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UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

SCHOOL OF PHARMACY

**PROJECT TITLE:**

# *IN VITRO* AND *IN VIVO* KINETIC CHARACTERISTICS OF CHITOSAN-PECTIN-BASED MATRIX OF LEVODOPA AND CARBIDOPA

**BY**

EMELIA PRISCILLA IMBEAH

(10339983)

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# DECLARATION

I, Emelia Priscilla Imbeah, hereby declare that this project, aside duly cited literature, is the outcome of my own ideas under the supervision of Dr. Seth Kwabena Amponsah and Dr. Ofosua Adi-Dako.

Emelia Priscilla Imbeah

(Student)

Signature…………………………… Date: 15/12/2021

Dr. Seth Kwabena Amponsah

(Supervisor)

Signature………………………………… Date: 15/12/2021

Dr. Ofosua Adi-Dako

(Supervisor)

Signature………………………………… Date: 15/12/2021

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# ABSTRACT

**Background:** Parkinson's disease is a progressive neurodegenerative disorder that causes disability usually among the elderly. Levodopa remains the drug of choice in the management of Parkinson's disease. Levodopa is extensively metabolized in the periphery by aromatic amino acid decarboxylases; thus, it is routinely co-administered with carbidopa, a peripheral decarboxylase inhibitor. Although the aforementioned combination therapy is effective, there is variable absorption and fluctuating plasma levels of levodopa after oral administration. New oral levodopa plus carbidopa (levodopa/carbidopa) formulations are needed to overcome this irregular absorption and maintain near constant plasma concentrations.

**Aim**: The aim of this study was to evaluate the kinetic characteristics of chitosan-pectin-based multiparticulate matrix of levodopa/carbidopa, using *in vitro* and *in vivo* models.

**Methodology:** Pectin was extracted from cocoa pod husk with hot aqueous solution. Preparation of the chitosan-pectin-based matrix was done by the wet granulation. The formulations were evaluated for drug-excipient compatibility, drug content, flowability and precompression properties and *in vitro* release. In evaluating *in vivo* pharmacokinetic and biodistribution characteristics, male Sprague Dawley rats were administered either chitosan-pectin based matrix of levodopa/carbidopa, Sinemet CR (a controlled release formulation of levodopa/carbidopa) or levodopa/carbidopa immediate release powder (20/5 mg/kg) via the oral route every 12 hours. After the third dose, tail vein samples and brain tissues were taken at predetermined times. Pharmacokinetic parameters ($C_{max}$, $T_{max}$, AUC and $t_{1/2}$) of levodopa were estimated for the various treatment, levels of levodopa in rat brains were estimated and compared.

**Results:** The yield of cocoa pod husk pectin extracted with hot aqueous solution was 7.91%.

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The excipients for drug formulation were compatible with levodopa/carbidopa. The content of levodopa and carbidopa in the various formulations were within the acceptance criteria (not less than 90% and not more than 110% of the stated amount) with the exception of F5. There was controlled and sustained release of levodopa and carbidopa *in vitro*. *In vivo* pharmacokinetic studies showed kinetic profiles of levodopa/carbidopa multiparticulate matrix as compared to the conventional control release formulation. The $AUC_{0-24}$ for optimized levodopa/carbidopa multiparticulate matrix (F3: 484.98 ± 18.70; F4: 535.60 ± 33.04), and $C_{max}$ (F3: 36.28 ± 1.52; F4: 34.80 ± 2.19 μg/mL) were relatively higher than Sinemet CR ($AUC_{0-24}$ 262.84 ± 16.73 and $C_{max}$ 30.62 ± 3.37 μg/mL).

**Conclusion:** Findings from the study suggest that chitosan-pectin based matrix of levodopa/carbidopa may have the potential to control and maintain therapeutic concentrations of levodopa in circulation over a period of time.

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# DEDICATION

This work is dedicated to my dear husband for his immense support, encouragement, love and help throughout this Master's program.

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# ACKNOWLEDGEMENT

I am thankful to God for his grace, mercies, divine favor, guidance and protection.

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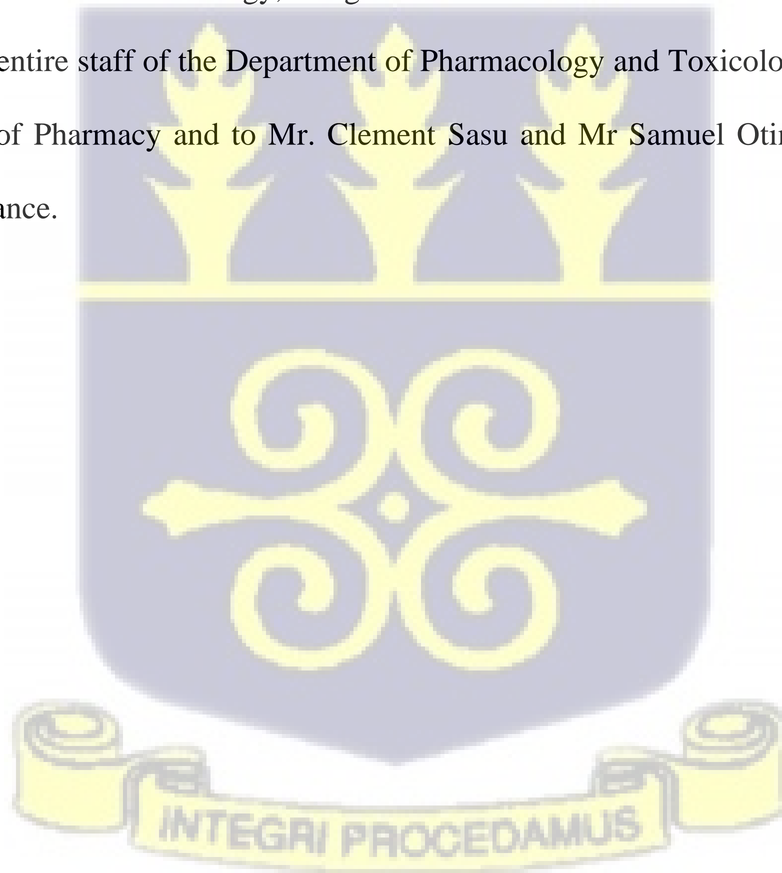

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| AUC | Area Under the Curve |
| BBB | Blood Brain Barrier |
| CD | Carbidopa |
| $C_{max}$ | Maximum Plasma Drug Concentration |
| CNS | Central Nervous System |
| COMT | Catechol-O-Methyl Transferase |
| CR | Controlled Release |
| DDI | Dopa Decarboxylase Inhibitors |
| EDTA | Ethylenediaminetetraacetic Acid |
| GPi | Internal Globus Pallidus |
| HCL | Hydrochloric acid |
| HPLC | High Performance Liquid Chromatography |
| $K_e$ | Elimination Rate Constant |
| LBs | Lewy Bodies |
| LD | Levodopa |
| LRRK2 | Leucine-rich Repeat Kinase 2 gene |
| MAO-B | Monoamine Oxidase-B |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |

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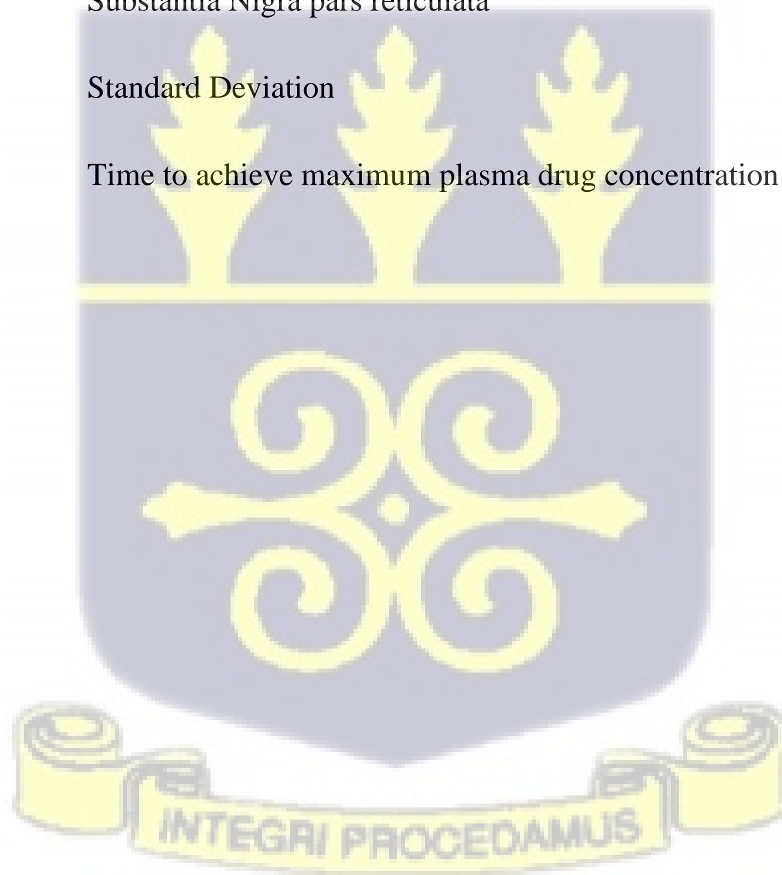
| | |
|---|---|
| MP | Microparticles |
| PD | Parkinson's Disease |
| PEC | Polyelectrolyte Complex |
| PRKN | Parkin gene |
| PINK1 | PTEN-Induced Putative Kinase 1 gene |
| PBS | Phosphate Buffered Saline |
| SD | Sprague-Dawley |
| SNCA | Alpha Synuclein |
| SNpr | Substantia Nigra pars reticulata |
| STDEV | Standard Deviation |
| $T_{max}$ | Time to achieve maximum plasma drug concentration |



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# CHAPTER ONE

## INTRODUCTION

### 1.1 Background

Parkinson's disease (PD) is one of the most common age-related neurodegenerative disorders (Tysnes & Storstein, 2017). PD is characterized by loss of dopaminergic neurons, and consequently low levels of dopamine in the brain (Beitz, 2014). Some cardinal symptoms associated with PD are: tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability, which occasionally can be given the acronym "TRAP". These symptoms begin gradually and worsen as the disease progresses (Kouli et al., 2018). Although less appreciated, non-motor symptoms such as dementia, autonomic dysfunction, sleep and sensory abnormalities, among others, also present in patients with PD.

Levodopa, a biological precursor of dopamine, is the drug of choice in the treatment of PD. After absorption and transport across the blood-brain barrier (BBB), levodopa is converted into dopamine by dopa decarboxylases (also known as aromatic amino acid decarboxylase), thus, restoring the level of dopamine in the depleted striatum (Kouli et al., 2018; Zahoor et al., 2018). When administered orally, levodopa exhibits low bioavailability (30%) and very low brain uptake due to its extensive metabolism by aromatic amino acid decarboxylase in peripheral circulation (Arisoy et al., 2020). Furthermore, the systemic conversion of levodopa to dopamine leads to unwanted side effects such as nausea, vomiting, cardiac arrhythmias, and hypotension (Salat & Tolosa, 2013). Hence, levodopa is routinely co-administered with dopa decarboxylase inhibitors such as carbidopa or benserazide.

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Usually, levodopa with carbidopa combination (levodopa/carbidopa) is effective. However, as the disease advances it becomes increasingly difficult to manage associated symptoms. Furthermore, motor fluctuations, that is, "end of dose wearing off" (periods with Parkinsonism), "on" (periods with near normal motor function and good antiparkinsonian effect) and "dyskinesia" (involuntary movement), may develop within months or years after initiating levodopa/carbidopa therapy (Salat & Tolosa, 2013). These motor complications are associated with the discontinuous or pulsatile stimulation of nigrostriatal dopaminergic neurons as a result of variable drug absorption and transit across the BBB, and the fluctuating plasma concentrations of levodopa due to its short half-life (Senek et al., 2017).

Following the discovery that intravenous levodopa infusions could reduce response fluctuations by maintaining constant and adequate plasma drug concentrations, controlled-release levodopa formulations were developed (Erni & Held, 1987; K. C. Yeh et al., 1989). Studies also suggest that maintaining constant dopamine levels in the central nervous system (CNS) lowers or prevents the emergence of motor fluctuations and dyskinesia in patients with Parkinson's disease (Gershanik & Jenner, 2012; Olanow et al, 2020; Wright & Waters, 2013). Thus, a number of studies have focused on the development of improved drug delivery systems in order to improve bioavailability of levodopa, maintain a near-constant plasma concentration of levodopa, and minimize unwanted motor complications of levodopa (Freitas et al., 2016; Garbayo et al., 2013). Among the various drug delivery systems includes the use of natural, biocompatible polymer matrices (Mohan et al., 2009; Ngwuluka et al., 2015; Sabel et al., 1990).

Polymers like chitosan and pectin are promising biopolymers and candidates for modified drug delivery systems (MDDS). Chitosan and pectin have good physicochemical characteristics, are biodegradable, readily available and possess minimal toxicity (Morris et al., 2010).

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Chitosan is a cationic polymer with outstanding mucoadhesive properties which makes it a perfect excipient for MDDS. The positively charged groups of chitosan readily interact with negatively charged mucous membranes of the gastrointestinal tract, thereby increasing adhesion, and thus improving contact time for drug absorption (Saikia et al., 2015). Furthermore, chitosan has been shown to have permeation enhancing properties (Bernkop-Schnürch & Dünnhaupt, 2012; Soliman et al 2014).

Despite afore-mentioned merits, chitosan tends to dissolve in acidic environment of the stomach, compromising its mucoadhesive capacity and resulting in an uncontrolled release of the active pharmaceutical ingredient. Studies suggest that in order to overcome this challenge, the structure of chitosan needs to be modified or combinations with other excipients have to be made (Bernkop-Schnürch and Dünnhaupt, 2012; Luo and Wang, 2014). One of the excipients that can be successfully combined with chitosan is pectin.

Pectin is a non-toxic, biodegradable, biocompatible, and an anionic polysaccharide present in the primary cell wall of plants (Cheikh et al., 2019; Morris et al., 2010). Recent studies have showed that coca pod husk could be a good source of pectins (Adi-Dako et al., 2018; Vriesmann et al. , 2012). Cocoa pod husk (CPH) pectin is extracted from pod husk waste after processing of cocoa beans. CPH pectin has the requisite physicochemical characteristics to be used as a multifunctional pharmaceutical excipient (Adi-Dako et al., 2016).

As an anionic polymer, pectin interacts with chitosan to form a polyelectrolyte complex (PEC). The intermolecular interaction between these polysaccharides of opposite charges has been applied in the design of drug delivery systems (García et al., 2015). Studies have shown that PECs have the ability of encapsulating drugs in the polymeric matrix at the molecular level, thereby enhancing the physicochemical and pharmacokinetic characteristics of drugs (Lu et al.,

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2010; Ngwuluka et al., 2015). Furthermore, pectin resists the action of digestive enzymes present in the upper part of the gastrointestinal tract and, in contrast with chitosan, is able to withstand low pH conditions (Cheikh et al., 2019). CPH pectin is reported to have the ability to swell at varying extents depending on the pH, ionic strength, and presence of salts in the medium. The swelling characteristics of CPH pectin makes it a suitable binder or matrix agent in controlled release formulations (Adi-Dako et al., 2018).

Based on available literature, it is possible that a combination of chitosan and pectin may have many advantages. Composites of chitosan and pectin have been used previously as carriers in drug delivery systems of diclofenac sodium, vancomycin, curcumin, among others (Cheikh et al., 2019; García et al., 2015; Hwang & Shin, 2018; Marudova et al., 2005; Marudova et al., 2004; Zambito & Di Colo, 2003). However, there is paucity of data on the *in vitro* and/or *in vivo* characteristics of chitosan-CPH pectin-based matrix of levodopa/carbidopa. Since the pharmacological effects of levodopa have been shown to correlate with its plasma concentration, this current study sought to formulate and evaluate the *in vitro* release and pharmacokinetic profile of chitosan and CPH-pectin-based composite of levodopa/carbidopa.

## 1.2 Problem Statement

Parkinson's disease (PD) is a neurodegenerative disease that affects one's ability to control the skeletal muscular system (Beitz, 2014). PD is estimated to affect over 6 million people worldwide (Dorsey et al., 2018). In Africa, reports suggest that prevalence ranges from 7/100,000 - 67/100,000 (Williams et al., 2018).

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Although the disease was discovered many years ago (1817), available therapies are unable to slowdown or stop the progression of the disease (Maiti et al., 2017). Therefore, treatment is aimed at relieving symptoms and improving the quality of life of patients. Levodopa, the gold standard in managing PD, may cause involuntary movements (dyskinesias) and motor fluctuations in patients (Salat & Tolosa, 2013). These motor complications have been associated with the discontinuous or pulsatile stimulation of dopaminergic neurons as a result of variable drug absorption and transit of levodopa across the BBB (Senek et al., 2017). It is widely believed that reducing the pulsatile stimulation of dopaminergic neurons lowers the risk of levodopa-induced motor complications (Schaeffer et al., 2014; Wright & Waters, 2013).

In order to improve the release profile and bioavailability of levodopa in formulations, alternative routes of administration (such as intravenous, transdermal, pulmonary and intraduodenal) have been explored (Salat & Tolosa, 2013; Sharma et al., 2014).

However, because of the chronic nature of the disease, oral administration remains the most convenient route (Ngwuluka et al., 2015). Several oral drug release systems including immediate release formulations, dual-release formulations, extended release, among others, have been developed, however, most of these are unable to provide constant and sustained delivery of levodopa (Freitas et al., 2016).

In an attempt to address the aforementioned challenges with PD management, this study sought to develop a sustained release formulation of levodopa and carbidopa using chitosan and CPH pectin as release modifiers.

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## 1.3 Justification

Recent studies have tried to explore new strategies to improve oral delivery of previously existing drugs for the management of PD (Dankyi et al., 2020; Margolesky & Singer, 2018; Ngwuluka et al., 2015; Senek et al., 2017). One of the novel approaches is the use of biopolymer matrices which act as carriers for sustained and extended release of drugs (Bukhary, Williams, & Orlu, 2020; L. N. M. Ribeiro et al., 2017).

Biopolymers like chitosan and pectin are readily available, eco-friendly immunocompatible, non-toxic and biodegradable. Also, their use as excipients for drug formulation is less expensive compared with synthetic polymers like polyglycolic acid (PGA), polylactic acid (PLA), among others.

Moreover, chitosan consists of several positively charged groups which readily interact with the negatively charged mucous membranes of the gastrointestinal tract, thereby increasing adhesion, and thus improving contact time for drug absorption (Saikia et al., 2015). Cocoa pod husk (CPH) pectin, an anionic polysaccharide, also has requisite physicochemical characteristics to be used as a multifunctional pharmaceutical excipient with remarkable properties (Adi-Dako et al., 2016).

As oppositely charged polymers, chitosan and pectin interact to form polyelectrolyte complex (PEC). Studies have shown that PECs have the ability to encapsulate drugs in a polymeric matrix at the molecular level thereby enhancing the physicochemical and pharmacokinetic characteristics of drugs (Lu et al., 2010; Ngwuluka et al., 2015). Chitosan-pectin based polyelectrolyte complexes have been employed in the design of modified drug delivery systems as well as site-specific drug delivery systems. In recent studies conducted by Cheikh et al., 2019 and Wang, 2017, chitosan-pectin based PECs were used to successfully encapsulate and sustain the release of aceclofenac and nisin, respectively.

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Furthermore, pectin resists the action of digestive enzymes present in the upper part of the gastrointestinal tract and, in contrast with chitosan, is able to withstand low pH conditions (Cheikh et al., 2019). Thus, combining chitosan and pectin in drug formulation could lead to products with enhanced characteristics.

There is currently no known study that has evaluated the *in-vitro* release profile and pharmacokinetic characteristics of chitosan-pectin based composites of levodopa/carbidopa for oral drug delivery. Therefore, in the present investigation, an attempt has been made to increase therapeutic efficacy, reduce frequency of administration, and improve patient compliance, by developing modified release formulation of levodopa/carbidopa using varying amounts of chitosan and CPH pectin composites as drug release modifiers.

## 1.4 Hypothesis

There is no difference in pharmacokinetic characteristics between chitosan-pectin-based matrix of levodopa/carbidopa and the levodopa/carbidopa immediate release formulation.

## 1.5 Aim

To evaluate the *in vitro* release and *in vivo* pharmacokinetic characteristics of chitosan-pectin-based matrix of levodopa/carbidopa.

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## 1.6 Specific Objectives

Below are the specific objectives of this research work:

1. To formulate chitosan-pectin based matrix of levodopa/carbidopa
2. To determine the physicochemical properties and *in vitro* release profile of chitosan-pectin based matrix of levodopa/carbidopa.
3. To estimate the pharmacokinetic and biodistribution profile of the chitosan-pectin-based matrix of levodopa/carbidopa using a rat model.

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# CHAPTER TWO

## LITERATURE REVIEW

## 2.1 Parkinson's Disease

### 2.1.1 History and Background

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by both motor and non-motor system manifestations (Beitz, 2014). Medically, the disease was first described as "Paralysis agitans (Shaking palsy)" by James Parkinson, an English surgeon, in 1817. Long before Parkinson made his observations, fragments of Parkinsonism had been captured in earlier descriptions. For instance, ancient Chinese sources and traditional Indian texts from as far as 1000 BC provided descriptions that suggest PD. Sylvius de la Boë touched on rest tremor and Sauvages described festination (Goetz, 2011). Parkinson in his paper (An Essay on the Shaking Palsy) captured the condition as: "involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured"

Later on, in the 19th century, Jean-Martin Charcot gave credit to James Parkinson by referring to the condition as "maladie de Parkinson" or Parkinson's disease. Charcot, however, was more thorough in his descriptions and was able to distinguish between rigidity, bradykinesia and muscle weakness associated with the disease (Obeso et al., 2017). Charcot's contribution to PD include not only his studies but also that of his students. He and his students described in full detail the arthritic changes, dysautonomia, and pain that can accompany Parkinson's disease. They also identified two prototypes, the tremorous and the rigid/akinetic form of PD.

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Ordenstein, one of Charcot's students, wrote his medical thesis on Parkinson's disease (1867) and introduced belladonna as a treatment option. Another student of his, Edouard Brissaud (1852-1909) was the first to describe midbrain lesions and propose damage to the subtantia nigra as the main anatomical change in Parkinson's disease (Goetz, 2011)

Based on his personal experience with 80 patients in the 1880s, William Gowers in his "Manual of Diseases of the Nervous System," correctly identified that men were more prone to PD than women and also detailed the joint deformities typical of the disease.

The findings of Brissaud, set the stage for other scientists like Trétiakoff and Foix and Nicolesco to further explore the pathologic studies of the midbrain in relationship to PD during the 1920s. These researchers observed that there were low levels of dopamine and degeneration of nerve cells in the *substantia nigra*. This made effective treatment of Parkinson's disease with dopamine agonist a possibility (Goetz, 2011).

Many clinicians from the 19th century to date, have attempted to further characterize and understand the nature of the disease, and have yielded incredible discoveries and advances in medical and surgical therapeutics (Obeso et al., 2017)

### 2.1.2 Epidemiology

PD is the most common age-related neurodegenerative disorder after Alzheimer's disease, affecting the elderly and middle aged (Tysnes & Storstein, 2017). Several epidemiological data on PD exist. However, direct comparison of prevalence estimates of PD is impossible because of the methodological differences between such studies. Generally, PD affects more than 1% of the population above 60 years, and nearly 4% of the population in older year groups (Tysnes & Storstein, 2017). About 80 % of individuals develop PD between the ages of 40 and 70 years.

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The average age at which PD presents is 60 years, with about 80 % of individuals developing the disorder between the ages of 40 and 70 years. However, young onset PD (developing symptoms before age 40 years) is reported in about 5 % of PD cases (Yao *et al.*, 2013).

### 2.1.3 Aetiology and Risk Factors

Parkinson's disease is multifactorial in nature. Environmental and genetic factors have been reported to play key roles. Age is by far the biggest risk factor for PD with a mean onset age of 60 years. Men have been shown to be at a greater risk of PD than women (Beitz, 2014). Although there are cross-cultural differences higher prevalence have been reported in Europe, South America, and North America compared with African, Arabic and Asian nations (Kalia & Lang, 2015).

#### *2.1.3.1 Genetics*

Studies have shown that about 10 -15% of PD patients have a very close relative who also has the disease and about 5% have Mendelian inheritance (Samii *et al.*, 2004). Also, the likelihood of an individual developing PD has been associated with poorly defined polygenic risk factors. Genes found to potentially cause PD are assigned a "PARK" name in the order of identification. Currently, 23 PARK genes have been linked to PD. Mutations in these PARK genes result in either autosomal recessive (e.g., PRKN, PINK1, and DJ-1) or autosomal dominant inheritance (e.g., SCNA, LRRK2, and VPS32) as shown in Table 1.1. However, the involvement of some of these genes in PD is still not well understood.

The most commonly inherited form of the disease is due to an autosomal dominant mutation in the leucine-rich repeat kinase 2 (LRRK2) protein. Mutations in LRRK2 have been associated with mitochondrial abnormalities as well as dysregulation of macro-autophagy. LRRK2

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mutations have also been implicated in sporadic and idiopathic PD. Although the exact mechanism by which LRRK2 is implicated in the pathogenesis of PD remains unclear, it is believed that LRRK2 kinase inhibitors may be beneficial for at least some forms of PD. Other genes that have been closely linked to PD are parkin (PRKN), PTEN-induced putative kinase 1 (PINK1), protein deglycase DJ-1 and alpha-synuclein (SNCA) (Davie, 2008; Lesage and Brice, 2009). More often than not, people carrying mutant forms of these genes end up developing PD.

Parkin is an E3 ubiquitin ligase. Mutations in this gene has been associated with an autosomal recessive form of Parkinson's disease. Mutations in PINK1, the protein responsible for recruiting Parkin to the mitochondrial membrane, also leads to an early-onset phenotype of Parkinson's disease. Protein deglycase DJ-1, also known as Parkinson disease protein 7, is a protein which in human beings is encoded by the PARK7 gene. Mutations in DJ-1 have been reported to cause an autosomal recessive, early-onset form of PD (Bonifati et al., 2003). A point mutation in the α-synuclein gene as well as duplications or triplications of the wildtyp*e* gene have been shown to cause neurodegeneration.

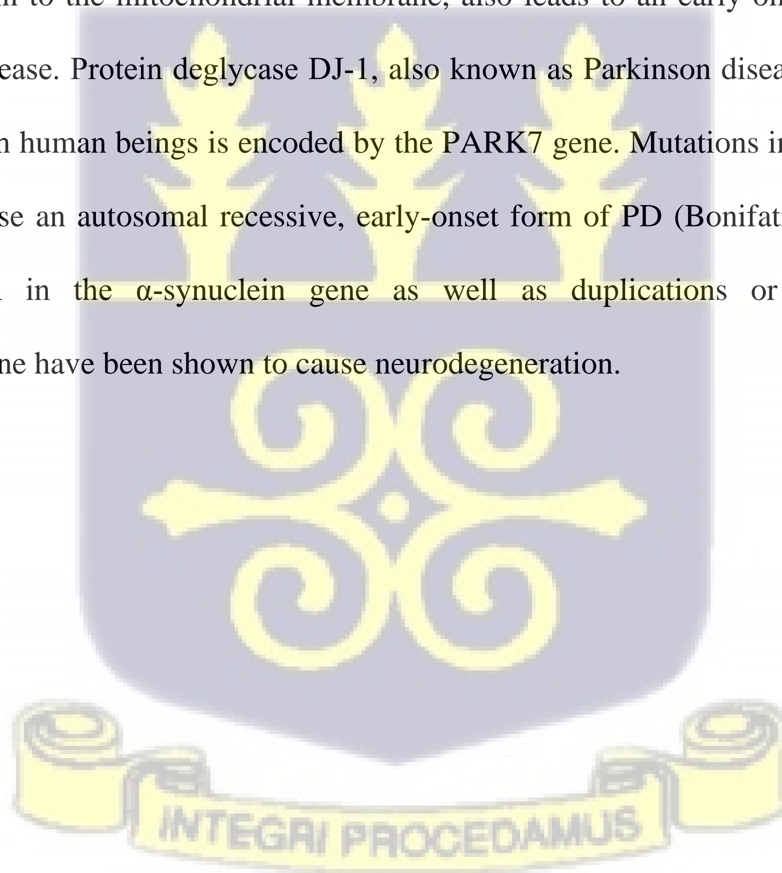

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**Table 1.1:** PARK-Designated Genes involved in Familial Parkinson's Disease

| PARK | Gene | OMIM reference | Inheritance | Description | Clinical features |
|---|---|---|---|---|---|
| PARK1 & PARK4 | *SNCA* | 168601 | AD | α-synuclein | Ranging from classical PD to early-onset cases with dementia, autonomic dysfunction, and rapid progression |
| PARK2 | *PRKN* | 600116 | AR | parkin RBR E3 ubiquitin protein ligase | Early-onset PD, slow progression, often features of dystonia |
| PARK5 | *UCHL1* | 613643 | AD | Ubiquitin C-terminal hydrolase L1 | Classical PD—only one family, findings not since replicated |
| PARK6 | *PINK1* | 605909 | AR | PTEN-induced putative kinase 1 | Early-onset PD, slow progression |
| PARK7 | *DJ-1* | 606324 | AR | Parkinsonism-associated deglycase | Early-onset PD, slow progression |
| PARK8 | *LRRK2* | 607060 | AD | Leucine-rich repeat kinase 2 | Classical PD with less frequent dementia and slower progression |
| PARK9 | *ATP13A2* | 606693 | AR | Cation-transporting ATPase 13A2 | Early-onset (adolescence), atypical parkinsonism with dementia, spasticity and supranuclear palsy (Kufor–Rakeb syndrome) |
| PARK11 | *GIGYF2* | 607688 | AD | GRB10 interacting GYF protein 2 | Classical PD |

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| | | | | | |
|---|---|---|---|---|---|
| PARK13 | *HTRA2* | 610297 | AR | HtrA serine peptidase 2 | Classical PD |
| PARK14 | *PLA2G6* | 612593 | AR | Calcium-independent phospholipase A2 enzyme | Early onset with atypical features (dystonia parkinsonism) |
| PARK15 | *FBX07* | 260300 | AR | F-box protein 7 | Early onset with atypical features (pallido-pyramidal syndrome) |
| PARK17 | *VPS35* | 614203 | AD | Vacuolar protein sorting-associated protein 35 | Classical PD |
| PARK18 | *EIF4G1* | 614251 | AD | Eukaryotic translation initiation factor 4 gamma 1 | Classical PD |
| PARK19 | *DNAJC6* | 615528 | AR | HSP40 Auxilin | Early-onset PD, slow progression |
| PARK20 | *SYNJ1* | 615530 | AR | Synaptojanin 1 | Parkinsonism with dystonia and cognitive decline |
| PARK21 | *DNAJC13* | 616361 | AD | Receptor mediated endocytosis 8 (RME-8) | Classical PD |
| PARK23 | *VPS13C* | 616840 | AR | Vacuolar protein sorting-associated protein 13C | Early-onset PD, rapid progression |

OMIM: Online Mendelian Inheritance in Man database, AD: autosomal dominant, AR: autosomal recessive. PARK3 PARK10, PARK12, PARK16, and PARK22 are considered risk factors or the genes that have not been identified yet and are not included in this table.

