the power of the analysis for each bioequivalence calculation was also calculated. The power is the probability of detecting at least 20 % difference between the least square means of the test and reference PK parameters.

Power = 1 – (probability of a type II error)

= probability of rejecting H_0 when H_a is true.

The 90 % confidence interval and the power of analysis for the PK parameters for lamivudine were calculated (Table 5.4). The 90 % confidence interval for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were 85.69 -102.54 %, 96.13 – 117.79 % and 82.63 – 106.72 % respectively. All these intervals within the 80 – 125 % bioequivalence limit. Therefore, lamivudine in the FDC granules for reconstitution and the reference lamivudine solution were bioequivalent.

The power of the analysis for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were 99.09 %, 97.41 % and 89.29% respectively. With respect to zidovudine, the 90 % confidence interval (power) for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were 83.33-109.15 % (86.32%), 99.94-124.54 % (95.43 %) and 98.22-122.43 % (92.90) respectively (Table 5.5). All these intervals were within the 80 – 125 % bioequivalence limit. Therefore, zidovudine in the test FDC granules and reference zidovudine syrup were bioequivalent. In the case of nevirapine, the 90 % confidence interval (power) for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ were 87.71-109.20 % (95.55%), 89.31-106.26 % (99.34 %) and 85.95-102.82 % (99.10) respectively (Table

5.6). The observed bioequivalence intervals for the PK parameters of nevirapine were within the 80 – 125 % bioequivalence limits. Hence, nevirapine in the FDC granules for reconstitution was bioequivalent with the nevirapine in the innovator suspension.

Therefore, combining the data for the three drugs, it is concluded that the fixed-dose combination granules for reconstitution was bioequivalent to the co-administered single component innovator products. The power for detecting at least 20 % difference between the test and reference products was greater than 86.32 %.

Table 5.4 Pharmacokinetic parameters, ratio, and 90 % confidence interval for lamivudine

Parameter	Test [T]	Ref [R]	Ratio[T/R]	90 % CI[a]	Power
C_{max} (ng/mL)[b]	828.13	883.47	93.74	85.69 -102.54	0.9909
AUC_{0-t} (ng*h/mL)[b]	3019.39	2837.52	106.41	96.13 – 117.79	0.9741
$AUC_{0-\infty}$ (ng*h/mL)[b]	3831.97	4080.57	93.91	82.63 – 106.72	0.8929
t_{max} (h)[c]	0.75 (0.50-1.50)	0.75 (0.50-1.50)			

a. CI: Confidence interval

b. Geometric least square mean

c. Median (range)

Table 5.5 Pharmacokinetic parameters, ratio, and 90 % confidence interval for zidovudine

Parameter	Test [T]	Ref [R]	Ratio[T/R]	90 % CI[a]	Power
C_{max} (ng/mL)[b]	575.44	603.38	95.37	83.33-109.15	0.8632
AUC_{0-t} (ng*h/mL)[b]	2935.26	2631.04	111.56	99.94-124.54	0.9543
$AUC_{0-\infty}$ (ng*h/mL)[b]	3941.48	3565.23	110.55	98.22-122.43	0.9290
t_{max} (h)[c]	0.75 (0.25-1.50)	0.50 (0.25-1.50)			

a. CI: Confidence interval

b. Geometric least square mean

c. Median (range)

Table 5.6 Pharmacokinetic parameters, ratio, and 90 % confidence interval for nevirapine

Parameter	Test [T]	Ref [R]	Ratio[T/R]	90 % CI[a]	Power
C_{max} (ng/mL)[b]	373.90	382.05	97.87	87.71-109.20	0.9555
AUC_{0-t} (ng*h/mL)[b]	19813.18	20338.82	97.42	89.31-106.26	0.9934
$AUC_{0-\infty}$ (ng*h/mL)[b]	30693.62	32649.68	94.01	85.95-102.82	0.9910
t_{max} (h)[c]	4.50 (0.75-12.0)	3.0 (1.5-6.0)			

a. CI: Confidence interval

b. Geometric least square mean

c. Median (range)

5.4 CONCLUSIONS

The validated HPLC-UV method for the bioanalytical assay had the requisite accuracy and precision for analyzing the plasma samples. Twenty-four healthy adults were enrolled and completed the clinical study. There were no reports of severe adverse events by any of the subjects enrolled in the study. The ANOVA tests showed that the model parameters – sequence, subject, period, and formulation – had no significant effect (p>0.05) on the observed mean C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for lamivudine, zidovudine and nevirapine. The observed 90 % confidence intervals for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for lamivudine, zidovudine and nevirapine were within the 80 – 125 % bioequivalence limits. Therefore, the fixed-dose granules for reconstitution and the co-administered reference products were bioequivalent.

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APPENDIX 1

SUMMARY

Granules and Suspension Formulation Development

The gradient high performance liquid chromatography method was validated for the pharmaceutical analysis of the drugs and the preservatives in the formulation. The method was suitable for the analysis of the fixed-dose combination granules and suspension without any interference from the formulation matrix.

There were no drug-drug interactions among the drugs and there were no drug-excipient interactions in the developed formulation. Sucralose was stable and compatible with the drug and suspending agent; therefore it could be used as high-intensity sweetener in oral powders for reconstitution into suspension. Furthermore, sucralose, the high intensity sweetener was preferred to neotame due to the lack of chromophores in the molecule and thus non-interference in HPLC-UV analysis.

The roller compaction process was suitable for producing granules of uniform potency as indicated by the results of content uniformity. Powder X-ray diffraction analysis showed that the roller compaction process did not induce any polymorphic transformations in the drugs. The roller compaction process parameters were optimized for ribbon strength, particle size distribution and granule flow. In addition, the

formulation optimization revealed that the Avicel RC591 and Aerosil 200 were important determinants of the viscosity of the suspension and the sedimentation rate.

The drugs and preservatives met the acceptance criteria for potency, and the granules had the desirable product characteristics. Dissolution conditions of 0.01N HCL medium and USP apparatus II at 100 rpm were identified to be discriminatory between granule formulations containing nevirapine anhydrous or nevirapine hemihydrate. The percent drug released for lamivudine, zidovudine, and nevirapine from all the three development batches and the clinical batch met the USP specifications for dissolution. The development batches and the GMP batch had the requisite potency and dissolution profiles.

Stability of the Granules for Reconstitution and the Reconstituted Suspension

The stability of the development batches of granules for reconstitution was evaluated at accelerated (40 $^{\circ}$C/75 %RH) and long-term (30 $^{\circ}$C/65 %RH) storage conditions. Lamivudine, zidovudine, and nevirapine were stable in the formulation during the six month period showing minimal degradation. The dissolution profiles of the drugs before and after stability studies did not show any significant changes. The potency and dissolution profiles met the USP criteria. In addition, after the stability testing of the clinical batch at long-term stability conditions, the potency and dissolution attributes met the USP specifications.

