

# **THE CHEMICAL SYNTHESIS, PHARMACEUTICAL PREPARATION AND TOXICITY ANALYSIS OF FLUORODOPA FOR POSITRON EMISSION TOMOGRAPHY (PET) BRAIN IMAGING IN SOUTH AFRICA**

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## DECLARATION

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The work described in this dissertation was carried out at the Department of Pharmacology, University of Pretoria, Pretoria, South Africa, under the supervision of Dr Duncan Cromarty. I declare that this dissertation is my own, unaided work submitted for the degree of Master of Science and that this dissertation does not incorporate, without written acknowledgement, any material that has previously been submitted for the award of any other degree or diploma in any university, college, or other educational institution; and to the best of my knowledge, this dissertation does not contain any material previously published or written by another person except where due reference is made in the text, including the disclosure of contributions for any work based in joint research or publications.



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## LIST OF ABBREVIATIONS

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|                      |  |
|----------------------|--|
| ADHA                 | Attention Deficit Hyperactivity Disorder                       |
| ALP                  | Alkaline phosphatase   |
| ALT                  | Aspartate transaminase   |
| ATP                  | Adenosine Triphosphate   |
| AUCC                 | Animal Use and Care Committee                                  |
| CDCl <sub>3</sub>    | Deuterated chloroform  |
| COMT                 | Cathocol-O-Methyl-Transferase                                  |
| D <sub>2</sub> O     | Deuterated H <sub>2</sub> O                                    |
| d <sub>6</sub> -DMSO | Deuterated Dimethylsulphoxide                                  |
| DMF                  | Dimethylformamide  |
| DMSO                 | Dimethylsulphoxide   |
| FDG                  | Fluoro Deoxy Glucose   |
| GGT                  | Gamma-glutamyltransferase                                      |
| HVA                  | Homo Vanilic Acid  |
| IV                   | Intravenous  |
| LDH                  | Lactate Dehydrogenase  |
| MAO                  | Mono-Amine-Oxidase   |
| MTT                  | (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) |
| NMR                  | Nuclear Magnetic Resonance                                     |
| NOAEL                | No Observed Adverse Effect Level                               |
| PBS                  | Phosphate Buffered Saline                                      |
| PD                   | Parkinsons Disease   |
| PET                  | Positron Emission Tomography                                   |



|       |   |
|-------|---|
| SEM   | Standard Error of Mean                            |
| SPECT | Single Photon Emission Computer Tomography        |
| UPBRC | University of Pretoria Biomedical Research Centre |
| VTA   | Ventral Tegmental Area                            |

## ABSTRACT

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Parkinson's disease (PD) impairs the quality of life of patients and causes substantial social and economic burden. However the currently available symptomatic treatments, although initially effective, do not satisfactorily control the progressive disability experienced by patients with PD in the long run. In order to develop effective treatments for patients that aim to attain the desired effect with as few adverse events as possible, it is crucial to be able to follow and understand the biological mechanisms underlying the continued neural degeneration and treatment failure. The efforts to understand the precise pathway by which neurodegenerative processes proceed and the development of approaches to modulate them offers the promise to eventually enable the prevention of these neurodegenerative diseases. This dissertation focused on two potential synthetic methods to produce pharmaceutical grade Fluorodopa, ultimately to be able to produce positron emitting  $^{18}\text{F}$ Fluorodopa in South Africa with its potential for studying neuronal mechanisms in the brain.  $^{18}\text{F}$ Fluorodopa allows a unique almost non-invasive *in vivo* approach to the evaluation of neurochemical function in the human brain and its local introduction will be a valuable addition to medical research within South Africa's borders. The successful implementation of safe and efficient non-radioactive models for Fluorodopa synthesis was achieved. The successful demonstration of locally synthesised Fluorodopa safety, as well as a low toxicity profile, both *in vitro* using cell cultures and *in vivo* in mouse models was achieved. These were both positive outcomes of objectives set out for this study.

# **CHAPTER ONE- GENERAL INTRODUCTION**

## **AIMS, OBJECTIVES, MOTIVATION & RESEARCH SCOPE**

## 1.1-INTRODUCTION

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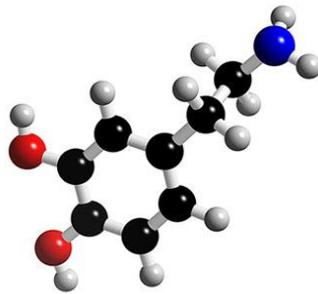
### *General introduction, the dopaminergic system*

The dopamine (DA) system in the brain has attracted considerable attention in neuroscience due to its involvement in fundamental brain functions and its postulated role in the pathophysiology of several neuropsychiatric disorders (*Valasco et al, 1998; Alvaro et al, 2001; Heimer et al, 2003; Sokoloff et al, 2006*). Parkinson's disease (PD) is the world's most common movement disorder which results in severe and progressive deficits in motor behaviour, predominantly as a consequence of degeneration of dopaminergic neurons within the substantia nigra (*Farde et al, 1986; Stern et al 1991; Jabcovik et al, 2008*). The exact cause of Parkinson's disease still remains unknown and consequently the pharmacotherapy for the disease consists predominantly of symptomatic treatment which is not successful in the long-term (*Pillon B, 1989; Rascol et al, 2002; Tintler R, 2002; Bertoni J, 2007*). A more rational and targeted treatment is needed, but this requires the introduction of compounds ameliorating the disease process itself. The need for such compounds has sparked the development of non invasive brain imaging methods to estimate the extent of the degeneration of the dopaminergic neurons in the living patient (*Farde et al, 1986*). The Dopa analogue 6-<sup>18</sup>Fluoro-L-Dopa (<sup>18</sup>Fluorodopa) is a positron emitting radiopharmaceutical with the ability to quantitatively assess the distribution, density and affinity for dopamine receptors *in vivo*, which has led to innovative research of neuropsychiatric disorders resulting from dopaminergic irregularities in the human brain. (*Garnett et al, 1983; Leenders et al, 1986; Moore et al, 2003*).

### *Dopamine, a neurotransmitter in the central nervous system*

Dopamine (DA), or 3,4-dihydroxyphenylethylamine (Figure 1.1), is an endogenous catecholamine that was first recognised as a neurotransmitter in 1957 (*Carlsson et al, 1958*). Electric impulses from a primary neuron results in an exocytotic process at the nerve terminal, which causes vesicles containing the neurotransmitter to be emptied into the synaptic cleft. The neurotransmitter binds reversibly to receptor proteins embedded in the membrane of a receiving neuron, which triggers a biochemical effect in the post synaptic neuron (*Jae et al, 2008*). Used by several billion neurons in the human brain, dopamine (DA)

plays a central and pivotal role in regulating the human body (Sedvall & Farde, 1995). Extensive research has shown that DA mediates several different functions in the brain including locomotor control, positive reinforcement, cognitive functions, personality traits and endocrine regulation (Missale et al, 1998; Vallone et al, 2000). Moreover it is these alterations in dopaminergic neurotransmission that have been implicated in the pathophysiology of neuropsychiatric disorders such as Parkinson's disease and schizophrenia (Sedvall & Farde, 1999; Kaasinen & Rinne, 2002; Sokolof et al, 2006).



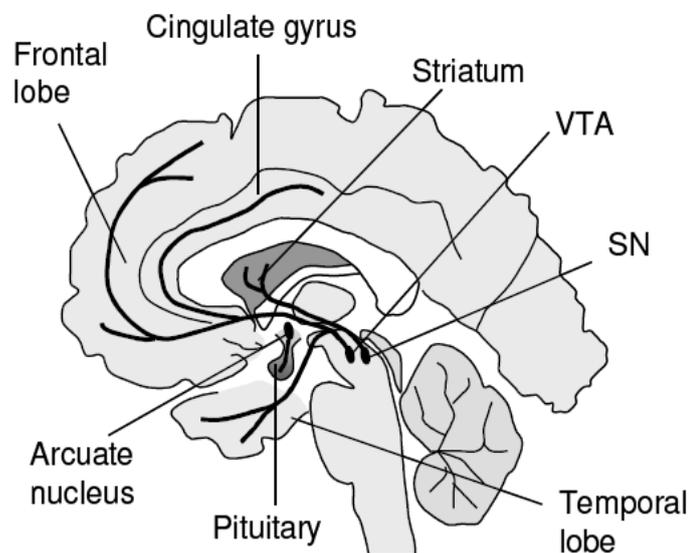
**Figure 1.1- The Ball and Stick Model of the chemical structure of Dopamine (DA), or 3,4-dihydroxyphenylethylamine:** NOTE: Black=Carbon, Red=Oxygen, Blue=Nitrogen, Grey=Hydrogen.

#### *Major dopaminergic pathways of the human brain*

The brain cells which secrete dopamine are called dopaminergic neurons. These neurons are predominantly found in three brain systems, or pathways; the mesencephalon, the diencephalon and the telencephalon. The projections originating from these nuclei form four major pathways in the brain (Figure 1.2) (Missale et al, 1998; Valloone et al, 2000; Alvaro et al, 2001) which include:

- ▶ The Nigrostriatal Pathway: Originates from the Substantia Nigra (SN) and innervates the dorsal striatum (caudate and putamen). The nigrostriatal pathway is vital in the regulation of movement and its degeneration is thought to play a central role in the pathogenesis of Parkinson's disease.
- ▶ The Mesocortical Pathway: Contains axons of dopaminergic cell bodies in the Ventral Tegmental Area (VTA) which project into different neocortical areas (frontal and temporal cortex) and the anterior cingulate gyrus. This pathway is involved in functions of learning and memory.

- ▶ The Mesolimbic Pathway: Also arises from the Ventral Tegmental Area (VTA), projecting to several limbic areas in the brain, including the nucleus accumbens, amygdala, anterior perforated substance, piriform and entohinal cortices. It is proposed that the mesolimbic pathway is responsible, in part, for motivated behaviour and emotions.
- ▶ The Tuberoinfundibular Pathway: Originates from the periventricular and arcuate nuclei in the hypothalamus and terminates in the mediance eminence of the hypothalamus. The tuberoinfundibular pathway is involved in the regulation of prolactin.

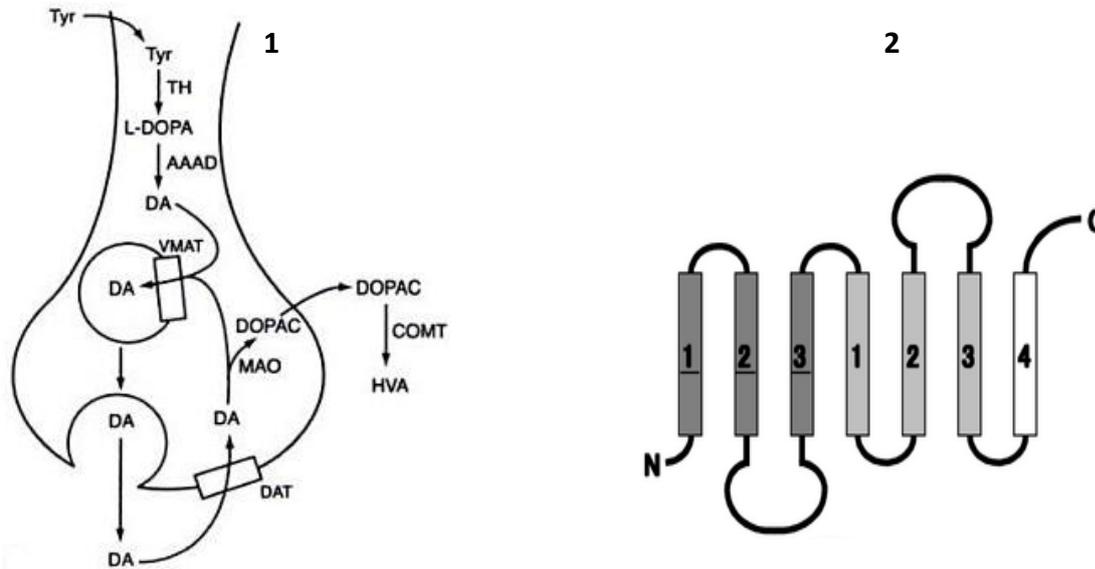


**Figure 1.2- The DA system in the human brain:** The four major dopaminergic pathways, originating from the Substantia Nigra (SN), Ventral Tegmental Area (VTA) and arcuate nucleus (shown in solid black lines). (Adapted from Alvaro et al, 2001)

### Biosynthesis of dopamine

The biochemical events that relate to the turnover of dopamine in nerve endings are summarised schematically in Figure 1.3. *In vivo*, dopamine is synthesised in two steps from the amino acid, L-tyrosine. In the first biosynthesis step, L-tyrosine is hydroxylated by the enzyme tyrosine hydroxylase to give L-3,4-dihydroxyphenylalanine (L-Dopa) (Holz et al 1996; Anthony et al, 2006). This amino acid is transported across the blood-brain barrier by

active transport mechanisms, after which it is accumulated in dopaminergic neurons (Hall et al, 1994). L-Dopa is subsequently decarboxylated to dopamine by the enzyme aromatic L-amino acid decarboxylase (Anthony A, 2006) and stored in vesicles.



**Figure 1.3- 1: Neuronal biochemical process of Dopamine (DA):** (Cooper et al, 1996; Hall et al, 1994; Anthony A, 2006). Neuronal uptake and initial conversion of tyrosine to DOPA by tyrosine hydroxylase (TH), which is in turn converted to DA by the enzyme aromatic L-amino acid decarboxylase (AAAD). The DA is pooled in the presynaptic vesicle. Neural stimulation results in exocytosis of the presynaptic vesicle with DA-release into the synaptic cleft with activation of postsynaptic DA-receptors. Reuptake of DA by the DA-reuptake transporter (DAT) followed by metabolism of the DA: sequential conversion by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) to give inactive homovanillic acid which is purged from the brain. **2: Dopamine receptor:** Schematic representation of the DA receptor with 7 transmembrane regions typical of the G protein coupled D<sub>2</sub> receptor.