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*2.1.3.2 Environmental Factors*

*2.1.3.2.1 Pesticides, herbicides and heavy metals*

In 1983, it was observed that several individuals after injecting themselves with a drug contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), exhibited typical signs of PD. Further studies unveiled that *in vivo*, MPTP undergoes metabolism into the neurotoxin, MPP+ (1-methyl-4- phenylpyridinium), which is a mitochondrial complex-I inhibitor that selectively damages dopaminergic cells in the substantia nigra (Langston *et al.*, 1983). This finding led to the notion that exposure to some environmental toxins could be associated to developing PD. Several other studies have shown a link between exposure to some chemicals in pesticides (e.g. Rotenone) and herbicides (e.g. Paraquate), and the incidence of Parkinson's disease (Kouli et al., 2018).

Many epidemiological studies have also shown a relationship between Parkinson's disease and exposure to welding and heavy metals such as lead, mercury, manganese, iron, copper, bismuth, aluminum, zinc, thallium, among others. It is hypothesized that these heavy metals accumulate in the substantia nigra and increases oxidative stress which in turn leads to the neurodegeneration (Lai *et al.*, 2002). Moreover, exposure to some of these heavy metals activate and trigger the expression of PARK genes (Bjorklund et al., 2018; Peng et al., 2010). These heavy metals have also been reported to have synergistic toxicity. A study conducted by Peng et al, 2007, demonstrate that combined environmental exposure to iron and paraquate (a herbicide) results in accelerated age-related degeneration of nigrostriatal dopaminergic neurons.

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*2.1.3.2.2 Cigarette smoking*

Several epidemiologic studies have shown a protective effect of smoking on PD. Although this theory is counter-intuitive, the results of these studies have been very consistent over the years, proving that cigarette smokers have a reduced risk of PD compared with non-smokers. This inverse relationship between cigarette smoking and PD have been reported to be dose dependent. (Kouli et al., 2018; Mappin-Kasirer et al., 2020; Tanner et al., 2002). A recent study conducted by Mappin-Kasirer et al, 2020 demonstrated that current smokers had 40% lower risk of PD compared with never smokers. Even among twin pairs (homozygotic and heterozygotic) who share the same DNA and often the similar environment, studies have shown that the twin without PD tended to smoke more than the twin with PD (Tanner et al., 2002; Wirdefeldt, Gatz, Pawitan, & Pedersen, 2005).

The reasons underlying this associated reduced risk are not yet fully understood. However, nicotine, the principal psychoactive chemical in cigarette smoke, is a cholinergic agonist. It stimulates the nicotinic acetylcholine receptor (nAchR), thereby mimicking acetylcholine. Different animal studies have demonstrated that nicotine and its receptors are involved in dopamine signaling and that nicotine protects against cell damage in dopaminergic neurons. It has been shown to have beneficial effects on sporadic and genetic models of PD. Nicotine has also been found to reduce MPTP-induced dopaminergic neurotoxicity (Costa et al., 2001; Maggio et al., 1998; Quik & Kulak, 2002).

Nevertheless, because the level of dopamine, the main compound responsible for addiction is reduced in Parkinson's disease, some researches have argued that PD patients are less likely to smoke cigarette (Ritz et al., 2014). Hence, it is difficult to ascertain whether smoking prevents PD or whether PD aids in preventing cigarette addiction.

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*2.1.3.2.3 Caffeine*

Accumulating evidence from epidemiological and animal studies suggest that caffeine has a protective effect on PD and that coffee drinkers have reduced risk of developing. A study by Noyce et al., 2012 reported a 25% risk reduction in developing PD among coffee drinkers. Several other studies have also firmly established that coffee drinkers have lower risk of developing PD than non-coffee drinkers (Ascherio et al., 2001; Hernán et al., 2002; Ren & Chen, 2020)

Caffeine, an adenosine $A_{2A}$ receptor antagonist, has been shown to confer neuroprotection against dopaminergic neurodegeneration mediated by MPTP, 6-hydroxydopamind (6-OHD), rotenone and expression of α-synuclein (α-Syn) in PD models. It is hypothesized that caffeine exerts its neuroprotective effects by modulating neuroinflammation, mitochondrial function and excitotoxicity (Ren & Chen, 20200. It has also been shown to modulate α-Syn degradation (Schepici et al., 2020).

*2.1.3.3 Infectious Organisms*

Studies have shown that patients with various viral (e.g., influenza virus, Coxsackie, Japanese encephalitis B, Herpes viruses, Hepatitis C virus, etc.), bacterial (e.g., *Helicobactor pylori*, *Clamydophilia pneumoniae*, *Borrelia burgdorferi,* among others) and fungal infections (e.g., *Malassezia)* might be at increased risk of Parkinson's disease. The risk of PD in these patients were however shown to vary with each infection (Smeyne et al., 2021; Wang et al., 2020). Although the pathogenic mechanisms by which these microorganisms cause PD remain incompletely understood, some of these pathogens have been reported to be neurotropic (tending to attack or affect the nervous system preferentially), causing neuroinflammation and neurodegeneration in the *substantia nigra* of the brain (Limphaibool et al., 2019).

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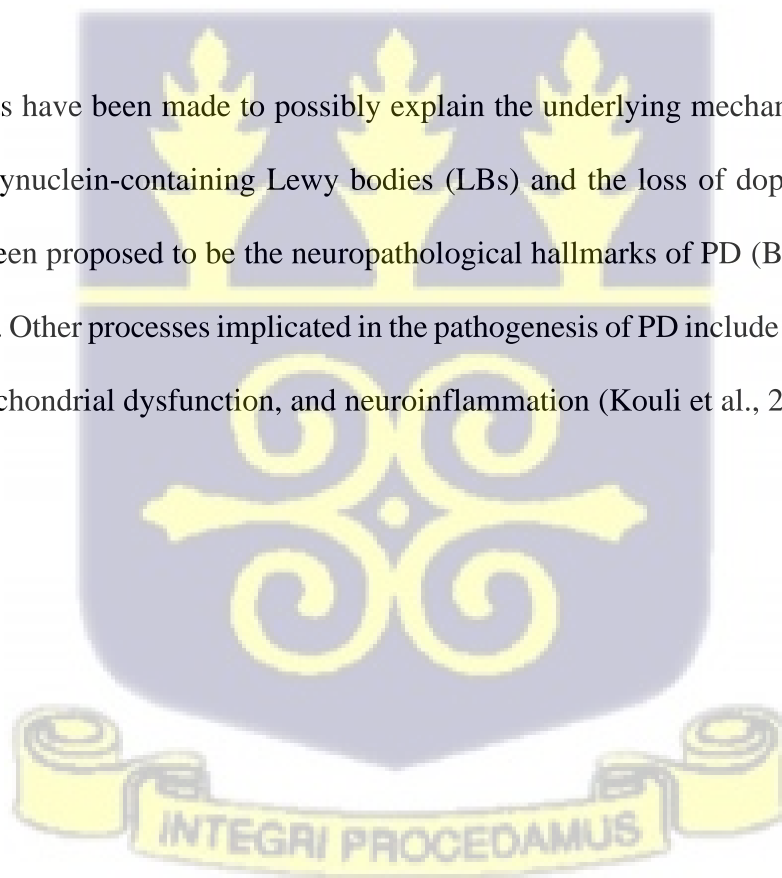
### 2.1.4 Pathophysiology

The pathology of Parkinson's disease remained unclear until the early 20$^{th}$ century. In 1912, Friedrich Heinrich Lewy, a German-born American neurologist, identified "neuronal cytoplasmic inclusions in a variety of brain regions" later known as Lewy bodies (Engelhardt & Gomes, 2017). In 1919, Konstantin Tretiakoff, a Russian neuropathologist, pointed out that the loss of neurons in the substantia nigra pars compacta (SNc) of the brain was the most critical abnormality in PD. The role of dopamine and its depletion from the basal ganglia was discovered by researchers in the late 1950s to be very key in understanding the pathophysiology of PD (Hornykiewicz, 2006). A diagram of the brain and the parts affected in PD is shown in Figure 2.1.

Several attempts have been made to possibly explain the underlying mechanisms of PD. The presence of α-synuclein-containing Lewy bodies (LBs) and the loss of dopamine producing neurons have been proposed to be the neuropathological hallmarks of PD (Beitz, 2014; Braak & Braak, 2000). Other processes implicated in the pathogenesis of PD include abnormal protein clearance, mitochondrial dysfunction, and neuroinflammation (Kouli et al., 2018).



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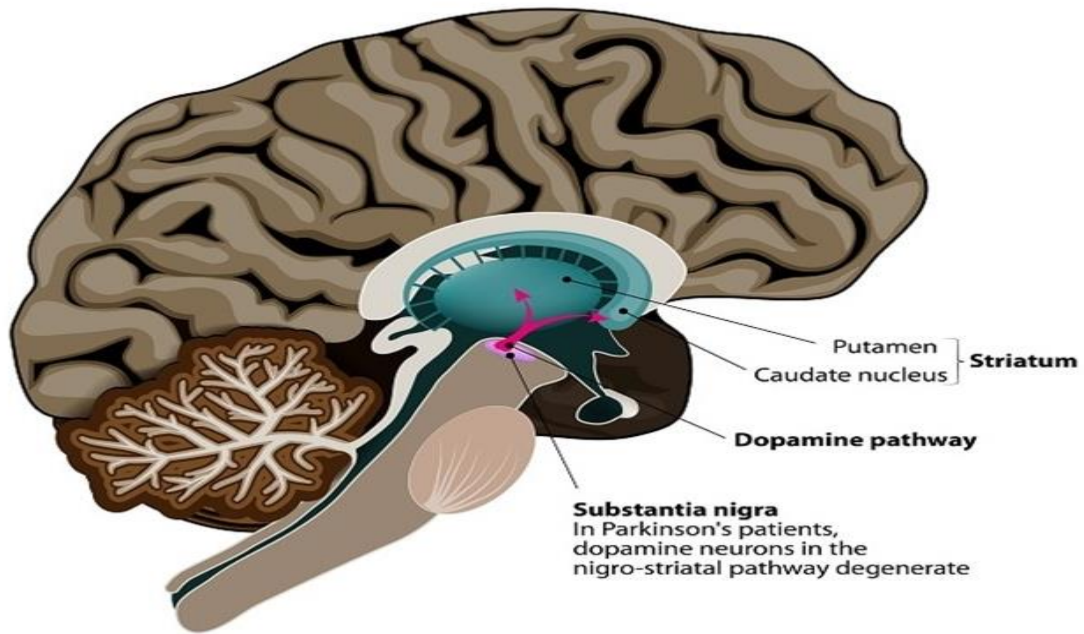


**Figure 2.1:** Diagram of the Brain showing the parts affected by Parkinson's Disease (Source: Designua/ Shutterstock.com)

*2.1.4.1 Neurodegeneration*

The major morphological change that occurs in the PD brain, is the loss of the darkly pigmented area in the substantia nigra pars compacta (SNpc) and locus coeruleus as shown in Figure 2.2.

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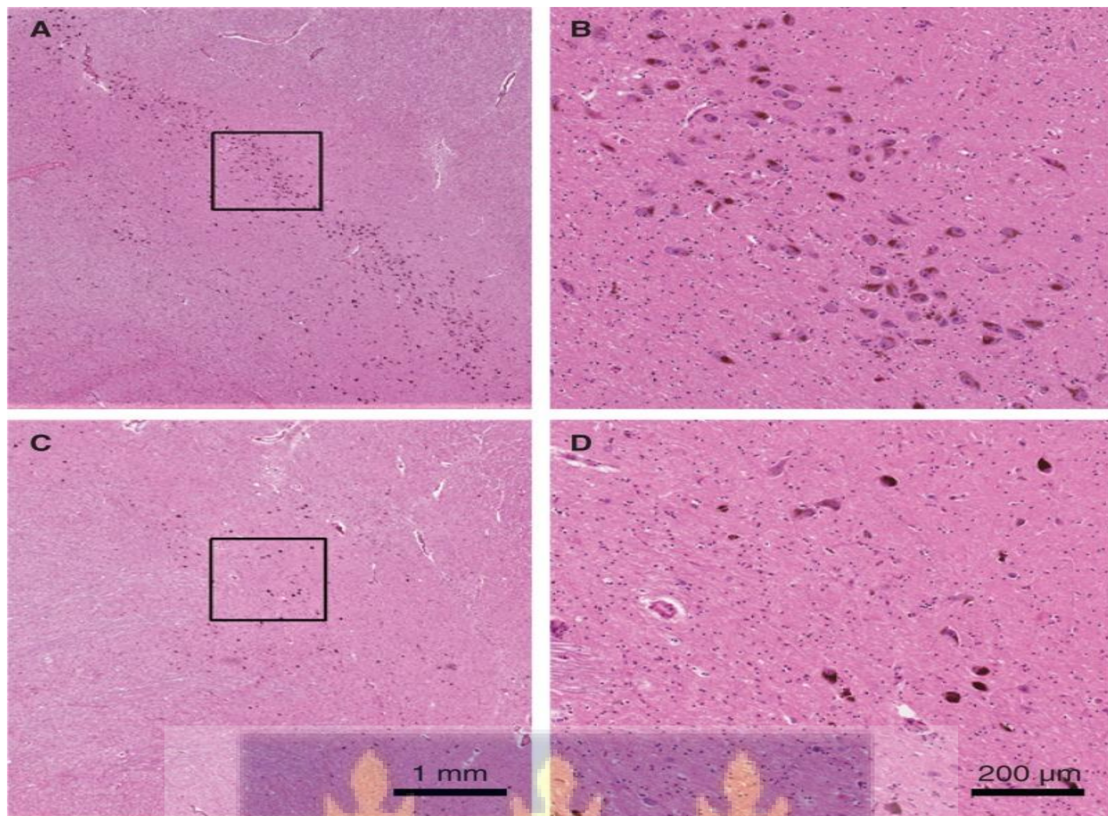


**Figure 2.2:** Coronal Section at the Level of the Substantia Nigra Pars Compacta (Snpc) Showing Dopaminergic Neurons in a Control (A and B) and a PD Brain (C And D) Stained by Hematoxylin and Eosin (Source: Kouli et al., 2018).

This pigmentation loss is directly linked to the death of dopaminergic (DA) neuromelanin-containing neurons in the SNpc and noradrenergic neurons in the locus coeruleus (Dickson, 2012). As a result, denervation of the nigrostriatal pathway occurs, leading to diminished dopamine levels in the striatum. The reduction in dopaminergic signaling accounts for the manifestation of the cardinal motor symptoms in PD such as bradykinesia, distal tremor, and muscle rigidity (Kouli et al., 2018). Apart from the SNpc, neurodegeneration has also been seen in several subcortical nuclei such as the locus coereleus, the dorsal motor nucleus of the vagus nerve, the raphe nuclei, among others and also the hypothalamus and the olfactory bulb (Giguère et al., 2018). Other neurotransmitter systems affected in PD include; the cholinergic,

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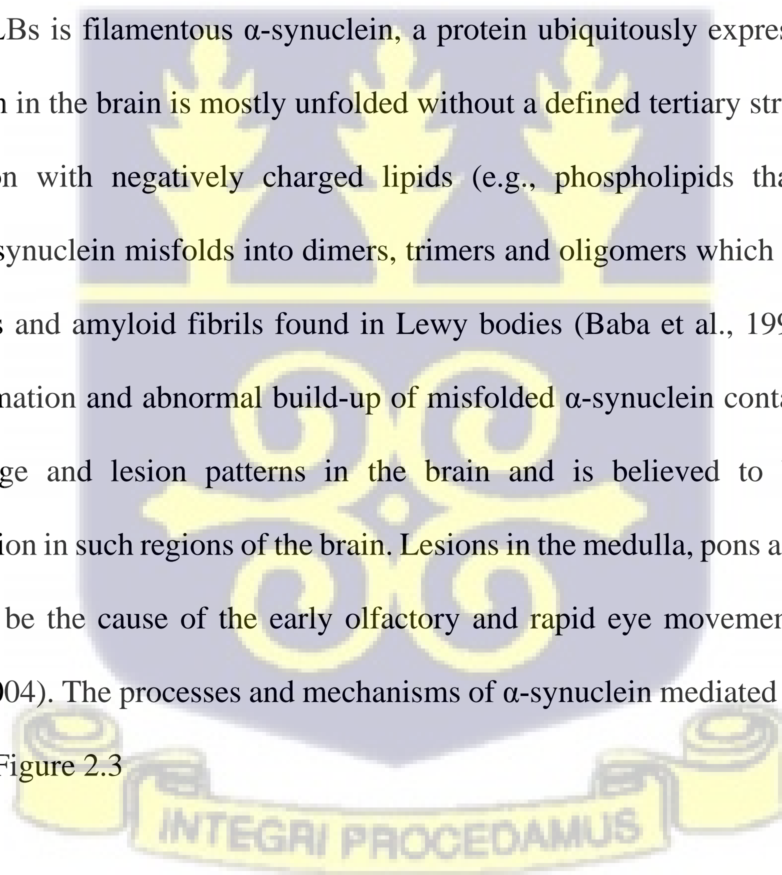
GABAergic, serotonergic, noradrenergic, adenosinergic, glutamatergic and histaminergic (Kalia et al., 2013). Degeneration in these pathways is thought to be responsible for some of the non-motor symptoms of PD that do not respond well to dopamine replacement therapies (Kouli et al., 2018)

*2.1.4.2 Lewy Bodies*

Lewy bodies (LBs) are intracellular cytoplasmic inclusions consisting of a granular and fibrillar core with a surrounding halo. LB are composed of number of proteins (such as ubiquitin, parkin, heat shock proteins, cytoskeletal proteins, etc.) lipids, proteasomal and lysosomal elements, and other materials (Braak *et al.*, 2003; Del and Braak, 2012). The principal structural constituent of LBs is filamentous α-synuclein, a protein ubiquitously expressed in the brain. Alpha synuclein in the brain is mostly unfolded without a defined tertiary structure. However, upon interaction with negatively charged lipids (e.g., phospholipids that make up cell membranes) α-synuclein misfolds into dimers, trimers and oligomers which further aggregate into protofibrils and amyloid fibrils found in Lewy bodies (Baba et al., 1998; Eliezer et al., 2001). The formation and abnormal build-up of misfolded α-synuclein containing LBs cause neuronal damage and lesion patterns in the brain and is believed to be the cause of neurodegeneration in such regions of the brain. Lesions in the medulla, pons and dorsal nucleus are reported to be the cause of the early olfactory and rapid eye movement features of PD (Braak *et al.*, 2004). The processes and mechanisms of α-synuclein mediated neuronal damage is described in Figure 2.3



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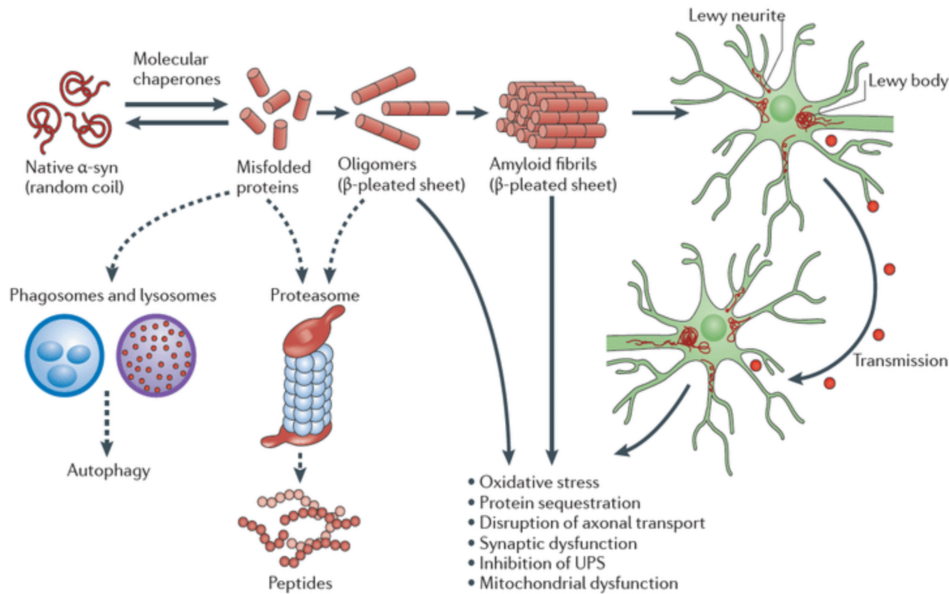


**Figure 2.3:** Proposed Mechanisms of α-Synuclein Pathology in PD
(Source: Researchgate.net, 3/10/21)

### 2.1.5 Clinical Presentation and Diagnosis

PD is characterized by four cardinal features under the acronym "TRAP": tremor at rest, rigidity, akinesia (or bradykinesia) and postural instability. These symptoms begin gradually and become worse as the disease progresses (Kouli et al., 2018). PD is also associated with non-motor symptoms (such as autonomic dysfunction, hyposmia, constipation, olfactory and sleep disorders, fatigue etc.) which often manifest as early as 12-14 years before diagnosis is made and can last for 4 to 6 years on average (Postuma et al., 2012). As the disease progresses, thermoregulatory dysfunction, neuropsychiatric symptoms and other clinical signs may occur. Neuropathic and nociceptive pain may also occur at either the early or later stages of the disease (Maetzler & Hausdorff, 2012; Postuma et al., 2012).

In majority of PD patients (about 90%), the disease begins in an insidious manner, for instance, difficulty while getting out of a chair, and is often unnoticed or misinterpreted. Sometimes

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diagnosis of PD is delayed as the non-motor symptoms preceding the disease is mistaken for signs of normal ageing. Accumulating evidence suggest that PD may begin in the peripheral autonomic nervous system and/or the olfactory bulb, with the pathology then spreading through the CNS, affecting the lower brainstem structures before getting to the substantia nigra (Katzenschlager et al., 2008; Schrag et al., 2015). This may thus explain the occurrence of non-motor symptoms in PD patients well before motor symptoms set in.

*2.1.5.1 Bradykinesia*

Bradykinesia, the most characteristic primary motor symptom of PD, has been defined as a reduction in the speed, gait and amplitude of a repetitive action involving voluntary movements (Grabli et al., 2012). Bradykinesia is reported to be a hallmark of basal ganglia disorders and may also occur in other disorders such as depression (Jankovic, 2008). Initial manifestations involve slowness in performing daily activities, slow movement and reaction times, difficulties with performing simultaneous tasks, impaired swallowing, loss of gesturing, decreased blinking, among others (Berardelli et al., 2001). Bradykinesia is usually assessed by making patients perform rapid, repetitive, alternating movements of the hand and heel taps and observing not only slowness but also decreased amplitude. Patients with bradykinesia have difficulty in initiating movements and fail to implement fast movements. Bradykinesia has been shown to be influenced by the emotional state of the PD patient (Jankovic, 2008).

*2.1.5.2 Tremor*

Deuschi *et al.*, defined tremor as "rhythmical, involuntary, oscillatory movement of a body part produced by alternating or synchronous contractions of antagonist muscles" (Deuschi *et al.*, 1998). It occurs as the initial symptom in about 60% of PD patients. Tremors have been classified into different types based on the position that underlines the tremor. Rest tremor

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basically occurs when the body is relaxed and is defined as rhythmic muscle contraction and relaxation occurring when there is no voluntarily activated muscle contraction (Deuschl et al., 1998). It is mostly seen in the distal part of an extremity but can also involve the jaw, chin, lips and legs. Typically, rest tremors tend to disappear with action and during sleep. Postural tremor occurs when a patient maintains an outstretched position against gravity (Jankovic et al., 1999). It may be one of the first signs of PD and is reported to be more prominent than rest tremor. The tremor of PD is different from essential tremor (previously known as benign essential tremor or familial tremor), one of the most common movement disorders. Both postural and rest tremors occur in the same (4–6 Hz) frequency range and are responsive to dopaminergic therapy in contrast to essential tremor (Váradi, 2020). Also, essential tremor may involve the head, neck and voice and is often reduced by the intake of alcohol, beta-blockers and botulinum toxin. Early-age essential tremor is reported to be a potential risk factor in developing PD (Shahed & Jankovic, 2007).

*2.1.5.3 Rigidity*

Rigidity occurs as inflexibility of the neck, limbs or trunk and is described as tension in the muscle, which displays small jerks or a ratchet-like quality when moved passively. It is the second most characteristic primary motor symptom of PD. Unlike bradykinesia, where the speed of the motion is reduced, in rigidity, movement is limited to a reduced range because of muscle stiffness and lack of relaxation capability (Váradi, 2020). Rigidity of PD can also affect the face, being displayed as a "masked" look (hypomimia) (J. Jankovic, 2008; Xia & Mao, 2012). Dopaminergic agonists have been shown to be efficient in reducing rigidity (Váradi, 2020).

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*2.1.5.4 Postural Instability*

Postural instability occurs after the onset of other clinical features, usually at the late stages of the disease. It manifests as a result of the loss of postural reflexes. Postural instability (along with freezing of gait) is the main symptom responsible for the falls observed in PD patients and its attendant risk of hip fractures. Assessment of postural instability is done by the pull test. In this test, patients are quickly pulled forward or backward by the shoulders, in order to assess the degree of propulsion or retropulsion respectively. The absence of any postural response or taking more than two steps backwards suggests an abnormal postural response.

### 2.1.6 Non-Motor Features of PD

Although less appreciated, non-motor symptoms also present in PD patients. Studies in recent years have shown that Lewy body neurodegeneration affect the enteric and peripheral nervous systems (PNS) not only through dopaminergic pathways but, also, GABAergic, cholinergic, noradrenergic, glutamatergic, serotonergic and histaminergic nerves (Váradi, 2020). The involvement of these nerves is expressed in a wide range of nonmotor symptoms (NMS), such as cognitive impairment (e.g., dementia, confusion, impaired judgement), autonomic dysfunction (e.g., orthostatic hypotension, gastrointestinal disturbance, sexual dysfunction) sleep disturbances (e.g. sleep apnea, restless legs syndrome), sensory abnormalities (e.g. paresthesia, pain, olfactory dysfunction and anosmia), mood disturbances (e.g., anxiety, depression, apathy) and neuropsychiatric symptoms. ((Beitz, 2014; Váradi, 2020). Generally, all PD patients experience some form of NMS, with an increased frequency observed as the disease progresses. Parkinson's disease is therefore, a complex disorder expressing both motor and nonmotor symptoms throughout the progression of the disease.

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### 2.1.6 Diagnosis

The differential diagnosis of PD can be very challenging, especially in the early stages of the disease when non-motor features and signs and symptoms of different forms of parkinsonism overlap greatly. Studies report error rates as high as 24% in the diagnosis of PD, even with movement-disorder specialists attending to most of the patients in such studies (Tolosa et al., 2006). Moreover, the cardinal motor features of PD may not manifest until approximately 50% to 80% of dopaminergic neurons are lost (Berg, 2012; Postuma *et al.*, 2012). There are no definitive tests for the diagnosis of PD, hence, the disease is often diagnosed based on clinical criteria.