The reconstituted suspension retained its potency throughout the study period. The pH and apparent viscosity of the suspension did not change with time. In addition, the Herschel Bulkley model adequately described the shear thinning parameters of the

suspension. The flow index obtained from the Herschel Bulkley small increments with time while the yield stress and consistency index showed marginal reductions. To conclude, the granules for reconstitution and the reconstituted suspension exhibited both physical and chemical stability.

Pilot Bioequivalence Clinical Study of Fixed-Dose Combination Tablets

The validated HPLC-UV method had the requisite accuracy and precision for the analysis of the plasma samples. The marketed test and reference fixed-dose combination lamivudine 150 mg/zidovudine 300 mg antiretroviral tablets were not bioequivalent. The sample size for the pivotal bioequivalence studies should be increased to enhance the likelihood of a bioequivalence outcome. Finally, due to some administrative inconvenience and logistic problems, the clinical site in Lagos, Nigeria could not be used for the Phase I clinical study. Hence an alternate location was identified for the-Phase1 bioequivalence clinical trial.

Bioequivalence Phase I Clinical Study of Fixed-Dose Combination Granules for Reconstitution

The validated HPLC-UV method for the bioanalytical assay had the requisite accuracy and precision for analyzing the plasma samples. Twenty-four healthy adults were enrolled and completed the clinical study. There were no reports of severe adverse events by any of the subjects enrolled in the study. The ANOVA tests showed that the model parameters – sequence, subject, period, and formulation – had no significant effect ($p > 0.05$) on the observed mean C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for lamivudine, zidovudine and

nevirapine. The observed 90 % confidence intervals for C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ for lamivudine, zidovudine and nevirapine were within the $80 - 125$ % bioequivalence limits. Therefore, the fixed-dose granules for reconstitution and the co-administered reference products were bioequivalent.

Effect of overencapsulation of Tablets of a BCS class I and class II drugs

While encapsulation resulted in a lag time of 2-3 min in disintegration compared to the unencapsulated tablets, the disintegration and dissolution of propranolol and rofecoxib was the same whether tablets were encapsulated in DB capsules or standard gelatin capsules. It can thus be expected that *in vitro* drug release will not be influenced by the type of capsule used for overencapsulation in the double blind clinical study.

DB capsules was not used in the bioequivalence studies of the ARV drugs because it was determined by the clinical research team that on-site packing or overencapsulation (instead at the clinical batch manufacturing site) violates GMP and GCP requirements. The bioequivalence between the unencapsulated and encapsulated tablet is not inferred in this study, however, the in vivo disintegration data of unencapsulated and overencapsulated tablets reported by Wilding et al. suggest that the in vitro disintegration lag time is negligible in vivo (2). Nevertheless, it is important to use DB capsules for test and comparator dosage forms to correlate in vitro data especially for the high soluble drugs (BCS class I). Similarly, the presence or absence of backfill had no significant effect on either disintegration or dissolution. In effect, capsules offer an easy and inexpensive way of blinding clinical trials while posing no threat to studies where the performance of two drugs is compared. The use of DB-type capsules offers the added

value of an advanced design that allows for overencapsulation of tablets with larger diameter and makes blind breaking in clinical trial virtually impossible.

FUTURE DIRECTIONS

The FDC formulation of lamivudine, zidovudine and nevirapine was stable at 40 ^{o}C/75 RH and 30 ^{o}C/65 %RH for six months. Although potency reduced during the stability studies, the peaks due to the degradation products were not detectable. To resolve this anomaly, HPLC instrument coupled to a mass spectrometer detector could be used for the detection of the degradation products formed. The mass spectrometer has much higher sensitivity than the UV detector and will facilitate the detection and identification of the possible degradation products formed in the solid state. Alternatively, if the degradation product is evolved as a gas, then gas chromatography linked to a mass detector could be used for identification. In addition, long-term stability studies should be continued for the determination of the actual shelf-life of the product.

The Phase 1 bioequivalence study assessed the pharmacokinetics parameters of the FDC granules in adults. The next step is to assess the bioavailability and bioequivalence in pediatric subjects. This study in pediatric subjects can be designed to evaluate pharmacokinetic parameters and safety. In addition, formulation acceptability in pediatric subjects should be assessed. Since pediatric patients dislike unpleasant tasting medicine, there is a need to conduct a taste test prior to the bioavailability and bioequivalence study in children. The taste test may require training of the investigators as well as the volunteers.

APPENDIX 2

THE EFFECT OF OVERENCAPSULATION ON DISSOLUTION AND DISINTEGRATION

The objective is to evaluate the effect of overencapsulation on *in vitro* disintegration and dissolution of BCS class I and II drugs. Overencapsulation was proposed earlier in the project as a method of blinding the tablets for the Phase I clinical study.

A2.1 Overencapsulating Dosage Forms for Clinical Trials

In the past decade, the number of comparative clinical trials has increased considerably. To meet the challenging time lines for the provision of clinical material, encapsulating tablets in hard gelatin capsules or overencapsulation is used as a quick and low cost technique to blind investigators and volunteers in a clinical study. Overencapsulation is a process of enclosing a capsule or a tablet in a larger capsule, and frequently backfilling with a pellets, granules, or powder to minimize or prevent rattling. It eliminates the need to outsource a matching placebo and complex double dummy study design. While standard hard gelatin capsules are widely used for overencapsulation of double blind clinical trials, they might not always provide the features required for the broad variety of clinical trial designs and comparator products. To overcome these

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limitations for certain trials, specific double blind capsules (DB capsule) have been developed. These DB capsules are two-piece hard gelatin capsules uniquely designed for double-blind clinical trials, in which the capsule body is completely covered by the elongated design of the cap. This makes it virtually impossible to open the capsule without causing clearly visible damage, and thus alerting investigators of blind breaking, and bias. The wider diameter of DB capsules offers an advantage of relative ease of containment of several shapes and sizes of tablets while the shorter length facilitates ease of swallowing. Whole tablets can be directly filled into DB capsules and no breaking or grinding of tablets is needed, eliminating the concern for inaccurate dosage or modification of intended performance of drug. Standard two-piece hard gelatin capsules can also be used for blinding tablets for clinical trials; however, in this case, the smaller diameter (compared to DB capsules) limits the size of tablet that can be filled into the capsule.

In studies involving overencapsulation, the questions can be asked; will the overencapsulation affect parameters such as dissolution, absorption or bioavailability. In comparative pharmacokinetic trials, the encapsulation of esomeprazole multiple-unit-pellet-system, 40 mg tablets using hard gelatin capsules did not influence the rate (C_{max}) and extent (AUC) of absorption in a study involving 49 volunteers.[112] Other *in vivo* studies have shown the equivalence of in-vivo disintegration time using gamma scintigraphy and therapeutic effect onset time between encapsulated and nonencapsulated sumatriptan tablets.[113] Although some studies have shown that encapsulation may not affect *in vitro* dissolution studies, it could delay absorption and *in vivo* efficacy of drugs that are intended for fast onset of action such as demonstrated in an open trial design with

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patient scoring the headache pain response to a migraine drug.[114] Dissolution could also be affected by low capsule fill weight,[115] type of filler and the solubility of the drug.[116] Using the right capsule size and excipients (backfill) similar to those in the tablet formulation could reduce the effect on dissolution. The solubility of the drug should be factored into the blinding process.