The transfer of signal from dopaminergic nerve endings to their post-synaptic cell is achieved chemically by dopamine itself and its specific receptor. When neurotransmission occurs dopamine is released from the storage vesicles into the synaptic cleft, exerting its action by binding reversibly to G protein coupled receptors (GPCR) on the postsynaptic neuron (Valone et al, 2000). After a receptor has been triggered, this G-protein may activate or inhibit the secondary messenger system of the cell. This messenger system then causes certain biochemical reactions to occur, thereby eliciting a biological response (Morris A, 1991; Baldwin J, 1993). Excess synaptic concentrations of and receptor released dopamine is then taken up by the presynaptic neuron and metabolised by the action of monoamine

oxidase (MAO) and catechol- O-methyl transferase (COMT) to the inactive homovanillic acid (HVA), which is then slowly purged from the brain (*Hall et al, 1994*).

### *Deficiencies in dopamine neurotransmission*

Imbalanced dopamine activity can cause brain dysfunction and disease. Since the 1970s, scientists have studied the correlation between dopamine malfunction and two major Central Nervous System (CNS) disorders, schizophrenia and Parkinson's disease. However more recent investigation has revealed that dysfunction of dopaminergic neurotransmission in the CNS is responsible for additional neuropsychiatric disorders including social phobia (*Schneier et al, 2000*), Tourette's syndrome (*Kienast & Heinz, 2006*), neuroleptic malignant syndrome (*Mikara et al, 2003*) and Attention-Deficit Hyperactivity Disorder (ADHD) (*Faraone & Khan, 2006*).

### *Parkinson's disease (PD)*

Parkinson's disease (PD) is the world's most common age related movement disorder and the second most common neurodegenerative disorder after Alzheimer's disease (*Huang L, 2003*). PD is a neurodegenerative disease that is characterised by progressive damage to dopaminergic neurons, which thereby become progressively disabled in their function (*Vallone et al, 2000; Wong R, 2002*). PD is a neurodegenerative disease centred in the substantia nigra ("black substance"), a brain area that projects to the striatum, which together form the nigrostriatal dopaminergic pathways (*Farde et al, 1986*). Anatomically this degenerative process is characterised by a progressive loss of the melanin pigmented (black) neurons of the substantia nigra (*Gelb D, 1999; Jancovic et al, 2001*). PD begins insidiously and gradually worsens in severity, usually only affecting one side of the body before spreading to involve the other side (*Braak H, 2000; Jancovic et al, 2001; NIH, 2006*). There is a steady worsening of symptoms over time, which progressively leads to disability with severe immobility. The early symptoms and signs of PD are rest tremor, bradykinesia, and rigidity, however as PD progresses over time, symptoms worsen: flexed posture, the freezing phenomenon, and loss of postural reflexes (*Le Potre et al 2006; Lieberman A, 2006; NIH, 2006*). While the motor symptoms of PD dominate the clinical picture, and even define the Parkinsonian syndrome itself, many patients with PD have other complaints that have

been classified as non-motor. These include fatigue, depression, anxiety, sleep disturbances, constipation, bladder and other autonomic disturbances (sexual and gastrointestinal), sensory complaints, decreased motivation and apathy, slowness in thinking (bradyphrenia), and a declining cognition that can progress to dementia (*Obesso et al, 2000; Ishihara et al, 2006*). With a growing deficiency of striatal DA concentration due to further damage of the nigrostriatal neurons, the condition and accompanying symptoms continue to worsen (*Braak H, 2000*). Without treatment, PD progresses over 5 to 10 years to a rigid akinetic state in which patients are incapable of caring for themselves. Death frequently results from complications of immobility, of which aspiration pneumonia and pulmonary embolism are most common (*Mayo Clinic, 2006*).

#### *Therapeutic treatments, attempting to restore the dopamine balance*

‘Restoring the balance’ at the level of the output structures of the basal ganglia can be achieved pharmacologically by supplementation with the dopamine precursor L-Dopa and/or with dopamine receptor agonists (which show similar or equivalent activity responses) such as bromocriptine and carbergoline (*Tolosa et al, 1998; Manyam et al 1999; Hornykiewitz, 2001*). In the late 1960’s shortly after it became apparent that patients with Parkinson’s disease were suffering from a dopamine deficit in the basal ganglia, the dopamine precursor L-Dopa (soon afterwards in combination with a peripheral decarboxylase inhibitor) was successfully given to supplement the depleted dopamine stores (*Tolosa et al, 1998; Cotzias et al, 1998*). Unfortunately however, long-term treatment with L-Dopa frequently resulted in fading of the therapeutic effect, in the development of serious motor side-effects such as on–off motor oscillations and in psychiatric complications (*Manyam et al 1999; Hornykiewitz, 2001*). Under those conditions, increasing the dose of L-Dopa to compensate for the loss of therapeutic efficacy only gave rise to more side-effects without beneficial outcome. Although long-term treatment with dopamine D<sub>2</sub> receptor agonists resulted in less dyskinesias, the therapeutic efficacy was likewise less dramatic as compared to the initial effects of L-Dopa, furthermore, increasing the dose of dopaminergic agonists would only give rise to adverse effects such as psychotic reactions (*Brooks et al, 2000; Shultz et al, 2002; Manyam et al, 2002* ). At present the generally accepted therapeutic protocol consists of a combination of a low dose of L-Dopa in combination with

a dopamine D<sub>2</sub> receptor agonist (Brooks et al, 2000; *Rascol et al, 2002; Tintler R, 2002*). This treatment regime in general results in optimal control of the symptoms with fewer side-effects, at least in the early stages of the disease. Nevertheless, this therapeutic strategy still has ineffective long-term results (*Rascol et al, 2002; Tintler R, 2002; Nutts J, 2005*).

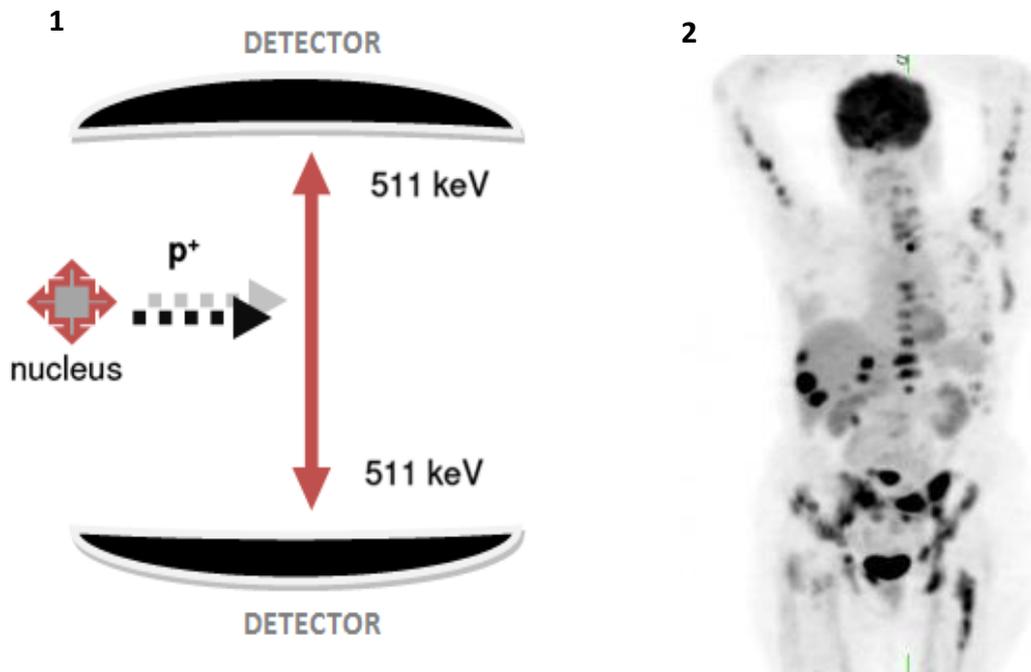
#### *Urgent need for a more causal treatment*

Although the symptomatic dopaminomimetic treatment approach is effective in the early stages of PD, the progressive decline in dopaminergic neurons and the development of pharmacodynamically and pharmacokinetically induced complications of therapy in advanced PD result in profound functional disability (*Rascol et al, 2002; Tintler R, 2002*). In addition, patients can progressively suffer from features that do not respond to dopaminergic therapy (*Manchua A, 2000; Mhangnoul et al, 2001*), and at present there is no treatment that can effectively influence the progressive course of the disease (*Kinast T, 2006*). The great hope and challenge for effective management of PD is the development of neuroprotective therapies to prevent further neuronal degeneration, thereby slowing down or halting disease progression. Growing insight into mechanisms underlying cell death in PD has provided several candidate drugs/interventions which can be considered as putative neuroprotective treatments (*Djaidetti R, 2002; Rovina et al, 2003; Bonuccelli U, 2006*).

#### *Advances in non-invasive tomographic imaging methods*

Advances in non-invasive imaging methods have recently afforded previously unavailable opportunities for the study of the central nervous system (CNS) function in living individuals (*Lainhinen A, 1992; Olga T et al, 2007*). PET and SPECT imaging techniques have been acknowledged as fundamental tools in the pathophysiological and neuropharmacological studies of the brain (*Brown A, 1999; Innus et al, 2003; Elp P, 2006*). For a given condition, a photon producing radionuclide is synthetically attached to a targeting molecule, usually one with established pharmacological activity, in a way that it will accumulate in the region of interest, when administered to the patient. Radioactivity (positron emission) originating from the radiotracer provides a signal that it is detected by a PET or SPECT camera (Figure 1.4). By a specialised reconstruction of the signal collected by the camera, the regional localisation of the radiotracer is determined over time and the resulting information

provides snapshots corresponding to the regional density of the protein or the process of interest (Kaaisinen V, 2001; Innus et al, 2003; Lasne et al, 2003). In PET, the data obtained by detection of tracer molecules is then used to reconstruct 3D images describing the distribution of the radiotracer in the body (Figure 1.4) (Eriksson L, 1989; Innus et al, 2003).

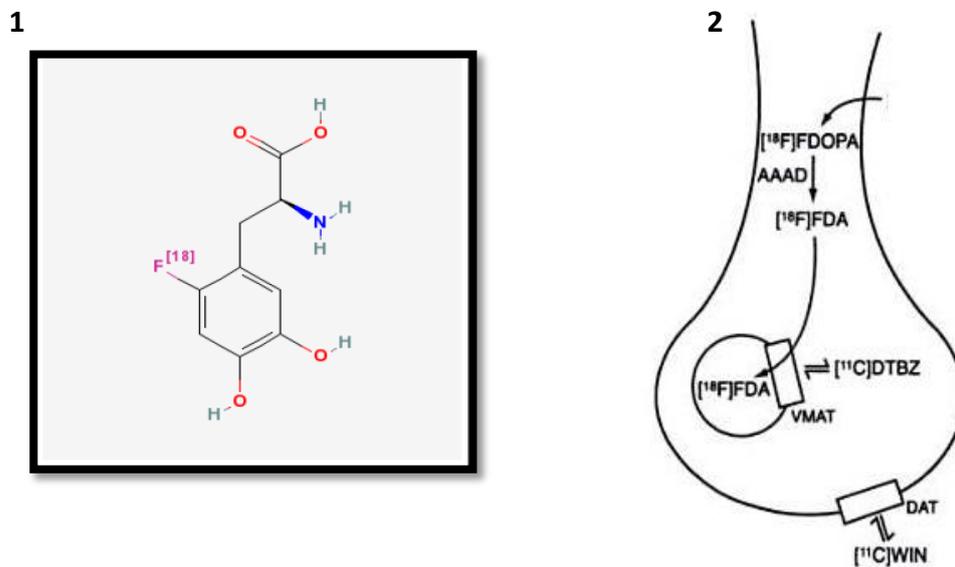


**Figure 1.4- 1- Physical Principles of PET:** The emitted positron ( $p^+$  and  $B^+$ ) travels a short distance before annihilation with an electron ( $e^-$ ). In the annihilation process, two  $\gamma$  photons (511KeV each) are formed which propagate in nearly opposite directions. These two  $\gamma$  photons are detected with opposed  $\gamma$  detectors. **2- PET 3D Image:** the data obtained by detection of tracer molecules is used to reconstruct 3D images describing the distribution of the radiotracer in the body.

### <sup>18</sup>Fluorodopa for brain imaging

In 1983 Garnett *et al* reported on the successful application of a <sup>18</sup>Fluorine labelled L-Dopa derivative for imaging nigrostriatal dopaminergic neurons. Known as 6-(<sup>18</sup>F)Fluoro-L-3, 4-dihydroxyphenylalanine or <sup>18</sup>Fluorodopa, this radiopharmaceutical has for over a decade been considered the “Gold Standard” for imaging dopaminergic neuronal loss in the living brain (Patricia *et al*, 1994; Alan L, 2004). <sup>18</sup>Fluorodopa (Figure 1.5) is used to monitor the conversion of L-dopa to dopamine through the aromatic acid decarboxylase in neuronal axons. <sup>18</sup>Fluorodopa is taken up by the nigrostriatal neurons in a manner similar to L-dopa and it is then decarboxylated to <sup>18</sup>Fluorodopamine, which subsequently follows the typical

metabolic pathway of dopamine (Figure 1.5). The area of accumulation and the level of radioactivity are measured, and the intensity of the radionuclide signal reflected in the activity and density of active DA neurons within the brain. <sup>18</sup>Fluorodopa not only provides us with an objective means of assessing the functional integrity of presynaptic nigrostriatal dopaminergic projections *in vivo* but also a means of assessing the integrity of dopamine receptors and their regional distribution (Volkow *et al*, 2001; Martinez *et al*, 2004). This data can provide information on early disease progression (Riperer P, 1999), medication related changes in receptor density (Mayo, 2006) and the involvement of receptors in the pathogenesis and treatment of Parkinson’s disease and related neuronal disorders (Volkow *et al*, 2001; Martinez *et al*, 2004).