Over the years, several clinical diagnostic tools and guidelines have been produced to effectively identify symptoms, classify and appropriately diagnose PD, especially in the early stages of the disease. Hoehn and Yahr in 1967 made the first attempt to describe the onset and progression of PD by providing a five (5)-stage descriptive scale known as the Hoehn and Yahr scale (Hoehn & Yahr, 1967). Each stage has a well-defined motor impairment and disability by which patients can be classified during disease progression. The original scale has since been modified with the addition of stages 1.5 and 2.5 to account for the intermediate course of Parkinson disease. The Hoehn and Yahr scale is still one of the most commonly used rating scales in PD progression (Goetz et al., 2004)

In 1988, the United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) provided its first form of clinical diagnostic criteria for PD. This guideline provides three different levels for classification including diagnostic, exclusion and supportive criteria. The diagnostic criteria involve the presence of bradykinesia and at least one of the following symptoms: muscular rigidity, 4–6 Hz rest tremor and postural instability (not caused by other disorders). The

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exclusion criteria consist of a history of repeated strokes or head injury, encephalitis, early severe autonomic involvement or dementia, Babinski sign, negative response to levodopa treatment and MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) exposure. The supportive criteria (at least three required) includes unilateral onset, rest tremor, progressive course, persistent asymmetry, excellent response to dopaminergic therapy, levodopa-induced dyskinesia, positive levodopa response five years or more and clinical course of ten years or more.

In order to distinguish PD from other related conditions, Gelb and his colleagues in 1999 described an improved criteria known as the Gelb criteria. This guideline differentiated three levels of PD namely; definite, probable and possible. However, the Gelb criteria restricted the diagnosis of definite PD to neuropathologic confirmation.

Subsequently, the importance of the non-motor features of PD and their effects on the quality of life of patients, necessitated the generation of newer, improved guidelines, that took into account nonmotor features. In 2009, Lees and his colleagues published a modified version of the Queen Square Brain Bank (QSBB) clinical diagnostic criteria (Lees et al., 2009). This guideline, mainly based on the UKPDSBB criteria, was extended to include for the first time nonmotor features of PD, such as hyposmia and hallucinations (Váradi, 2020).

The International Parkinson and Movement Disorder Society (MDS) task force in 2015, published a novel clinical diagnostic criteria intended for clinical practice and PD research known as MDS-PD (Postuma et al., 2015). The development of MDS-PD criteria was largely based on the UKPDSBB and Gelb criteria. Several nonmotor features of PD (such as sleep, autonomic, psychiatric and olfactory dysfunction) are captured as additional diagnostic features.

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The differential diagnosis of PD must include a comprehensive history and physical examination. Clinicians should review the patient's history to assess symptoms and to rule out alternative diagnoses, such as multiple-system atrophy, essential tremor, and other diseases that have presentations similar to those of PD. Also, a complete medication history and evaluation should be done to identify drug induced parkinsonism (DIP) and to avoid treating patients inappropriately. Moreover, laboratory studies may be done to rule out nutritional deficiencies and other abnormalities such as thyroid disease. When a patient's history suggests possible exposure to a neurotoxin, toxin screening should be done (DeMaagd & Philip, 2015) .

### 2.1.7 Management of PD

Therapies currently employed in managing PD neither slowdown nor stop the progression of the disease. Therefore, the primary goal of medical management of PD is to control symptomatic motor and non-motor features of the disease, with the aim of minimizing adverse events (AEs) and improving the quality of life of patients (DeMaagd & Philip, 2015). Appropriate management of PD requires the services of a multidisciplinary team comprising of neurologists, movement disorder specialists, general practitioners, pharmacists, nurses, physiotherapists and social workers (Van der Marck & Bloem, 2014). The patient and his or her relatives must also be actively involved in taking management decisions (Politis et al., 2010)

In order to maximize the clinical outcomes and ensure effective management, both pharmacological and non-pharmacological strategies need to be employed. Pharmacologic treatment involve the use of dopamine precursors (drugs metabolized to yield dopamine) such as levodopa; dopamine agonists (drugs that activate dopamine receptors) example ropinirole;

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centrally acting antimuscarinic drugs (e.g., trihexyphenidyl, benztropine) or drugs that prevent the breakdown of endogenous dopamine such as catechol-O-methyl transferase (COMT) inhibitors (e.g., entacapone) and monoamine oxidase B inhibitors, such as selegiline (Gunay et al., 2015).

The choice of drugs depends on a number of factors, such as the stage of disease, age of the patient, level of functional disability, cognitive status, AEs associated with the agent, among others. Levodopa, compared with the other therapeutic agents, is more efficacious, less costly and better tolerated (Salat & Tolosa, 2013). Currently, levodopa, combined with a dopa decarboxylase (DDC) inhibitor such as carbidopa or benserazide, is the standard treatment for the motor symptoms of PD.

## 2.2 LEVODOPA/CARBIDOPA FOR MANAGING PD

### 2.2.1 Levodopa

Levodopa, chemically known as L-3,4-di-hydroxyphenyl-alanine, is an aromatic amino acid synthesized and used in normal human biology. It is synthesized from L-tyrosine by the enzyme, tyrosine hydroxylase, as a biological precursor to dopamine. The schematic representation of the biosynthesis of levodopa by hydroxylation is shown in Figure 2.4 below.

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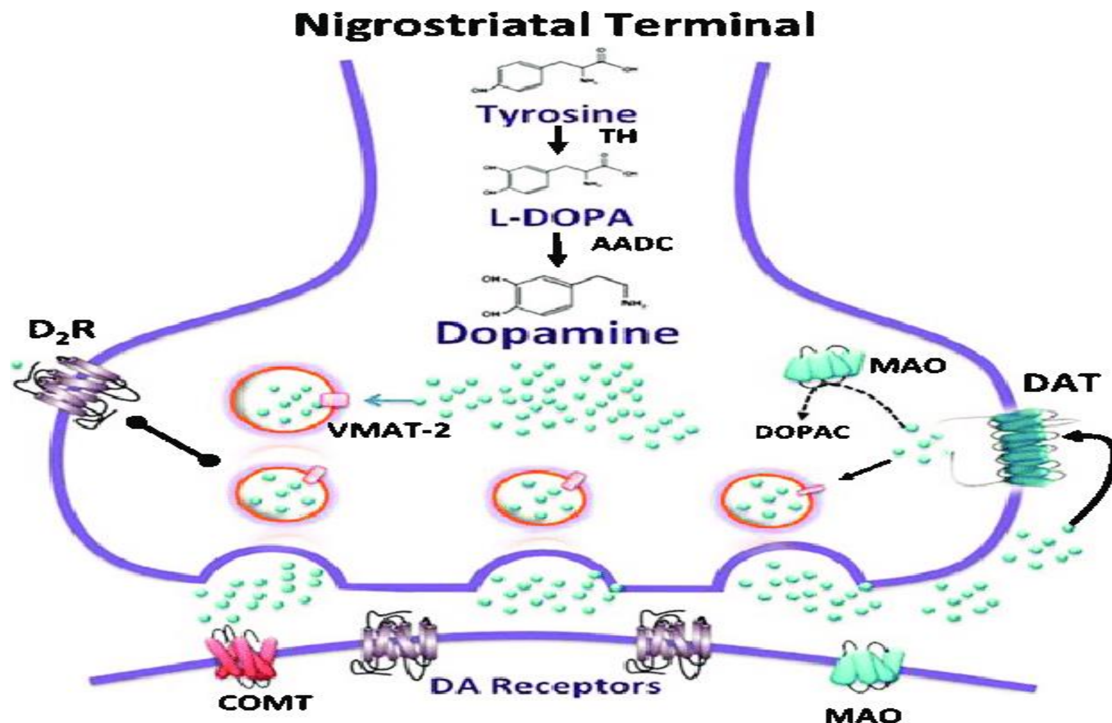


**Figure 2.4:** Synthesis of Levodopa and Dopamine in Dopaminergic Neuron
*TH=Tyrosine hydroxylase, AADC= Aromatic amino acid decarboxylase, DAT=DA transporter, D1=D1-like DA receptor, D2=D2-like DA receptor. VMAT=Vesicular monoamine transporter.*

Levodopa is also manufactured as a prodrug and used as a dopamine replacement agent in the management of PD and other related conditions. Levodopa, classified as Class I agent, has a high solubility and high permeability, according to Biopharmaceutics Classification System (BCS). It is a white crystalline compound, shown to be slightly soluble in water, with solubility increasing below pH 3 and above pH 8. Its empirical formula is $C_9H_{11}N0_4$ and its structural formula is shown in Figure 2.5.

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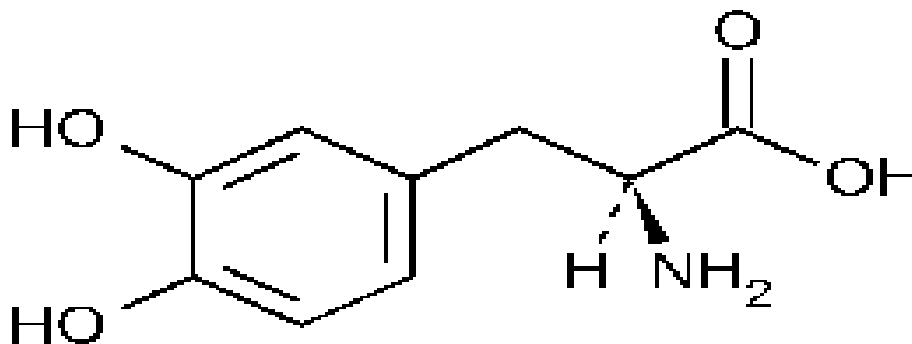


**Figure 2.5:** Chemical structure of Levodopa
(Source: www.newdruginfo.com)

Unlike dopamine, levodopa is able to cross the blood-brain barrier (BBB). After transport across the BBB, levodopa is converted into the neurotransmitter dopamine by aromatic amino acid decarboxylases (AADC) also known as dopa decarboxylases (DDC) thus, increasing levels of dopamine in the depleted striatum (Kouli et al., 2018; Zahoor et al., 2018). These enzymes are not only present in the CNS but are widely distributed in liver, gastrointestinal tract (GIT), kidneys, spleen, heart, adrenals and lungs.

When administered orally, only 1% of the given dose of levodopa reaches the brain because of metabolism and rapid plasma clearance chiefly by peripheral AADC. Levodopa is also shown to undergo O-methylation, transamination, and oxidation into other metabolites (Elroby et al., 2012). The peripheral conversion to dopamine and other metabolites is reported to greatly reduce the half-life and bioavailability of levodopa. Furthermore, the systemic conversion to dopamine leads to unwanted side effects such as nausea, vomiting, cardiac arrhythmias, and hypotension (Salat & Tolosa, 2013).

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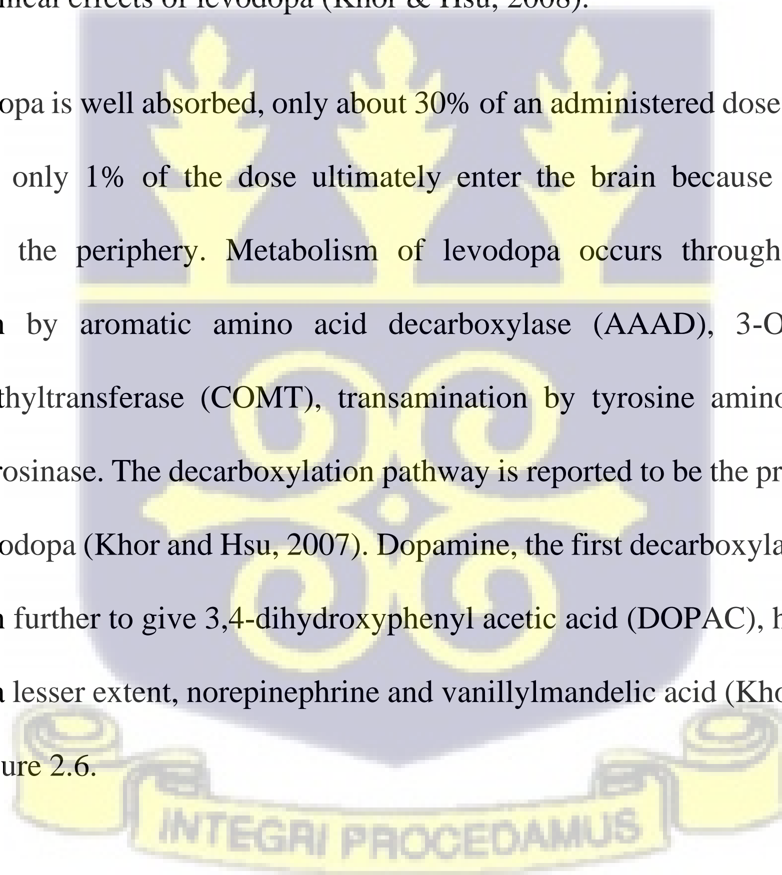
*2.2.1.1 Pharmacokinetics of Levodopa*

Levodopa is almost completely absorbed following oral administration, with just about 2% of drug seen in faeces. Absorption of levodopa is facilitated through a saturable L-neutral amino acid transport system (Morgan *et al.*, 1971). A high protein meal appears to affect the absorption of levodopa although this effect varies with different formulations. Evidence from several studies show that the clinical effect of levodopa is reduced by a daily diet containing protein in excess of 1.6 g/kg or a single protein load of approximately 28 g (Carter et al., 1989; Simon et al., 2004; Tsui et al., 1989). The findings of positron emission tomography study suggest that a protein-rich diet may compete with the uptake of levodopa into the brain, thereby, reducing the clinical effects of levodopa (Khor & Hsu, 2008).




Although levodopa is well absorbed, only about 30% of an administered dose reaches systemic circulation and only 1% of the dose ultimately enter the brain because it is extensively metabolized in the periphery. Metabolism of levodopa occurs through four pathways; decarboxylation by aromatic amino acid decarboxylase (AAAD), 3-O-methylation by Catechol-O-methyltransferase (COMT), transamination by tyrosine aminotransferases and oxidation by tyrosinase. The decarboxylation pathway is reported to be the principal metabolic pathway for levodopa (Khor and Hsu, 2007). Dopamine, the first decarboxylation product may be broken down further to give 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), and to a lesser extent, norepinephrine and vanillylmandelic acid (Khor and Hsu, 2007) as shown in Figure 2.6.

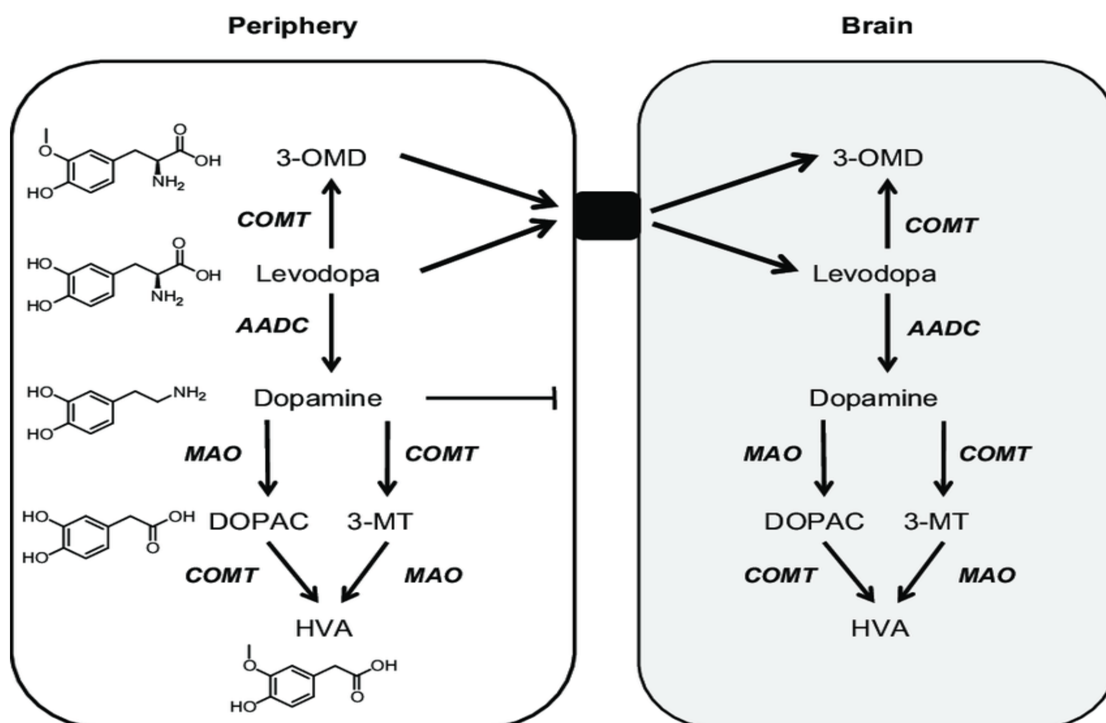



**Figure 2.6:** Levodopa metabolism in the periphery and the CNS
*where COMT: -catechol O-methyl transferases, MAO: monoamine oxidases, 3-OMD: 3-O methyldopa, DOPAC: 3,4 dihydroxyphenyl acetic acid, HVA: homovanillic acid, 3-MT: 3-methyldopa (Source: Palma et al., 2013).*

When given alone, levodopa has a plasma half-life of about 50 minutes but this may be increased to 90 minutes when co-administered with DDC inhibitors such as carbidopa and benserazide (Khor & Hsu, 2008). Levodopa has a high volume of distribution and is not highly bound to plasma protein (Hinterberger and Andrews, 1972). Levodopa is mainly excreted via urine.

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2.2.2 Carbidopa

Dopa decarboxylase inhibitors like carbidopa do not cross the blood-brain barrier even when high doses are administered (Lotti & Porter, 1970). These agents primarily stay in the periphery and block levodopa metabolism, thereby slowing the plasma clearance of levodopa, reducing the rate of the first-pass metabolism, increasing the bioavailability and prolonging its plasma half-life. Clinical studies suggest that, the peripheral half-life of levodopa increases from 30 min to about 90 min when co-administered with DDC inhibitors. Also, the required levodopa dose is reduced by 60–80% (Cedarbaum, 1987; Hauser, 2009). Moreover, reduced peripheral decarboxylation of levodopa to dopamine decreases the characteristic peripheral side effects of dopamine (e.g., nausea, vomiting, anorexia) (Obeso et al., 2017).

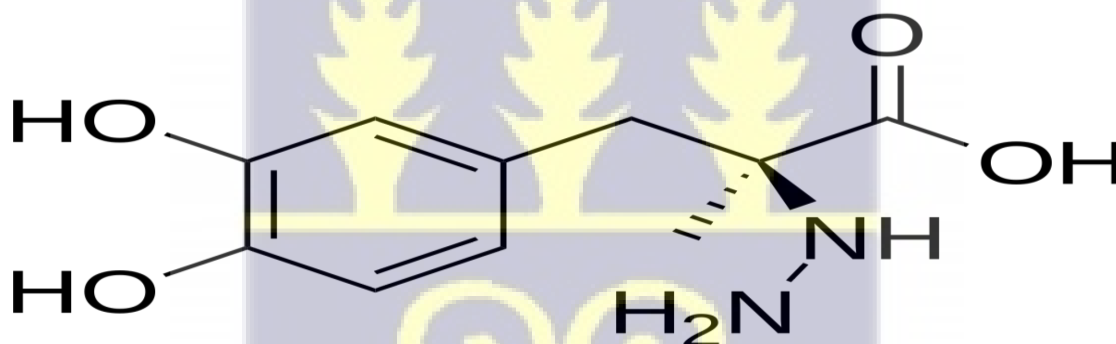


**Figure 2.7:** Structure of Carbidiopa
(Source: enwikepedia.org)

In 1975, combinations of levodopa with either carbidopa or benserazide were made commercially available (Tolosa *et al.*, 1998) and till date, is approved by the Food and Drugs Authority (FDA) for the treatment of PD.

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*2.2.2.1 Pharmacokinetics of Carbidopa*

Compared with levodopa, carbidopa is absorbed quite slowly, taking about 2 hours (for an immediate release formulation) or close to 2.8 hours (for a controlled release formulation) to peak in plasma. About 40 to 70 % of an orally administered dose of carbidopa is absorbed (Vickers *et al.*, 1974). The bioavailability of carbidopa is not affected by the co-administration of Levodopa (Khor & Hsu, 2008). Also, carbidopa is not highly protein bound. The percentage plasma protein binding is found to be 36±1.6% (Khor & Hsu, 2008).

Carbidopa undergoes metabolism to give four metabolites namely; 2-methyl-3-methoxy-4-hydroxy-phenylpropionic acid, 2-methyl-3,4-dihydroxy-phemnylpropionic acid, 3-hydroxy-α-methyl-phenylpropionic acid, and 3,4-didydroxy-phenylacetone (Khor and Hsu, 2007). The half-life of carbidopa is about 2 hours when co-administered with levodopa. When administered alone, it has been shown to have a similar half-life of 2.08 (Vickers *et al.,* 1974). Carbidopa is largely excreted in urine.

### 2.2.3 Motor Fluctuations and Dyskinesia in Parkinson Disease

Motor fluctuations and dyskinesia are major complications of levodopa therapy, affecting many patients especially as the disease progresses.

In the 1960s, it became clinically apparent that majority of PD patients who responded to levodopa also developed dyskinesias and motor fluctuations. Studies have shown that about 40% to 75% of PD patients develop these complications after 4–6 years of levodopa therapy (Hauser, 2009; Lloyd *et al.*, 1975) although they may occasionally manifest just after few weeks or months of treatment.

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Motor fluctuations refers to alterations between periods of positive response to levodopa ("on"), and periods marked by reemergence of parkinsonian symptoms ("off") as the response to levodopa begins to wear off. Different types of motor fluctuations are observed in PD patients namely; wearing off, unpredictable "off", failure of an "on" response, freezing of gait and acute akinesia (Jankovic, 2008; Obeso et al., 2017) . The "wearing off" effect is the first and most commonly encountered among patients with PD. "Wearing off" is characterized by the recurrence of parkinsonian symptoms as the effect of exogenous levodopa reduces near the end of the dose interval. "Wearing off" is often observed in patients usually three to four hours after a given dose (Liang, 2018).

Levodopa induced dyskinesia (LID) is defined as abnormal involuntary movement occurring as a result of the use of levodopa. LID involves a variety of involuntary movements or postures, such as chorea, ballism, dystonia and myoclonus, which emerge at various times in relation to levodopa dosing. Levodopa induced dyskinesia is classified as peak-dose dyskinesia, wearing-off or off-period dyskinesia, and diphasic dyskinesia (Pandey & Srivanitchapoom, 2017)

*2.2.3.1 Mechanism of Motor fluctuations and Levodopa Induced Dyskinesia*

Although PD is characterized by the progressive degeneration and loss of nigrostriatal dopaminergic neurons, studies have shown that in the early stage of the disease, there are still sufficient neurons to maintain consistent striatal dopamine concentrations and continuous activation of the striatal dopamine receptors (Pfeiffer, 2005; Olanow *et al.*, 2006). The remaining dopaminergic neurons take up exogenous levodopa and convert it to dopamine, which is then stored and released slowly into the synapse over time. These neurons are also able to re-uptake, recycle and control the release of dopamine to ensure constant dopamine

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concentrations. Thus, buffering the effects of the fluctuating plasma levels of levodopa due to its short half-life.

However, as neurodegeneration progresses, this conversion, storage, and release mechanism becomes compromised. Also, the buffering capacity greatly reduces (Hauser, 2009). The conversion of exogenous levodopa to dopamine then takes place mainly at the non-dopaminergic sites (e.g., glial cells, serotonergic neurons etc.) which lack the capacity of storing dopamine (Melamed *et al.*, 2000). Hence, fluctuations in plasma levodopa concentration may result in fluctuations in striatal dopamine concentration and pulsatile stimulation of dopamine receptors, leading to motor complications such as dyskinesia and motor fluctuations (Olanow *et al.*, 2006). Ultimately, patients become largely reliant on a continual in-flow of levodopa into the brain to obtain a clinical response as dopamine levels in the synapse begin to reflect levels of levodopa in the peripheral circulation.

Moreover, the pulsatile stimulation of dopamine receptors by levodopa-derived dopamine have been proven to narrow the therapeutic window of levodopa over time (Salat & Tolosa, 2013). Eventually, patients require higher levodopa doses for symptom relief. Higher levodopa doses lead to higher peaks (Cmax) of levodopa concentration and this, together with the dopaminergic degeneration in the CNS, is believed to result in motor complications.

Overstimulation of dopamine receptors following levodopa administration have been shown to cause dyskinesia. The development of motor complications has also been associated with variable absorption of levodopa in the small intestine as a result of poor gastric emptying, competing dietary protein, slow intestinal transit times, among others.

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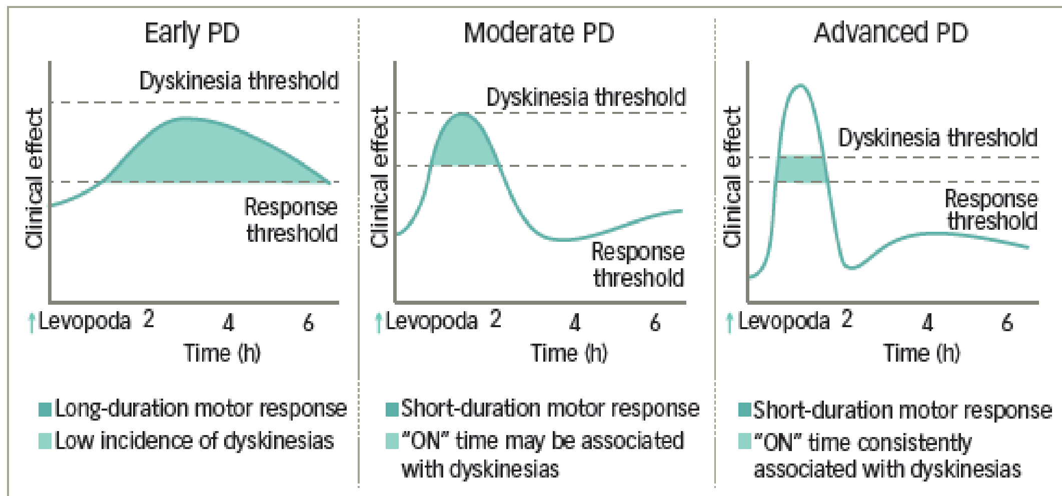


**Figure 2.8:** Changes in Motor Response Associated with Chronic Levodopa Therapy (Source: Longo *et al.,* 2011)

Studies have demonstrated that, continuous administration of levodopa leads to a reduction in dyskinesia and motor fluctuations(Gershanik & Jenner, 2012; Olanow et al., 2006). Similarly, administration of short-acting dopamine agonists has been shown to induce more dyskinesia than administration of the same agonist in a more continuous fashion (Stocchi et al., 2002). Based on these findings, research has focused on attempting to provide more sustained dopamine concentrations in the CNS.

### 2.2.4 Levodopa Drug Delivery Systems

In order to improve its bioavailability, maintain a near-constant plasma concentration and minimize the unwanted motor complications of levodopa, several studies have focused on the development of improved drug delivery systems for levodopa (Ngwuluka et al., 2010).

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First, immediate release drug delivery systems composed of levodopa in combination with peripheral dopa decarboxylase (DDC) inhibitor such as carbidopa or benserazide were formulated. These combinations, namely Sinemet® and Madopar® respectively, were shown to greatly reduce the extracerebral metabolism of levodopa (increasing the half-life from 50min to 1.5hrs) and side-effects such as nausea and vomiting but were ineffective in controlling dyskinesias and motor fluctuations associated with long-term use of levodopa.

Catechol-O-methyl transferase (COMT) inhibitors such as entacapone and tolcapone were then added to levodopa/carbidopa in a single tablet to block the second metabolic pathway (O-methylation). Although the addition of a COMT inhibitor increased the plasma level of levodopa by 35% and the half-life from 1.5h to 2.4h, dopaminergic side effects such as dyskinesias increased, thereby necessitating the levodopa dose to be reduced.

Levodopa oral disintegrating tablets (ODTs) were also introduced to make up for the reduced duration of clinical response that occurred with the use of immediate release drug formulations. These tablets enabled patients to take smaller and more frequent doses. Drug dosages were also tailored to the needs of individual patients. However, due to the frequency of dosing, most patients fail to comply, therefore, making it difficult to achieve constant delivery of the drug. Additionally, liquid formulations of levodopa were developed to facilitate rapid onset of action. These formulations were shown to give a rapid onset of action (within 5 min) and are therefore given to reduce the delay in the On' effect which has been observed with the use of controlled release (CR) formulations. However, their effects only last for a very short period (1-2hrs) and requires frequent administration (Ngwuluka et al., 2010). Non-compliance is therefore, a major challenge with the use of this formulation.

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In order to reduce the interval between doses and to solve the "wearing off" problem encountered with levodopa, controlled release (CR) formulations were introduced. A typical example is Sinemet CR, which currently is the most commonly prescribed medication for PD. Pharmacokinetic studies have shown that steady state levodopa plasma levels do not fluctuate as much with Sinemet CR compared to the immediate release formulation, Sinemet. However, the bioavailability of Sinemet CR is about 30% less than Sinemet (Yeh et al., 1989). Additionally, Sinemet CR is absorbed slowly, and although the absorption occurs over an extended period, there is a late onset of drug action. After administration, it takes about 2-4hrs to reach peak plasma concentration and the peak concentrations may be lower than that obtained with immediate release (IR) formulations. Hence, patients may have to take an IR formulation in the morning and a CR formulation or combination IR and CR during the day in order to produce a rapid onset of action (Gasser et al., 1998). Furthermore, CR formulations are often associated with a problem of variable bioavailability and consequently variable efficacy (Goole & Amighi, 2009).