The antiretroviral drugs lamivudine and zidovudine are BCS Class 1 compounds whereas nevirapine is a BCS class II compound. Propranolol, a highly soluble highly permeable BCS class I chiral drug marketed in the racemic mixture exists as polymorph forms I and II. Crystallization solvent, grinding and compression have been reported to cause changes of the crystalline state, causing differences in dissolution performance.[117, 118] Although propranolol is not hygroscopic, presence of moisture in the backfill could cause interparticle surface interaction that could somewhat affect performance of the encapsulated tablet. Rofecoxib, a low solubility and high permeability BCS class II drug is susceptible to oxidation and photolysis.[119] The influence of moisture on the disintegration and dissolution of the encapsulated tablets has not been studied for either class of drugs.

The model independent similarity factor (f_2) may be used to compare the dissolution profiles of two drug products (Eq. A1). This is a measurement of the similarity in the percent dissolution between the two curves. An f_2 greater than 50 implies two dissolution profiles are similar in the percent of drug dissolved per time (9). The FDA recommends using only one sample beyond 85% dissolution because the value of f_2 is sensitive to the number of sampling points used in the computation.[120, 121]

$$f_2 = 50 \bullet \log\{[1+(1/n)\sum_{t=1}^{n}(R_t - T_t)^2]^{-0.5} \bullet 100\} \qquad \text{Eq. A1}$$

R_t and T_t are dissolution values of the reference and test batches respectively at time t, and n is the number of points. The dissolution profiles can be determined using the traditional approach of high performance liquid chromatography (HPLC) method or the data can be acquired in "real time" using a fiber optic diode array probes or system (FOPS). The latter method was used by Bijlani and Adeyeye in the monitoring of dissolution of a multiparticulate ibuprofen system.[122] They reported that it was much faster and accurate than the HPLC method.

The objective of the study was to investigate the effect of overencapsulation of tablets of a BCS class I drug (propranolol) or class II drug (rofecoxib) on *in-vitro* disintegration and dissolution. DB® capsules were compared with Coni-Snap capsules in the presence or absence of backfill. The dissolution was carried out using the Delphian FOPS method. The long-term stability of the overencapsulated products was examined.

A2.2 Experimental

A2.2.1 Materials

Propranolol hydrochloride USP tablets (blue color), 20mg, and rofecoxib (VIOXX®) tablets (yellow color), 25mg, were purchased from Pliva Inc., East Hanover, NJ and Merck & Co. Inc., Whitehouse Station, NJ respectively. DB capsules (DBcaps® Size B) and standard gelatin capsules (Coni-Snap® size 00) were supplied by CAPSUGEL (Greenwood, SC). Microcrystalline cellulose (Avicel® PH102) was donated by FMC Biopolymer (Philadelphia, PA), while Foremost Farms (Baraboo, WI) supplied anhydrous lactose. Vankel dissolution apparatus and Delphian® fiber optic dissolution monitoring system from Delphian Inc. (Woburn, MA) were used.

A2.2.2. Experimental Design

Two blocks of 2 x 2 x 2 randomized full factorial design were used to determine the effect of three independent parameters (Table A2.1). Two levels i.e. 2 drug types (propranolol and rofecoxib), 2 capsule types (DBcaps® and Coni-Snap®); 2 filler levels (microcrystalline cellulose/lactose 1:1 mixture and no filler) were used in the design.

A2.2.3 Capsule Backfilling

A predetermined weight of a 1:1 physical mixture of anhydrous lactose:microcrystalline cellulose was filled into the capsule body. The tablet was placed on the backfill and the capsule cap was snapped on to lock. In cases where backfill was not required, only the tablet was filled into the capsule.

A2.2.4 Weight Variation

Twenty capsules from each batch were weighed and the average weight, standard deviation and %RSD computed. To remove bias, the hand filling of the capsules was blinded from the analyst who conducted the weight variation experiment. The samples were also coded.

Table A2.1 Blocks of 2 x 2 x 2 randomized full factorial design

Run	Batch	Drug	Capsule	Filler
1	1a	R	DB	None
2	2a	R	ConiS	MCC:Lactose
3	3a	P	ConiS	MCC:Lactose
4	4a	P	DB	None
5	5a	P	DB	MCC:Lactose
6	6a	R	DB	MCC:Lactose
7	7a	R	ConiS	None
8	8a	P	ConiS	None
9	1b	P	ConiS	None
10	2b	R	ConiS	MCC:Lactose
11	3b	R	DB	MCC:Lactose
12	4b	R	ConiS	None
13	5b	R	DB	None
14	6b	P	DB	None
15	7b	P	DB	MCC:Lactose
16	8b	P	ConiS	MCC:Lactose

A2.2.5 Disintegration Test

Vankel disintegration apparatus, running at 30 dips per minute (dpm) and simulated

gastric fluid (pH 1.2) without pepsin was used for disintegration test. Two randomly

selected capsules from a batch were tested at a time. This was repeated three times,

making a total of six capsules from each batch. The average D-time was then computed.

Two capsules were run at a time to allow video recorder camera (Kodak MDS 100) to

capture the process of capsule disintegration.

A2.2.6 Dissolution Analysis

The Vankel dissolution apparatus and Delphian RAINBOW^{TM} Dynamic Dissolution

Monitor System consisting of six photodiode array (PDA) probes, cell length 10mm,

were used. Simulated gastric fluid (pH 1.2) without pepsin, degassed at 40°C was used as

the medium at 37°C.

Linearity: A spectrophotometric linearity test was performed to test each individual probe

for both linearity and reproducibility. Using the blank dissolution medium, the fiber optic

system first acquired the 100% transmittance. This was followed by calibration involving

collecting the transmittance from three replicates of six respective standard solutions.

Percent difference and %RSD were calculated, and linearity determined.

The dissolution of propranolol was determined using the USP Apparatus I (basket

method) at 50 rpm in 900mL of simulated gastric fluid. A filtered solution of 20mg

propranolol in 900ml simulated gastric fluid was used as the standard. Peak absorbance wavelength and baseline correction wavelength were 288nm and 370nm respectively.

In the case of rofecoxib, the USP Apparatus II (paddle method), at 50 rpm, in 900mL of simulated gastric fluid was used. A filtered solution of 25 mg rofecoxib in 900ml simulated gastric fluid was used as the standard. Peak absorbance wavelength and baseline correction wavelength were 268 nm and 370 nm respectively. Replicates of eight batches were tested making a total of 16 runs.