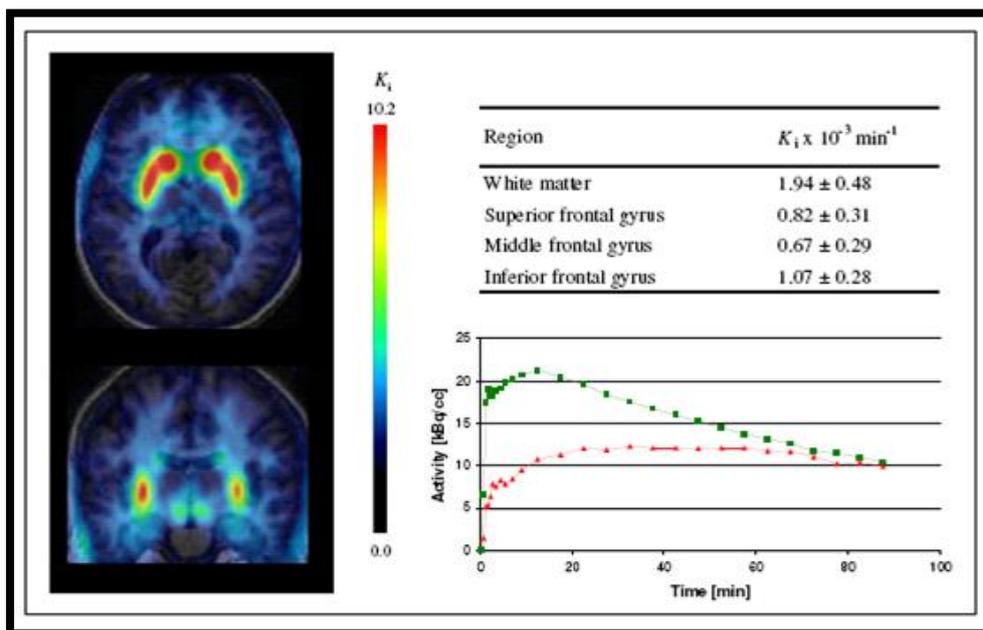


**Figure 1.5: 1- The chemical structure of <sup>18</sup>Fluorodopa. 2- Neuronal biochemical process of <sup>18</sup>Fluorodopa:** <sup>18</sup>Fluorodopa is taken up by the nigrostriatal neurons in a manner similar to L-dopa where it is then decarboxylated to <sup>18</sup>Fluorodopamine, which is subsequently metabolised like dopamine. The level of radioactivity during the process is measured, and the intensity of the signal is reflected in the activity and density of the active neurons (Hughes *et al*, 1992; Manyam B, 2004; Freed *et al*, 2004).

#### Indications for <sup>18</sup>Fluorodopa in neurology

PET with <sup>18</sup>Fluorodopa is indicated for detecting loss of functional dopaminergic neuron terminals in the striatum and can be used for diagnosis of Parkinson’s disease as well as differentiation between essential tremor and Parkinsonian syndromes (Brown *et al*, 1999). The typical pattern of <sup>18</sup>Fluorodopa uptake in PD is a severe impairment of uptake in the

posterior part of the putamen (less than half of normal), less impairment in the anterior putamen (60% of normal) and a relatively normal  $^{18}\text{F}$ Fluorodopa uptake in the caudate (85% of normal) (Brooks *et al*, 1990; Kuwabara *et al*, 1995; Lee *et al*, 2000). The significant difference in kinetics of  $^{18}\text{F}$ Fluorodopa uptake and washout between representative healthy subjects and Parkinsonian patients with PET (Figure 1.6) may assist clinicians in distinguishing underlying idiopathic PD in people with exposure to dopamine receptor-blocking drugs, since those patients with low  $^{18}\text{F}$ Fluorodopa uptake may be more likely to have long lasting or progressive Parkinsonism after cessation of the offending drug (Cedarbaum *et al*, 2003).  $^{18}\text{F}$ Fluorodopa also provides an objective measure of disease progression in PD (Hughes *et al*, 1992; Manyam B, 2004; Freed *et al*, 2004) which can be particularly useful for assessing changes in the rate of disease progression with a variety of therapeutic interventions (Hughes *et al*, 1992; Manyam B, 2004; Freed *et al*, 2004).



**Figure 1.6-**  $^{18}\text{F}$ Fluorodopa PET scan: Mean parametric image of the  $K_i$  of healthy subjects.  $K_i$  values are greater in cortical white matter than adjacent gray matter regions. The kinetics of white matter (■) uptake and washout from the representative healthy subject is significantly different from the reference region occipital cortex (■) (Adapted from Dieperink *et al*, 2003).

### <sup>18</sup>Fluorodopa, additional clinical applications

<sup>18</sup>Fluorodopa has also been used to monitor the efficacy of foetal dopamine cell implantation into the striatum of PD and has demonstrated the quantitative dopamine changes following transplantation (Sawle G et al, 2002; Freed et al, 2004; Redmond et al, 2004; Redmond et al, 2007). <sup>18</sup>Fluorodopa is also particularly useful for imaging of brain tumours because of the high Dopa uptake in tumour tissue and the low Dopa uptake in normal brain tissue (Saha G, 1994; Couturier, 2005; Becheral A, 2008). In primary brain tumours, imaging of amino acid transport in brain lesions with <sup>18</sup>Fluorodopa has been shown to be more sensitive than 2-Fluoro-(<sup>18</sup>F) Deoxyglucose (FDG) PET (Becheral A, 2008) for this application. A head to head comparison by Chen and co-workers, (2006) demonstrated a significantly greater sensitivity of <sup>18</sup>Fluorodopa over <sup>18</sup>FDG for the detection of the recurrence of high grade gliomas in the brain. <sup>18</sup>FDG is however used successfully for diagnosis in many other oncological applications. In about one-third of endocrine pancreatic tumours (EPT) patients did not present with any hormone-related symptoms. Publications by Hoegerle and co-workers (2001) and Becherer and co-workers (2004) have shown a potential clinical usefulness of <sup>18</sup>Fluorodopa PET in this setting. Furthermore, <sup>18</sup>Fluorodopa PET has also been shown to be a very effective, non-invasive method for the detection of a focal form of hyperinsulinism in infants with very high accuracy (Hernandez, 2001; Santiagom et al, 2006; Kauhanen et al, 2007).

## 1.2-MOTIVATION FOR STUDY

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Parkinson's disease has major economic and public health impacts in the developed world. The seriousness of this problem is projected to grow over the next 30 years with the approaching demographic bulge of elderly baby-boomers and an increase in average age in the populations throughout the world (Maras C, 2005). PD impairs the quality of life of patients and causes substantial social and economic burden (Wherton et al, 1997; Rubinstein et al, 1997; Sheif et al, 2000; Siderolf et al, 2000). The currently available symptomatic treatment, although initially effective, does not satisfactorily control the increasing disability experienced by patients with PD in the long run (Rascol et al, 2002; Tintler R, 2002; Stern et al, 2003). A more rational treatment is needed, but this requires the introduction of compounds ameliorating the disease process itself. For over ten years  $^{18}\text{F}$ Fluorodopa has been considered the gold standard for imaging degeneration of the dopaminergic system in the living brain (Patricia et al, 1994; Alan L, 2004). With the capacity of diagnosing Parkinson's prior to the onset of clinical symptoms and monitoring neuroprotective compounds on a longitudinal basis (Hoffman et al, 1982),  $^{18}\text{F}$ Fluorodopa forms a unique *in vivo* approach to the evaluation of neurochemical function in the human brain. However with a half life of only 109 minutes, importation of  $^{18}\text{F}$ Fluorodopa from Europe or further is not economically feasible. The local synthesis of  $^{18}\text{F}$ Fluorodopa therefore will be a valuable addition to medical research tools within our region.

Prior to its introduction however, it is important to compare the available techniques for its synthesis using non radioactive material to assess the best reaction sequence and what the cleanup requirements would be, taking into consideration the isotope half-life for when the  $^{18}\text{F}$ Fluorodopa is finally synthesised using the chosen synthesis. Furthermore a suggested pharmaceutical quality control protocol for the production and the testing of the *in vitro* and *in vivo* toxicity profile of the synthesised product were to be established.

## 1.3- AIMS AND OBJECTIVES

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### *-Chapter two- The nucleophilic and electrophilic synthesis of Fluorodopa*

Evaluate, compare and assess two principal synthesis methods for the production of Fluorodopa: the electrophilic fluorodestanylation method developed by Namavari and co-workers, 1992 and the nucleophilic displacement method developed by Machula and co-workers, 2005.

- ▶ Evaluate, compare and assess the synthesis steps in the non radioactive production of Fluorodopa by the electrophilic synthesis route (*Namavari et al, 1992*).
- ▶ Evaluate, compare and assess the synthesis steps in the non radioactive production of Fluorodopa by the nucleophilic synthesis route (*Machula et al, 2005*).
- ▶ Purify, chemically, the final product of the electrophilic and nucleophilic synthesis routes with solid phase extraction (SPE) and semi preparative thin layer chromatography (TLC).
- ▶ Purify, enantiomerically, the final product of the nucleophilic synthesis route with chiral thin layer chromatography.
- ▶ Confirm, enantiomerically, the final product of the nucleophilic and electrophilic synthesis routes with chiral thin layer chromatography.
- ▶ Confirm, chemically, the final product of the nucleophilic and electrophilic synthesis routes with High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) imaging.

### *-Chapter three- Radiopharmaceutical preparation and quality control*

To design a rapid “in house” preparative separation and quality control procedure for Fluorodopa that takes into account local factors that might affect product quality.

- ▶ To design and implement a safe and reliable synthetic quality control protocol for Fluorodopa, prepared for intravenous human administration.
- ▶ Design and implement the use of a “Production Quality Control Sheet” that includes the necessary regulatory parameters for Fluorodopa production.

- ▶ Formulate complete standard operating procedures (SOP) for monitoring the quality control of Fluorodopa:
  - ❖ Chemical purity, with High Performance Liquid Chromatography (HPLC).
  - ❖ Enantiomeric purity, with chiral thin layer chromatography (TLC).
  - ❖ Endotoxin levels with the limulus lysate test.
  - ❖ Sterility with the thioglycollate culture method.

*-Chapter four- in vitro toxicity investigation of Fluorodopa*

Determine the toxicological effect of Fluorodopa when administered to *in vitro* human lymphocytes.

- ▶ Assess the cellular toxicity of non-radioactive Fluorodopa over a concentration gradient (0,004 $\mu$ M-500 $\mu$ M) on PHA stimulated human lymphocytes *in vitro*.
- ▶ Assess the cellular toxicity of non-radioactive Fluorodopa over a concentration gradient (0,004 $\mu$ M-500 $\mu$ M) on resting human lymphocytes *in vitro*.
- ▶ Establish a dose response curve for chemical risk assessment of Fluorodopa on PHA stimulated human lymphocytes *in vitro*.
- ▶ Establish a dose response curve for chemical risk assessment of Fluorodopa on resting human lymphocytes *in vitro*.
- ▶ Calculate the concentration of Fluorodopa that produces:
  - ❖ No Observed Effect Concentration (NOAEL)
  - ❖ 10% inhibition of cellular viability (IC<sub>10</sub>)
  - ❖ 50% inhibition of cellular viability (IC<sub>50</sub>)
  - ❖ 90% inhibition of cellular viability (IC<sub>90</sub>)
  - ❖ Total lethal concentration (LD<sub>100</sub>)

*-Chapter five- in vivo toxicity investigation of Fluorodopa*

Assess whether a single administered intravenous dose of 1 $\mu$ mole/kg(bodyweight) non-radioactive Fluorodopa is toxic *in vivo*.

- ▶ Assess the immediate *in vivo* effects of a single administered dose of 1µmole/kg(body weight) non-radioactive Fluorodopa (maximum recommended dose given for Fluorodopa PET brain imaging) in mice for 60 minutes following intravenous administration.
- ▶ Assess the *in vivo* toxicity of a single administered intravenous dose of 1µmole/kg (bodyweight) non-radioactive Fluorodopa in mice over a period of seven days.
- ▶ Ascertain whether there are any significant observational differences between Fluorodopa dosed and vehicle control mice following a single administered intravenous dose of 1µmole/kg(body weight) non-radioactive Fluorodopa over a period of seven days. Observational parameters include:
  - ❖ General behaviour
  - ❖ Food and water intake
  - ❖ Weight gain
- ▶ Demonstrate that there are no significant variations in clinical toxicity markers between non-radioactive Fluorodopa dosed and vehicle control mice for seven days following a single administered intravenous dose of 1µmole/kg (body weight) Fluorodopa. Measured parameters include:
  - ❖ Haematological findings
  - ❖ Histopathological examinations
  - ❖ Blood chemistry analysis

*-Chapter six- Conclusion and Future Prospects*

- ▶ Summary of research outcomes and concluding remarks
- ▶ *Future considerations for the production of radioactive <sup>18</sup>Fluorodopa*

*-Chapter seven- Supporting documents*

- ▶ References and supporting documents

## 1.5-STATISTICS

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Dr Samuel Manda, Senior Biostatistician at the Medical Research Council (MRC) assisted with study design, and statistical analysis.