Dual release (DR) formulations were also introduced in order to overcome the delayed action observed with the use of CR formulations (Rubin, 2000). These drug delivery systems combine the advantages of a rapid onset of action as well as a sustained effect. However, when DR formulations were compared with CR formulations, the mean Dyskinesia Rating Scale severity score was similar for both formulations (2.8±2.5 vs. 2.7±3.1) implying that there may be variable bioavailability with DR formulations as well. Furthermore, gastro-retentive drug delivery systems have been developed. However, these formulations are not recommended because of the risk of these systems staying longer than desired in the gastric region of humans (Ngwuluka et al., 2015).

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Intravenous infusion of levodopa has also been developed. However, studies revealed that levodopa is irritating to the veins and could not be administered continuously for more than 7-10 days via the intravenous route (Shoulson et al., 1975; Hardie et al., 1984). Intraduodenal levodopa infusion has also been proven to be a successful therapeutic approach for PD (Kurlan et al., 1988). However, its use is greatly limited because of the frequent dislocation of the distal part of the tube from the duodenum into the stomach resulting in sudden failure of clinical response with recurring motor fluctuations (Nyholm et al., 2005).

Clearly, most of these drug delivery systems are unable to provide constant and sustained delivery of levodopa over a prolonged period to ensure its optimal absorption and subsequent CNS bioavailability.

## 2.3 BIOPOLYMERS AS DRUG DELIVERY SYSTEMS

The oral route is by far the most preferred route of drug administration especially for the management of chronic diseases. However, factors such as extensive presystemic metabolism, erratic gastric emptying and limited absorption via specific segments of the gastrointestinal tract, among others, restrict the therapeutic potential of many drugs (Hua, 2020). In order to enhance the absorption, bioavailability, pharmacokinetic and biodistribution profile of drugs following oral administration, current research has explored new strategies. One such strategy is to incorporate drugs into polymer matrices to form polymer composites.

In recent years, biopolymers such as chitosan, pectin, alginate, guar gum, gelatin, dextran, xanthan, and other polymers have gained significant attention not only in the pharmaceutical industry, but also in the food and biomedical sectors (Martau, Mihai, & Vodnar, 2019).

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Biopolymers are polymers derived from natural sources. They are either entirely biosynthesized by living organisms or chemically synthesized from biological material (Liu, Lin, Astruc, & Gu, 2019; Martau et al., 2019). Generally, three types of polymers have been classified namely; polysaccharide, protein and lipid biopolymers.

Polysaccharide biopolymers like chitosan and pectin are abundant in nature and readily available, making them relatively less excipients. Furthermore, because these polymers are non-toxic, biocompatible, biodegradable, and flexible, they have significant potential in drug delivery systems.

### 2.3.1 Chitosan

Chitosan (CS) is a family of linear polysaccharides prepared from the partial deacetylation of chitin, the principal component of the exoskeletons of crustaceans such as crabs and shrimps, fungus cells walls and insect cuticles, by alkaline hydrolysis. It is composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) as shown in Figure 2.9

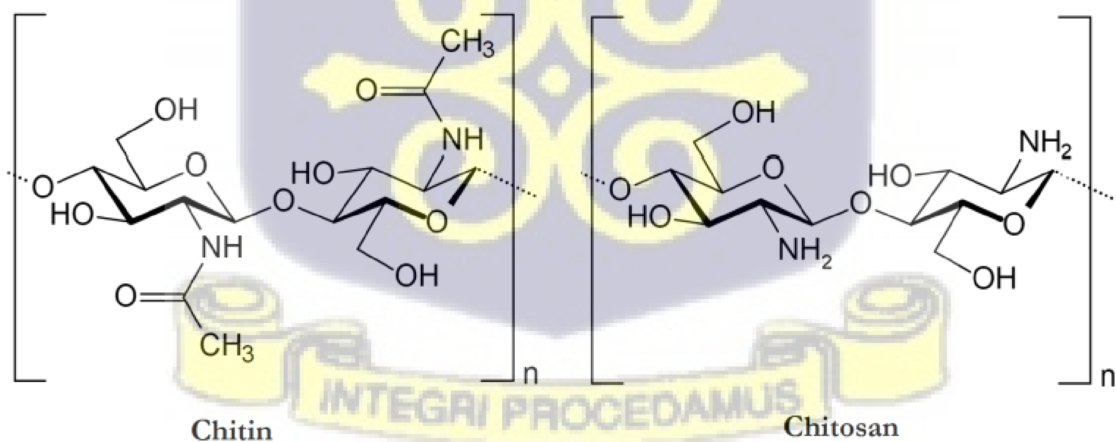


**Figure 2.9:** Chemical Structure of Chitin and Chitosan

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The degree of acetylation (DA) and molecular weight of chitosan depends largely on the natural source, the reaction parameters and the conditions used to isolate and deacetylate chitin (Quiñones, Peniche, & Peniche, 2018). Hence, commercially, CS is available in a range of molecular weights, degrees of deacetylation and different types of salts such as glutamate, hydrochloride and lactate (Syed et al., 2014). CS has free hydroxyl (–OH) and amino (–$NH_2$) groups in its structure that allow for hydrogen bonding and chemical modifications that enhances some of its properties for certain applications.

Chitosan is a biocompatible, biodegradable, non-toxic and non-immunogenic material with wound healing capacity, hemostatic and antimicrobial activity. It easily forms films and can be processed into gels, fibres, microspheres, microparticles and nanostructures (Morris et al , 2010). These exceptional biological, physical and chemical properties make CS an excellent polymer for use not only in pharmaceutics but also in cosmetics and food industry (Morris et al., 2010). Generally, CS is preferred to other cationic polymers such as polylysine, polyarginin, or polyethyleneimine because it is comparatively less toxic.

The primary amine group in chitosan accounts for its various properties such as cationic nature, muco-adhesion, permeation enhancement, controlled drug release, in situ gelation, antimicrobial effects, among others (Bernkop-Schnürch & Dünnhaupt, 2012).

Mucoadhesion refers to the adhesion between two materials, at least one of which is a mucosal surface (Phanindra et al., 2013). The cationic polyelectrolyte nature of chitosan provides a strong electrostatic interaction with negatively charged mucosal surfaces. This makes it a perfect excipient able to improve the drug residence time in a specific mucous tissue, enhance absorption at the site and provide sustained drug release. The significance of this mucoadhesive

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property of chitosan and chitosan nanoparticles has been demonstrated in earlier work by Fernandes et al., (2013), Hejjaji et al., (2018), Silva et al., (2017) and Ways et al., (2018).

Furthermore, chitosan has been shown to have permeation enhancing properties (Bernkop-Schnürch & Dünnhaupt, 2012; Soliman et al 2014). Studies have shown that its positive charges interact with the cell membrane resulting in a structural reorganization of tight junction-associated proteins thereby allowing the paracellular transport of macromolecular drugs (Yeh et al., 2011). Drug-loaded chitosan nanoparticles have also been used successfully to deliver drugs to the brain to treat diseases such as Alzheimer's disease, Parkinson's disease, epilepsy, stroke, among others (Fan et al., 2018). Its mucoadhesive property creates an interface of electrostatic interaction with the negative charges of the glycocalyx and the phospholipids of the epithelial membrane in the BBB (Peptu et al., 2014). Permeation of such drugs is also enhanced by the opening of tight junctions whereas some of these drug-bound nanoparticles cross the BBB into the brain due to their nanometric particle size (Cortés et al., 2020)

Despite the above-mentioned useful properties, chitosan tends to dissolve in the acidic environment of the stomach, compromising its mucoadhesive capacity and therefore, resulting in an uncontrolled release of the carried drug. Studies suggest that in order to overcome this challenge, the structure of chitosan needs to be modified or combinations with other excipients be made (Bernkop-Schnürch and Dünnhaupt, 2012; Luo and Wang, 2014). One of the excipients that can be successfully combined with chitosan is pectin.

### 2.3.2 Pectin

Pectin is a non-toxic, biodegradable, biocompatible, anionic polysaccharide present in the primary cell wall of plants (Cheikh et al., 2019). Pectin extracts are mainly made up of linear chains of (1, 4)-α-D-galacturonic acid residues partially esterified with methyl groups.

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Depending on the degree of esterification DE (that is, the substitution degree of D-galacturonic carboxyl groups by methoxyl groups (–$OCH_3$), pectins can be classified as low esterified (also known as low methoxyl) pectin (LEP, DE < 50%) or high esterified (high methoxyl) pectin (HEP, DE > 50%) (Cheikh et al., 2019).

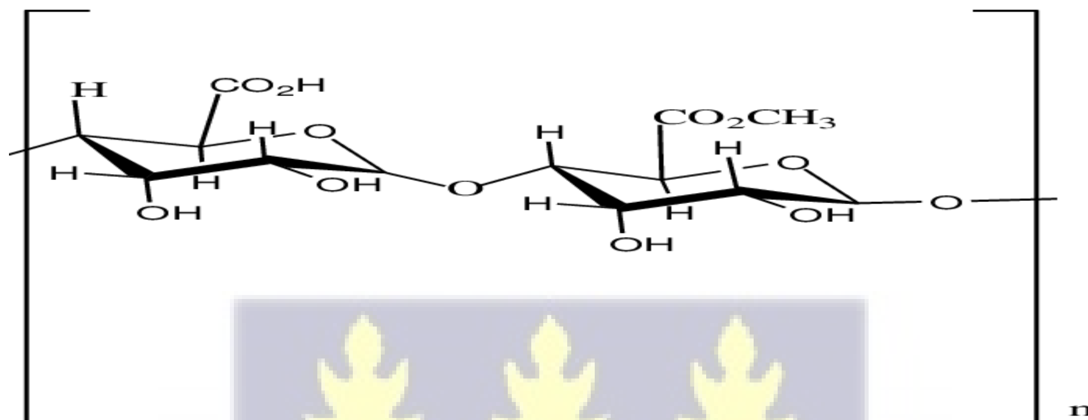


**Figure 2.10:** Chemical Structure of Pectin (Source: Researchgate.com)

Cocoa pod husk (CPH) pectin is extracted from pod husk waste after processing of cocoa beans. Previous studies shows that CPH pectin has the requisite physicochemical characteristics to be used as a multifunctional pharmaceutical excipient with remarkable properties (Adi-Dako et al., 2016).

As an anionic polymer, pectin interacts with chitosan to form a polyelectrolyte complex (PEC). The intermolecular interaction between these polysaccharides of opposite charges has been applied in the design of drug delivery systems (García et al., 2015). Studies have shown that PECs have the ability of encapsulating drugs in the polymeric matrix at molecular level thereby enhancing the physicochemical and pharmacokinetic characteristics of drugs (Ngwuluka et al.,

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2015). Furthermore, pectin resists the action of digestive enzymes present in the upper part of the gastrointestinal tract and, in contrast with chitosan, is able to withstand the low pH conditions (Cheikh et al., 2019).

In the last years, the properties of chitosan-pectin based polyelectrolyte complexes have been extensively investigated and used as carriers for specific drug delivery systems (Cheikh et al., 2019; Ghaffari et al., 2007; Maciel et al., 2015; Syed et al., 2014; Wang, 2017).

### 2.3.4 Chitosan-Pectin Based PEC

Polyelectrolyte complexes (PECs) are multifunctional formulations formed when solutions of two oppositely charged biopolymers (i.e., a polycation and a polyanion or their corresponding salts) are mixed together. Coulomb's (electrostatic) interactions between charged microdomains of the two oppositely charged polyelectrolytes lead to the formation of polyelectrolyte complexes (PECs). Hydrogen bonds, hydrophobic interactions, van der Waals interactions, and dipole interactions also contribute to complexing (Ghaffari et al., 2007; Kulkarni et al., 2016; Quiñones et al., 2018). The resulting complexes (also known as polysalts) precipitate or separate from the solution, forming a complex-rich liquid phase. However, under certain conditions, the polyelectrolytes with weak ionic groups and significantly different molecular weights at non-stoichiometric mixing ratios generate water-soluble PECs on a molecular level (Kulkarni et al., 2016).

Studies have proven that the formation of PEC leads to the formation of stable nanodispersions with dimensions in a colloidal size range (Mao et al. 2006, Sun et al. 2008). Formation of PECs

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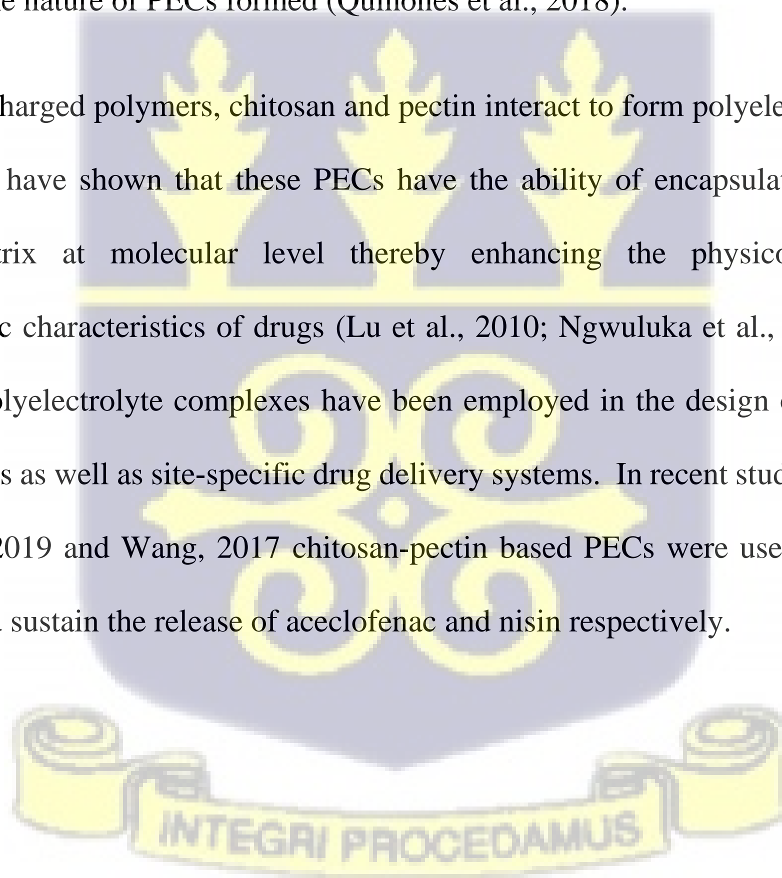
is simple and easy. PECs are able to maintain the activity and stability of embedded drugs under normal physiological conditions (Tsai et al., 2014; Wang, 2017).

PECs are classified as stoichiometric (S-PECs; PECs generated by polymers in an equimolar ratio) or non-stoichiometric (N-PECs) (N-PECs generated when one polymer is in excess compared to another) (De Robertis et al., 2015; Kulkarni et al., 2016). The structure and stability of PECs formed depends on a number of factors such as polyelectrolytes concentration, the mixing order, mixing ratio (*Z*), the degree of ionization the polyions, their charge densities and charge distribution on the polymer chains, among others. The interaction time, ionic strength, temperature as well as the pH of the medium have also been reported to greatly affect the nature of PECs formed (Quiñones et al., 2018).

As oppositely charged polymers, chitosan and pectin interact to form polyelectrolyte complex (PEC). Studies have shown that these PECs have the ability of encapsulating drugs in the polymeric matrix at molecular level thereby enhancing the physicochemical and pharmacokinetic characteristics of drugs (Lu et al., 2010; Ngwuluka et al., 2015). Chitosan-pectin based polyelectrolyte complexes have been employed in the design of modified drug delivery systems as well as site-specific drug delivery systems. In recent studies conducted by Cheikh et al., 2019 and Wang, 2017 chitosan-pectin based PECs were used to successfully encapsulate and sustain the release of aceclofenac and nisin respectively.



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# CHAPTER THREE

## METHODS USED

### 3.1 Materials and Methods

Levodopa powder (99.6%), carbidopa powder (99.8%) and chitosan (low molecular weight) were purchased from Sigma-Aldrich (USA). Pectin was extracted from cocoa pod husk. Microcrystalline cellulose was purchased from Sigma Aldrich (USA). Sinemet CR tablet (100/25mg) (Merck) was purchased from Medimart Pharmacy, Accra. All solvents used for chromatographic assay were of HPLC purity. All other reagents used were of analytical grade and purchased from approved suppliers.

### 3.2 Study Design

The study design employed was experimental. It involved the extraction of CPH pectin, formulation of chitosan-CPH pectin-based composite of levodopa/carbidopa, in vitro assays, in vivo (rat model) pharmacokinetic and biodistribution evaluation.

### 3.3 Study Location

The formulation and characterization of composite matrix of levodopa and carbidopa were done at the Pharmaceutics Research Laboratory of the School of Pharmacy, University of Ghana, Legon. The pharmacokinetic aspect of this research was done at the Animal Experimentation Unit of the School of Biomedical and Allied Health Sciences, University of Ghana, Korle-Bu. Content analysis and determination of drug levels (in plasma and brain

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tissue) were done at Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, Legon, Ghana.

## 3.4 Collection and Extraction of Cocoa Pod Husk (CPH) Pectin

Ripe cocoa pods were harvested from *Theobroma cacao* L. trees in the experimental plantation of Cocoa Research Institute of Ghana (CRIG), Tafo, Ghana. The pulp and seeds were then removed, and the fresh whole pod husks peeled to avoid pigmentation. Afterwards, the peeled husks were minced with a mechanical blender and prepared for extraction. The extraction of pectin from fresh CPHs was done according to a procedure previously described by Vriesmann *et al*, (2012) with minor modifications. Hot aqueous extraction of the fresh peeled minced husks (1.05 g/mL) was carried out at 50°C in a water bath. The extract was precipitated with 90% (v/v) ethanol and filtered twice with two-fold linen cloth. Afterwards, the pectin precipitate was washed two times with 70% ethanol, followed by 95% ethanol, and kept for a while under a fume extractor (for evaporation of residual solvent). The extraction process was repeated to exhaustion and the extract allowed to dry under vacuum. The hot water-soluble extract was then dried at 40°C overnight.  Pectin yield was then determined. The dried pectin sample was stored in aluminum foil in a desiccant until used.

### 3.4.1 Characterization of Extracted Hot Water Soluble CPH-Pectin

#### *3.4.1.1 Fourier Transform Infrared Spectroscopy (FTIR)*

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FTIR spectra of extracted hot water-soluble pectin was done using Attenuated Total Reflectance FTIR spectrometer (Perkin Elmer UATR-II, Spectrum Two). Attenuated total reflectance (ATR)spectrum was measured over the wavelength range of 4000-500 $cm^{-1}$ and at

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a resolution of 1.0 cm[1]. The powder sample was placed onto the ATR crystal surface and the sample spectrum determined.

*3.4.1.2 Swelling Index of Hot Water Soluble CPH-Pectin*

The swelling index of the CPH-pectin was determined as described elsewhere (Adi-Dako et al., 2016). One gram of the sample was accurately weighed into a 100 mL measuring cylinder and the initial volume occupied noted as $V_1$. Twenty-five (25) milliliters of distilled water was then measured, added to the sample and shaken intermittently for 1 hour. Afterwards, the sample was allowed to stand for 3 hours. The final volume occupied was noted as $V_2$. The swelling capacity was calculated using the equation:

$$\text{Swelling Capacity} = \frac{V_2}{V_1} \times 100$$

## 3.5 Preparation of Chitosan-Pectin Based Composite Matrix of Levodopa and Carbidopa

The respective amounts of powders with the exception of chitosan and pectin, were accurately weighed and mixed by geometric dilution in a mortar. Briefly, chitosan was weighed and dissolved in 1% glacial acetic acid. In order to form a viscous dispersion, the mixed powder was triturated with the chitosan solution until a uniform mixture was obtained. Pectin was dispersed in adequate amount of hot water and added with stirring until a slurry mixture was obtained. The mixture was then dried at 30 degrees Celsius (30°C) for 8 hrs. Afterwards, the dried mass was milled, passed through sieve number 40, weighed and stored at room

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temperature in glass containers. The various formulations (F1, F2, F3, F4 and F5) prepared are summarized in Table 3.1

**Table 3.1:** Composition of Chitosan-Pectin based Multiparticulate Matrix of Levodopa and Carbidopa

| Ingredient | F1 (mg) | F2 (mg) | F3 (mg) | F4 (mg) | F5 (mg) |
|---|---|---|---|---|---|
| Levodopa | 100 | 100 | 100 | 100 | 100 |
| Carbidopa | 25 | 25 | 25 | 25 | 25 |
| CS | 100 | 100 | 100 | 100 | 100 |
| CPH-PECTIN | - | 50 | 100 | 100 | 100 |
| HA | - | - | - | 10 | - |
| $CaCl_2$ | - | - | - | - | 50 |
| MCC | 200 | 150 | 100 | 90 | 50 |
| TOTAL | 425.0 | 425.0 | 425.0 | 425.0 | 425.0 |

CS = chitosan, MCC microcrystalline cellulose, HA = hydroxyapatite, $CaCl_2$ = calcium chloride, F1 = formulation 1, F2 = formulation 2, F3 = formulation 3, F4 = formulation 4 and F5 = formulation 5

3.5.1 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra of chitosan, pectin, pure levodopa, carbidopa and the chitosan-pectin based matrix were obtained using Attenuated Total Reflectance FTIR spectrometer (Perkin Elmer UATR-II, Spectrum Two). ATR spectra were measured over the wavelength range of 4000-500 $cm^{-1}$ at a resolution of 1.0 $cm^{1}$. The powder samples were placed onto the ATR crystal surface individually and the sample spectrum obtained. For each sample, the spectrum was run 4 times and the average taken. After each determination, the sample was scraped from the

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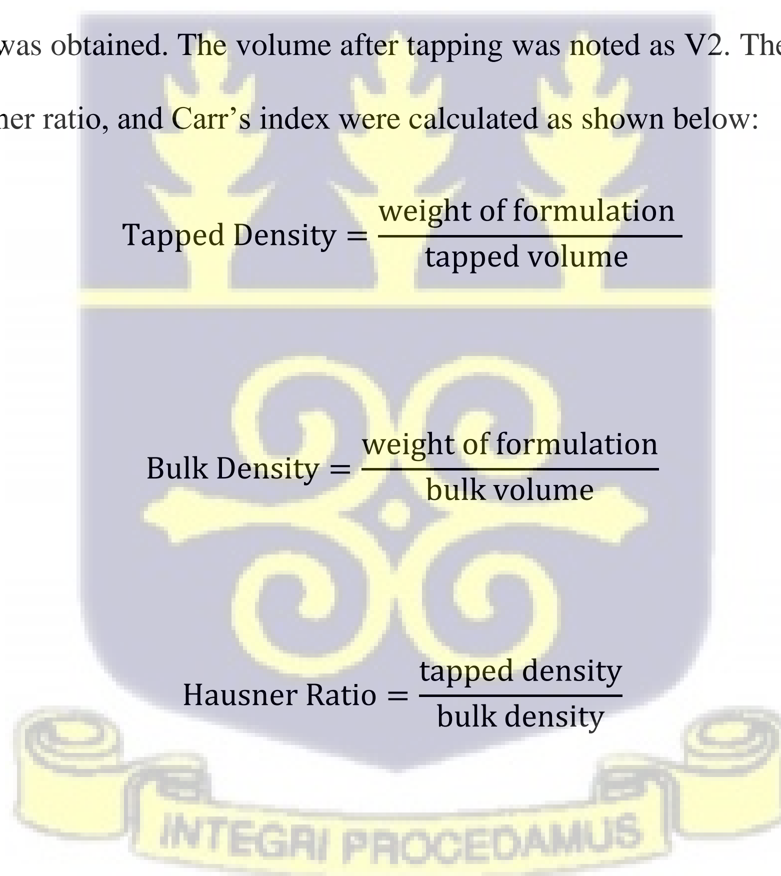
crystal surface and the surface cleaned with acetone. The characteristic peaks were recorded for each of the sample. The spectra for chitosan, pectin, pure levodopa, pure carbidopa and the chitosan-pectin matrix were superimposed. The similarities and differences in the spectra were then determined.

### 3.5.2 Flow Properties of Chitosan-Pectin Based Multiparticulate Matrix

The flow properties of the various formulations were determined by first weighing 3 g of the formulations into a 100 ml graduated measuring cylinder and noting the volume occupied as V1. The samples in the cylinder were then tapped until the powders were consolidated and a constant value was obtained. The volume after tapping was noted as V2. The bulk and tapped densities, Hausner ratio, and Carr's index were calculated as shown below:



$$\text{Tapped Density} = \frac{\text{weight of formulation}}{\text{tapped volume}}$$

$$\text{Bulk Density} = \frac{\text{weight of formulation}}{\text{bulk volume}}$$

$$\text{Hausner Ratio} = \frac{\text{tapped density}}{\text{bulk density}}$$

$$\text{Carr's Compressibility Index} = \frac{(\text{tapped density} - \text{bulk density})}{\text{tapped density}}$$

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In order to determine the angle of repose, 5 g of the formulations were weighed, transferred into a funnel clamped to a retort stand (with the tip about 10 cm from a plane paper placed on the base) and allowed to flow freely unto the paper surface. The height, H, of the cone formed was noted. A circle was drawn around the base of the cone formed and the radium, R, obtained. The angle of repose was determined by the formula;

$$\tan\theta = \frac{H}{R}$$

$$\theta = \tan - 1\left(\frac{H}{R}\right)$$

### 3.5.3 Content Analysis

The drug content of the chitosan-pectin based matrix was determined by accurately weighing 1 mg of the formulation (in triplicate) into Eppendorf tubes. One millilitre (1 ml) of distilled water was pipetted and added to each sample. Afterwards, the suspensions were vortex mixed for 10 min sonicated for 10 min in order to completely dissolve the drugs and then centrifuged at 10,000 rpm for 10 min. Portions of the supernatant (500 µL) were pipetted into HPLC auto-sampler vials. The drug content was assayed by injecting 20 µL into an HPLC system (described below) for the analysis. The concentration of levodopa and carbidopa in each solution was calculated using the straight-line equations of the standard calibration plots as shown below:

Straight line equation: $Y = mx + b$

Where, Y is the peak area of levodopa or carbidopa in the standard solution, m is the slope of the straight line, x is the concentration (µg/mL) of the levodopa or carbidopa in a sample solution, and b is the Y-axis intercept.

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## 3.6 High Performance Liquid Chromatography (HPLC) for Levodopa and Carbidopa Levels

### 3.6.1 Chromatographic Conditions

The HPLC analysis for drug content determination; in vitro release and in vivo kinetic studies was done according to a validated method for the simultaneous assay of both levodopa and carbidopa previously described by Shohreh et al., (2019) with minor modifications. The chromatographic procedure was carried out using Agilent Technologies system 1100 (Jos. Hansen, Hamburg/Germany) with the following chromatographic conditions: A stainless steel column (250 mm × 4.6 mm) packed with stationary phase ODS C18 reverse phase, mobile phase comprised of 10 mM phosphate buffer (pH of 4.0) and methanol (90:10 v/v), injection volume of 20 µL, a flow rate of 0.6 ml/min, run time of 10 min, at 30°C and a detection wavelength of 280 nm. All solvents used were of HPLC grade and were filtered with 0.45 µm filters prior to use.

### 3.6.2 Preparation of Stock and Working Solutions

Stock standard solution (1 mg/ml) of levodopa and carbidopa were prepared by weighing and dissolving 1 mg levodopa and 1 mg carbidopa in 1000µL distilled water, separately. The respective solutions were vortex mixed for 10 min and sonicated for 20 min in order to completely dissolve the drugs. In order to make 200 µg/ml solutions, 200 µL of the respective solutions were pipetted into separate Eppendorf tubes and diluted with 800 µL of distilled water. The retention time and peak area of these two standard solutions were determined by injecting 20 µL into the HPLC system. Afterwards, two-fold serial dilutions of the stock standard solutions were prepared to give 9 standard solutions with concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/mL each for levodopa and carbidopa. The same volume of

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standard solutions of levodopa and carbidopa were mixed together, giving standard working solutions 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/mL each for levodopa and carbidopa. Twenty microliters (20 µL) of the standard working solutions were injected and the retention times and peak areas for levodopa and carbidopa determined. Standard calibration curves of levodopa and carbidopa were obtained by plotting area under curve values against concentration (µg/mL). Straight-line equations were also generated and used to determine and calculate the concentration of levodopa and carbidopa in a given sample. Straight line equation:

$Y = mx + b$

Where, Y is the peak area of levodopa or carbidopa in the standard solution, m is the slope of the straight line, x is the concentration (µg/mL) of the levodopa or carbidopa in a sample solution, and b is the Y-axis intercept.

### 3.7 *In Vitro* Drug Release Study

Drug dissolution was conducted in phosphate buffer pH 6.8 (simulated gastrointestinal environment) (Dankyi et al., 2020; Ngwuluka et al., 2013). Samples of the various chitosan-pectin based formulations (F1, F2, F3, F4 and F5) were weighed and transferred into empty gelatin capsule shells (size 0). The filled capsules were then placed in baskets of USP Dissolution Apparatus 1. The dissolution media consisted of 750 ml of phosphate buffer, pH 6.8, rotating at 50 rpm and at a temperature 37 ± 2°C. Samples (5 ml) were withdrawn at time 0, 0.5, 1, 2, 4, 8, 12 and 24 hr and immediately replaced with an equal volume of fresh medium. The quantity of drug released was determined using HPLC as described above. The release experiment was performed in triplicate.

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*In vitro* drug release studies was also performed in phosphate buffer pH 4.5 (for optimized formulations F3 and F4 only) in order to mimic the duodenum-jejunal environment where absorption of levodopa actually occurs (Popa et al., 2020; Rosebraugh et al., 2019) and to observe the behaviour of the chitosan-pectin matrix in this media.