A2.2.7 Statistical Analysis

The f2 similarity factor (Eq. 1) was used to compare the dissolution profiles. Six and 16 time points were used for propranolol and rofecoxib respectively, due to differences in the dissolution rates of the two drugs. In comparing the dissolution profiles for encapsulated and unencapsulated tablets, the data was normalized for a lag time of 2 min (13). The significance of effects of individual parameters and their interaction on disintegration time and dissolution were determined using least squares regression model (p-value < 0.05).

A2.3 Results and Discussion

A2.3.1 Weight Variation

The weights of the samples showed no randomness, the reason being that the contributors to the total sample weight (drug weight, capsule weight, and filler weight) were fixed with fairly little variation from the mean of the individual parameters. This uniformity was reflected by the low %RSD (0.49 – 1.31%), indicating the process of

encapsulation was accurate and efficient. Rofecoxib had a higher overall weight because the original average weight (200 mg) of the tablets was higher than that of propranolol (110 mg).

A2.3.2 Disintegration

Review of the video of the process of capsule disintegration did not reveal any differences between DB and standard gelatin capsules. There was an average time lag of 60 s before the capsules ruptured and the range was 34 s to 70 s. Some capsules did not rupture, instead the capsule cap separated from the body, exposing the content to the medium. The individual parameters, i.e., capsule type, drug type and filler showed no effect on disintegration D-time (Table A2.2). The effects due to interactions among parameters on disintegration time were also not significant.

The D-time for rofecoxib was slightly higher than for propranolol, possibly due to its lower solubility; the average D-times were 3.82 ± 0.28 and 3.69 ± 0.44 min respectively. Comparing the capsule effect, the average D-times were 3.85 ± 0.28 and 3.67 ± 0.43 min for DB and standard gelatin capsules respectively. However, these differences were not statistically significant ($p = 0.05$). In the case of the filler, average D-times were 3.75 ± 0.36 and 3.77 ± 0.39 min for presence and absence of filler respectively, an indication that the filler had no effect on disintegration time.

As expected, the D-time for encapsulated tablets was greater than that for plain tablets. D-times for unencapsulated tablets were 1.80 min and 1.93 min compared to 3.69 and 3.82 min for the overencapsulated propranolol and rofecoxib tablets respectively. This is due to time lag required for capsules to rupture and expose content to

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disintegration medium. All the samples passed USP specification of D-time of less than 30min. The residual plot (Fig A2.1) was random without any trend indicating that the parameters had no effect on disintegration time.
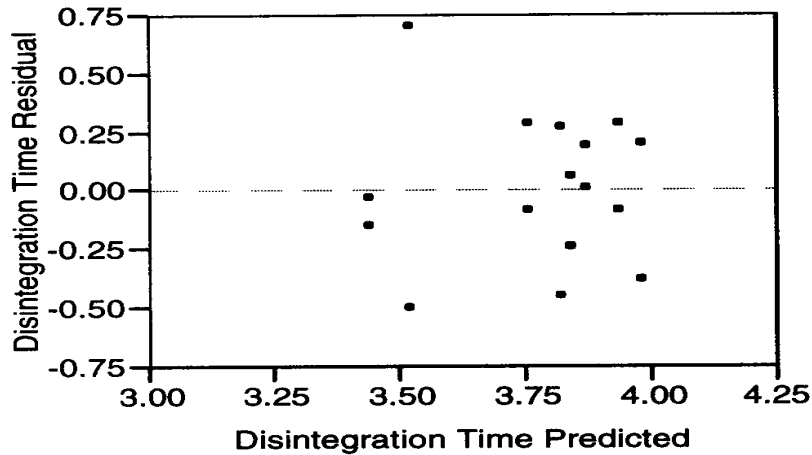
Fig A2.1 Residual plot of observed and predicted disintegration time

Table A2.2. Significance of effects of individual parameters and the interaction on disintegration time, irrespective of capsule type.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob>F
Drug	1	1	0.072900	0.4382	0.5246
Capsule	1	1	0.119025	0.7155	0.4195
Drug*Capsule	1	1	0.297025	1.7856	0.2143
Filler	1	1	0.000225	0.0014	0.9715
Drug*Filler	1	1	0.030625	0.1841	0.6780
Capsule*Filler	1	1	0.000900	0.0054	0.9430

"DF is degrees of freedom, F Ratio is F-statistic for testing that the effect is zero, and Nparm is the number of parameters associated with the effect".

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A2.3.3 Calibration of Fiber-Optic Probes

All the six Delphian fiber optic probes had good linearity (coefficient of determination R^2 0.9991±0.0004) for propranolol and rofecoxib (Fig A2.2). The % RSD of each probe was less than 2.0 % indicating reproducibility for the different concentration levels. The probes passed the percent difference between six probes.
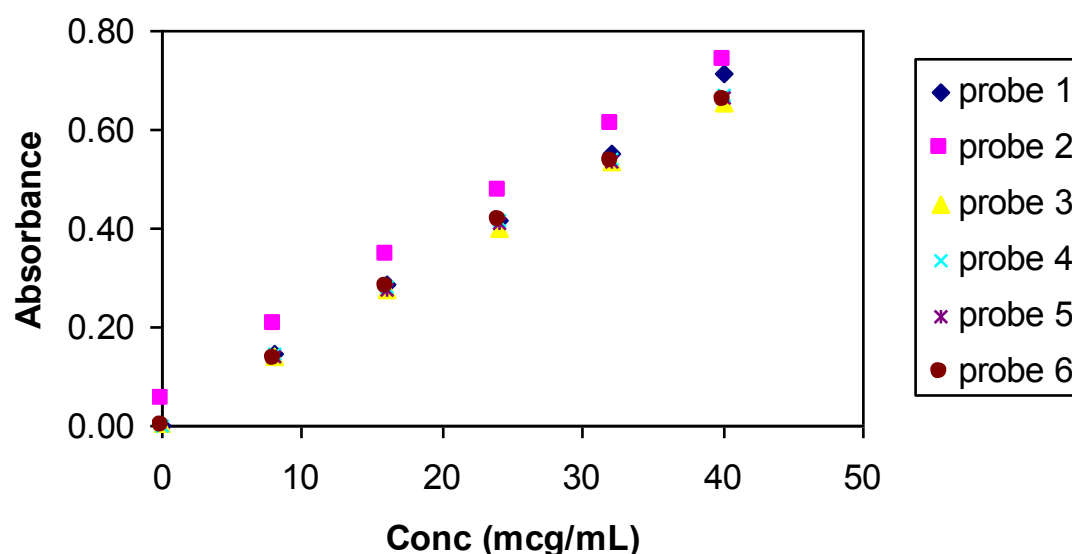
Fig A2.2 Linear range of the six probes.

A2.3.4 Dissolution

The time taken for 80 % of the drug to dissolve (T_{80}) was also used to statistically compare the results of dissolution profiles. From the significance of effects table (Table A2.3), it was observed that the drug used had an effect on T_{80} (p <0.0001). Although the interaction between drug and capsule appeared significant (p = 0.0219), this is of no formulation consequence considering the fact that the drugs belong to two different BCS classes.