## 1.6-ETHICAL APPROVAL

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Animal ethics was obtained from the University of Pretoria's Animal Use and Care Committee for the acute *in vivo* toxicity study. (Project Number HO-25 08) on 1<sup>st</sup> July 2008.

## **1.7-BUDGET**

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The University of Pretoria, Department of Pharmacology and PET Labs Pharmaceuticals covered all experimental costs. No Financial or material contributions were received from any other sources.



## **CHAPTER TWO- CHEMICAL SYNTHESIS**

### **THE non radioactive ELECTROPHILIC AND NUCLEOPHILIC SYNTHESIS OF FLUORODOPA**

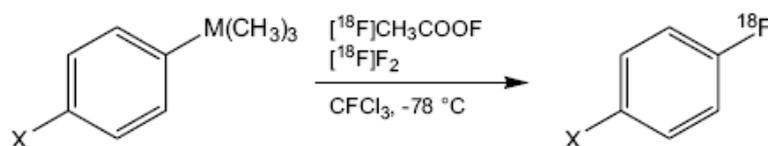
## 2.1-INTRODUCTION

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### *General introduction, fluorine chemistry*

Elemental fluorine is in many ways unique in both chemical characteristics and usefulness in the pharmaceutical industry (Couturier *et al* 1986; Kilbourn *et al*, 1990; Snider *et al*, 2003). Fluorine has a very small steric size and exhibits very high carbon fluorine bond energies; as fluorine is extremely electronegative such substitutions can often produce useful changes in the physiochemical and biological properties of these compounds (Pike *et al*, 1988; Kilbourn *et al*, 1990; Snider *et al*, 2003). The well understood chemistry, the 109 minute  $^{18}\text{F}$  half-life and relatively widespread availability of  $^{18}\text{F}$  allows for various synthetic options to be considered. The substitution of hydrogen or hydroxyl groups in small molecule drugs or natural bioactive molecules with  $^{18}\text{F}$  Fluorine has resulted in several useful diagnostic radiopharmaceuticals.

One such molecule is  $^{18}\text{F}$ Fluorodopa that has been synthesised via various chemical synthetic routes with different points of introduction of the fluorine isotope. Several electrophilic (Figure 2.1) (Adam M, 1980; Chirakal, 1984; Chaly T, 1986; Ding *et al*, 1990; Luxen *et al*, 1992; Namavari *et al*, 1992; Ishiwata *et al*, 1993) and nucleophilic (Figure 2.2) (Lemaire *et al*, 1990, 1992, 1993, 1994, 1995; Machula *et al*, 2005) methods of Fluorine incorporation into dopamine have been described and reviewed.



**Figure 2.1:**  $^{18}\text{F}$ -labelling via electrophilic demetallation reactions ( $M = \text{Sn, Ge, Si}$ ;  $X = \text{OCH}_3, \text{CH}_3, \text{H, F, CF}_3, \text{NO}_2$ ) (Luxen *et al* 1992; Bergman & Solin, 1997)

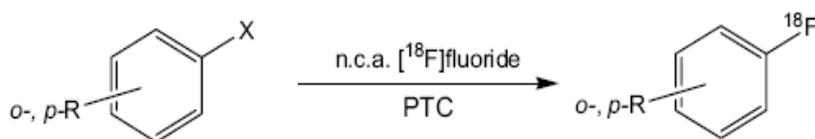
### *Electrophilic fluorination of Fluorodopa*

Electrophilic fluorination is the process by which fluorine is delivered to an electron donating reactant, such as an alkene, aromatic ring or carbanion by a formal “positive-fluorine” reagent to form a covalent carbon fluorine bond (*Beridge & Tewson, 1986*). These reactions have proved to be efficient and fast (*Namavari et al, 1992; Luxen et al 1992; Bergman & Solin, 1997; Bergman et al, 1997*). The electrophilic synthesis of Fluorodopa has been well described: Fluorodopa has been achieved by the direct fluorination of protected dopa derivatives (*Firnau et al, 1984; Adam et al, 1986; Chaly & Diksic, 1986*), however this method has the drawback of producing regioisomers that require further separation by HPLC (*Chirakal et al, 1986; Adam et al, 1986; Coenen et al, 1998*). To overcome the problem of nonregioselective fluorination, several fluoro-demetalation reactions have been successfully developed (*Luxen & Barrio, 1988; Adam et al, 1983; Coenen & Moerlem, 1987*). These methods are more attractive and are generally preferred because of the high regioselectivity of the process and easy product purification by single pass preparative HPLC.

This significant advantage has been fully exploited and well described in the fluorodemercuration reaction (*Luxen et al, 1986, 1987, 1989, 1990; Adam & Jivan, 1988*) however this fluorination method is accompanied by concerns of potential toxicity due to residual mercury. The Fluorodestanylation reaction, a variation of this method, makes use of a simple aryltin derivative and overcomes the major concern of possible mercury contamination. It has been described as the most complete among all the fluoro-demetalation reactions (*Coenen & Moern, 1987; Adam et al, 1983, 1984*). It is for these reasons that an adapted fluorodestanylation method described by Namavari and co-workers (*Namavari et al, 1992*) was used for the routine production of cold electrophilic Fluorodopa.

### *Nucleophilic fluorination of Fluorodopa*

Nucleophilic substitution usually comprises at least two chemical steps: first the introduction of the Fluorine followed by multi deprotection steps, which is not only time consuming but requires complicated final separations. Nucleophilic methods using the no



**Figure 2.2: A generalised nucleophilic aromatic  $^{18}\text{F}$ -fluorination using activated arenes** ( $X = \text{NO}_2, \text{N}(\text{CH}_3)_3$  and (counter ions:  $\text{TfO}^-$ ,  $\text{TsO}^-$ ,  $\text{ClO}_4^-$ ,  $\text{I}^-$ ),  $\text{Br}$ ,  $\text{Cl}$ ,  $\text{I}$ ;  $\text{R} = \text{NO}_2, \text{CN}, \text{CHO}, \text{RCO}, \text{COOR}, \text{Cl}, \text{Br}, \text{I}$ ;  $\text{PTC} = [\text{K}_{2.2.2}]\text{CO}_3, [\text{K}_{2.2.2}]\text{C}_2\text{O}_4/\text{CO}_3, [\text{K}_{2.2.2}]\text{HCO}_3, \text{R}_4\text{N}^+.\text{Rb}^+$ ) (Berridge & Tewson 1986)

carrier added fluoride ion (Ding *et al*, 1990; Lemaire *et al*, 1992, 1993, 1994; Chirakal *et al*, 1995; Machula *et al*, 2005) have the potential to provide a high absolute yield and a high specific activity, comparable to the leading electrophilic substitution methods. The most promising nucleophilic labelling approaches were published by Lemaire and co-workers, (1992, 1993, 1994, 1995), however more recently Machula and co workers, (2005) have reported on an improved synthesis, a variation of Lemaire's method that claims higher regioselectivity (only one of the three positional isomers) and stereoselectivity (more than 96%), using nucleophilic displacement of the nitro groups of 3,4-dimethoxy-2-nitrobenzaldehyde. This method was used for the production of cold nucleophilic Fluorodopa in the study.

#### *Fluorodopa, conditions for preparation*

The synthesis of Fluorodopa however cannot be considered in isolation from its ultimate use in man because its intravenous use dictates the strict conditions that must be met by its preparative methods: (Adam M, 1980; Chirakal, 1984; Chaly T, 1986; Berridge & Tewson, 1986; Kilbourn M, 1990; Snyder & Kilbourn, 2003)

- ▶ The labelled  $^{18}\text{F}$  Fluorodopa has to be synthesised in the L-form. The carrier mediated transport system in the blood brain barrier transports only L-amino acids.
- ▶ The Fluorine must be in the 6 position on the aromatic ring of L-Dopa so that the molecule retains its *in vivo* biochemical properties.
- ▶ An enantiomeric purity of more than 95%
- ▶ An isomeric purity of over 95%.

Furthermore the preparation of  $^{18}\text{F}$ Fluorodopa should fulfil the following requirements:

- ▶ An overall radiochemical yield (not decay corrected) of production >10% (Adam et al, 1986).
- ▶ A total chemical processing time not exceeding 120 minutes after fluorine introduction.
- ▶ An easy availability of the starting material showing high chemical stability and reproducible purity.

## 2.2- MOTIVATION FOR RESEARCH WORK

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The chemistry and utility of Fluorodopa has been discussed in numerous reviews owing to its widespread use as a PET imaging radiopharmaceutical (*Fowler & Wolf, 1990; Halpem 1995; Ruth & Adam, 1996*). Although several electrophilic (*Firnau et al, 1984; Adam et al, 1986; Chaly & Diksilic, 1986; Luxen et al, 1986*) and nucleophilic (*Lemaire et al, 1990, 1992, 1993, 1994, 1995; Demege et al, 1990, 1991; Machula et al, 2005*) methods have been described and reviewed, little emphasis has gone into the direct comparative evaluation of the two synthesis methods. The rapidly increasing demand for its local production has created the need for a simple, reliable procedure for its routine production in South Africa. Prior to its introduction however, it is important to assess the available non radioactive techniques for its synthesis, and conclude on the method most preferable for the future implementation of a radioactive model in a production setting in South Africa.

## 2.3-MATERIALS AND METHODS

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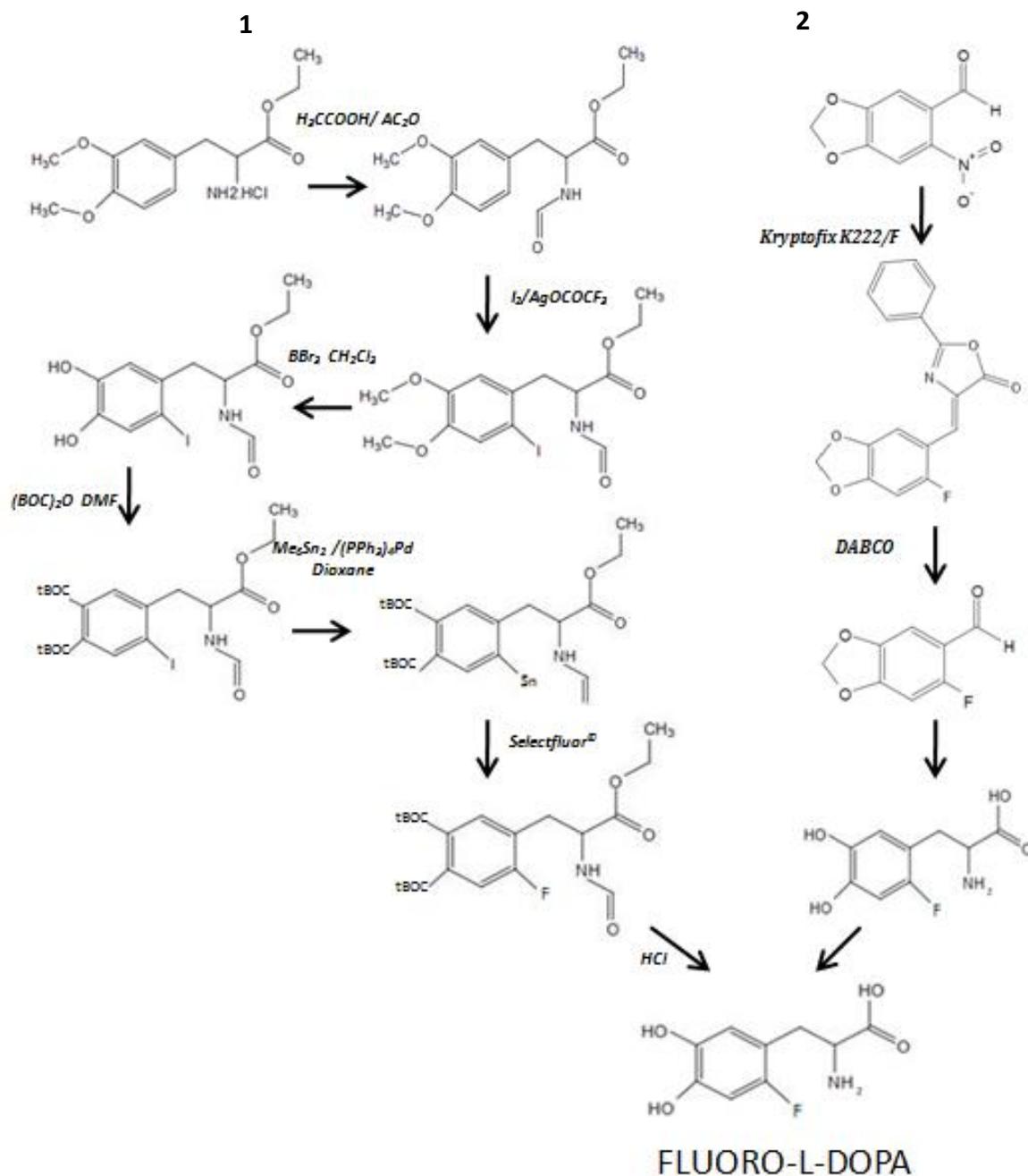
### *Materials*

3,4-dihydroxyphenyl-L-alanine, hydrogen chloride (HCl) (37%), formic acid (96%), triethylamine, magnesium sulphate, boron tribromide, silver trifluoroacetate, hexamethylditin, tetrakis triphenylphosphine palladium, anhydrous 1,4 dioxane, di-t-butyl dicarbonate (tBoc), DMF, ethanol absolute, methanol, chloroform, DMSO, DMF, ethyl acetate, 6-nitropiperonal, 1,4-diazabicyclo[2,2,2]octane (DABCO), K222 (Kryptofix), hydriodic acid (distilled and buffered), hypophosphoric acid, red phosphorus, hippuric acid, t-butanol, acetic anhydride, potassium carbonate, THF, acetic acid, sodium hydroxide, sodium biphosphate, copper sulphate, hexane and sodium carbonate were all purchased at the highest available quality through Sigma Aldrich Pty Ltd. Macherey & Nagel Chiral TLC plates were purchased from Separations, South Africa. Non radioactive L-Fluorodopa (analytical standard) was purchased from ABX Pharmaceuticals International.