## 3.8 *In Vivo* Pharmacokinetic Evaluation of Levodopa/Carbidopa Multiparticulate Matrix

### 3.8.1 Animal Acquisition and Housing

Twenty (20) male Sprague-Dawley (SD) rats (Hsd; SD strain), weighing 150-200 g and 6-8 weeks old were obtained from the Department of Animal Experimentation, School of Biomedical and Allied Health Sciences, University of Ghana, Korle-Bu. The animals were housed in stainless steel cages at the animal house. Each rat occupied a minimum space of 2 cubic feet (61 cm x 31 cm) having soft wood shavings as bedding for their comfort. Animals were fed with normal pellet diet (AGRIMAT Kumasi), given water *ad libitum*, and kept under optimal laboratory conditions (temperature 25 ± 1°C, relative humidity 60-70%, and 12-hour light-dark cycle). The feeding and water troughs were cleaned regularly to prevent contamination. Prior to experimentation, the animals were made to acclimatise to this environment for two weeks. All animal procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals ( Institute for Laboratory Animal Research, 2011).

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### 3.8.2 Animal Groupings for Pharmacokinetic Study

The SD rats were put into 4 groups consisting of 5 rats each by simple random sampling. Group 1 received optimized levodopa/carbidopa composite formulation F3, Group 2 were given optimized formulation F4, Group 3 received Sinemet CR (a controlled release formulation of levodopa/carbidopa) and Group 4 were given levodopa/carbidopa powder (serving as an immediate release formulation).

### 3.8.3 Drug Administration and Sample Collection

Animals were made to fast overnight prior to administration of formulations. Rats in each group were given a dose of (20/5 mg/kg) of levodopa/carbidopa of the respective formulations via the oral route. Treatments were given every 12 hr. After administration of the third dose, tail vein samples were taken into Ethylenediaminetetraacetic acid (EDTA) tubes at time intervals of 0.25, 0.5, 1, 2, 4, 8, 12 and 24hr. In order to minimize the oxidation of levodopa in blood samples, a solution of 25% sodium metabisulfite in water was prepared and added in a ratio of 1: 10 (v/v) to rat blood samples collected. Blood samples were centrifuged at 4,500 rpm for 10 min to separate plasma. The plasma was carefully pipetted, transferred into fresh cryotubes and stored at -20°C until analysis.

### 3.8.4 Determination of Levels of Levodopa and Carbidopa in Plasma

#### *3.8.4.1 Plasma Fortification for Method Validation*

A 100 µL aliquot of blank plasma (plasma free of analytes) was transferred into fresh Eppendorf tubes (n=3). The plasma samples were then spiked with 100 µL of the standard

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analyte solution to obtain the desired concentrations. The spiked samples were vortexed for 2 min. To the spiked samples, 50 µL of 0.1 M perchloric acid was added, vortexed for 2 min, centrifuged at 10,000 rpm for 10min and the supernatant transferred into an auto-sampler vial for HPLC analysis (Ribeiro et al., 2015).

*3.8.4.2 Sample Preparation Procedure*

Levodopa and carbidopa in rat plasma were extracted using protein precipitation method with perchloric acid as the precipitating agent. To 100 µL of rat plasma, 50 µL of 0.1 M perchloric acid were added. The mixture was vortexed for 2 min. Afterwards, centrifugation was done at 10,000 rpm and the supernatant transferred into an auto-sampler vial. An injection of 20 µL of the supernatant was made directly into the analytical column for immediate HPLC analysis (previously described). The chromatograms were compared with those obtained from blank plasma spiked with standard analyte solutions. The peak areas for levodopa and carbidopa at their respective retention times were noted (Huang et al., 2014).

## 3.9 *In Vivo* Biodistribution Study

### 3.9.1 Animal Grouping and Drug Administration

Rats were put into 5 groups consisting of 6 rats each. Group 1 received optimized levodopa/carbidopa formulation F3, Group 2 were given optimized formulation F4, Group 3 received Sinemet CR (a controlled release formulation of levodopa/carbidopa), Group 4 were given levodopa/carbidopa powder (immediate release formulation) and Group 5 received normal saline (as negative control). Animals were made to fast overnight prior to administration of treatments. Rats in each group were given a dose of (20/5 mg/kg) of

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levodopa/carbidopa of the respective formulations via the oral route. Treatments were given every 12 h.

### 3.9.2 Sample Collection and Preparation

Following administration of the third dose, 3 rats from each of the 5 groups were anaesthetized with diethyl ether (by inhalation) at predetermined times of 1 and 2 hr after treatment. The limbs of the anaesthetized rats were fixed unto an autopsy table. The heart of the rats was exposed by opening the thoracic cavity. The vascular bed was then perfused with 10 ml normal saline via the left ventricle in order to prevent blood flow to the brain. Rat brains were excised and flushed with normal saline three times to remove the blood remains. Brain tissues were blotted after washing, weighed and stored immediately at -80°C until use.

### 3.9.3 HPLC Analysis of Levodopa in Brain Tissue

Each brain tissue was homogenized with double amount of phosphate buffer (pH 7.4) on ice. Afterwards, 200 µL of organ homogenate was taken and extracted with 1 mL mixture solution of methanol and chloroform (4:1) (Han et al., 2005). The mixture was then vortexed for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and transferred into fresh auto sampler vials.

The concentrations of levodopa in brain tissue were quantified by HPLC according to a method described by Huang et al (2014) with minor modifications. Separation was performed using a stainless-steel column (250 mm × 4.6 mm) packed with stationary phase ODS C18 reverse

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phase (Agilent Technologies system 1100; Jos. Hansen, Hamburg/Germany) with column temperature of 30°C and a flow rate of 1ml/min. Mobile phase comprised of 10 mM phosphate buffer (pH of 4.0) and methanol (90:10 v/v), injection volume of 20 µL, run time of 10 min, and a detection wavelength of 280 nm. The column was equilibrated with the mobile phase for 20min before the analysis. Results were calculated and expressed as µg/g.

### 3.10 Ethical Considerations

The study protocol was approved by the Ethics and Protocol Review Committee for College of Health Sciences, University of Ghana, with a Protocol Identification Number: *CHS-Et/M.5 – 4.4/2020 – 2021.*

### 3.11 Data Analysis

All test results were expressed as mean and standard deviation from mean (STDEV) or standard error from the mean (SEM). Statistical test of significance was taken as $p < 0.05$ and performed on all continuous data using unpaired t-test for two independent sample means, and one-way analysis of variance (ANOVA) for comparison of more than two independent sample means. Pharmacokinetic parameters for levodopa and carbidopa in the 4 groups were determined by non-compartmental analysis. The maximum plasma drug concentration ($C_{max}$) and the time to achieve this peak ($T_{max}$) of levodopa were extrapolated from concentration-time curves. The elimination rate constant ($K_e$) was determined by linear regression analysis of the terminal-linear part of the log plasma concentration-time curves. Area under the concentration-time curve (AUC) was calculated by the linear trapezoidal rule.

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For comparative assessment of biodistribution, a one-way ANOVA was performed to assess whether there is any significant difference in the levels of levodopa in rat brains. A p value $< 0.05$ was considered statistically significant.

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# CHAPTER FOUR

## RESULTS

### 4.1 Fourier Transform Infra-Red (FT-IR) Studies

Figure 4.1 is indicative of the infra-red spectrum of CPH pectin, showed a broad absorption band at 3280 $cm^{-1}$ and at 2932 $cm^{-1}$, 1735 $cm^{-1}$ and 1596 $cm^{-1}$. Other absorption bands were observed between and 1500 and 1428 $cm^{-1}$.

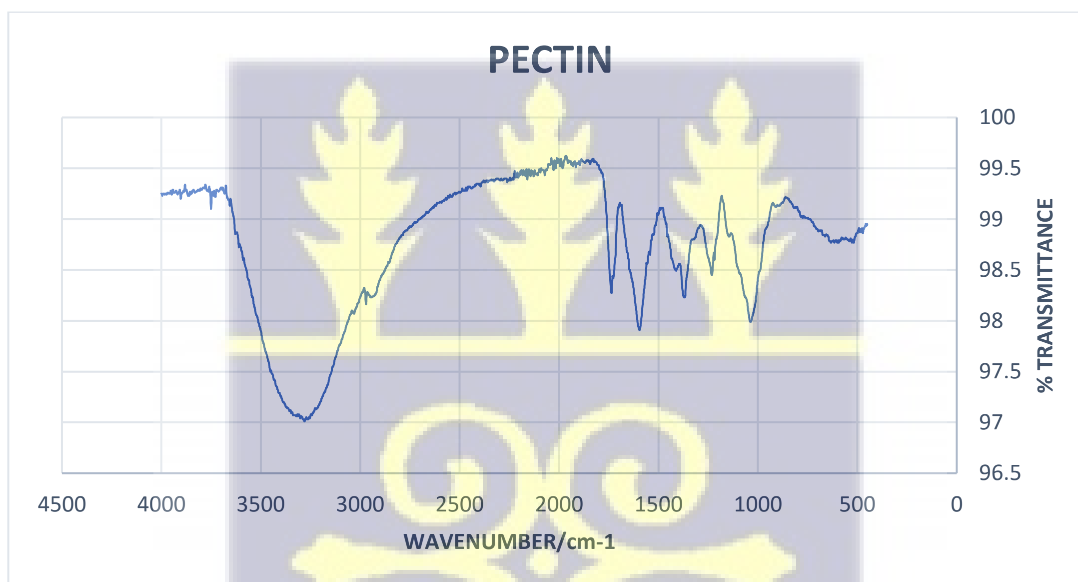


**Figure 4.1:** FTIR of Pectin

### 4.2 Swelling Index of Hot Water Soluble (HWS) CPH Pectin

The swelling index of HWS CPH pectin in 0.1M HCl, phosphate buffer (pH 6.8) and water (pH 7.0) were calculated to be 102%, 193% and 224% respectively.

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## 4.3 Characterization of Chitosan-Pectin Based Matrix of Levodopa/Carbidopa

### 4.3.1 FTIR - Drug Excipient Compatibility Study

FT-IR studies was carried out to know how compatible Levodopa and Carbidopa are with the excipients. The results of the study, shown in Figure 4.2, revealed that there are no interactions between levodopa, carbidopa and excipients since the characteristic peaks of levodopa and carbidopa are retained in the various formulations (optimized formulations F3 and F4). The FT-IR spectra of the individual drugs, excipients, formulations F3 and F4, with their characteristic absorption bands, is shown in Appendix 8.

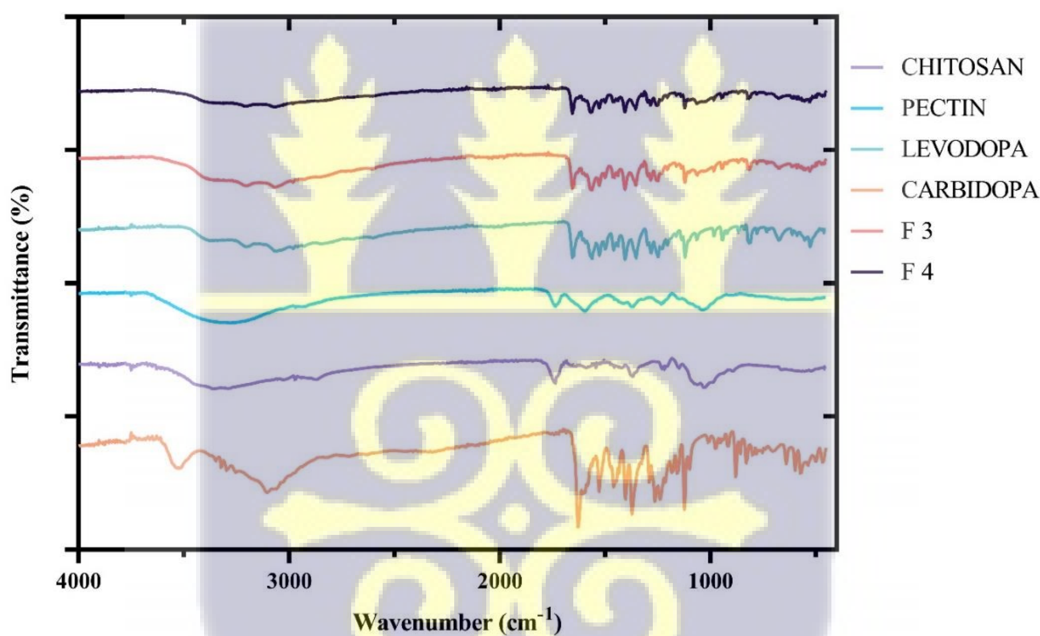


**Figure 4.2:** FT-IR Spectra of Levodopa, Carbidopa, Cocoa Pod Husk Pectin, Chitosan and Optimized Formulations F3 and F4

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### 4.3.2 Flow and Precompression Parameters

The bulk and tapped densities of the various chitosan-pectin based formulations were determined and used to calculate the Hausner ratio and Carr's compressibility index. The angle of repose was also determined. All the formulations were less cohesive and had satisfactory flow properties (Table 4.1a) as shown in the reference table (Table 4.1b).

**Table 4.1a:** Flow Properties of Chitosan-Pectin Based Formulations

| Formulation | Bulk Density ($kg/m^3$) | Tapped Density ($kg/m^3$) | Hausner Ratio | Carr's Index | Angle of Repose (°) |
|---|---|---|---|---|---|
| F1 | 566.04 | 697.67 | 1.23 | 18.87 | 27.15 |
| F2 | 545.45 | 750.00 | 1.38 | 27.27 | 23.5 |
| F3 | 612.24 | 714.29 | 1.17 | 14.29 | 24.64 |
| F4 | 588.24 | 697.67 | 1.19 | 15.69 | 28.61 |
| F5 | 517.24 | 666.67 | 1.29 | 22.41 | 24.44 |

**Table 4.1b:** Reference Ranges for Hausner ratio, Carr's Index and Angle of Repose

| Flow Character | Carr's Index (%) | Hausner Ratio | Angle of Repose (°) |
|---|---|---|---|
| **Excellent** | ≤ 10 | 1.00-1.11 | < 20 |
| **Good** | 11 -15 | 1.12 -1.18 | 20-30 |
| **Fair** | 16 -20 | 1.19 -1.25 | ………. |
| **Passable** | 21 -25 | 1.26 -1.34 | 30 -34 |
| **Poor** | 26 -31 | 1.35 -1.45 | ……… |
| **Very Poor** | 32 -37 | 1.46-1.59 | >35 |
| **Very very poor** | > 38 | >1.6 | ………. |

**(Balaji et al., 2020)**

### 4.3.3 Drug Content Analysis

Content analysis was done to determine the actual amount of levodopa and carbidopa in the chitosan-pectin based multiparticulate matrix. Peak areas generated from the HPLC analysis were substituted into the equation of the straight line obtained from the standard calibration curves (shown in Figure 4.4a and 4.4b). This was used to calculate for the content of levodopa and carbidopa in the various formulations (shown in Table 4.2a and 4.2b).

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**Table 4.2a:** Content Analysis - Levodopa Portion

| Formulation | Run 1 % Content | Run 2 % Content | Run 3 % Content | Average % Content ± STDEV |
|---|---|---|---|---|
| F1 | 128.28 | 97.74 | 95.59 | 107.20 ± 10.56 |
| F2 | 83.32 | 101.27 | 89.16 | 91.25 ± 5.29 |
| F3 | 91.33 | 91.62 | 91.73 | 100.70 ± 0.12 |
| F4 | 93.99 | 93.50 | 93.03 | 93.51 ± 0.28 |
| F5 | 125.13 | 110.50 | 108.40 | 114.68 ± 5.26 |

**Table 4.2b:** Content Analysis - Carbidopa Portion

| Formulation | Run 1 % Content | Run 2 % Content | Run 3 % Content | Average % Content ± STDEV |
|---|---|---|---|---|
| F1 | 108.93 | 97.42 | 107.82 | 104.73 ± 3.67 |
| F2 | 99.47 | 88.83 | 101.47 | 96.59 ± 3.93 |
| F3 | 92.21 | 98.85 | 90.39 | 99.03 ± 2.57 |
| F4 | 88.90 | 95.20 | 91.77 | 91.96 ± 1.82 |
| F5 | 112.73 | 113.52 | 117.94 | 114.73 ± 1.62 |

Levodopa/Carbidopa extended-release formulations should contain not less than 90.0% and not more than 110.0% of the stated amount of levodopa and carbidopa (United States Pharmacopoeia, 2018).

## 4.4 HPLC Analysis of Levodopa and Carbidopa

### 4.4.1 Chromatogram for Levodopa and Carbidopa

A simple isocratic mobile phase comprising of 10 mM phosphate buffer and methanol 90:10 (%v/v) was used to separate the analytes completely with a UV-visible detector at 280nm. As seen in Figure 4.3a, 4.3b and 4.3c, levodopa and carbidopa peaks appeared at 3.5 and 5.1 min separately, while their retention times in mixture solution changed to 3.7 and 5.5 min

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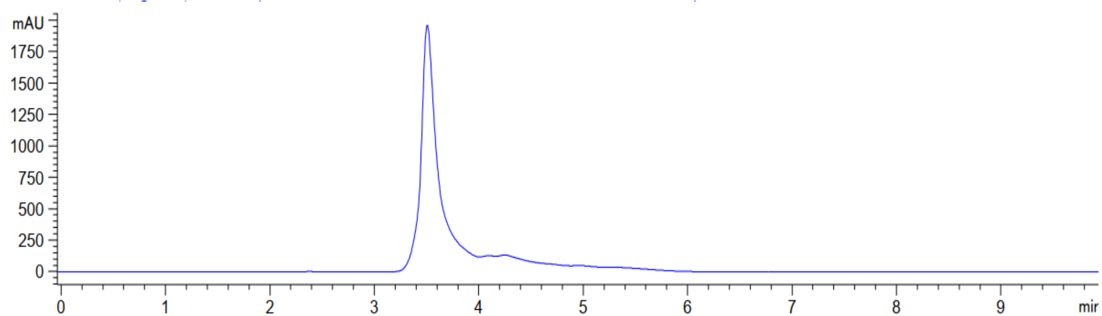
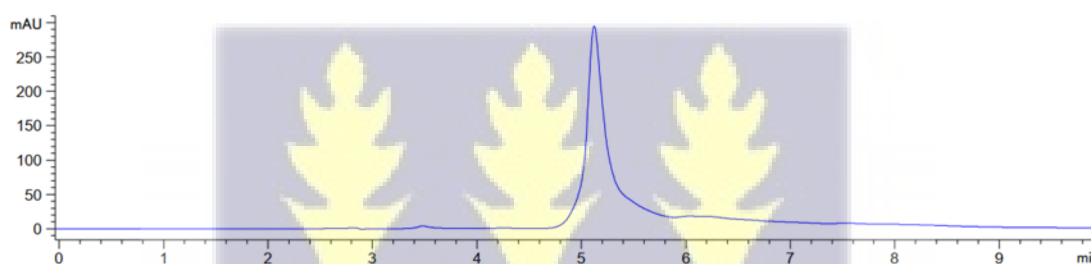
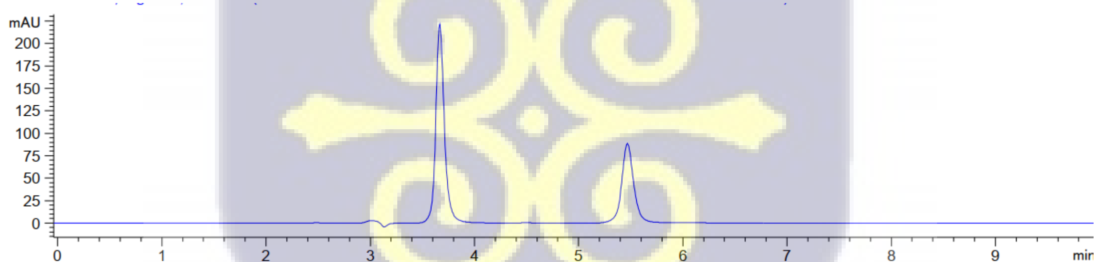
respectively with no interference of tablet excipients. Levodopa and carbidopa were separated completely and showed good resolution and suitable retention times. The total run time was 10 min.




(a)




(b)




(c)

**Figure 4.3:** Representative Chromatograms of Levodopa only (a), Carbidopa only (b) and their respective Peaks when Standard Solutions were mixed (c)

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### 4.4.2 Standard Calibration Curve for Levodopa and Carbidopa

Nine (9) serially diluted solutions of Levodopa and Carbidopa were analyzed (Table 4.3). Standard calibration curves of levodopa and carbidopa were obtained by plotting peak areas (obtained from the HPLC analysis) against concentration (µg/mL). The correlation coefficients ($R^2$) of both Levodopa and Carbidopa were 0.994 and 0.9948 respectively.

**Table 4.3:** Concentrations and Peak Areas of Serially Diluted Standard Levodopa and Standard Carbidopa Solutions

| Solution (Serial dilutions) | Levodopa | | Carbidopa | |
|---|---|---|---|---|
| | Concentration (µg/ml) | Peak Area | Concentration (µg/ml) | Peak Area |
| 1 | 100 | 2678.1 | 100 | 1646.2 |
| 2 | 50 | 1441.9 | 50 | 725.2 |
| 3 | 25 | 828.8 | 25 | 316.9 |
| 4 | 12.5 | 479 | 12.5 | 160.6 |
| 5 | 6.250 | 292 | 6.250 | 82.9 |
| 6 | 3.125 | 93.6 | 3.125 | 40.7 |
| 7 | 1.563 | 47.59 | 1.563 | 19.3 |
| 8 | 0.781 | 24.2 | 0.781 | 12.1 |
| 9 | 0.391 | 14.4 | 0.391 | 8 |

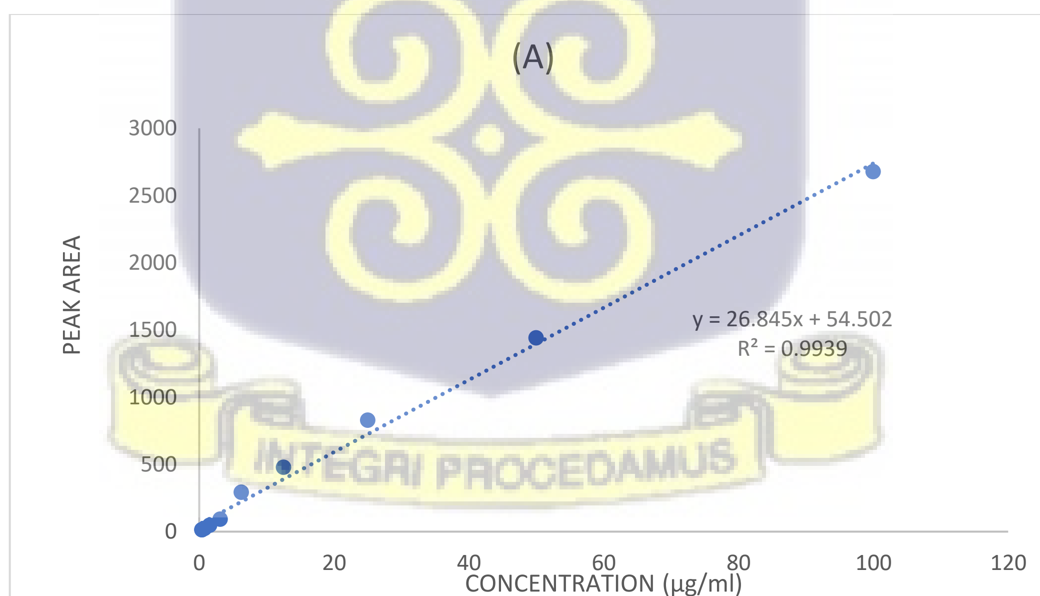


**Figure 4.4a:** Levodopa Standard Curve showing the Equation of the Line (y =26.845x + 54.502) and the Correlation Coefficient ($R^2$ = 0.994)

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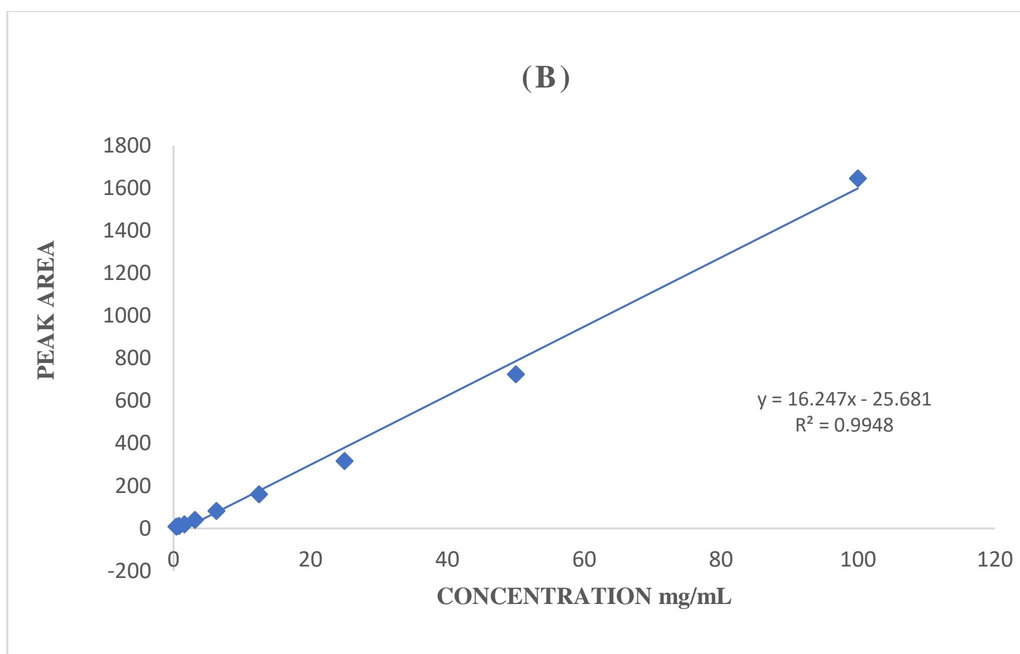


**Figure 4.4b:** Carbidopa Standard Curve showing the Equation of the Line (y = 16.247x -25.681) and the Correlation Coefficient ($R^2$ = 0.9948)

## 4.5 *In Vitro* Drug Release Studies in Simulated Intestinal Fluid

*In vitro* release studies of the chitosan-pectin based levodopa/carbidopa formulations were performed in phosphate buffered saline (pH 6.8) and in phosphate buffered saline pH 4.5 (for only F3 and F4) using Dissolution Apparatus 1 equipment. The release of levodopa and carbidopa from the chitosan-pectin based matrix was found to be pH dependent as shown in Figures 4.5 a, b, c and d. In general, the release of both drugs from the chitosan-pectin based matrix was controlled and sustained over the 24-hr period. The levels of carbidopa, however, declined sharply in phosphate buffer, pH 6.8, after 4 hr for all formulations. Results of the *in vitro* drug release profile of levodopa/carbidopa matrix is summarised in Table 4.4 a, b, c and d.