The effects due to filler (p = 0.5716) and type of capsule (p = 0.7614) were not significant. The average T_{80} times were 14.39 and 13.80 min for presence and absence of filler respectively. The differences were not statistically significant. The same weight of backfill was used for all capsule types although the DBcaps size B capsules were smaller in volume than Coni-Snap size 00 capsules. Differences in T_{80} for the two capsules types were not significant, thus the volume of fill did not have an effect on dissolution profile.

Table A2.3 Significance of effects of individual parameters and their interaction on dissolution T_{80}, irrespective of capsule type.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Filler	1	1	1.3631	0.3447	0.5716
Capsule	1	1	0.3875	0.098	0.7614
Drug	1	1	838.8264	212.1287	<0.0001
Drug*Capsule	1	1	30.2775	7.6568	0.0219
Capsule*Filler	1	1	6.4643	1.6347	0.2330
Drug*Filler	1	1	0.5006	0.1266	0.7302

"DF is degrees of freedom, F Ratio is F-statistic for testing that the effect is zero, and Nparm is the number of parameters associated with the effect".

A2.3.5 F2 Similarity Factor

The dissolution profiles were also compared using the f_2 similarity factor. A lag time of 2 min was observed for the dissolution profiles of encapsulated and unencapsulated tablets, and this was incorporated into f_2 computations.[123] An f_2 greater

than 50 is an indication that two dissolution profiles are similar. Propranolol attained dissolution plateau faster than rofecoxib due to differences in solubility of the drugs in the dissolution medium. For propranolol tablets, the f_2 between standard gelatin and DB capsules was 60.67 indicating that the dissolution curves of the two were similar (Fig A2.3). The f_2 between unencapsulated propranolol tablets and DB capsules or standard gelatin capsules were 59.48 and 53.94 respectively. The similarity factor between rofecoxib tablets encapsulated in DB and CS capsules was 62.48 (Fig A2.4). The f_2 between unencapsulated rofecoxib tablets and DB capsules or unencapsulated rofecoxib tablets and standard gelatin capsules were 56.97 and 54.92 respectively.
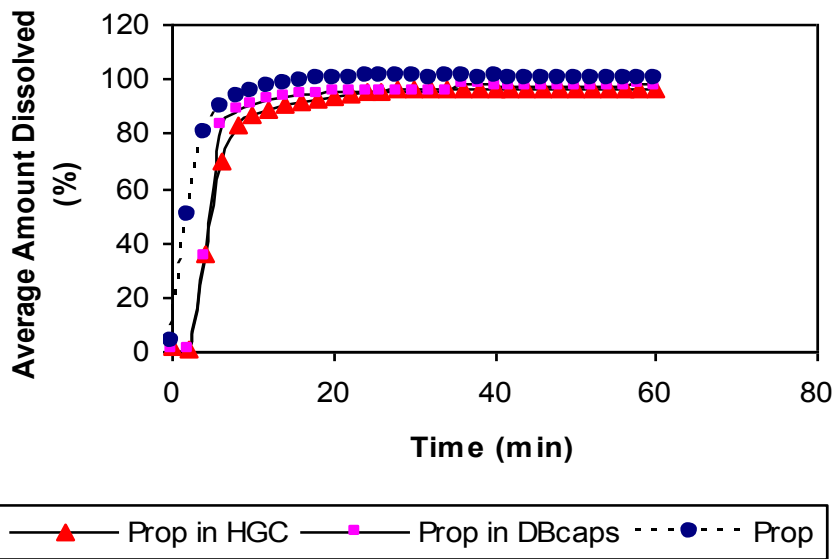
Fig A2.3 Dissolution profiles of propranolol tablets (prop), in Coni-Snap capsule (prop in CS) and in DBcaps (prop in DB).
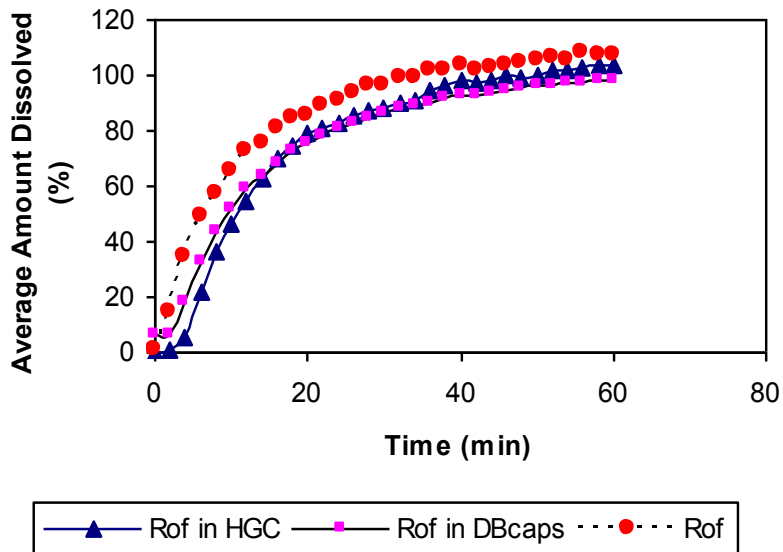
Fig A2.4 Dissolution profiles of rofecoxib tablets (Rof), in Coni-Snap capsule (Rof in CS) and in DBcaps (Rof in DB).

A2.3.6 Long-term Stability

On storing the encapsulated tablets at room temperature and humidity conditions for one year, there were no differences in the dissolution profiles of propranolol and rofecoxib tablets encapsulated using DB or standard gelatin capsules. This implies that the double walled DB capsules could be used for blinding drug candidates for a clinical study.

A2.4 Conclusions

While encapsulation resulted in a lag time of 2-3 min in disintegration compared to the unencapsulated tablets, the disintegration and dissolution of propranolol and rofecoxib was the same whether tablets were encapsulated in DB capsules or standard gelatin capsules. It can thus be expected that *in vitro* drug release will not be influenced by the type of capsule used for overencapsulation in the double blind clinical study.   The bioequivalence between the unencapsulated and encapsulated tablet is not inferred in this study, however, the in vivo disintegration data of unencapsulated and overencapsulated tablets reported by Wilding et al. suggest that the in vitro disintegration lag time is negligible in vivo (2). Nevertheless, it is important to use DB capsules for test and comparator dosage forms to correlate in vitro data especially for the high soluble drugs (BCS class I). Similarly, the presence or absence of backfill had no significant effect on either disintegration or dissolution. In effect, capsules offer an easy and inexpensive way of blinding clinical trials while posing no threat to studies where the performance of two drugs is compared. The use of DB-type capsules offers the added value of an advanced design that allows for overencapsulation of tablets with larger diameter and makes blind breaking in clinical trial virtually impossible.