### *Experimental*

The electrophilic chemical synthesis is based on the methodology reported by Namavari *et al* (1992) with only minor modifications. 3,4-dihydroxyphenyl-L-alanine (starting compound for the electrophilic synthesis) was synthesised at PET Labs Pharmaceuticals according to the method described by Luxen *et al* (1986). The nucleophilic chemical synthesis is based on the methodology reported by Machula *et al* (2005) with no modifications. 2-phenyl-5-oxazolone, which is not available commercially, was synthesised at the Department of Pharmacology, University of Pretoria according to the method described by Vandenberg and co-workers, (1967).

The chemical synthesis of Fluorodopa

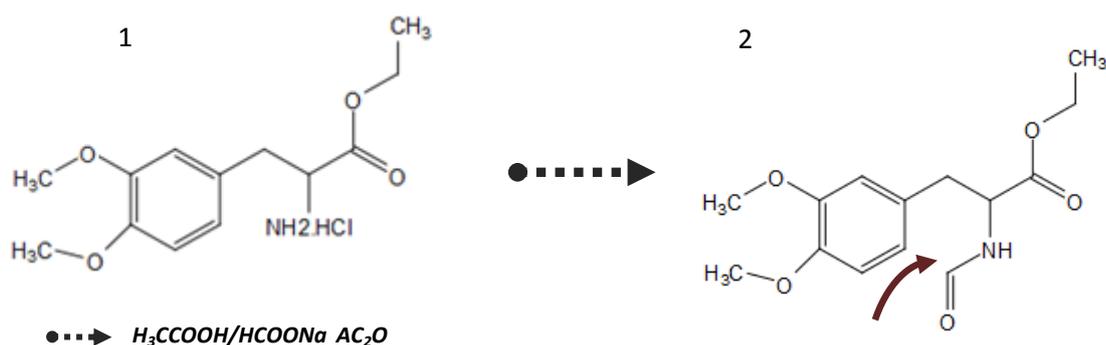


**Figure 2.3: The synthesis steps in the production of Fluorodopa-** The two most commonly used chemical synthesis methods for the production of Fluorodopa. **1-** The nine step electrophilic synthesis based on the methodology used by Namavari et al, 1992 **2-** The four step nucleophilic synthesis based on the methodology used by Machula et al, 2005.

### The electrophilic synthesis of Fluorodopa

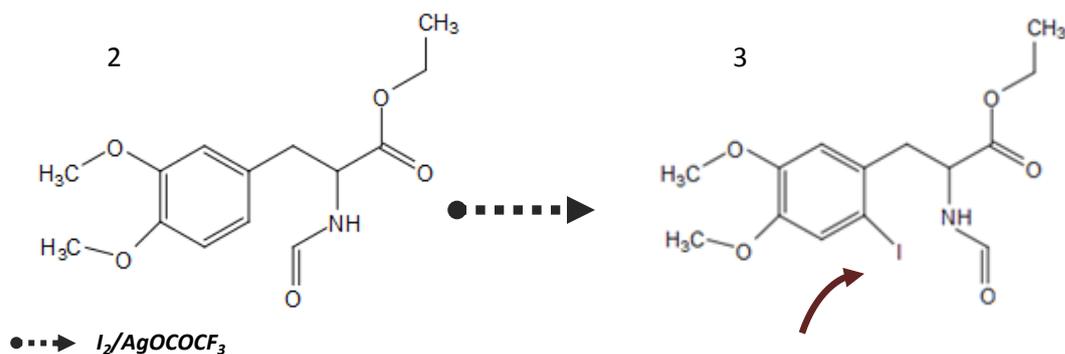
#### Step One- Synthesis of N-formyl-3,4-dimethoxy-L-phenylalanine ethyl ester (2)

20ml of acetic anhydride was added to a mixture of 3,4 dimethoxy-L-phenylalanine ethyl ester hydrochloride (1) (5.0 g, 18.3 mmol) and sodium formate (1.44 g, 21.2 mmol) in 85% formic acid (50 ml), which was cooled in an ice bath and stirred at room temperature for 10 hours. After the addition of absolute ethanol (40ml) the mixture was stirred at room temperature for an additional 3 hours before concentrating the solution under vacuum. The yellow residue that formed was dissolved in ethyl acetate, filtered and the insoluble material washed with ethyl acetate (100ml). The combined filtrates were then washed successively with aqueous 10% HCl (100ml), saturated aqueous NaHCO<sub>3</sub> (100ml) and saturated aqueous NaCl (100mL), and then dried under vacuum to afford N-formyl-3,4-dimethoxy-L-phenylalanine ethyl ester as a white solid (2.1g, 41%, 24 hours).



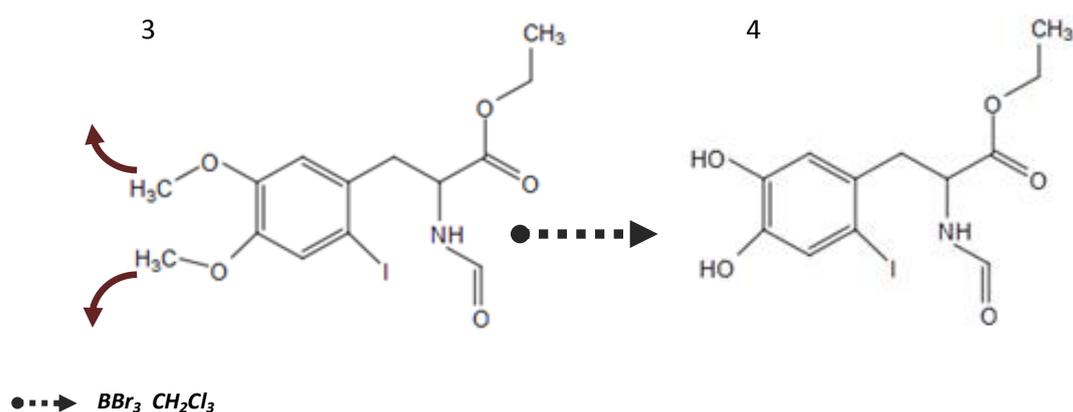
#### Step Two- Synthesis of N-formyl-3,4-dimethoxy-6-iodo-L-phenylalanine ethyl ester (3)

Iodine (2.1 g, 83 mmol) was added to a solution of N-formyl-3,4-dimethoxy-L-phenylalanine ethyl ester (2) (2.0 g, 75mmol) and silver trifluoroacetate (2.1 g, 75 mmol) in methylene chloride (100 ml) and the reaction mixture was stirred at room temperature for 48 hours. The yellow precipitate that formed was filtered and then washed with methylene chloride (100ml). The combined filtrates were then washed with 1 M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (2 x 75 ml), water (2 x 75 ml) and concentrated under vacuum to afford N-formyl-3,4-dimethoxy-6-iodo-L-phenylalanine ethyl ester (3). (1.8g, 59%, 72 hours)



*Step Four- Synthesis of formyl-3,4-dihydroxy- 6-iodo- L-phenylalanine ethyl ester (4)*

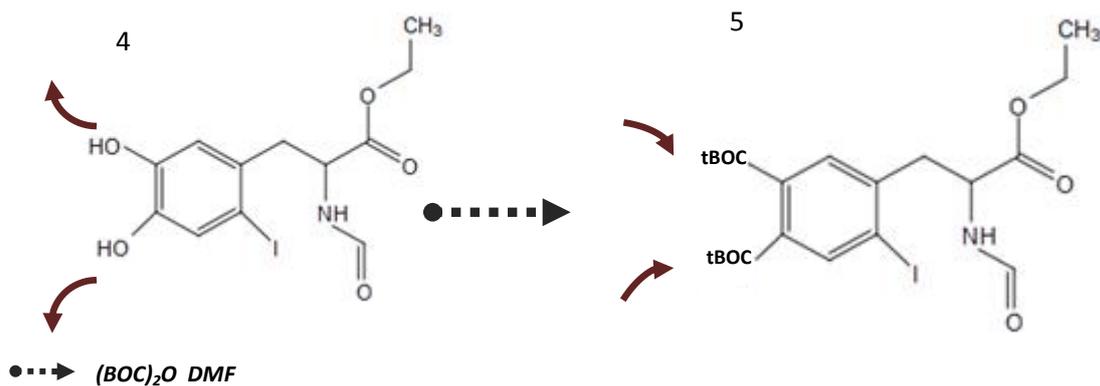
A solution of *N*-formyl-3,4-dimethoxy-6-iodo-*L*-phenylalanine ethyl ester (3) (2 g, 4.9 mmol) in methylene chloride (35 ml) was cooled to  $-78^\circ\text{C}$  with dry ice in acetone.  $BBr_3$  (17.5 ml of a 1 M solution in methylene chloride) was added and the mixture was stirred for 15 minutes. The cooling bath was then removed and the stirring continued until the mixture reached ambient temperature. The reaction mixture was then be poured into ice water (100 ml), stirred at room temperature for an additional 30 min and the methylene chloride layer was separated. Evaporation of the methylene chloride under reduced pressure afforded *N*-formyl 3,4-dihydroxy- 6-iodo- *L*-phenylalanine ethyl ester (4) as a yellow syrup. (1.1g, 54%, 3 hours)



*Step Five-Synthesis of N-formyl-3,4-di-t-butoxycarbonyloxy-6-iodo-L-phenylalanine ethyl ester (5)*

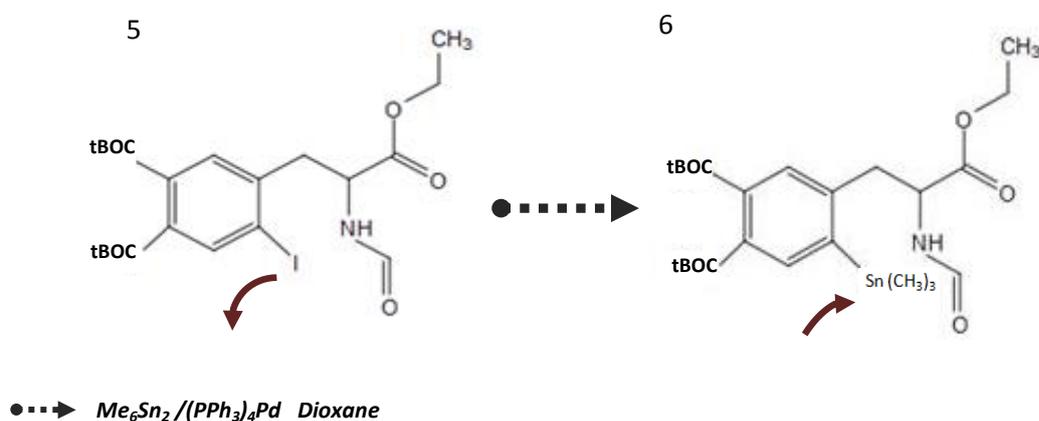
A solution of di-*t*-butyl dicarbonate (tBoc<sub>2</sub>O) (1.21 g, 6.47 mmol) in anhydrous DMF (10 ml) was added drop wise to a solution of *N*-formyl-3,4-dihydroxy- 6-iodo- *L*-phenylalanine ethyl

ester (4) (0.82 g, 2,16 mmol) in anhydrous DMF (10 ml) and triethylamine (0,36 ml, 2.59 mmol). After stirring the reaction mixture at room temperature for 16 hours, ethyl acetate (75 ml) was added and the reaction mixture was washed with saturated aqueous NaCl solution (3 x 50 ml) and water (3 x 50 ml). Evaporation of the organic phase after drying with anhydrous Na<sub>2</sub>SO<sub>4</sub> afforded formyl-3,4-di-t-butoxycarbonyloxy-6-iodo-L-phenylalanine ethyl ester (5) as a colourless oil. (0.72g, 69%, 24 hours)



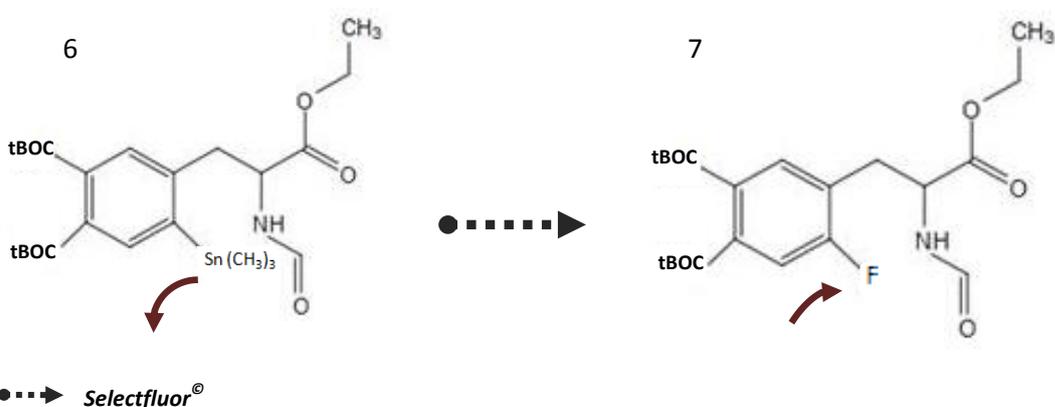
*Step 6- Synthesis of N-formyl-3,4-di-t-butoxycarbonyloxy-6-(trimethylstannyl)-L-phenylalanine ethyl ester (6)*