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**Table 4.4a:** Cumulative Release of Levodopa in Phosphate Buffer pH 6.8

| **Average Cumulative Levodopa Release in Phosphate Buffer pH 6.8 (%) ± STDEV** | | | | | | |
|---|---|---|---|---|---|---|
| **Time (hr)** | **Animal Code** | | | | | |
| | **F1** | **F2** | **F3** | **F4** | **F5** | **SINEMET** |
| 0.5 | 55.58 ± 3.43 | 73.34 ± 0.41 | 50.53 ± 2.39 | 15.45 ± 1.62 | 33.03 ± 0.60 | 4.4 ± 0.82 |
| 1 | 73.79 ± 1.26 | 91.71 ± 1.90 | 66.02 ± 2.35 | 41.61 ± 3.64 | 40.17 ± 2.06 | 12.73 ± 1.53 |
| 2 | 78.91 ± 0.45 | 96.13 ± 0.81 | 80.90 ± 2.07 | 50.90 ± 1.35 | 50.79 ± 0.71 | 33.95 ± 9.71 |
| 4 | 93.30 ± 3.69 | 95.70 ± 3.41 | 90.43 ± 2.40 | 74.36 ± 0.81 | 66.46 ± 2.30 | 57.01 ± 2.42 |
| 8 | 99.17 ± 2.22 | 100.19 ± 1.35 | 99.32 ± 0.22 | 86.93 ± 5.49 | 77.81 ± 0.55 | 90.91 ± 5.29 |
| 12 | 100.93 ± 1.29 | 102.46 ± 0.38 | 101.95 ± 2.50 | 99.71 ± 4.41 | 73.50 ± 3.64 | 95.82 ± 1.65 |
| 24 | 102.38 ± 1.57 | 107.88 ± 3.00 | 103.71 ± 2.32 | 93.40 ± 2.52 | 72.15 ± 1.40 | 100.64 ± 1.82 |

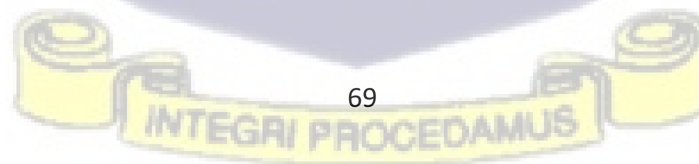

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**Table 4.4b:** Cumulative Carbidopa Release from formulations in Phosphate Buffer, pH 6.8

| **Average Cumulative Carbidopa Release in Phosphate Buffer pH 6.8 (%) ± STDEV** | | | | | | |
|---|---|---|---|---|---|---|
| **Time (hr)** | **Animal Code** | | | | | |
| | **F1** | **F2** | **F3** | **F4** | **F5** | **SINEMET** |
| 0.5 | 42.60 ± 1.33 | 46.42 ± 3.59 | 41.34 ± 2.39 | 36.37 ± 2.11 | 40.29 ± 2.78 | 13.77 ± 1.66 |
| 1 | 43.96 ± 2.30 | 58.09 ± 2.06 | 56.05 ± 3.65 | 51.19 ± 2.35 | 45.43 ± 1.42 | 21.16 ± 1.50 |
| 2 | 48.39 ± 1.40 | 52.15 ± 0.88 | 56.66 ± 1.98 | 60.68 ± 0.98 | 47.52 ± 1.08 | 32.41 ± 0.67 |
| 4 | 51.81 ± 0.74 | 52.24 ± 4.17 | 70.10 ± 2.80 | 65.90 ± 2.26 | 49.37 ± 1.65 | 53.48 ± 1.55 |
| 8 | 51.48 ± 1.16 | 43.82 ± 2.52 | 57.15 ± 1.86 | 37.90 ± 5.64 | 54.11 ± 1.99 | 70.74 ± 0.73 |
| 12 | 36.04 ± 2.16 | 38.61 ± 1.88 | 48.41 ± 2.03 | 26.46 ± 4.01 | 33.68 ± 3.95 | 55.13 ± 2.53 |
| 24 | 28.83 ± 2.92 | 25.54 ± 4.15 | 29.51 ± 1.37 | 22.89 ± 3.35 | 26.85 ± 1.68 | 37.84 ± 2.60 |



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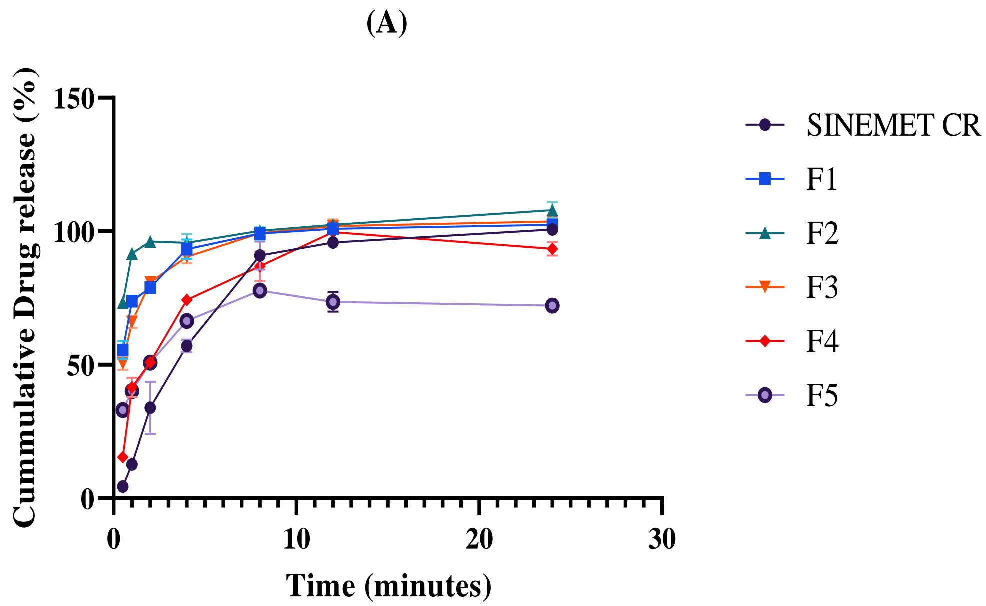


**Figure 4.5a:** Release Profile of Levodopa from Formulations in Phosphate Buffer pH 6.8

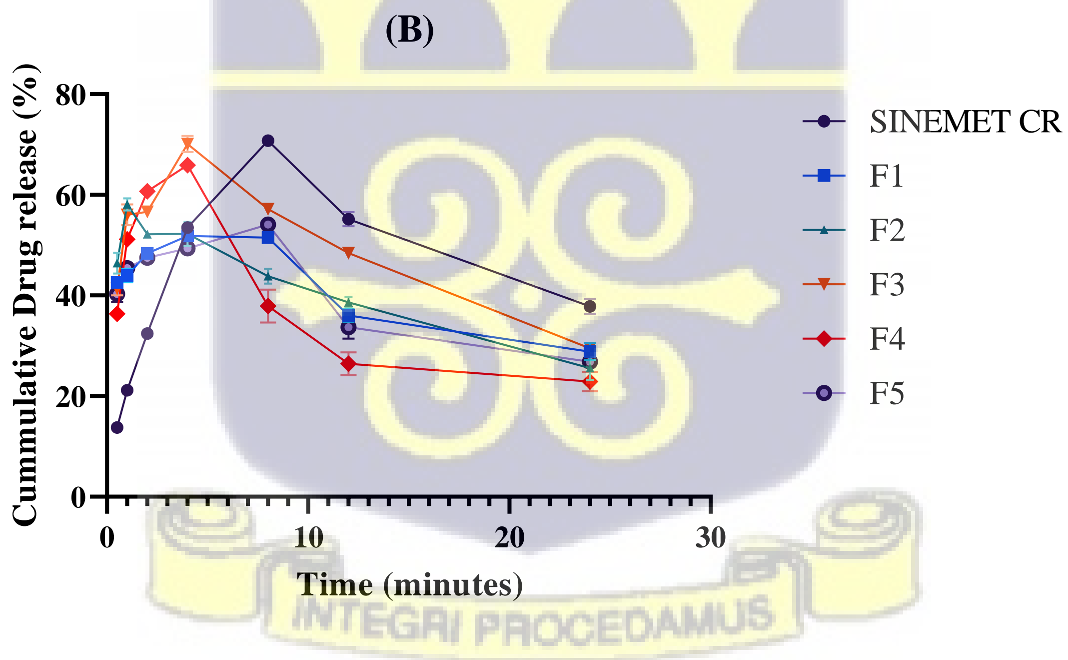


**Figure 4.5b:** Release Profile of Carbidopa from Chitosan-Pectin based formulations in Phosphate Buffer pH 6.8

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**Table 4.4c:** Cumulative Release of Levodopa from Optimized Formulations, F3 and F4 in Phosphate Buffer at pH 4.5

| **Average Cumulative Levodopa Release in Phosphate Buffer pH 4.5 (%) ± STDEV** | | |
|---|---|---|
| **Time (hr)** | **Animal Code** | |
| | **F3** | **F4** |
| 0.5 | 7.71 ± 0.33 | 15.97 ± 3.81 |
| 1 | 18.87 ± 3.0 | 20.89 ± 6.37 |
| 2 | 39.86 ± 3.24 | 51.78 ± 1.08 |
| 4 | 87.51 ± 7.41 | 79.35 ± 4.72 |
| 8 | 79.20 ± 5.72 | 103.1 ± 4.59 |
| 12 | 71.53 ± 1.94 | 95.92 ± 3.93 |
| 24 | 52.89 ± 2.44 | 84.30 ± 9.53 |

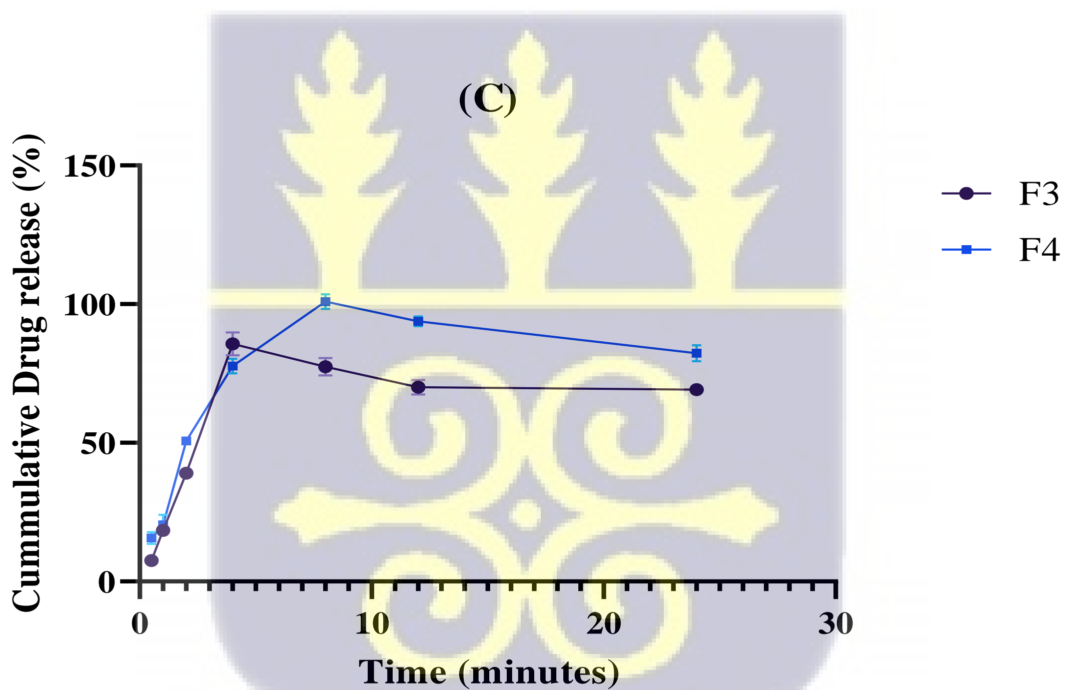


**Figure 4.5c:** Release Profile of Levodopa from F3 and F4 at pH 4.5

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**Table 4.4d:** Cumulative Release of Levodopa from Optimized Formulations, F3 and F4 at pH 4.5

| Average Cumulative Carbidopa Release in Phosphate Buffer 4.5 (%) ± STDEV | | |
|---|---|---|
| **Time (hr)** | **Animal Code** | |
| | **F3** | **F4** |
| 0.5 | 16.36 ± 0.82 | 32. 88 ± 3.12 |
| 1 | 25.96 ± 1.07 | 46. 80 ± 2.08 |
| 2 | 45.36 ± 3.67 | 75. 70 ± 4.42 |
| 4 | 88.36 ± 2.96 | 85. 10 ± 2.64 |
| 8 | 76.16 ± 2.47 | 100. 74 ± 1.78 |
| 12 | 53.96 ± 2.08 | 97. 55 ± 2.73 |
| 24 | 51.36 ± 0.06 | 87. 41 ± 1.57 |

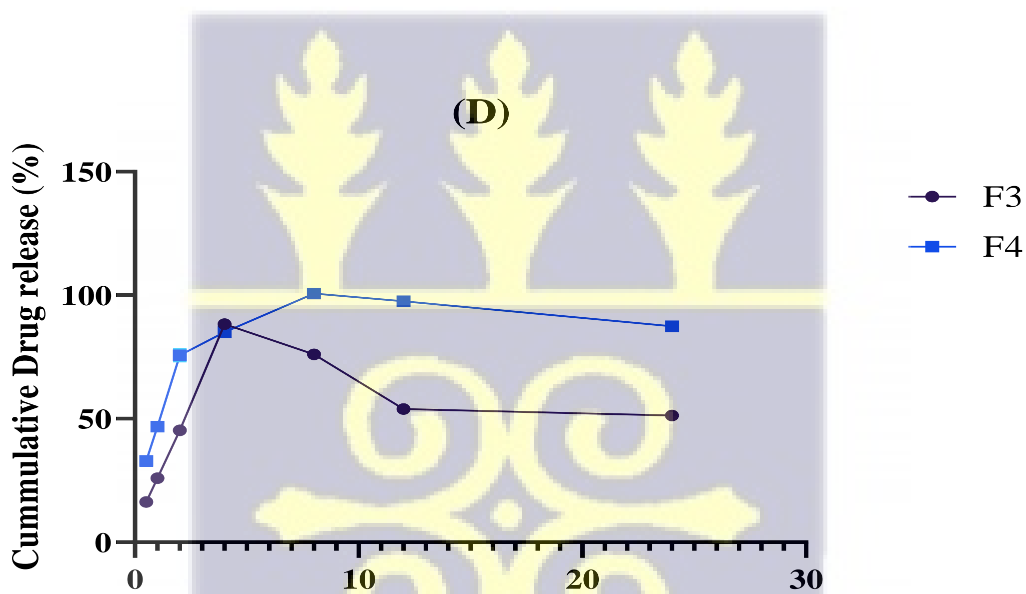
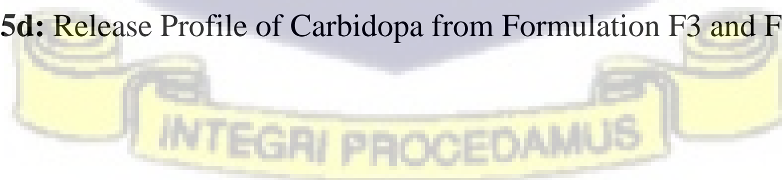



**Figure 4.5d:** Release Profile of Carbidopa from Formulation F3 and F4 at pH 4.5



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## 4.6 *In Vivo* Pharmacokinetic Evaluation of Levodopa/Carbidopa Multiparticulate Matrix

### 4.6.1 Concentration-time Curves of Levodopa in the Four Treatment Groups

Plasma samples of each of the five (5) rats in each group were analysed separately and the average concentration at different time points determined. From the concentration-time plots (Figure 4.6a). From the graph, the maximum concentration (Cmax) and the time it takes to reach Cmax (Tmax) were higher for the optimized chitosan-pectin based matrix compared with the positive control (Sinemet CR). Formulation 4 had the highest $T_{max}$.

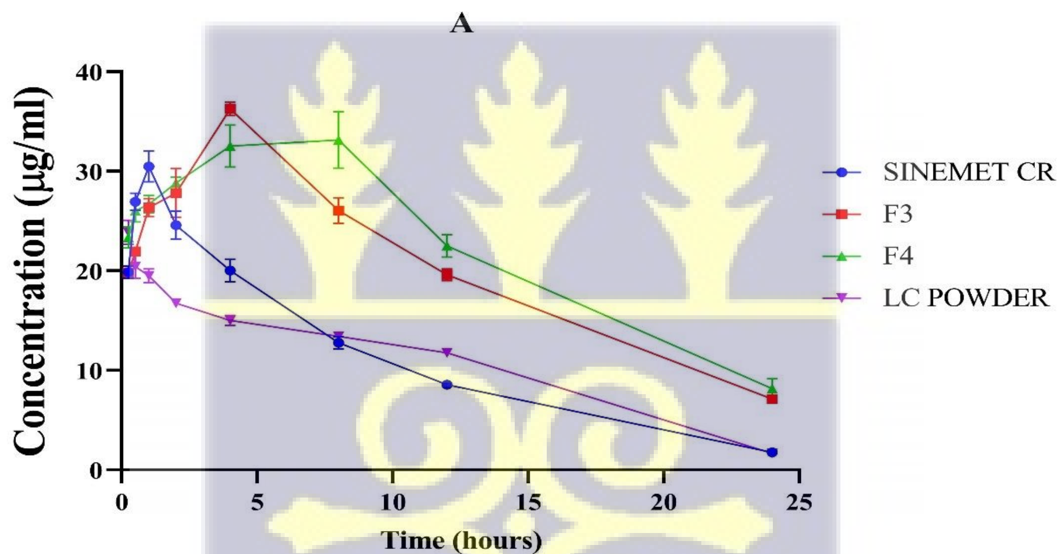


**Figure 4.6a:** Concentration-Time Curves of Levodopa for the Four Treatment Groups of SD Rats

*LC=Levodopa + Carbidopa Powder (immediate release formulation)*

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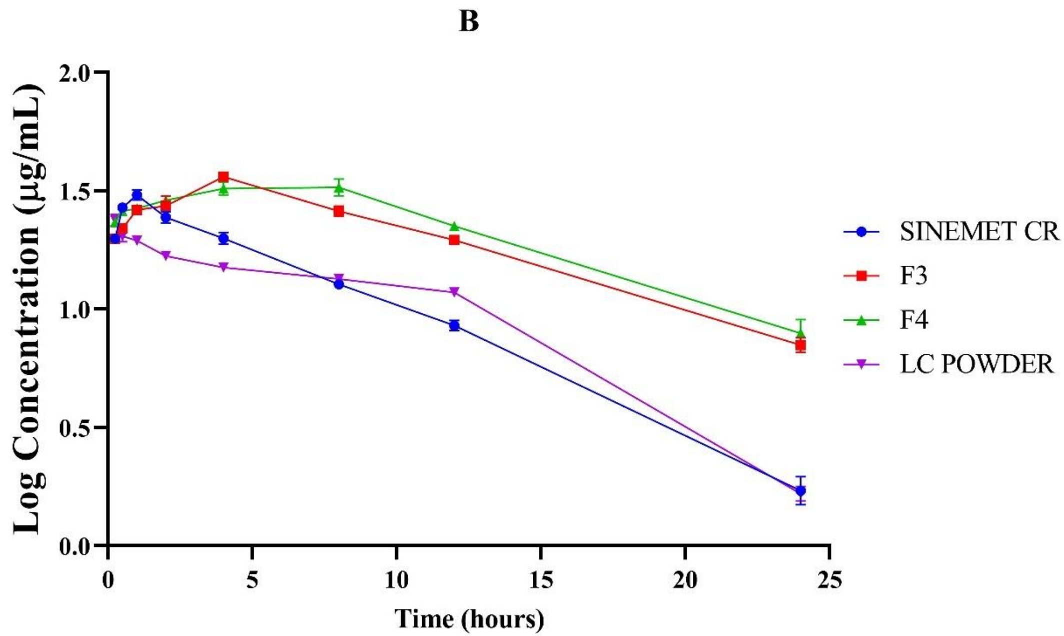


**Figure 4.6b:** Log Concentration-Time Curves of Levodopa for the Four Treatment Groups of Rats

4.6.2 Pharmacokinetic Parameters of Levodopa

The pharmacokinetic parameters of levodopa in the 4 treatment groups derived from the concentration-time curve and the log concentration-time plots is shown in Table 4.5. One way ANOVA performed on the pharmacokinetic parameters showed that $T_{max}$, $C_{max}$, Area under curve ($AUC_{0-24}$), AUC to infinity ($AUC_{0\rightarrow\infty}$), elimination rate constant ($K_e$) and plasma half-life ($t_{1/2}$) varied significantly ($p < 0.05$) between the 4 treatment groups.

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**Table 4.5:** Pharmacokinetic Parameters of Levodopa in the 4 Treatment Groups (± SEM)

| PK parameter | Sinemet | F3 | F4 | LC | p- value |
|---|---|---|---|---|---|
| T max (hr.) | 0.9 (0.22) | 4 (0.00) | 4.4 (2.19) | 0.40 (0.34) | <0.0001 |
| C max (μg/ml) | 30.62 (3.37) | 36.28 (1.52) | 34.80 (2.19) | 24.00 (2.42) | <0.0001 |
| $AUC_{0-24}$ (μg/hr/ml) | 262.84 (16.73) | 484.98 (18.70) | 535.60 (33.04) | 252.39 (135.468) | <0.0001 |
| $AUC_{0-\infty}$ | 275.60 (16.89) | 572.13 (36.46) | 647.40 (83.55) | 262.83 (10.35) | <0.0001 |
| $K_e$ (1/hr) | 0.13 (0.03) | 0.09 (0.02) | 0.09 (0.03) | 0.16 (0.01) | 0.0003 |
| $T_{1/2}$ (hr.) | 5.34 (0.95) | 8.33 (0.01) | 8.84 (2.95) | 4.27 (0.35) | <0.0001 |

*4.6.2.1 Post hoc Analysis with Tukey's Multiple Comparison*

A pairwise comparison was done using the Tukey's multiple comparison test, to detect the difference of pharmacokinetic parameters between a given pair of treatment groups. The results of this test are shown in Appendix 7.

*4.6.2.1.1 Maximum Concentration of Levodopa reached (Cmax)*

The Tukey's multiple comparison test between the $C_{max}$ of Sinemet CR and F4 showed that the maximum concentration after administration ($C_{max}$) did not differ significantly ($p = 0.07$). A comparison between the $C_{max}$ of the optimized formulations, F3 and F4 and levodopa/carbidopa immediate release powder showed a statistically significant difference ($p < 0.0001$) respectively. Also, $C_{max}$ was found to differ significantly between Sinemet CR and F3 ($p = 0.0109$) and between levodopa/carbidopa immediate release powder and Sinemet CR ($p = 0.0031$).

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*4.6.2.1.2 Time Taken to Reach Maximum Concentration (Tmax)*

A Statistical comparison with Tukey's multiple comparison test between Sinemet CR and levodopa/carbidopa powder showed that the time taken to reach maximum concentration after administration ($T_{max}$) did not differ significantly ($p = 0.99$). A comparison of $T_{max}$ between formulation 3 (F3) and formulation 4 (F4) also showed no statistically significant difference ($p = 0.94$). However, there was a significant difference between the $T_{max}$ of Sinemet CR and F3 ($p = 0.001$) and between Sinemet CR and F4 ($p = 0.0003$).

*4.6.2.1.3 Area under the Concentration-Time Curves ($AUC_{0-\infty}$ and $AUC_{0-24hr}$)*

The area under the concentration time curves from time 0 to 24hrs ($AUC_{0-\infty}$) and from time 0 to infinity ($AUC_{0-24hr}$) between the formulations were also compared. A statistical comparison of the $AUC_{0-\infty}$ and $AUC_{0-24hr}$ between F3 and Sinemet CR and between F4 and Sinemet CR were all found to differ significantly ($p < 0.0001$) in all cases. Also, the comparison of the $AUC_{0-\infty}$ and $AUC_{0-24hr}$ between F3 and LC powder and between F4 and LC powder were all found to differ significantly ($p < 0.0001$) in all cases.

*4.6.2.1.4 Elimination Half-Life ($T_{1/2}$)*

A statistical comparison between the half-life ($T_{1/2}$) of Sinemet CR and F3 showed a statistically significant difference ($p < 0.0001$). The difference between the $T_{1/2}$ of Sinemet CR and F4 was also found to be significant ($p < 0.0001$). A comparison between F3 and F4 showed that the time taken for the plasma concentration to be halved ($T_{1/2}$) does not differ significantly ($p = 0.92$).

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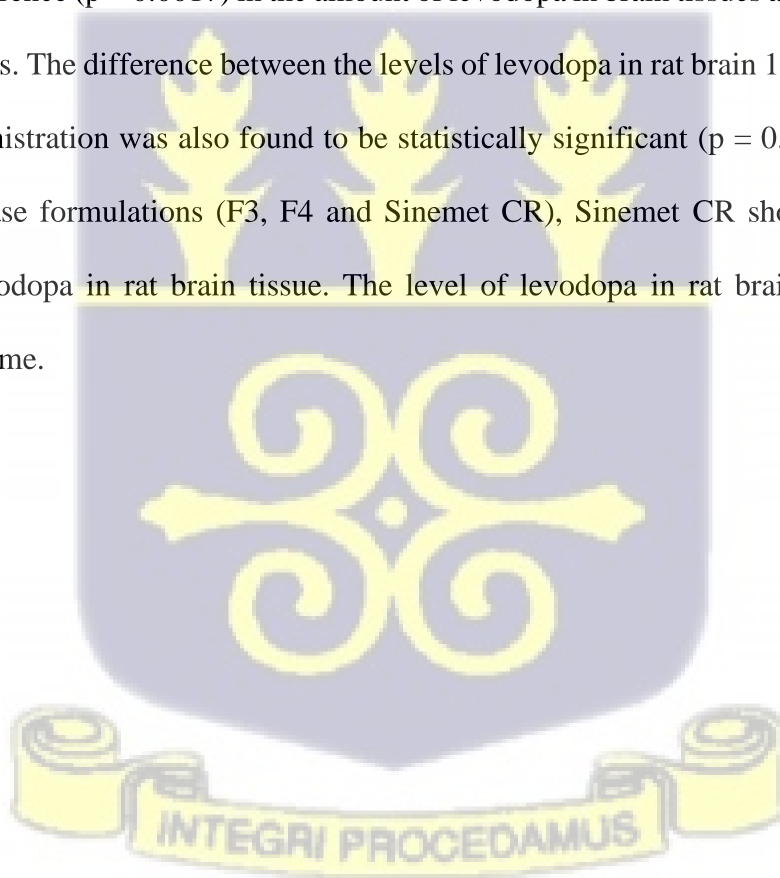
*4.6.2.1.5 Elimination rate constant ($K_e$)*

A comparison between the elimination rate constant of the optimized formulations (F3 and F4) and levodopa/carbidopa immediate release powder were found to differ significantly ($p = 0.0008$ in both cases).

## 4.7 Biodistribution Study

Biodistribution of levodopa to the brain was studied to assess whether there is any significant difference in the levels of levodopa reaching rat brains from the various treatment groups. A two-way ANOVA on the biodistribution data (shown in Appendix 9), showed a statistically significant difference ($p = 0.0017$) in the amount of levodopa in brain tissues among the various treatment groups. The difference between the levels of levodopa in rat brain 1 hr and 2 hrs after oral drug administration was also found to be statistically significant ($p = 0.0276$). Of all the controlled release formulations (F3, F4 and Sinemet CR), Sinemet CR showed the highest amount of Levodopa in rat brain tissue. The level of levodopa in rat brains was found to decrease with time.



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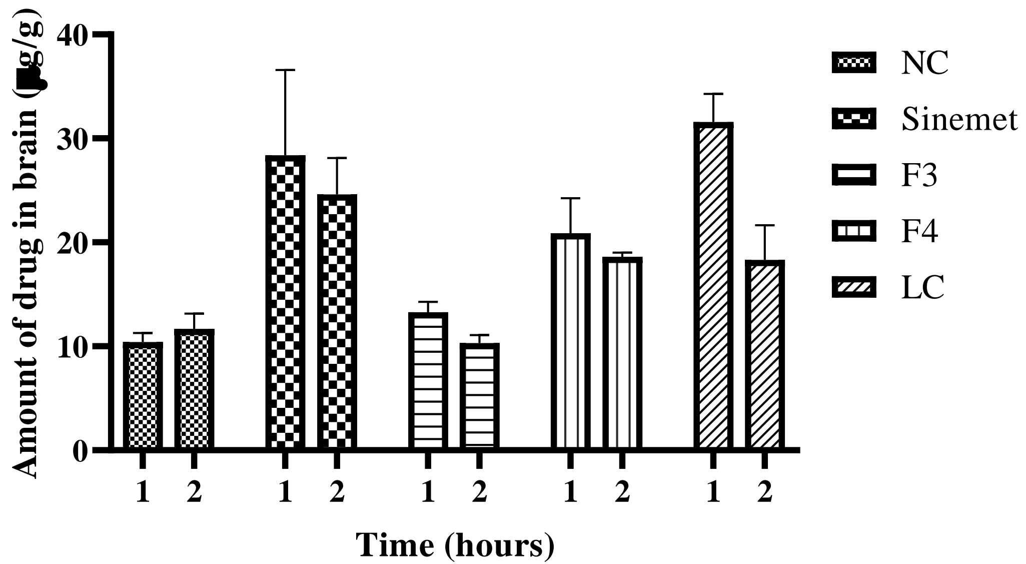
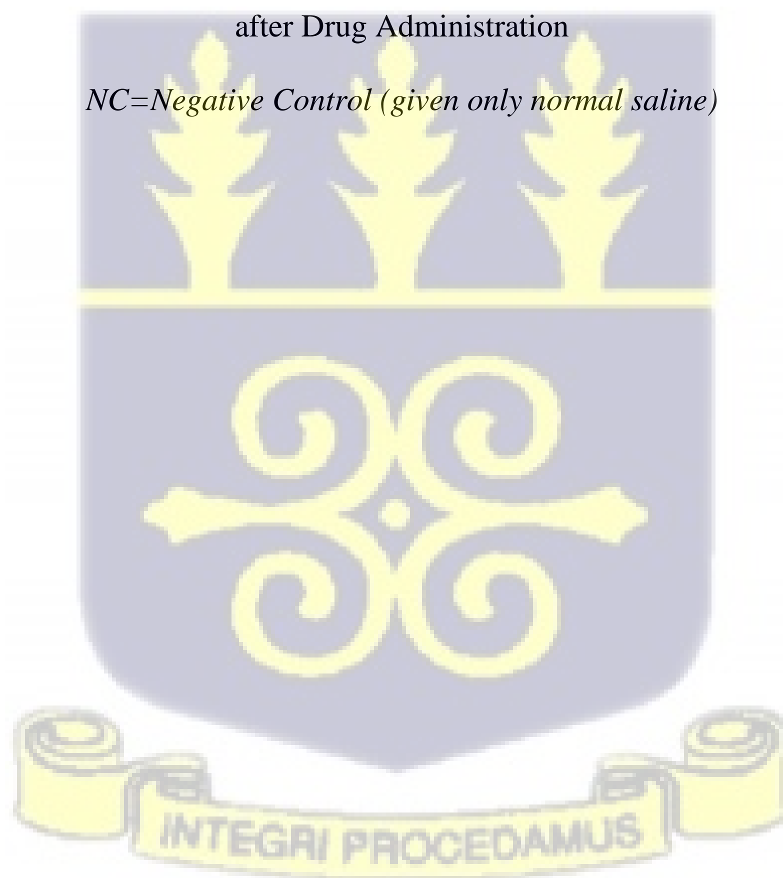



**Figure 4.7:** A Chart of the Amount of Levodopa in Rat Brain (µg/g) One and Two Hours after Drug Administration

*NC=Negative Control (given only normal saline)*



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# CHAPTER FIVE

## DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

### 5.1 Discussion

The extraction of pectin from cocoa pod husk (CPH) and its use as biocompatible, biodegradable and cheaper excipient in drug formulation is an environmentally and economically friendly means of managing CPH waste after harvesting the beans from the pods. In the current study, the yield of hot water-soluble pectin from CPH was 7.91%. Previous studies by Adi-Dako et al., (2016) and Mollea et al., (2008), reported extraction yields of 23.30% and 6.5% respectively for hot water soluble pectin. The extraction yields CPH pectin with hot water (pH 7.0) have been shown to depend largely on factors such as origin of cocoa pods, extraction time, as well as pre-treatment methods employed (Mollea et al., 2008; Sahari et al., 2003). Although, the quantities of CPH pectin recovered with hot water (pH 7) is relatively lower when compared with other solvents, it is safer and more advantageous over extraction with solvents such as citric acid, nitric acid and hydrochloric acid because no corrosive effluents are released into the environment (Adi-Dako et al., 2016).