Appendix 3

Approval Letter from Duquesne University IRB (Chapter 4)

DUQUESNE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
424 RANGOS BUILDING ◆ PITTSBURGH, PA 15282-0202

Dr. Paul Richer
Chair, Institutional Review Board
Phone (412) 396-6326 Fax (412) 396-5176
e-mail: richer@duq.edu

December 16, 2005

Mr. Fredrick Yirenkyi Esseku
Graduate School of Pharmaceutical Sciences
Department of Pharmaceutics
Duquesne University
Pittsburgh PA 15282

Re: **"Bioavailability/bioequivalence studies of generic and innovator lamivudine/zidovudine/nevirapine fixed dose combination (FDC) projects used in Nigeria"**
Protocol #05/103

Dear Mr. Esseku:

Thank you for submitting the revisions requested by the IRB.

After review by IRB representatives, Dr. Chris O'Neil and Dr. Monica Skomo, along with the entire board, the study is approved under the federal Common Rule, specifically 45-Federal Code of Regulations 46.101 and 46.111.

The Board wants to emphasize that you or a member of your team should read through consent forms with subjects, attending to medical terminology that might need to be explained in everyday terms.

Please remember that in accordance with federal regulations, you must produce two original signed copies of all consent forms, one for you and one for the person signing. We will enclose the first page of the consent form stamped with approval and expiration dates. Please use the stamped form as master for copies you use.

This approval and also the consent and assent forms will be renewed in one year as part of the IRB's continuing review.   The IRB will provide you with a questionnaire to complete and we will approve the forms for another year if you are still using them.

Approval Letter from Duquesne University IRB (Chapter 4)

If, prior to the annual review, you propose any changes in your procedure or consent process, you must inform the IRB Chair of those changes and wait for approval before implementing them. In addition, if any procedural complications or adverse effects on subjects are discovered before the annual review, they immediately must be reported to the IRB Chair before proceeding with the study.

When the study is complete, please provide the IRB with a summary, approximately one page. Often the completed study's Abstract suffices. Please keep a copy of your research records, other than those you have agreed to destroy for confidentiality, over a period of three years after the study's completion.

Thank you for contributing to Duquesne's research endeavors.

If you have any questions, feel free to contact me at any time.

Sincerely yours,

Paul Richer
Chair, IRB
Duquesne University

C: Dr. Chris O'Neil
       Dr. Monica Skomo
       Dr. Christiana Adeyeye
       IRB Records

Appendix 4

Approved Consent Form (Chapter 4)

DUQUESNE UNIVERSITY

600 FORBES AVENUE ♦ PITTSBURGH, PA 15282

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE:	Bioavailability/Bioequivalence Studies of Generic and Innovator Lamivudine/Zidovudine/Nevirapine Fixed Dose Combination (FDC) Products Used in Nigeria
INVESTIGATOR:	Fredrick Yirenkyi Esseku Graduate School of Pharmaceutical Sciences Department of Pharmaceutics Duquesne University, Pittsburgh PA 15282, 412-361-2381
ADVISOR:	Professor Moji Christianah Adeyeye Division of Pharmaceutical Sciences School of Pharmacy Duquesne University Pittsburgh, PA 15228, 412-396-5133
SOURCE OF SUPPORT:	This study is being performed as partial fulfillment of the requirements for the Masters degree in Pharmaceutics at Duquesne University, and, is partially supported by a grant from the Fulbright Organization and Center for International Exchange of Scholars (CIES)
PURPOSE:	You are being asked to participate in a research project that seeks to investigate the similarity between generic and brand name drugs (lamivudine/zidovudine/nevirapine) used to treat AIDS patients. The research is neither a treatment study nor a diagnostic study for HIV/AIDS. If qualified, you will be invited to the laboratory of the Clinical Pharmacy Department, Faculty of Pharmacy where you will be given the drugs to take as directed. The drugs are approved by Nigeria's National Agency for Food and Drug Administration Control (NAFDAC) and are commercially available in pharmacy stores.

DUQUESNE UNIVERSITY
INSTITUTIONAL REVIEW BOARD

APPROVAL DATE: 12-16-05
EXPIRATION DATE: 12-16-06

Following administration of the drugs by swallowing, five (5) ml of your blood will be drawn through a small catheter placed in a vein in one arm at the following specified intervals: just before dosing and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 48 & 72 hr post dose (17 samples making a total of 85 ml will be collected in three days). Sampling will be repeated on two other days at 14 days intervals to provide a total of 51 samples (255 ml). A catheter will be inserted in your arm at times 0, 24, 48 and 72 hours. A total of 4 vein punctures using 4 catheters will be made during each study period adding up to 12 vein punctures through out the study. Volunteers may leave after the 12 hr sampling and return at the later times for sampling. You will be required to stay in the clinic and not allowed to eat any food except the one provided at the clinic. This will be repeated on two other days when you will be asked to take either the generic or branded product. In addition, you will be asked to allow Professor Adeyeye, Professor Igwilo and Dr. Silva to interview you.

These are the only requests that will be made of you.

Approved Consent Form (Chapter 4)

RISKS AND BENEFITS:
You will be asked to do a free blood screening to determine if you can participate in the study. Exclusion criteria includes age range 18-45 years, not pregnant, and not mentally impaired among other conditions as listed in the questionnaire. This will be followed by confidential laboratory tests for: hemoglobin concentration, neutrophhil and platelet count, liver function (aspartate aminotransferase, alanine aminotransferase), renal function (creatinine clearance). Volunteers with test results outside the population range or 10% within the extremes of the range would be excluded. Female volunteers will be screened for pregnancy (by urine test) prior to enrollment. Individuals with abnormal laboratory tests results would be notified and advised to seek medical attention. If you meet enrollment criteria, then you will be enrolled to take part in the study.

The effects of blood drawing are occasionally some pain, bleeding and/or bruising where the catheter was inserted. Occasionally the area around the wound may swell. You may feel some discomfort, such as nausea, headache, muscle aches, insomnia (sleeplessness) and fatigue as side effects from the drugs. You can take Panadol® (acetaminophen) if you experience these effects. Panadol® is ordinarily used to treat minor aches and pains. However, you should let us know if this is the case. Gastrointestinal effects such as nausea, vomiting and abdominal pain may also occur with the use of the drugs. Skin rash may also occur but it may not be severe. If it is, you should contact the doctor to whom you will be directed.

COMPENSATION:
You will be paid 4000 Naira (USD 28) for the entire study. 1500 Naira will be paid at the end of the first day and the remaining 2500 Naira will be paid at the end of the last sampling on the last day of the study. However, participation in the project will require no monetary cost to you. An envelope is provided for return of your response to the investigator.

CONFIDENTIALITY:
Identifying data will be kept by investigators, but will not be linked to any survey or research instruments i.e. your name will never appear on any survey or research instruments. Volunteers would be identified by unique numbers known only to investigators. No identity will be made in the data analysis. All written materials and consent forms will be stored in a locked file in the researcher's home. Your response(s) will only appear in statistical data summaries. All materials will be destroyed by shredding two years after the completion of the research.