Hexamethylditin (0.43 g, 1.31 mmol) was added to a mixture of N-formyl-3,4-di-t-butoxycarbonyloxy-6-iodo-L-phenylalanine ethyl ester (5) (0.51 g, 0.88 mmol) and tetrakis-triphenylphospene palladium (0.05 g) in anhydrous 1,4-dioxane (12 ml) and stirred under reflux in a nitrogen atmosphere for 6 hours. After cooling, the black reaction mixture was filtered and the insoluble material was washed with ethyl acetate. Evaporation of the combined filtrates afforded N-formyl-3,4-di-t-butoxycarbonyloxy-6-(trimethylstannyl)-L-phenylalanine ethyl ester (6). (0.29g, 72%, 11 hours)



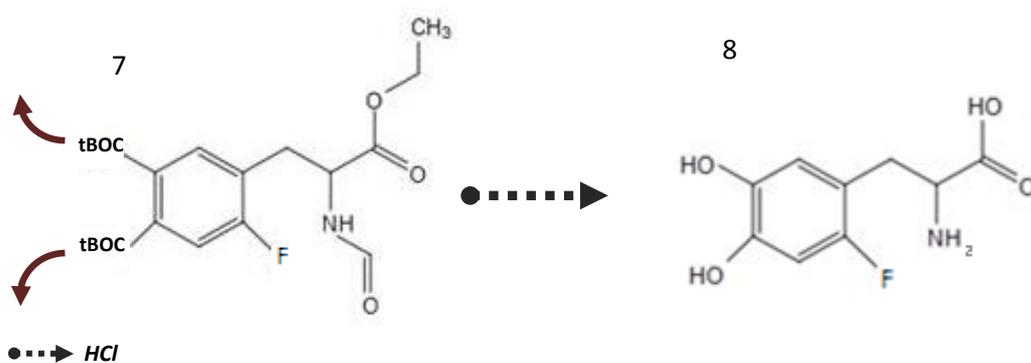
*Step Seven- Synthesis of N-Formyl-3,4-di-t-butoxycarbonyloxy-6-fluoro-L-phenylalanine ethyl ester (7)*

The non radioactive electrophilic fluorinating agent, Selectfluor<sup>®</sup> (0.2g 300mmol), was added to a solution of N-formyl-3,4-di-t-butoxycarbonyloxy-6-(trimethylstannyl)- L-phenylalanine ethyl ester (6) (0,1 g 200 mmol) in DMSO (20 ml) at room temperature and was stirred for 30 minutes. The reaction mixture was then diluted with methylene chloride (25ml) and the organic phase was washed with 1 M Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (2 x 20 ml), water (2 x 20ml) and dried over MgSO<sub>4</sub>. Evaporation of the solvents under vacuum afforded N-Formyl-3,4-di-t-butoxycarbonyloxy-6-fluoro-L-phenylalanine ethyl ester (7). (23mg, 24%, 45 minutes)



*Step Eight- Synthesis of fluoro-3,4-dihydroxy-L-phenylalanine (Fluorodopa) (8)*

N-formyl-3,4-di-t-butoxycarbonyloxy-6-fluoro-L-phenylalanine ethyl ester (7) (0.02g) was treated with 6 N HCl (10 ml) and heated under reflux for 20 minutes. Evaporation of HCl under vacuum afforded 6-fluoro-3,4-dihydroxy-L-phenylalanine (Fluorodopa) as a hydrochloride, ready for purification. (10mg, 65%, 30 minutes)



### *Step Nine- Purification of Fluorodopa with semi-preparative TLC*

The crude electrophilic Fluorodopa product was purified using semi-preparative silica TLC plates using 96% EtOH as a mobile phase according to Fluorodopa reference R<sub>f</sub> values and against an authenticated Fluorodopa reference standard. The reference area was scraped from the TLC Plate and extracted with methanol (500µl) ready for HPLC analysis (5mg, 30 minutes).

## *The nucleophilic synthesis of Fluorodopa*

### *Production of Nucleophilic Fluorine*

The production of N.C.A [<sup>18</sup>F] Fluoride for labelling of FDG is normally carried out at PET Labs Pharmaceuticals on a Siemens 11MeV Cyclotron. The Fluoride is produced via bombardment of an isotopically enriched [<sup>18</sup>O] water target (1.3 ml) with a 11 MeV proton beam using the <sup>18</sup>O(p,n)<sup>18</sup>F nuclear reaction. To test whether the same method and procedure of radioactive fluoride production could be used in the final labelled synthesis of Fluorodopa, cold fluoride (as KF) was dissolved in water in the same sealed glass vials as those used during the radioactive fluorine production.

### *Step One- Synthesis of the Fluoro-Hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (Kryptofix K222) Complex (i)*

A 5 ml sealed glass vial equipped with a stirring bar and a septum was used for the synthesis. 2.5mls of aqueous potassium fluoride solution 3.4mg was added to 7 mg K<sub>2</sub>CO<sub>3</sub> and 22 mg Hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (K222) and injected into the sealed vial. Under nitrogen bubbling, this solution was held for 3 minutes on an aluminium heating block at 150°C after which 500µl of CH<sub>3</sub>CN was added and allowed to dissolve. The solution was then evaporated to dryness to yield the Kryptofix/fluorine complex. (10 minutes). This procedure mimics that which would be used during the synthesis of the radioactive product.

### *Step Two- Synthesis of 6-Fluoro 1,3-benzodioxole-5-aldehyde (ii)*

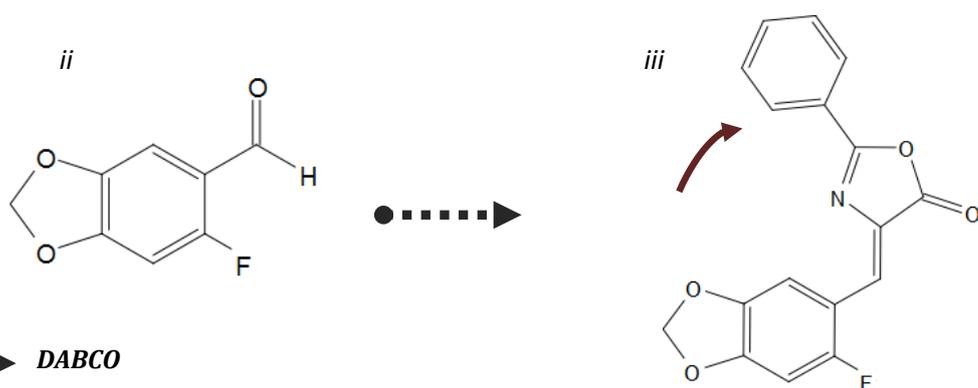
A solution of 15 mg of 1,3- nitro-benzodioxole 6-nitro-5-aldehyde (i) in 1 ml of DMSO was added to the previously produced and dried Fluoro-Hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (Kryptofix K222) Complex (i). The solution was heated to 130°C for 20 min, then diluted into 15 ml of water and passed through two C-18 SEPPAK solid phase extraction (SPE) cartridges in series. After successively washing with 5 ml of 10% HCl, 10 ml H<sub>2</sub>O and 0.5ml of tetrahydrofuran (THF) as a predrying agent, the cartridges were swept with a N<sub>2</sub> flow for 20 seconds and then eluted into a conical vial through a small column (5cm by 1cm) of dry Na<sub>2</sub>CO<sub>3</sub> with 3 x 1.5ml ethanol to give 6-Fluoro-1,3-benzodioxole-5-nitroaldehyde (ii). (25 minutes)



•••→ **Kryptofix K222/F**

### Step Three-Synthesis of 6-Fluoro-1,3-benzodioxole-5-azlactone(iii)

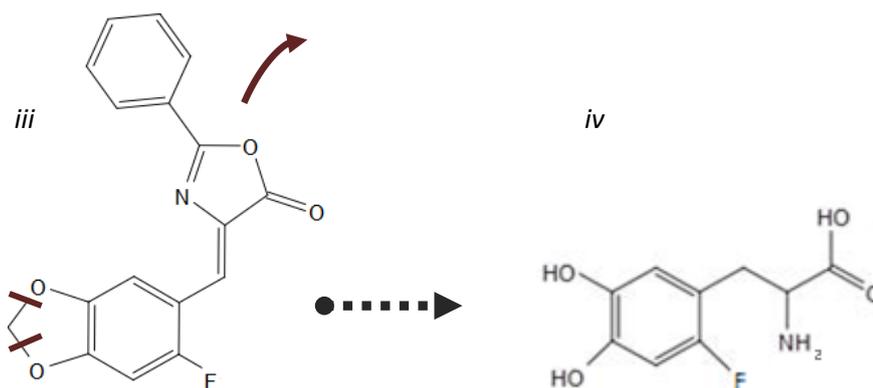
A solution of 2-phenyl-5-oxazolone (50 mg, 0.31 mmol) and 1,4-diazabicyclo[2,2,2]octane (DABCO) (100 mg, 0.81 mmol) in 1 ml ethanol was added to the ethanol solution containing the 6 Fluoro-1,3-benzodioxole-5-nitroaldehyde (ii). The mixture was refluxed at 90°C for 10 min after which the solvent was evaporated under nitrogen flow to give 6-Fluoro-1,3-benzodioxole-5-azlactone (iii). (25 minutes).



•••→ **DABCO**

### Step Four- Synthesis of 6- Fluoro-3,4-dihydroxyphenylalanine (Fluorodopa) (ix)

Reduction and hydrolysis of Fluoro-1,3-benzodioxole-5-azlactone (iii) was carried out in ethanol (3ml) using redistilled hydriodic acid (1.0 ml, stabilized with 30  $\mu$ L hypophosphorous acid) and red phosphorous (100 mg). The mixture was then refluxed under nitrogen for 15 minutes for complete hydrolysis. After partial neutralization with 1 ml of 6N NaOH, the red phosphorus was removed by filtration and the solution was passed through a C-18 Sep-Pak cartridge to remove apolar compounds, producing approximately 3.5ml of pre-purified 6-Fluoro-3,4-dihydroxy-L-phenylalanine (Fluorodopa) ready for purification. (30 minutes)



•••→ *HI/P*

#### *Step Five- Purification of Fluorodopa with semi-preparative TLC*

The crude nucleophilic Fluorodopa product (3mg, 11%) was purified using semi-preparative silica TLC plates according to the same methodology used in the electrophilic cleanup. The reference area was scraped from the TLC Plate and extracted with methanol (500 $\mu$ L) ready for enantiomeric purification. The separation was performed according to reference R<sub>f</sub> values of an authenticated Fluorodopa reference standard (30 minutes).

#### *Step Six- Chiral separation with Thin Layer Chromatography (TLC)*

The racemic mixture was separated with chiral thin layer chromatography, using methanol/water/acetonitrile 30:30:100 (v/v/v) as a mobile phase. The Fluorodopa product was streaked along the base of the plate (1cm from the base) and left to dry for a few minutes in a well ventilated area. Once the plate had dried it was developed in a vapour saturated TLC chamber. The L isomer was isolated according to R<sub>f</sub> value, against a Fluoro-DL-Dopa and Fluoro-L-Dopa (>97%) standard. (2mg, 7%, 40 Minutes)

#### *Determining Chemical purity of the Fluorodopa products*

After purification, the Fluorodopa products were filtered through a 0.22 $\mu$ m membrane filter and injected onto the HPLC column(C-18, 150 x 4.6mm, 5 $\mu$ m particle size). Elution was with 1% acetic acid and 5% methanol in water, at a flow rate of 0.8ml/min. The peaks were monitored with a UV detector at 282nm. The <sup>1</sup>H (360.14 MHz), <sup>13</sup>C (90.57 MHz), and <sup>19</sup>F (338.87 MHz) NMR spectra were recorded on a Bruker AM-360 WB spectrometer and the

measured chemical shifts were specified in parts per million (ppm). The distribution of the isomers (2, 5 and 6) of Fluorodopa were determined from the integrated intensity of the  $^{19}\text{F}$ -NMR signals and the proportion of each isomer was expressed as a percentage of the combined intensities of the signals. Enantiomeric purity was performed on the ChiralPlate (Macherey-Nagel, Duren, FRG). The plates were developed using varying methanol/water/acetonitrile ratios and the enantiomers were identified using Fluoro-DL-dopa and a Fluoro-L-dopa (>97%) standards and visualised using the ninhydrin reagent.