Data obtained from FT-IR spectroscopy of CPH pectin showed a broad absorption band $3280cm^{-1}$ corresponding to hydroxyl (-OH) stretching, a band at $2932cm^{-1}$ corresponding to the tension of C-H due to vibration of methyl ester groups ($CH_3$). The sharp absorption band at 1735 $cm^{-1}$ and 1596 $cm^{-1}$ were indicative of the presence of esterified carboxyl group (COO) and non-esterified carboxyl groups (COO-R) in CPH pectin, respectively. Absorption signals between 1623 and 1428 $cm^{-1}$ have been shown to correspond to wavelength features for polygalacturonic acid (Monsoor et al., 2001; Santos et al., 2013). Thus, the pectin extracted

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from CPH was rich in polygalacturonic acid. Previous studies by Adi-Dako et al., (2016) and Hennessey-Ramos et al., (2021) on CPH pectin, showed similar spectrum.

Over the years, natural polymers such as arginine, chitosan, dextrin, among others have been employed in the formulation of controlled and sustained release formulations (De Robertis et al., 2015; Sung & Kim, 2020). In this study, chitosan and CPH pectin were employed in the formulation of a matrix for the sustained release of levodopa/carbidopa. Drug-excipients compatibility test was done by FT-IR in order to detect and predict any potential chemical or physical interactions that could affect the quality, physicochemical properties and release of the active pharmaceutical ingredients (Dave et al., 2015).

In the FT-IR spectrum of LD (appendix 8d), characteristic peaks appearing between 3500-3200cm$^{-1}$ corresponded to O-H stretching, Bands between 3000cm – 2850 cm$^{-1}$ corresponded to symmetric and asymmetric -C-H (aliphatic) stretches. Secondary amine (-$NH_2$) stretches were visible at 1560 cm$^{-1}$, and a sharp peak at 1633cm$^{-1}$ corresponded to the presence of a carbonyl (C=O) (Ledeti et al., 2017). FT-IR spectrum of carbidopa (appendix 8c), showed visible absorption bands between 3500-3200cm$^{-1}$ and 3100 -3000cm$^{-1}$ corresponding to O–H stretches and -C–H stretches respectively. Carbonyl peak was visible at 1627cm, $NH_2$ (secondary amine) absorption band appeared at 1560 cm$^{-1}$ and absorption bands appearing between 1450 – 1400cm-1 corresponded to phenyl group C=C vibrations (Bukhary et al., 2020). The spectrum of chitosan showed characteristic absorption bands of C=O stretching and amidic N-H bending between 1700 - 1650cm$^{-1}$ and 1500 -1400 cm$^{-1}$ respectively (Bigucci et al., 2008) whereas the spectrum of pectin showed characteristic peaks as described previously (appendix 8 a and b). FT-IR results of optimized formulations (F3 and F4) showed similar peaks for the specific functional groups present in levodopa and carbidopa as shown in Figure 4.2. This finding suggests that there was no structural change or interference to levodopa or

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carbidopa with the excipients used. Previous reports by Bigucci et al., (2008) and Gadalla et al., (2016) also revealed that chitosan and pectin were compatible with vancomycin and progesterone respectively and could be safely used in drug formulation.

In capsule formulation, the ease of flow of powders is of great significance because free flowing powders ensure reproducible capsule dosator filling, thus ensuring weight uniformity and consistency in the physical properties of capsules (Adi-Dako et al., 2016). The flow and precompression parameters of the chitosan-pectin based formulations studied were Hausner ratio, Carr's index and angle of repose. For Hausner ratio, values close to 1.2 are suggestive of free-flowing and less cohesive powders whilst values greater than 1.6 are indicative of cohesive powders with poor flowability. In general, Carr's compressibility index above 32 and angles of repose above 35° are indicative of powders with poor and unsatisfactory flow properties (Balaji M et al., 2020). In this study, formulation F1, F2, F3, FF4 and F5 were found to be less cohesive and had satisfactory flow properties as shown in Table 4.1a.

Drug content analysis was made to evaluate the efficacy of process of formulation. According to the United States Pharmacopoeia (2018), levodopa/carbidopa combination product should contain not less than 90% and not more than 110% of the stated amount of levodopa and carbidopa. In the current study, the content of levodopa and carbidopa in all formulations with the exception of F5 were within the stated acceptance criteria as shown in Table 4.2a and b.

In order to determine the extent and rate of levodopa/carbidopa release and to predict the absorption of these drugs within living organisms, in vitro drug release studies were performed. In the current study, in vitro drug release studies was performed in phosphate buffered saline pH 6.8 and at 37℃ in order to mimic physiologic environment (Dankyi et al., 2020; Ngwuluka et al., 2013). The release of the levodopa and carbidopa from the matrix was found to be

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controlled and sustained over 24 hrs. As shown in Table 4.4a, levodopa was rapidly released from formulation F1 (containing only chitosan) and formulation F2 (containing 100mg chitosan and 50mg pectin). Increasing the amount of pectin in F3 (containing 100mg chitosan and 100mg pectin) resulted in a reduced release rate of levodopa from the chitosan-pectin matrix. The results obtained are consistent with those of El-Gibaly (2002) who also reported a more delayed release of drug when concentration of pectin in a matrix was increased.

The addition of hydroxyapatite and calcium chloride salts to the chitosan-pectin matrix as seen in F4 (containing 100mg chitosan, 100mg pectin and 10mg hydroxyapatite) and F5 (containing 100mg chitosan, 100mg pectin and 50mg $CaCl_2$) further delayed the release of both levodopa and carbidopa from these formulations. Hydroxyapatite is a calcium-rich mineral. Calcium ions serve as cross-linking agents for pectin and earlier reports suggest that the binding of calcium ions with pectin delay drug solubility and increases gel strength of polymer matrices (Adi-Dako et al., 2018; El-Gibaly, 2002; Wu et al., 2007). Findings from *in vitro* drug release profiles of F4 and F5 also suggested that, calcium ions from $CaCl_2$ provided better cross-linking with pectin than hydroxyapatite. The release profile of formulation 5, was however unsatisfactory as only 77.8 % of levodopa was released from the multiparticulate matrix.

In comparison with Sinemet CR (shown in Table 4.4a), the release of levodopa from the chitosan-pectin matrix was found to be biphasic, with averagely 50% of drug released in the first 1 hour (as against ~ 12% for Sinemet CR) and the remaining drug released in a controlled fashion over the next 24 hours. Thus, the chitosan-pectin matrix may be used to rapidly achieve therapeutic levodopa plasma concentrations and maintain them over longer periods than Sinemet CR.

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In general, the release of carbidopa from the chitosan-pectin multiparticulate matrix and Sinemet CR increased gradually over 4 hours and 5 hours respectively and declined sharply beyond these time points. This finding was found to be consistent with earlier reports by Ngwuluka et al., 2013 and Rosebraugh et al., 2019. Carbidopa is reported to be unstable at pH 6.8 (Rosebraugh et al., 2019), hence significant degradation might have occurred during the *in vitro* drug release studies, accounting for the observation made in Figure 4.4b.

Based on the aforementioned content analysis data and drug release profiles of chitosan-pectin based formulations (F1, F2, F3, F4 and F5), F3 and F4 were selected as optimized formulations for further investigations.

The release patterns of levodopa and carbidopa in the optimized formulations (F3 and F4) were further investigated in phosphate buffer pH 4.5, mimicking the conditions of the duodenum-jejunal region of the gastrointestinal tract where absorption of levodopa occurs. The release of levodopa and carbidopa was found to be controlled and sustained. In this medium, maximum release of levodopa and carbidopa from F3 and F4 occurred after 4hrs and 8hrs respectively.

The in vivo pharmacokinetic (PK) characteristics and biodistribution profiles of the optimized chitosan-pectin based matrix of levodopa and carbidopa were investigated in male SD rats. In the current study, only the PK parameters and biodistribution of levodopa were considered since it is the main drug of concern when it comes to the management of PD. Again, carbidopa does not cross the BBB but works in the periphery to prevent conversion of levodopa to dopamine by dopa decarboxylases (Khor & Hsu, 2008; Olanow et al., 2006).

Findings from the concentration-time plots of levodopa showed that, higher levodopa plasma concentrations ($C_{max}$) were achieved with F3 and F4 compared with the levodopa/carbidopa immediate release powder and Sinemet CR. Previous studies by Kaur & Kaur (2012) and Zhao

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et al., (2020) showed similar results for carvedilol and anthracyanins with the use of chitosan-pectin complex. Levels of levodopa in plasma have been shown to be directly correlated with the amount reaching the brain (Connolly & Lang, 2014) hence a higher $C_{max}$ may result in an increase in the levels of levodopa reaching the brain and may be advantageous in the management of PD symptoms.

Another important pharmacokinetic parameter is the time taken to reach peak plasma concentrations ($T_{max}$). From table 4.5, $C_{max}$ for F3 and F4 were reached after 4.0hr and 4.4hr whereas Cmax for Sinemet and the immediate release powder was reached after 0.9hr and 0.4 hr respectively. Thus, the *in vivo* absorption of levodopa from the chitosan-pectin based matrix was slower compared with Sinemet CR and the immediate release powder.

Evaluation of the area under concentration-time plots from time 0 to 24hrs ($AUC_{0-24}$) and to infinity ($AUC_{0-\infty}$) for F3, F4, Sinemet CR and levodopa/carbidopa immediate release powders showed that F3 and F4 had higher AUCs, about twice the AUC for Sinemet CR and the immediate release powder (Table 4.5). AUC values give a reflection of the total drug exposure after administration (DiPiro *et al.*, 2005). The high AUC values of F3 and F4 could be attributed to the outstanding mucoadhesive property conferred in the matrix by chitosan. Chitosan, a mucoadhesive polymer, is rich in positively charged groups which readily interact with negatively charged mucous membranes of the gastrointestinal tract, thereby increasing adhesion, and thus improving contact time for drug absorption (Saikia et al., 2015). Furthermore, chitosan has been shown to have permeation enhancing properties (Bernkop-Schnürch & Dünnhaupt, 2012; Soliman et al 2014). Since AUC is the most reliable measure of a drug's bioavailability, it can be inferred from the AUC values that the chitosan-pectin based matrix of levodopa/carbidopa had greater bioavailability than Sinemet CR (the conventional controlled release product on the market) and levodopa/carbidopa powder.

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The elimination half-lives ($T_{1/2}$) of F3, F4, Sinemet CR and the levodopa/ carbidopa immediate release powder were also investigated. $T_{1/2}$ is indicative of how a drug is cleared from the plasma and is defined as the time taken for plasma concentration of a drug to decrease by half the original concentration (DiPiro et al., 2006). In the current study, it was observed, that $T_{1/2}$ was highest for F4, followed by F3, Sinemet CR and lastly, levodopa/carbidopa immediate release powder. $T_{1/2}$ has been shown to play a key role in determining whether accumulation of drug will occur under multiple dosage regimen (DiPiro et al., 2006). The finding of this study suggests that it takes a relatively longer time for levodopa within the chitosan-pectin based matrix to be cleared from plasma. This property may be of great benefit in preventing the "on" and "off" phenomenon observed with the use of conventional levodopa/carbidopa formulations.

Results from the biodistribution studies showed that the levels of levodopa in rat brain tissues decreased with time, after oral administration. As shown in Figure 4.7, higher levels of levodopa from the immediate release powder and Sinemet CR were observed within 1 hour after oral administration. The relatively lower levels of levodopa in the brains of rats administered F3 and F4 is consistent with the pharmacokinetic profiles of these formulations. Thus, lower brain levels of levodopa is expected since formulations F3 and F4 delayed the release of levodopa into plasma.

## 5.2 Conclusion

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Drug release from the chitosan-pectin based matrix of levodopa/carbidopa was controlled and sustained over time. The chitosan-pectin based multiparticulate matrix of levodopa/carbidopa exhibited a modest improvement in the pharmacokinetic profile of levodopa compared with

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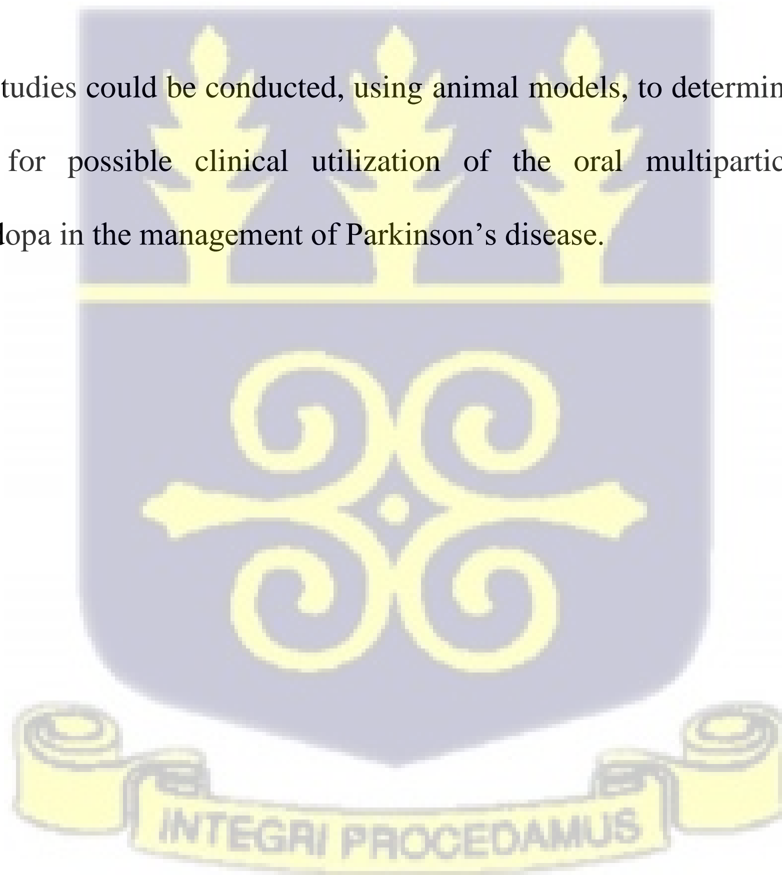
Sinemet CR and levodopa/carbidopa immediate release powder. The mucoadhesive nature of the matrix and sustained delivery of levodopa from the matrix can be employed in overcoming the irregular gastric emptying and motor fluctuations ('on' and 'off' phenomenon) associated with oral administration of levodopa

## 5.3 Recommendations

Further investigations should be done to ascertain and confirm the biodistribution of the multiparticulate matrix of levodopa/carbidopa in the brain in order to know the levels of levodopa reaching the brain after oral administration.

Also, efficacy studies could be conducted, using animal models, to determine suitable dosing concentrations for possible clinical utilization of the oral multiparticulate matrix of levodopa/carbidopa in the management of Parkinson's disease.



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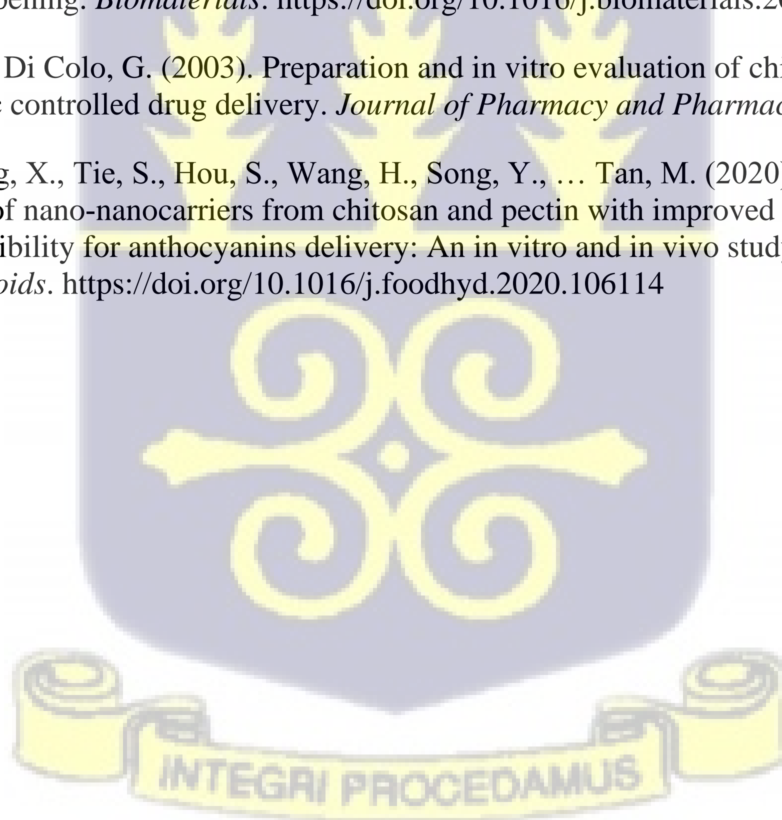

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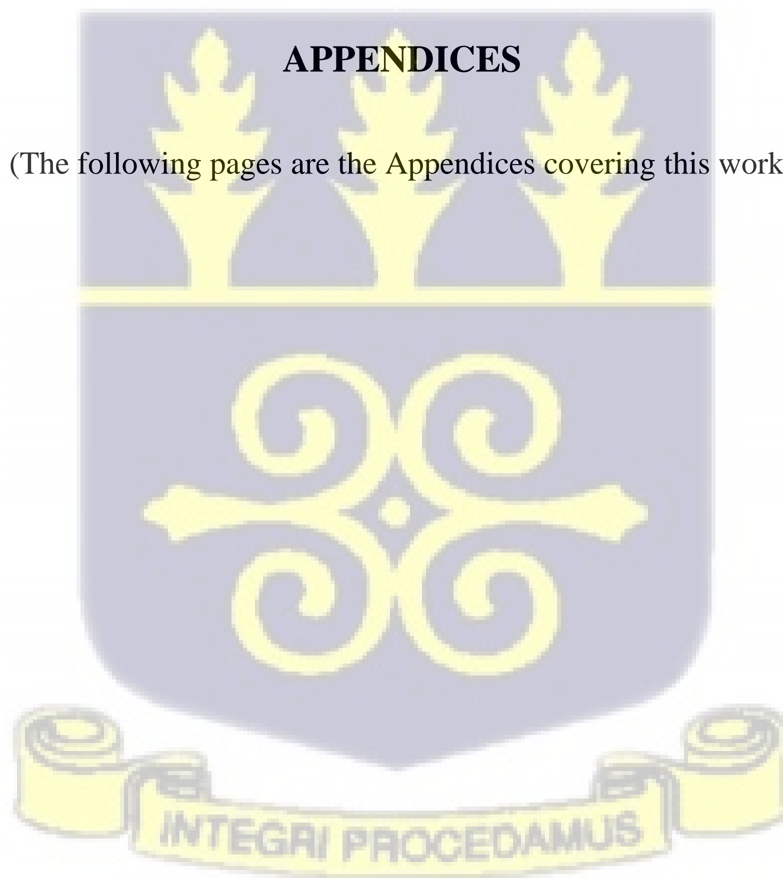


# APPENDICES

(The following pages are the Appendices covering this work)

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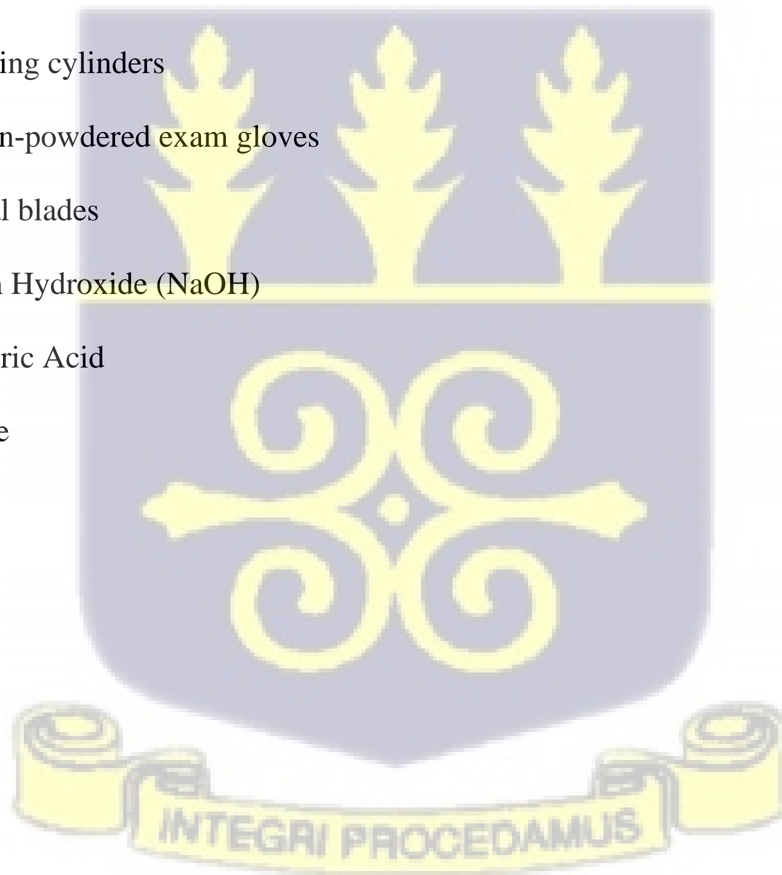
# APPENDIX 1

## REAGENTS AND EQUIPMENT

### Appendix 1a: List of Reagents

- Potassium dihydrogen phosphate
- Absolute ethanol
- Chloroform
- Diethyl ether
- Eppendorf tubes
- Cryotubes
- Measuring cylinders
- Nile non-powdered exam gloves
- Surgical blades
- Sodium Hydroxide (NaOH)
- Perchloric Acid
- Acetone



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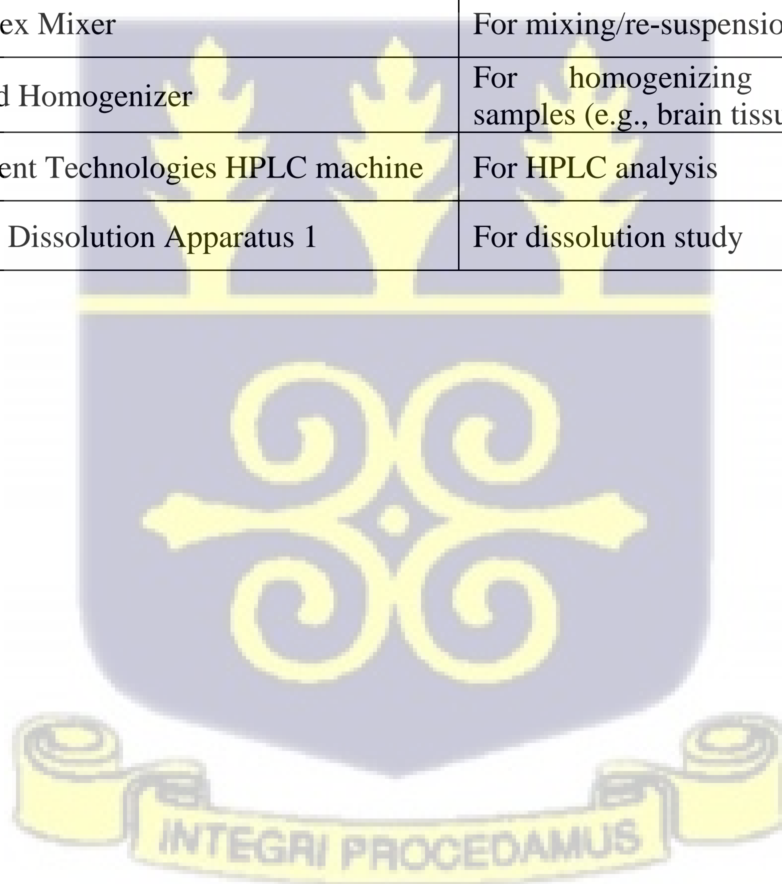
## Appendix 1b: List of Equipment

**Table A1:** List of Equipment

| SN | EQUIPMENT | USE |
|---|---|---|
| 1. | Perkin Elmer ATR FTIR Spectrometer | For Fourier transform infra-red spectroscopy |
| 2. | Mettler Toledo Analytical balance | For weighing Levodopa, Carbidopa, excipient, salts and other solids |
| 3. | Ultrasonic Washer Sonicator | Uses ultrasound to agitate and breakdown solid particles in solution |
| 4. | Mikro 200 Centrifuge | For spinning at high speed in order to separate various components of fluids and for centrifuging blood samples before separation |
| 5. | Micropipettes | For drawing/dispensing solutions |
| 6. | Vortex Mixer | For mixing/re-suspension of liquids |
| 7. | Hand Homogenizer | For homogenizing biological samples (e.g., brain tissue) |
| 8. | Agilent Technologies HPLC machine | For HPLC analysis |
| 9. | USP Dissolution Apparatus 1 | For dissolution study |



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# APPENDIX 3

## CALCULATION OF ANIMAL EQUIVALENT DOSE (AED) OF LEVODOPA/CARBIDOPA

Human dose Levodopa/Carbidopa combination drug = 200/50 mg b.i.d, respectively

Average human weight = 60 kg

Therefore,

$$\text{The dose of Levodopa per kg body weight} = \frac{200\ \text{mg}}{60\ \text{kg}} = 3.33\ \text{mg / kg}$$

$$\text{The dose of Carbidopa per kg body weight} = \frac{50\ \text{mg}}{60\ \text{kg}} = 0.83\ \text{mg / kg}$$

$$\text{Animal Equivalent Dose } (\text{AED}) = \text{Human Dose } (\text{per kg BW}) \times \text{Correction factor } (\text{Km})$$

But

$$\text{Km} = \frac{\text{Average body weight of species}}{\text{Body surface area}}$$

For rats;

Average body wight = 0.155kg and Body surface area =0.025

$$\therefore \text{Km for rats} = \frac{0.155}{0.025} = 6.2$$

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$$\text{Therefore, the AED of Levodopa for rats} = 3.33\ \text{mg/kg} \times 6.2$$
$$= 20.646\ \text{mg/kg}$$
$$\approx 20.0\ \text{mg/kg}$$

$$\text{AED of Carbidopa for rats} = 0.83\ \text{mg/kg} \times 6.2 = 5.146\ \text{mg/kg}$$
$$\approx 5.0\ \text{mg/kg}$$

$$\text{Animal equivalent dose} = 20/5\ \text{mg/kg}$$

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# APPENDIX 4

## CALCULATION OF LEVODOPA/ CARBIDOPA CONTENT

Standard calibration equation of Levodopa is $y = 26.845x + 54.502$

Where;

y-intercept = 54.502, slope = 26.845, y = peak areas obtained from HPLC and x = concentration of levodopa to be determined

$$\therefore \text{ concentration of levodopa in any sample } (x) = \frac{(y - 54.502)}{26.846}$$

Amount of Formulation weighed for content analysis =1mg

Using one of the optimized formulations F3 as an example, from the triplicate test done,

Peak area of Run 1 = 6492.5

$$\therefore \text{ Concentration of levodopa } = \frac{(6492.5 - 54.502)}{26.846}$$

$$= 239.821\,\mu g / mL$$

$$= 0.23981\,mg / mL$$

If 1mg of F3 contains 0.23981mg,

Then

$$\text{the total amount in } 425\,mg \text{ (total weight of formulation per capsule)} = 0.23981 \times 425$$

$$= 101.92\,mg$$

Expected Amount of Levodopa per capsule is 100mg

$$\therefore \text{ Content of Levodopa in F3 } = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

***Run 1***,

$$\%\text{ Content } = \frac{101.92}{100} \times 100\%$$

$$= 101.92\%$$

Peak area of Run 2 = 6321.0

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$$\text{Concentration of levodopa} = \frac{6321.0 - 54.502}{26.846}$$
$$= 233.433\ \mu g/mL$$
$$= 0.23343\ mg/mL$$

If 1mg of F3 contains 0.23343, then

$$\text{the total amount in } 425\,\text{mg (total weight of formulation per capsule)} = 0.23343 \times 425$$
$$= 99.209\ mg$$

Expected Amount of Levodopa per capsule is 100mg

Therefore,

$$\therefore \text{ Content of Levodopa in F3} = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

***For run 2***,

$$\%\ \text{Content} = \frac{99.209}{100} \times 100\%$$
$$= 99.21\%$$

Peak area of Run 3 = 6406.8

$$\text{Concentration of levodopa} = \frac{6406.8 - 54.502}{26.846}$$
$$= 236.629\ \mu g/mL$$
$$= 0.23662\ mg/mL$$