RIGHT TO WITHDRAW:
You are under no obligation to participate in this study. You are free to withdraw your consent to participate at any time.

SUMMARY OF RESULTS:
A summary of the results of this research will be supplied to you, at no cost, upon request.

VOLUNTARY CONSENT:
I have read the above statements and understand what is being requested of me. I also understand that my participation is voluntary and that I am free to withdraw my consent at any time, for any reason. On these terms, I certify that I am willing to participate in this research project.

I understand that should I have any further questions about my participation in this study, I may call

2 of 3

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Approved Consent Form (Chapter 4)

Moji Christianah Adeyeye, Ph.D
441 Mellon Hall
Division of Pharmaceutical Sciences
School of Pharmacy
Duquesne University Pittsburgh, PA 15228 USA
412-396-5133
adeyeyechri@duq.edu

Department of Pharmaceutics and Pharmaceutical Technology
Faculty of Pharmacy University of Lagos, Idi-Araba, Lagos, Nigeria
0803-664-4581

OR
Professor Fola Tayo
Chair of Institutional Review Board, Faculty of Pharmacy
Department of Clinical Sciences, Faculty of Pharmacy
University of Lagos
Idi-Araba, Lagos, Nigeria
Tel: 0803-335-8444

OR
Paul Richer, Ph.D
Chair of the Duquesne University Institutional Review Board
Office of Research
424 Health Science Bldg
Duquesne University Pittsburgh, PA, 15228 USA
412-396-6326
richer@duq.edu

_____   _____
Participant's Signature   Date

_____   _____
Researcher's Signature   Date

Appendix 5

Approval Letter from Duquesne University IRB (Chapter 5)

DUQUESNE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

424 RANGOS BUILDING ♦ PITTSBURGH, PA 15282-0202

Dr. Paul Richer
Chair, Institutional Review Board
Human Protections Administrator
Phone (412) 396-6326 Fax (412) 396-5176
e-mail: richer@duq.edu

July 8, 2009

Re: Three-way crossover bioequivalence study of pediatric formulations of lamivudine/zidovudine/nevirapine using adult volunteers (Protocol #09-56)

Mr. Frederick Esseku
School of Pharmacy
422 Mellon Hall
Duquesne University
Pittsburgh PA 15282

Dear Mr. Esseku:

Thank you for submitting the research proposal and revisions to the IRB.

After review by IRB members, Dr. Larry Block and Dr. Khalid Kamal, along with the entire Board, the study is approved under the federal Common Rule, specifically 45-Federal Code of Regulations #46.101 and 46.111.

The consent form is stamped with approval and expiration date. This approval will be renewed in one year as part of the IRB's continuing review. You will need to submit a progress report to the IRB at the address shown above. The report will involve supplying answers to a number of questions that will be sent to you. In addition, if you are still using assent/permission forms, you will need to obtain renewed approvals. In correspondence about this study, please refer to the protocol number shown after the title above.

If, prior to the annual review, you propose any changes in your procedure or consent process, you must inform the IRB Chair of those changes and wait for approval before implementing them. In addition, if any unanticipated problems or adverse effects on subjects are discovered before the annual review, they immediately must be reported to the IRB Chair before proceeding with the study.

When the study is complete, please provide the IRB with a summary, approximately one page. Often the completed study's Abstract suffices. Keep a copy of your research records, other than those you have agreed to destroy for confidentiality, over a period of five years after the study's completion.

If you have any questions, feel free to contact me at any time.

Sincerely yours,

Paul Richer, Ph.D.

C: Dr. Larry Block
       Dr. Khalid Kamal
       Dr. Christianah Adeyeye
       IRB Records

Appendix 6

Approved Consent Form (Chapter 5)

DUQUESNEUNIVERSITY

600 FORBES AVENUE ♦ PITTSBURGH, PA 15282

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE: **Three-Way Cross-over Bioequivalence Study of Pediatric Formulations of Lamivudine/Zidovudine/Nevirapine Using Adult Volunteers**

INVESTIGATOR: Fredrick Esseku & Anjali Joshi
Graduate School of Pharmaceutical Sciences
422 Mellon Hall
Duquesne University,
Pittsburgh, PA 15282
412.396.5279

ADVISOR: Moji Christianah Adeyeye, Ph.D.
Graduate School of Pharmaceutical Sciences
441 Mellon Hall
Duquesne University,
Pittsburgh, PA 15282412.396.5133

PURPOSE:
The purpose of the study is to investigate the bioavailability/bioequivalence (the way the drug is absorbed and distributed in the blood) of two pediatric drug products (tablet and reconstitutable suspension) of lamivudine/zidovudine/ nevirapine, compared to the innovator (brand) product in healthy adult volunteers. I am being asked to participate in this study as the volunteer. If I participate in this study, I will be given two test drugs and one reference drug and blood samples will be collected from me many times for analysis. These are the only requests that will be made. This study is being performed as partial fulfillment of the requirements for the doctoral degree in Pharmaceutics at Duquesne University by Anjali Joshi and Fredrick Esseku

RISKS AND BENEFITS:
If I participate in the study, I may be exposed to following risks:
The study medications (lamivudine, zidovudine and nevirapine) are approved by the US Food and Drug Administration. The drugs are also approved by the Nigeria National Agency for Food and Drug Administration and Control. The common side effects associated with the drugs are severe skin rash, headache, nausea, dizziness, muscle ache, fatigue, diarrhea, and cough.) The side effects do not occur frequently. Blood drawing may sometime cause some pain, swelling, bleeding and/or bruising where the catheter is inserted. In rare cases, blood clot or infections may also occur. I will be given Panadol® (acetaminophen) to relieve pain. If I experience any side effects I will immediately inform the medical doctor (Dr. Gbadero). If I experience severe rash, I will be withdrawn from the study and treated immediately with drugs for the rash under Dr. Gbadero's supervision.

Revised: September, 2008

Duquesne University
Institutional Review Board
Protocol #09-56
Approval Date: July 8, 2009
Expiration Date: July 8, 2010

Approved Consent Form (Chapter 5)

By participating in this study, I will be contributing to the development of drugs for treating HIV/AIDS in young children. Taking HIV/AIDS drugs will not make me ill with HIV/AIDS. Adults like me are being tested in order to know how well the drugs will perform in adults first before giving them to children.

ELIGIBILITY CRITERIA:
I am informed the following constitutes the eligibility criteria for participation in the study:
(a)Male and female, aged between 22 and 55 years, (b) Smokers and alcoholics will not be enrolled.
I will be asked to undergo physical examination and laboratory test for heart, kidney, and liver function. Other laboratory tests will also be performed to check for anemia, evidence of pregnancy (females), HIV/AIDS, and hepatitis B and C. I should be competent and willing to sign informed consent form voluntarily after being given all the detailed information about the study.