## 2.4-RESULTS

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### *The electrophilic synthesis*

The product from the formylation of the protected L-dopa derivative 3,4 dimethoxy-L-phenylalanine ethyl ester hydrochloride (1) by formic acid and acetic anhydride was dried over anhydrous  $MgSO_4$ , and then concentrated under reduced pressure to afford N-formyl-3,4-dimethoxy-L-phenylalanine ethyl ester (2) as a white solid (2.1g, 41%, 24hours). Optimisation of this synthesis step: increase in the reaction temperature ( $40^\circ C$ ), running the experiment under anhydrous conditions and increasing the reaction time (10hrs) aided somewhat, however yields were still dramatically lower than those reported by other authors. The iodination of N-formyl-3,4-dimethoxy-L-phenylalanine ethyl ester (2) by trifluoroacetyl hypoiodite was found to be reasonable, giving exclusively the monoiodo derivative N-formyl-3,4-dimethoxy-6-iodo-L-phenylalanine ethyl ester (3) in good yields (1.1g, 54%, 3hours). The dimethoxy groups were easily hydrolysed to afford N-Formyl-3,4-dihydroxy-6-iodo- L-phenylalanine ethyl ester (4) as a pale yellow solid (0,72g, 69% 24hours). The reaction of the iodo derivative formyl-3,4-di-t-butoxycarbonyloxy-6-iodo-L-phenylalanine ethyl ester (5) with hexamethylditin gave the substituted tin precursor N-formyl-3,4-di-t-butoxycarbonyloxy-6-(trimethylstannyl)- L-phenylalanine ethyl ester (6) as a shelf-stable crystalline white solid in good yields (0.29mg 72% 11hours). N-formyl-3,4-di-t-butoxycarbonyloxy-6-(trimethylstannyl)- L-phenylalanine ethyl ester (6) was fluorinated with Selectfluor in DMSO for 20 minutes with relative ease.(23mg, 24%, 45minutes) The last step of the synthesis, which required the removal of the protecting groups, was performed in HCl (10mg, 30minutes).

### *The nucleophilic synthesis*

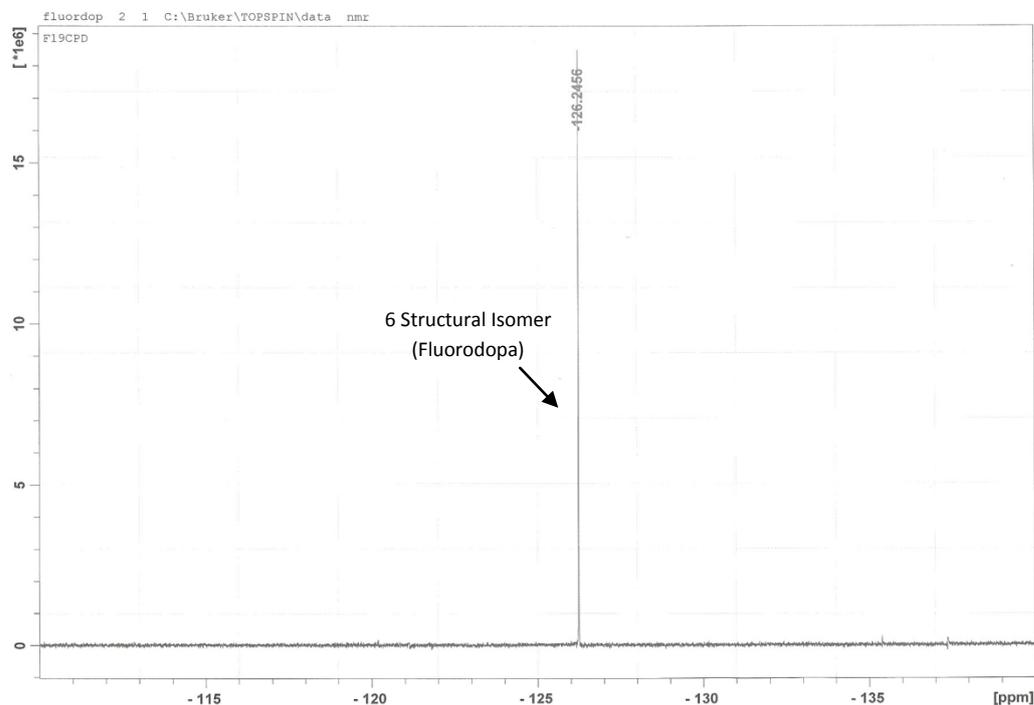
Fluorination of 3,4-dimethoxy-2-nitrobenzaldehyde was conducted in DMSO in the presence of the Fluorine/Kryptofix 2.2.2 aminopolyether complex. The condensation of the fluorinated aldehyde with phenyloxazolone was conducted in the presence of a base catalyst (DABCO) after which hydrolysis with HI/P was performed. The first purification was via C18- Sep-Pak SPE which allowed for the isolation of Fluoro-1,3-benzodioxole-5-azlactone free from the large excess of unreacted compounds(30minutes). The second purification

with C-18 Sep-Pak SPE was to remove apolar compounds(30minutes). As a result, the final purification of the amino acid from the precursor was greatly simplified. The racemic mixture was then separated with chiral thin layer chromatography, using methanol/water/acetonitrile 30:30:100 (v/v/v) as a mobile phase. (2mg, 6%, 40 minutes).

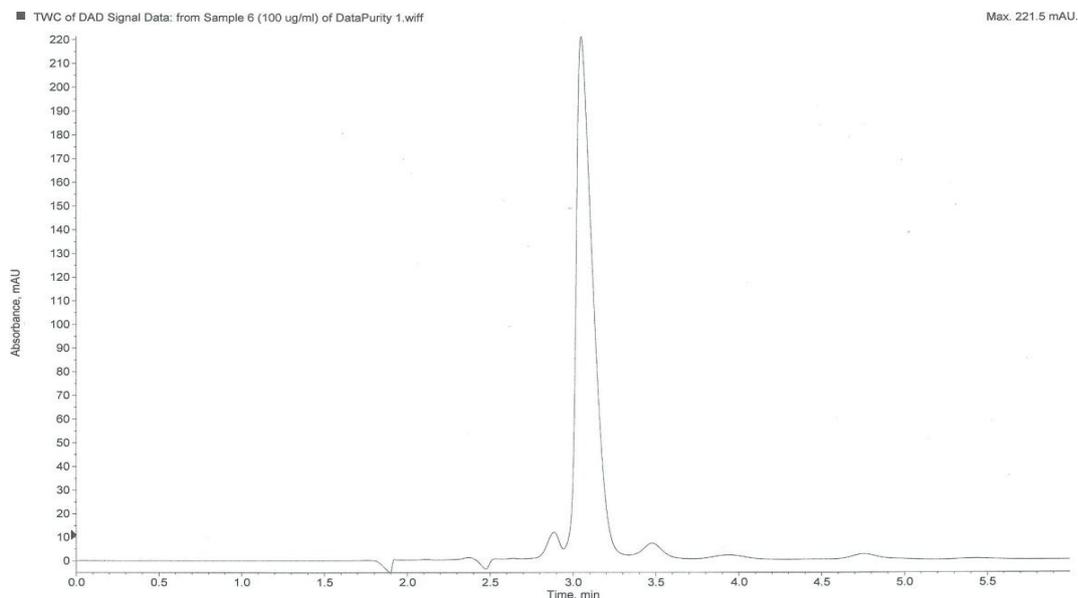
### *Identification of Fluorodopa*

Figure 2.5 and 2.8 illustrates the typical HPLC chromatograms obtained for the separation of Fluorodopa via the two synthetic routes: although the retention time of both the electrophilic and nucleophilic Fluorodopa correlated with the authenticated Fluorodopa standard, both samples presented with contaminants eluting just prior to the main Fluorodopa peak. In addition, the nucleophilic synthesis exhibited several small unidentified peaks (<10%). The scans were performed at 282nm according to reported procedures (Namavari et al, 1992), and under these conditions, both the electrophilic and nucleophilic products appeared to be chemically pure (>90%). However with a broader wavelength bandwidth one would assume that further contaminants would possibly be detected. <sup>19</sup>F NMR (Figure 2.5 and 2.8) and <sup>1</sup>H NMR spectroscopy (Table 2.1) revealed the characteristic peaks consistent with the peaks present in the authenticated Fluorodopa standard however the intensity of the peaks in the H spectrum of the electrophilic product were comparatively low due to water present in the sample giving the typical water peak at 4.64ppm, probably as a result of insufficient drying in the final synthetic step. In addition there was an unidentified peak within the nucleophilic Fluorodopa <sup>1</sup>H spectrum at (4.78-4.80 PPM), most likely contamination from a previous synthesis step. The 5- and 2- fluorine isomers of Fluorodopa were not observed in the F-NMR spectrum of the electrophilic product (Figure 2.4) suggesting total regioselectivity for the 6- isomer of Fluorodopa for this synthesis, but the presence of two small additional peaks in the F NMR spectrum of the nucleophilic synthesis product (Figure 2.7) demonstrated the presence of small fractions of the Fluorine regioisomers of 6-Fluorodopa (at positions 2 and 5 respectively). Expressed as relative F signal intensities, the percentage of the 6 isomer (Fluorodopa) was approximately 90% for the nucleophilic synthesis route, inconsistent with the reported claims of total position 6 regioselectivity for this synthesis. The enantiomeric TLC separation results for Fluorodopa are shown in Figure 2.6: the products from the electrophilic and nucleophilic

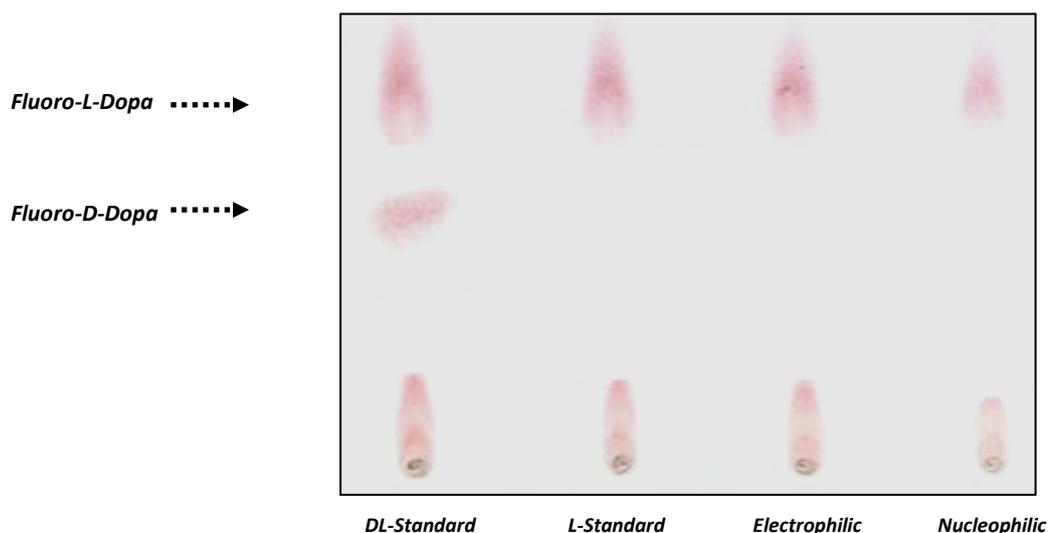
syntheses were found to be essentially enantiomerically pure, exhibiting only the L-isomer of Fluorodopa with the same  $R_f$  value as the L-isomer of an authenticated Fluoro-L-Dopa standard.



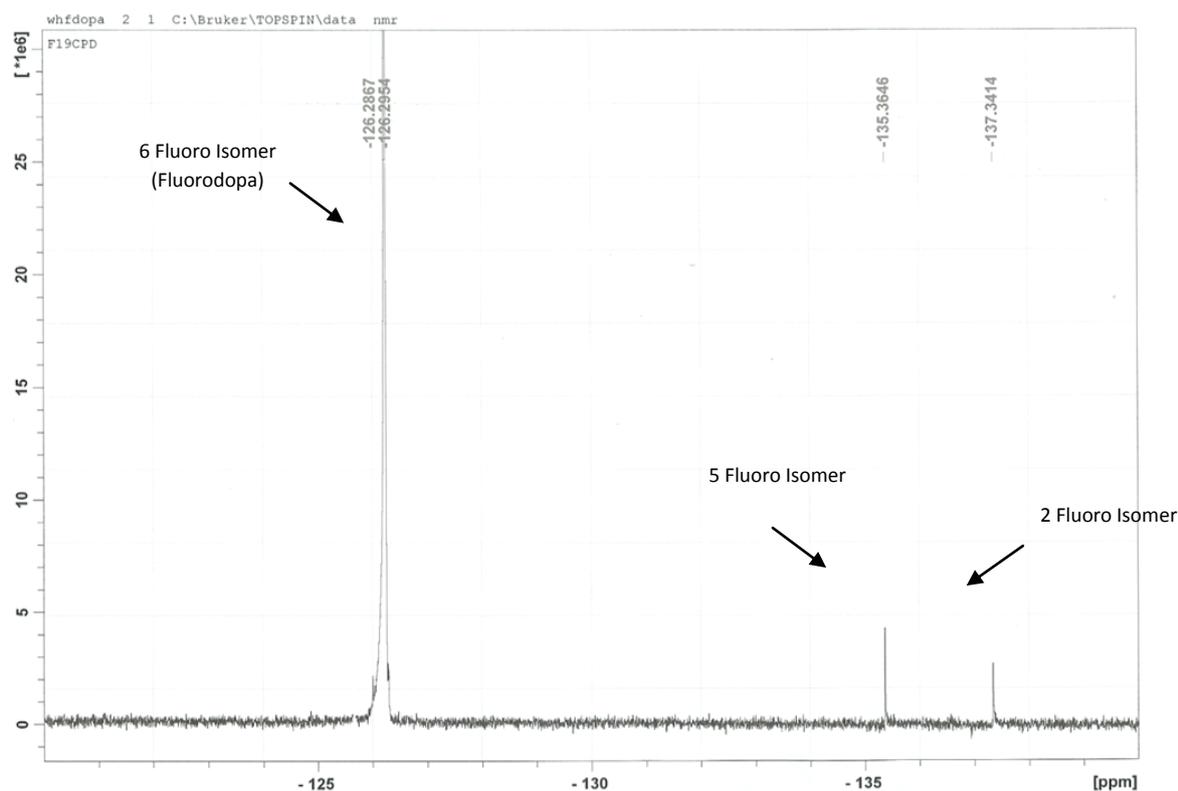
**Figure 2.4- Chemical purity of Fluorodopa (electrophilic):**  $^1F$  Nuclear Magnetic Resonance chromatogram of Fluorodopa in  $D_2O$ . Peak shifts were consistent with an authenticated Fluorodopa standard.