If 1mg of F3 contains 0.23662mg, then

$$\text{the total amount in } 425\,\text{mg (total weight of formulation per capsule)} = 0.23662 \times 425$$
$$= 100.57\ mg$$

Expected Amount of Levodopa per capsule is 100mg

$$\therefore \text{ Content of Levodopa in F3} = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

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***For run 3***,

$$\% \text{ Content } = \frac{100.57}{100} \times 100\%$$
$$= 100.57\%$$

$$\textbf{Average \% of Levodopa Content of F3} = \frac{(101.92 + 99.21 + 100.57)}{3} = 100.7\%$$

For Carbidopa Content,

Standard calibration equation for carbidopa is $y = 16.247x - 25.681$

Where y-intercept = 25.681, slope = 16.247, y = peak areas obtained from HPLC and x = concentration of Carbidopa

$$\therefore \text{ concentration of Carbidopa in any sample } (x) = \frac{(y + 25.681)}{16.247}$$

Amount of Formulation weighed for content analysis =1g

Using one of the optimized formulations F3 as an example, from the triplicate test done,

Peak area of Run 1 = 943.1

$$\text{Concentration of Carbidopa} = \frac{(943.1 + 25.681)}{16.247}$$
$$= 59.6283\ \mu g / mL$$
$$= 0.05963\ mg / mL$$

If 1mg of F3 contains 0.05963mg, then

the total amount in 425mg (total weight of formulation per capsule) = 0.05963 x 425
$= 25.32mg$

Expected Amount of Carbidopa per capsule is 25 mg

Therefore,

$$\therefore \text{ Content of Carbidopa in F3 } = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

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***Run 1,***

$$\%\ \text{Content} = \frac{25.32}{25} \times 100\%$$
$$= 101.28\%$$

Peak area of Run 2 = 933.4

$$\text{Concentration of Carbidopa} = \frac{(933.4 + 25.681)}{16.247}$$
$$= 59.03127\ \mu g / mL$$
$$= 0.0590\ mg / mL$$

If 1mg of F3 contains 0.0590, then

$$\text{the total amount in 425mg (total weight of formulation per capsule)} = 0.0590 \text{ x } 425$$
$$= 25.0883\ mg$$

Expected Amount of Carbidopa per capsule is 25mg

$$\therefore \text{Content of Carbidopa in F3} = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

***For run 2,***

$$\%\ \text{Content} = \frac{25.09}{25} \times 100\%$$
$$= 100.35\%$$

Peak area of Run 3 = 938.25

$$\text{Concentration of Carbidopa} = \frac{(938.25 + 25.681)}{16.247}$$
$$= 56.16846\ \mu g / mL$$
$$= 0.056168\ mg / mL$$

If 1mg of F3 contains 0.05617 then

$$\text{the total amount in 425mg (total weight of formulation per capsule)} = 0.05617 \text{ x } 425$$
$$= 23.872\ mg$$

Expected Amount of Carbidopa per capsule is 25mg

$$\therefore \text{Content of Carbidopa in F3} = \frac{\text{Actual amount of Drug}}{\text{Expected}} \times 100\%$$

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***For run 3***,

$$\% \text{ Content } = \frac{23.872}{25} \times 100\%$$
$$= 95.48\%$$

$$\textbf{Average \% of Carbidopa Content of F3} = \frac{(101.28 + 100.35 + 95.48)}{3} = 99.0\%$$

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# APPENDIX 5

## CALCULATION FOR AVERAGE PERCENTAGE CUMULATIVE RELEASE OF LEVODOPA

Drug release studies was done in triplicate. From the peak areas obtained by HPLC, the concentrations of levodopa released were calculated by interpolation from the calibration equation. Average concentration of levodopa released at each time point was then determined.

$$\text{Average Cumulative Release}(\%) = \frac{\text{Average concentration of levodopa released into the dissolution medium}}{\text{expected concentration}} \times 100\%$$

$$\text{Expected concentration} = \frac{\text{actual amount of levodopa in the weighed 425mg of polymeric matrix used for in vitro release test}}{\text{volume of dissolution medium}}$$

$$\text{Expected concentration} = \frac{100\,\text{mg}}{750\,\text{mL}} = 0.0133333\,\text{mg/mL} = 133.3333\,\mu\text{g/mL}$$

Calculation of Average Cumulative Drug Release at different time points:

**Table A5:** Calculated Values of Average Cumulative Drug Release at Different Time Points

| Time (t) | Average Concentration | Calculated Value |
|---|---|---|
| 0.5 hrs | 5.8699 | $\%\text{ Average Cumulative Release} = \frac{5.8699}{133.3333} \times 100\% = 4.40\%$ |
| 1 hr | 16.9752 | $\%\text{ Average Cumulative Release} = \frac{16.9752}{133.3333} \times 100\% = 12.73\%$ |
| 2 hrs | 45.7227 | $\%\text{ Average Cumulative Release} = \frac{45.7227}{133.3333} \times 100\% = 33.95\%$ |
| 4 hrs | 76.0141 | $\%\text{ Average Cumulative Release} = \frac{76.0141}{133.3333} \times 100\% = 57.01\%$ |
| 8 hrs | 121.2106 | $\%\text{ Average Cumulative Release} = \frac{121.2106}{133.3333} \times 100\% = 90.91\%$ |
| 12 hrs | 127.7649 | $\%\text{ Average Cumulative Release} = \frac{127.7649}{133.3333} \times 100\% = 95.82\%$ |
| 24 hrs | 134.1905 | $\%\text{ Average Cumulative Release} = \frac{134.1905}{133.3333} \times 100\% = 100.64\%$ |

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The same approach was employed in determining the average cumulative carbidopa release in the dissolution media used.

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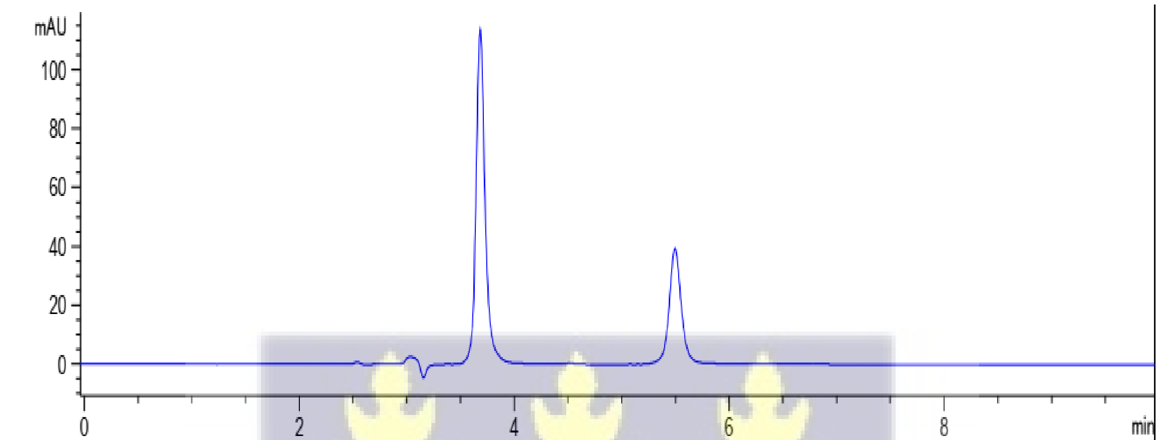
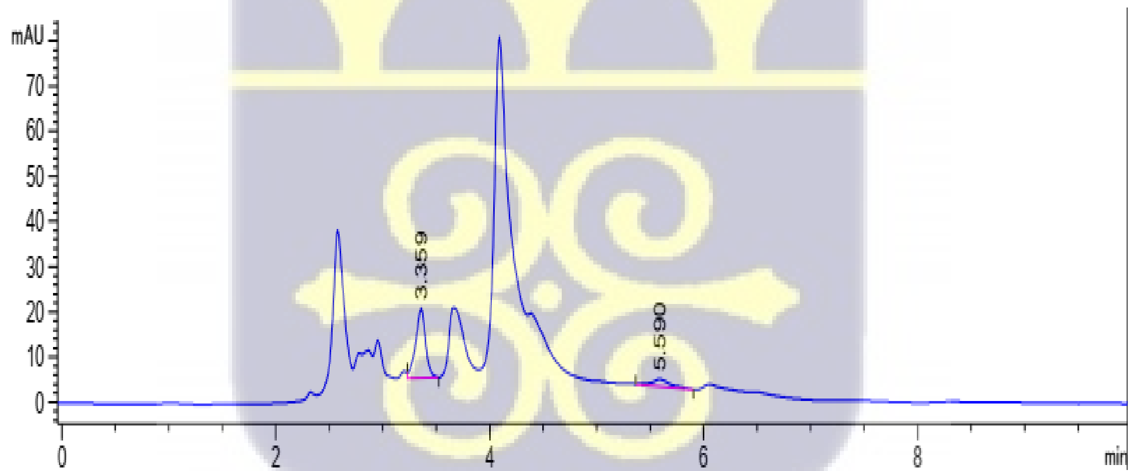
# APPENDIX 6

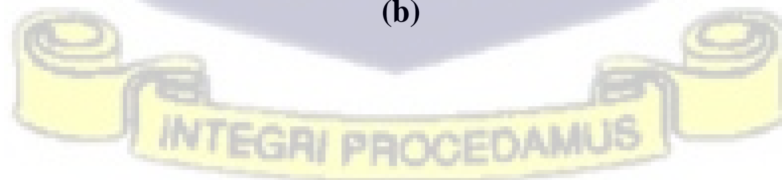
## REPRESENTATIVE CHROMATOGRAMS GENERATED FROM THE HPLC ANALYSIS OF IN VITRO DISSOLUTION MEDIA, PLASMA SAMPLES AND BRAIN SAMPLES




**(a)**




**(b)**



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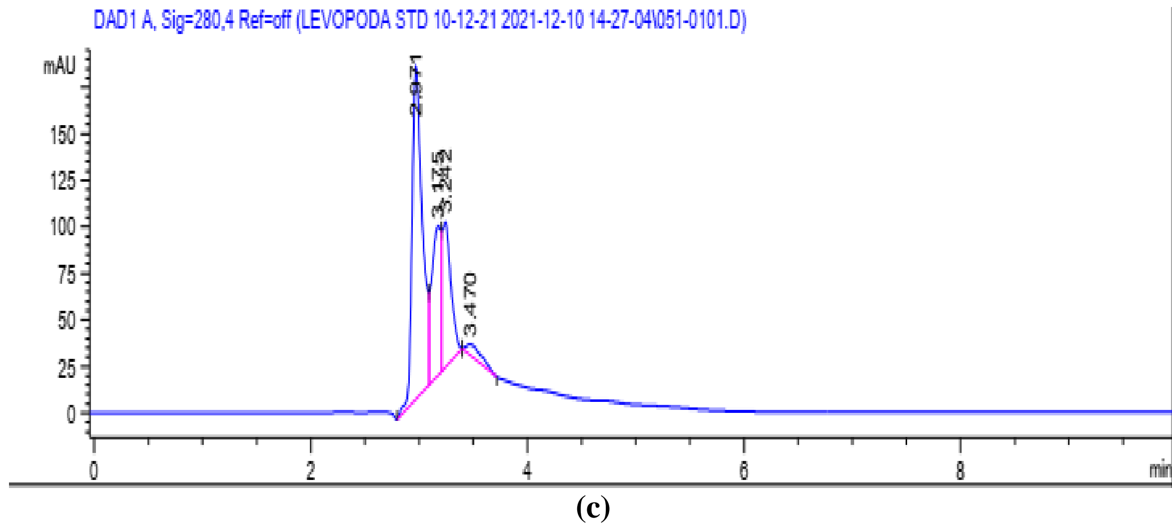
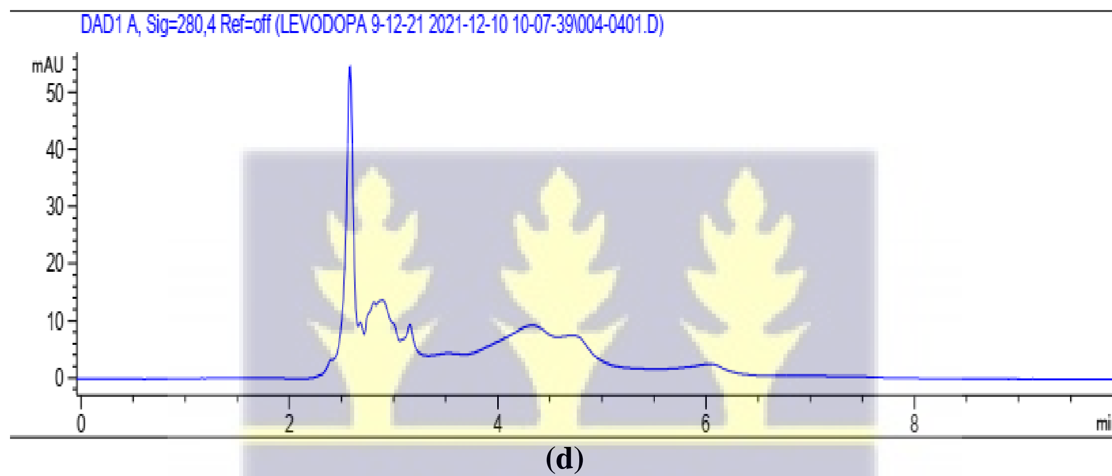



(c)




(d)

**Figure A6.1 Chromatograms showing the Peak Areas of (a) Levodopa and Carbidopa in Dissolution Media (0.1M Hcl), (b) Levodopa and Carbidopa in Rat Plasma, (c) Levodopa Standard in A Mixture of Methanol and Chloroform (4:1); and (d) Levodopa in Rat Brain Tissues**

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# APPENDIX 7

## POST HOC ANALYSIS WITH TUKEY'S MULTIPLE COMPARISON

### Time taken to reach maximum concentration ($T_{max}$)

| ANOVA summary | |
|---|---|
| F | 18.53 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.7765 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | **Mean Diff.** | **95.00% CI of diff.** | **Significant?** | **Adjusted P Value** |
| SINEMET vs. F3 | -3.400 | -5.415 to -1.385 | Yes | 0.0010 |
| SINEMET vs. F4 | -3.800 | -5.815 to -1.785 | Yes | 0.0003 |
| SINEMET vs. LC | 0.2000 | -1.815 to 2.215 | No | 0.9917 |
| F3 vs. F4 | -0.4000 | -2.415 to 1.615 | No | 0.9402 |
| F3 vs. LC | 3.600 | 1.585 to 5.615 | Yes | 0.0005 |
| F4 vs. LC | 4.000 | 1.985 to 6.015 | Yes | 0.0002 |

### Half- Life ($T_{1/2}$)

| ANOVA summary | |
|---|---|
| F | 50.29 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.9041 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | **Mean Diff.** | **95.00% CI of diff.** | **Significant?** | **Adjusted P Value** |
| SINEMET vs. F3 | -7.990 | -10.26 to -5.725 | Yes | <0.0001 |
| SINEMET vs. F4 | -8.500 | -10.77 to -6.235 | Yes | <0.0001 |
| SINEMET vs. LC | -3.930 | -6.195 to -1.665 | Yes | 0.0007 |
| F3 vs. F4 | -0.5100 | -2.775 to 1.755 | No | 0.9161 |
| F3 vs. LC | 4.060 | 1.795 to 6.325 | Yes | 0.0005 |
| F4 vs. LC | 4.570 | 2.305 to 6.835 | Yes | 0.0002 |

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**Elimination Rate Constant ($K_e$)**

| ANOVA summary | |
|---|---|
| F | 11.58 |
| P value | 0.0003 |
| P value summary | *** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.6847 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | Mean Diff. | 95.00% CI of diff. | Significant? | Adjusted P Value |
| SINEMET vs. F3 | 0.04000 | -0.0004609 to 0.08046 | No | 0.0532 |
| SINEMET vs. F4 | 0.04000 | -0.0004609 to 0.08046 | No | 0.0532 |
| SINEMET vs. LC | -0.03000 | -0.07046 to 0.01046 | No | 0.1884 |
| F3 vs. F4 | 0.000 | -0.04046 to 0.04046 | No | >0.9999 |
| F3 vs. LC | -0.07000 | -0.1105 to -0.02954 | Yes | 0.0008 |
| F4 vs. LC | -0.07000 | -0.1105 to -0.02954 | Yes | 0.0008 |

**Area under curve from time 0 to infinity ($AUC_{0-\infty}$)**

| ANOVA summary | |
|---|---|
| F | 90.81 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.9445 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | Mean Diff. | 95.00% CI of diff. | Significant? | Adjusted P Value |
| SINEMET vs. F3 | -296.5 | -380.9 to -212.1 | Yes | <0.0001 |
| SINEMET vs. F4 | -371.8 | -456.2 to -287.4 | Yes | <0.0001 |
| SINEMET vs. LC | 11.77 | -72.63 to 96.17 | No | 0.9778 |
| F3 vs. F4 | -75.27 | -159.7 to 9.129 | No | 0.0893 |
| F3 vs. LC | 308.3 | 223.9 to 392.7 | Yes | <0.0001 |
| F4 vs. LC | 383.6 | 299.2 to 468.0 | Yes | <0.0001 |

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**Area under curve from time 0 to 24hr ($AUC_{0-24}$)**

| ANOVA summary | |
|---|---|
| F | 238.8 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.9782 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | **Mean Diff.** | **95.00% CI of Diff.** | **Significant?** | **Adjusted P Value** |
| SINEMET vs. F3 | -223.0 | -261.6 to -184.4 | Yes | <0.0001 |
| SINEMET vs. F4 | -273.6 | -312.2 to -235.0 | Yes | <0.0001 |
| SINEMET vs. LC | 8.610 | -29.96 to 47.18 | No | 0.9180 |
| F3 vs. F4 | -50.62 | -89.19 to -12.05 | Yes | 0.0084 |
| F3 vs. LC | 231.6 | 193.0 to 270.2 | Yes | <0.0001 |
| F4 vs. LC | 282.2 | 243.6 to 320.8 | Yes | <0.0001 |

**$C_{max}$**

| ANOVA summary | |
|---|---|
| F | 24.87 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R squared | 0.8234 |

| Tukey's multiple comparisons test | | | | |
|---|---|---|---|---|
| | **Mean Diff.** | **95.00% CI of Diff.** | **Significant?** | **Adjusted P Value** |
| SINEMET vs. F3 | -5.660 | -10.12 to -1.198 | Yes | 0.0109 |
| SINEMET vs. F4 | -4.180 | -8.642 to 0.2817 | No | 0.0704 |
| SINEMET vs. LC | 6.620 | 2.158 to 11.08 | Yes | 0.0031 |
| F3 vs. F4 | 1.480 | -2.982 to 5.942 | No | 0.7793 |
| F3 vs. LC | 12.28 | 7.818 to 16.74 | Yes | <0.0001 |
| F4 vs. LC | 10.80 | 6.338 to 15.26 | Yes | <0.0001 |

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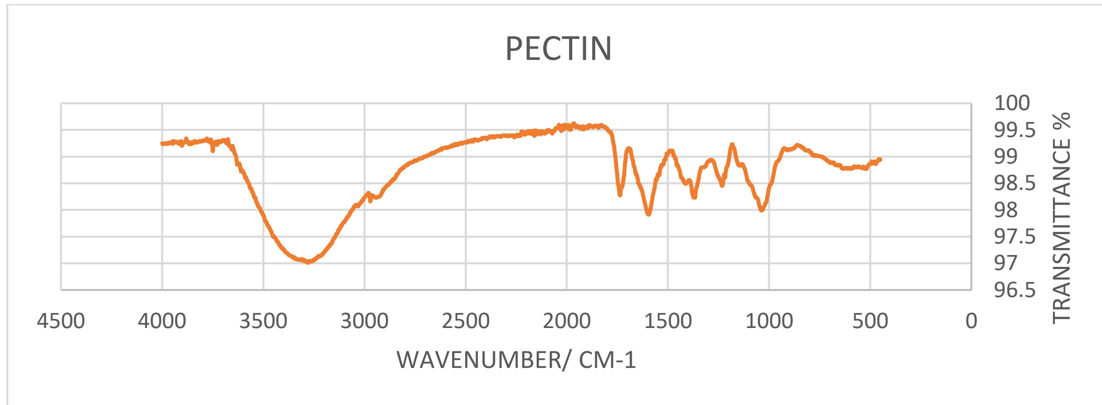
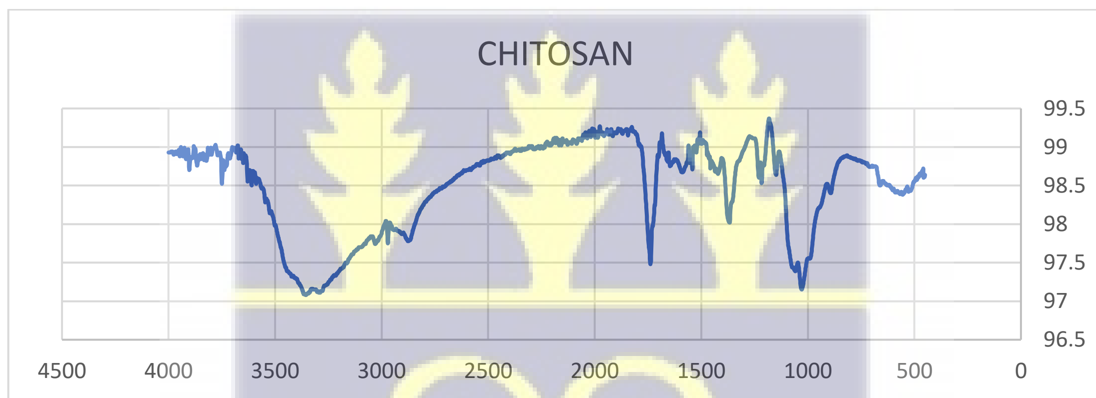
# APPENDIX 8

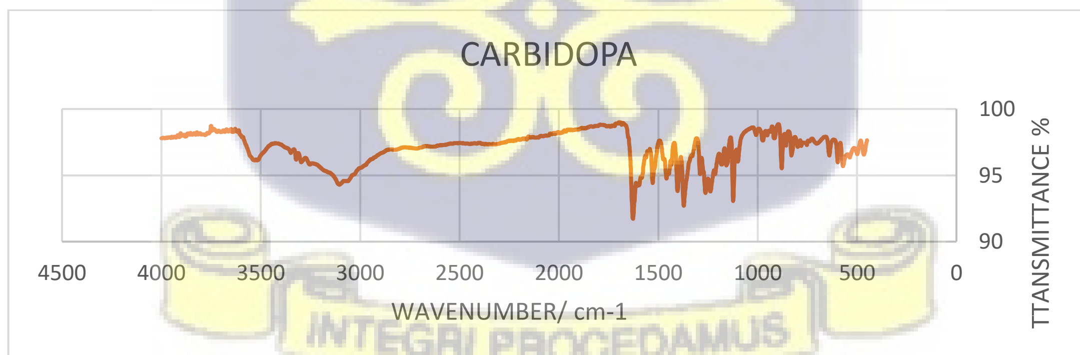
## FT-IR SPECTRA FOR DRUG-EXCIPIENTS COMPATIBILITY STUDY




**(a)**




**(b)**




**(c)**

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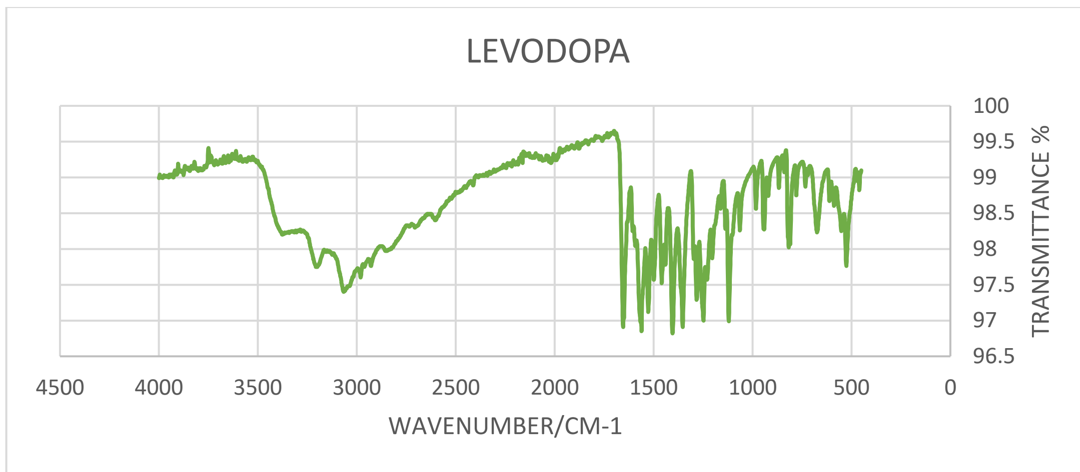
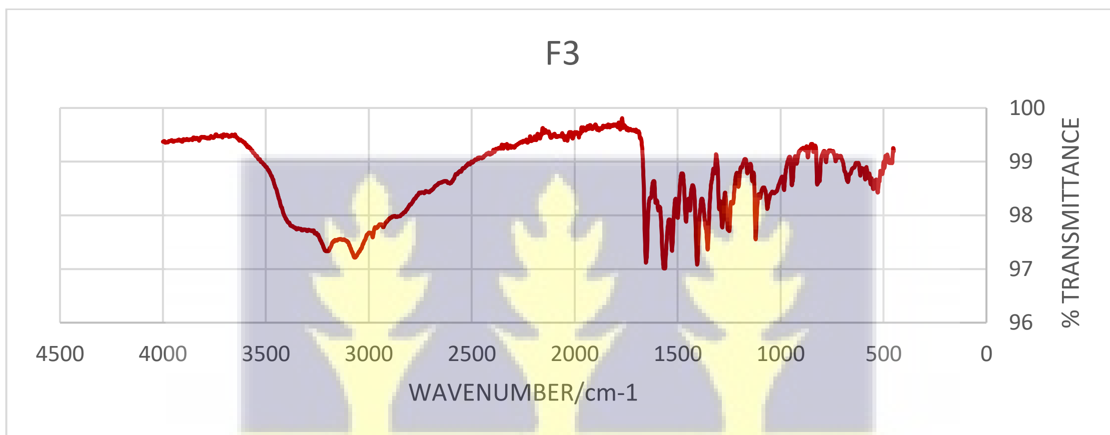
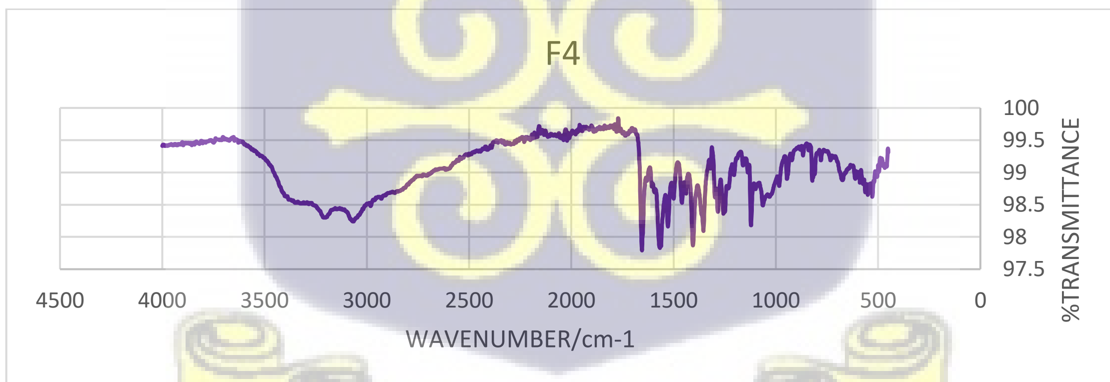



**(d)**




**(e)**




**(f)**

**FIGURE A8.1 Individual FT-IR Spectra of Levodopa, Carbidopa, Chitosan, CPH Pectin and Optimized Formulations (F3 and F4)**

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# APPENDIX 9

## TWO-WAY ANOVA AND POST HOC ANALYSIS OF BIODISTRIBUTION STUDY

| Two-way ANOVA | Ordinary | | | |
|---|---|---|---|---|
| Alpha | 0.05 | | | |
| | | | | |
| **Source of Variation** | **% of total variation** | **P value** | **P value summary** | **Significant?** |
| Interaction | 6.567 | 0.4813 | ns | No |
| Row Factor | 10.27 | 0.0276 | * | Yes |
| Column Factor | 46.77 | 0.0017 | ** | Yes |

| Tukey's Multiple Comparisons Test | Mean Diff. | 95.00% CI Of Diff. | Significant? | Summary |
|---|---|---|---|---|
| NC vs. Sinemet | -10.46 | -20.66 to -0.2490 | Yes | * |
| NC vs. F3 | 4.237 | -5.969 to 14.44 | No | ns |
| NC vs. F4 | -3.704 | -13.91 to 6.502 | No | ns |
| NC vs. LC | -8.915 | -19.12 to 1.292 | No | ns |
| Sinemet vs. F3 | 14.69 | 4.486 to 24.90 | Yes | ** |
| Sinemet vs. F4 | 6.751 | -3.455 to 16.96 | No | ns |
| Sinemet vs. LC | 1.541 | -8.666 to 11.75 | No | ns |
| F3 vs. F4 | -7.941 | -18.15 to 2.265 | No | ns |
| F3 vs. LC | -13.15 | -23.36 to -2.946 | Yes | ** |
| F4 vs. LC | -5.211 | -15.42 to 4.995 | No | ns |