EXPERIMENTAL PROCEDURE:
After screening for eligibility, I will be assigned to one of the three treatment groups, A, B and C. Each group will be given at a period, one of the two test drugs or the reference drug in a random order.
For dosing and blood withdrawals, I will be hospitalized for 24 hrs. One needle attached to a catheter will be inserted in one arm for blood sample collection during hospitalization for 24 hours. I will be given the drug with 240ml of water. I will be provided with standard meal (lunch and dinner) during the day of dosing. Thereafter I will make daily visits to the hospital for six days for blood samples collection and will come back after a week for the next period. Fresh needle punctures will be made for each daily visit to the hospital for the sample collection. This will be repeated for the next two periods. I cannot consume alcohol, tobacco or caffeine containing products, herbal or other drugs 72 hours before and during the study. I should not eat 8 hours before taking the medication.
Details of the study which last for about 5 weeks are explained in table below.

Table: Treatment, dosing and blood sampling schedule for the entire study period

Treatment Period	Visit or Trip to Hospital	Blood Sampling Schedule	Drug Products
Period 1	Day 1: Dosing and Blood Sampling	5ml blood sample before dosing, and at 15 min, 30 min and 1, 1.5, 2, 3, 4, 5, 6, 12, 16 and 24hrs after dosing	Test Product 1 or Test Product 2 or Reference Product
	Daily visit on day 2,3,4,5,6,7 for Blood Sampling (1hr visit/per day)	1 sample each day	
Period 2	Day 15: Dosing and Blood Sampling	5ml blood sample before dosing, and at 15 min, 30 min and 1, 1.5, 2, 3, 4, 5, 6, 12, 16 and 24hrs after dosing	Test Product 1 or Test Product 2 or Reference Product
	Daily visit on day 16,17,18,19,20,21 for blood Sampling (1hr visit/per day)	1 sample each day	
Period 3	Day 29: Dosing and Blood Sampling	5ml blood sample before dosing, and at 15 min, 30 min and 1, 1.5, 2, 3, 4, 5, 6, 12, 16 and 24hrs after dosing	Test Product 1 or Test Product 2 or Reference Product
	Daily visit on day 30,31,32,33,34,35,36: for blood Sampling. (1hr visit/per day)	1 sample each day	
Total	21 Visits or Trips	57 blood draws, 5ml each (Total Blood Volume: 285ml) 21 needle punctures	

COMPENSATION:

Revised: September, 2008

Approved Consent Form (Chapter 5)

I will be paid $50 (or 10,000 Naira) and fed a standard diet during the 3-way crossover study ($50). I will be paid 40% of the total compensation at the start of the study, 30 % after second dose and the final 30% upon completion of the study However, participation in the project will require no monetary cost to me. I will be given a standard diet during hospitalization. If I withdraw at anytime during the study period, I will be paid based on the duration of my participation.

CONFIDENTIALITY:
My name as it appears on the questionnaire will be given an identification number and will not be disclosed to other people aside from Dr. Gbadero and Prof. Adeyeye. All other documents will have only the identification number. My name will not be used in any public document. I have been given the assurance that all written materials and the consent form will be stored in a safe place and locked up in the researcher's office. All materials will be destroyed by shredding five years after the completion of the research.

RIGHT TO WITHDRAW:
I am under no obligation to participate in this study. I am free to withdraw my consent to participate at any time without any restrictions.

SUMMARY OF RESULTS:
A summary of the results of this research may be supplied to me, at no cost, upon request.

VOLUNTARY CONSENT:
I have read the above statements and understand what is being requested of me. I also understand that my participation is voluntary and that I am free to withdraw my consent at any time, for any reason. On these terms, I certify that I am willing to participate in this research project

For any health related questions I may have, during or after the study, I may contact Dr. Dosu Gbadero at Baptist Medical Centre, Ogbomoso, Oyo State at 08035530907.

I understand that should I have any further questions about my participation in this study, during or after the study period, I may call:

Moji Christianah Adeyeye Ph.D.
Duquesne University, Pittsburgh, PA 15282
234-0802-395-6910

Dr. Adenike Adeniran
Chair, Baptist Medical Centre Ethics Board, Ogbomoso, Oyo State
234-08035530907

Dr. Paul Richer
Chair of the Duquesne University Institutional Review Board
0091.412.396.6326

_____   _____
Participant's Signature   Date

_____   _____
Researcher's Signature   Date

Revised: September, 2008

Duquesne University
Institutional Review Board
Protocol #09-56
Approval Date: July 8, 2009
Expiration Date: July 8, 2010

Approval Letter from Roosevelt University IRB (Chapter 5)

DATE: 6/25/10

TO: Moji Christianah Adeyeye

FROM: Judith Gouwens, Chair, Institutional Review Board

I am pleased to inform you that the Roosevelt University Institutional Review Board has considered and approved your study, "Three-way Crossover Bioequivalence, Mouth Disintegration and Taste Assessment Study of Pediatric Formulations of Lamivudine/Ziovudine/Nevirapine Using Adult Volunteers."

Your project number is 2010-73.

Please remember that you are required to re-submit your project for continuing review if your data collection or data analysis exceeds a period of one year from the above date. If any adverse event is associated with your study, it must be reported promptly to the IRB.

We wish you success in your project.

Members of the Institutional Review Board:

Judith Gouwens, Chair
LaVonne Downey
Gregory Hauser
Martin Jason
Kathleen Kane-Willis
Christa Marshall
Cami McBride
Josetta McLaughlin
Deborah Pavelka
Richard Ruby
Susan Torres-Harding
Janett Trubatch

Approval Letter from BUTH Ethics Board (Chapter 5)

Baptist Medical Centre

Department_____
P. O. Box 15, Tel: 038-720162, 720071, 720121
Mobile: 0803 410 2152, 0803 407 6631
OGBOMOSO, NIGERIA

email: nbcbmco@yahoo.co.uk
ogbomosobaptistmedicalcentre@yahoo.co.uk

21st May, 2009

Moji Christiana Adeyeye, PH.D.
Professor of Pharmaceutics and Manufacturing Science
441 Mellon Hall School of Pharmacy
Duquesne University
Phittsburg, PA 15282.

Dear Madam,

ETHICAL APPROVAL FOR STUDY TITLED : THREE-WAY CROSS OVER BIO EQUIVALENCE STUDY OF PAEDIATRIC FORMULATIONS OF LAMIVUDINE/ZIDOVUDINE/NEVIRAPINE.

The Ethical Committee of the Baptist Medical Centre, Ogbomoso reviewed the revised protocol for above study and are of the opinion that the issues warranting previous withholding of consent have been satisfactorily addressed.

The Committee hereby approves your request to proceed with the study. You should however inform the committee of any changes that may occur during the study before instituting such changes.

Yours Sincerely,

Dr. Adenike Adeniran
Chairman
BMC Ogbomoso Ethical Committee

"Jesus went about teaching, preaching and healing" (Matt. 4:23)
"...whatsoever ye do, do all to the glory of God" (I Cor. 10:31)