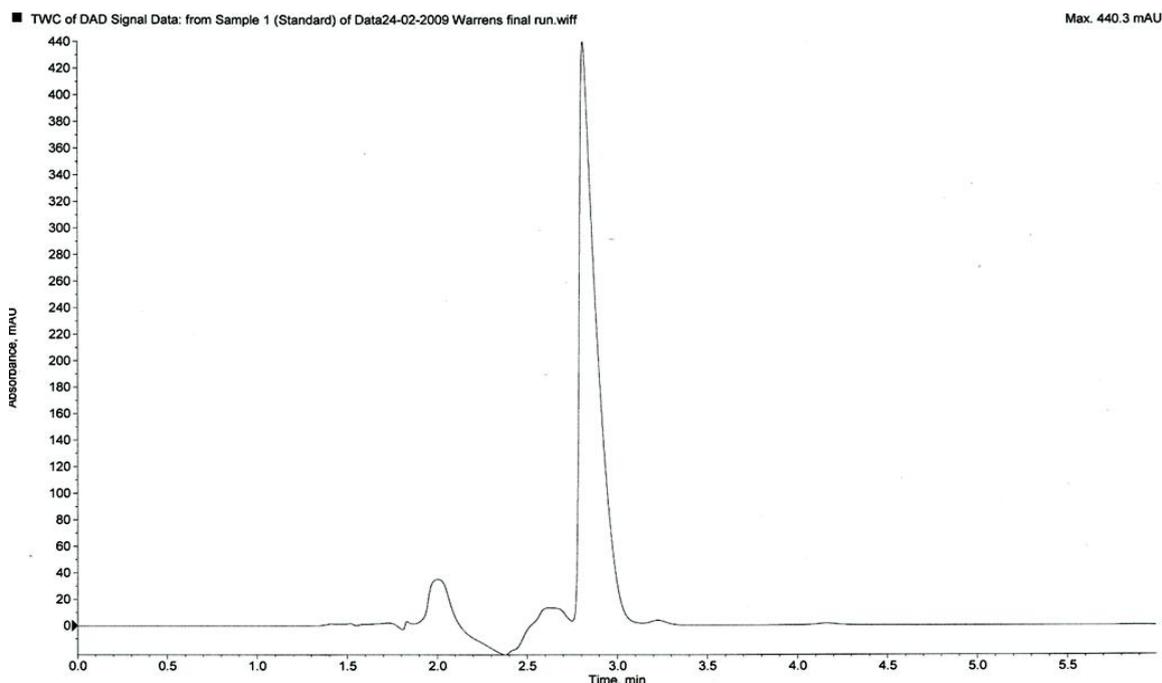
**Figure 2.5- Chemical purity with High Performance Liquid Chromatography (HPLC):** This figure illustrates a typical chromatogram obtained from the product of the electrophilic synthesis of Fluorodopa. The chromatogram obtained from the test product is in agreement with the Fluorodopa reference standard although interference prior to the Fluorodopa peak is visible (<10%).



**Figure 2.6- Enantiomeric purity of Fluorodopa:** This figure illustrates a typical ChiralPlate identification of Fluorodopa for the electrophilic (lane three) and nucleophilic (lane four) synthesis. Both the electrophilic and nucleophilic products display only the L isomer, but do not display the D isomer, confirmed against a Fluoro-DL-Dopa (lane one) and a Fluoro-L-Dopa standard (lane two).



**Figure 2.7- Chemical purity of Fluorodopa (nucleophilic):** <sup>19</sup>F Nuclear Magnetic Resonance chromatogram of Fluorodopa in D<sub>2</sub>O. Peaks were consistent with an authenticated Fluorodopa standard.



**Figure 2.8- Chemical purity with High Performance Liquid Chromotography (HPLC).** This figure illustrates a typical chromatogram obtained from the nucleophilic synthesis of Fluorodopa. The chromatogram obtained from the nucleophilic product is in agreement with the Fluorodopa reference standard although interference prior to the Fluorodopa peak is visible (<10%).

**Table 2.1- <sup>1</sup>H Proton Nuclear Magnetic Resonance Spectral Data (ppm)**

| Product               | CH <sub>2</sub>                          | CH               | Aromatic protons                                  |  |
|-----------------------|--|------------------|---|--|
|                       |  |                  | H-2   | H-5  |
| Electrophilic product | 2.83-2.87 (dd)(15Hz)<br>3.12-3.16 (12Hz) | 3.92-3.99 (21Hz) | 6.61-6.63(6Hz)<br>6.65-6.68 (9Hz)<br>(ABX split)  | 6.70-6.72 (6Hz)<br>6.74-6.82 (24Hz)<br>(ABX split) |
| Nucleophilic product  | 3.10-3.14(dd)(12Hz)<br>3.18-3.22(15Hz)   | 3.96-3.98 (15Hz) | 6.60-6.62(6Hz)<br>6.64-6.67(9Hz)<br>(ABX split)   | 6.73-6.75 (6Hz)<br>6.77-6.87 (30Hz)<br>(ABX split) |
| Standard              | 3.14-3.19(dd)(12Hz)<br>3.22-3.26 (15Hz)  | 4.21-4.27 (18Hz) | 6.62-6.64 (6Hz)<br>6.66-6.69 (9Hz)<br>(ABX split) | 6.71-6.74 (9Hz)<br>6.77-6.82 (27Hz)<br>(ABX split) |

NOTE: <sup>1</sup>H NMR Spectrum 300MHz in D<sub>2</sub>O and pH 7.00

### Discussion

The fluorodestanylation reaction promised to overcome many of the shortfalls of the previous fluorodemercuration methods (Namavari et al, 1992; Luxen et al, 1992): mercury residue is no longer a concern and higher regiospecificity has been reported. The electrophilic synthesis uses a reportedly reliable and high yielding procedure for preparing an aryltin compound suitable for reacting with radioactive fluorine gas or fluorination compounds to produce moderate yields of isomerically pure high specific activity

radiolabelled Fluorodopa that could easily be chemically purified. However the method itself did have limitations. By making use of aqueous Selectfluor, a mild reacting form of electrophilic Fluorine, the synthesis appeared to be relatively simple, however in practice electrophilic Fluorine is extremely reactive and one can expect radiochemical yields to diminish due to radiofluorines high specific activity leading to low absolute concentrations of the fluoride and competing side reactions with the fluorination reagent. The use of acetyl hypofluorite and related compounds has assisted to reduce the corrosive nature of  $F_2$  to some degree, however the use of any chemical form of electrophilic  $^{18}F$  Fluorine in practice would require specialist training.

The nucleophilic synthesis of Machula and co-workers, (2005) offers a number of advantages over previously reported nucleophilic methods; most notable is the significant improvement in yields over the work published by Le Maire and co-workers. The accessibility of Fluorine by means of protons from a single particle cyclotron would produce Fluorine as an aqueous solution opposed to the aggressive Fluorine gas equivalent for the electrophilic synthesis. The nucleophilic synthesis offers an easy availability of low cost starting materials and with a relatively straightforward synthesis procedure that could make its automation possible. The synthesis itself was relatively straightforward, requiring uncomplicated temperature manipulation with simple separation via C18 Sep-Pak SPE extraction and cleanup. The labelling method lead to high but not complete regiospecificity of the Fluorine and chemical purity comparable to that of the electrophilic synthesis, however it produced an enantiomeric mixture of Fluorodopa that required tricky and time consuming chiral separation. A further disadvantage is that the synthetic pathway required that the Fluorine be introduced at a very early stage in the chemical synthesis, which then required chemical manipulation of this precursor through several steps before isolating the final product nearly two hours later. With a half life of only 109 minutes, this would result in the loss of over half the activity when working with  $^{18}F$  Fluorodopa.

## 2.5 CONCLUSION

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The process of electrophilic demetalation by Namavari and co-workers, (1992) allowed the direct use of the cold fluorinating agent Selectfluor by efficient reactivity with an aryltin derivative to produce a regiospecific and enantiomerically pure product that was chemically pure. Yields obtained from the cold electrophilic synthesis support the possibility of a large scale production of Fluorodopa with good chemical, radiochemical and enantiomeric purities. However electrophilic Fluorine is extremely reactive in any form and one can expect radiochemical yields to diminish due to radiofluorine decay. Furthermore the synthesis would require specialised equipment and training in the use of radioactive Fluorine gas.

The Nucleophilic method appeared to have potential for higher absolute yield and higher activity, however early Fluorine labelling in a time consuming multistep synthesis (160 minutes) and a cleanup procedure meant that in practice there would be significant loss of radioactivity. Although the product was chemically pure, comparable to that of the electrophilic synthesis, it required a tricky chiral separation to isolate enantiomerically pure Fluorodopa which would further contribute to decreased activity in production setting. The successful development of chiral auxiliaries or chiral catalytic phase transfer procedures may assist to ensure a less complicated and more timely synthesis for its nucleophilic production in the future, however for the time being the electrophilic synthesis of Fluorodopa appears more attractive for the future implementation of radioactive Fluorodopa in a production setting in South Africa.



# **CHAPTER THREE- QUALITY CONTROL PHARMACEUTICAL QUALITY CONTROL (QC) OF FLUORODOPA FOR INTRAVENOUS HUMAN ADMINISTRATION**

## 3.1-INTRODUCTION

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### *General introduction, quality control*

Radiopharmaceuticals and pharmaceuticals alike require safety evaluation, quality assurance and quality control procedures (Krohn *et al* 1977; Meyer *et al* 1992; Miller M, 1999). The special status of short lived radiopharmaceuticals is based on the fact that they have to be produced near the site of their use and that there is limited time between their production and application or use to perform all the currently accepted quality control tests. This holds true not only for tests of sterility and pyrogenicity but also for some tests of quality control: chemical purity, enantiomeric purity, pH and physical appearance. As any production procedure is prone to variability or vulnerable to unforeseen circumstances, this makes it imperative to employ a range of quick validation techniques to test the final product (Berridge *et al* 1986; Theobald A, 1999).

### *Design of a quality assurance programme*

A quality assurance programme that takes into account all aspects of preparation is the best way to guarantee a product of the required quality. A quality control programme should include the following parameters:

- ▶ *Physical Appearance*

Physical appearance relates to the colour, clarity or turbidity of a PET radiopharmaceutical and should be checked by visual inspection, or more ideally by spectrophotometric analysis.

- ▶ *pH:*

The pH of a PET radiopharmaceutical for human administration should ideally be 7.4, but both slightly acidic and slightly basic pH values are tolerated due to the buffer capacity of the blood (Robert A, 2002). Fluorodopa itself should be kept at a pH of 6 to ensure it retains its original specifications.

### ► *Chemical Purity*

Chemical purity is the fraction of the radiopharmaceutical in the form of the desired chemical molecule whether all of it is radiolabelled or not. The presence of contaminants may lead to an unintended biodistribution of the radiopharmaceutical and dosimetric problems. Any deviations from the molecules normal distribution may result in erroneous diagnostic outcomes (*Adam et al, 1988; Kilbourn M, 1990 Meyer et al 1992; Miller M, 1999; Theobald A, 1999*), it is therefore essential that Fluorodopa is produced chemically pure, free from impurities and isomeric contaminants.

### ► *Enantiomeric Purity*

Chirality has become a major concern in the modern pharmaceutical industry, attributed largely to a heightened awareness that enantiomers of a racemic drug may have different pharmacological, pharmacokinetic and pharmacodynamic effects. As the blood brain barrier transport system only accepts L- isomers, it is essential that Fluorodopa (Fluoro-L-dopa) is administered as an enantiomerically pure L-isomer (*Ward et al, 1986; Weiner et al, 1987; Snider & Kilbourn 2003*).

### ► *Sterility*

Sterility indicates the absence of any viable bacteria or microorganisms in a radiopharmaceutical preparation. All radiopharmaceuticals must be sterile prior to human administration, which is normally accomplished by filtering the product through a sterile 0.22 µm membrane filter, or heating the sample under increased pressure (*Chirakal et al, 1984; Adam et al 1988; Lemaire et al, 1993*). Sterility testing for PET radiopharmaceuticals are performed “after the fact” due to the long incubation times required for microbial growth to be confirmed.

### ► *Pyrogenicity*

Pyrogens are polysaccharides produced by the metabolism of microorganisms and have been identified as a direct and contributory cause of death of many hospitalised patients (*Todar et al, 2007*). More particularly, endotoxins are known to cause febrile reactions in animals with symptoms of extremely high fever, vasodilation, diarrhoea, and in extreme

cases can cause fatal shock (*Snider & Kilbourn 2003; Todar et al, 2007*). The limulus lysate test (LAL) for pyrogens is now the most accepted validated and regulatory test for the detection of pyrogenic endotoxins (*Liebsch et al, 1995*). The FDA now requires an LAL test for intravenous drugs as well as for the screening of prosthetic devices such as heart valves or hip replacements (*FDA, 2008*).

▶ *Documentation system*

Good documentation is an essential part of the quality assurance system, and should exist for all aspects of production (*British Pharmacopoeia, 2007*). Its aims are to define the specifications and procedures for testing all materials and methods of manufacture and control. The processing records of regular production batches provides a complete account of the manufacturing history of each batch of radiopharmaceutical produced, showing it has been manufactured, tested and dispensed according to written procedures (*Ward et al, 1986; Weiner et al, 1987; British Pharmacopoeia, 2007*). This must cover aspects of traceability of all compounds used as well as how, when, where and by whom the testing was performed.

## 3.2-MOTIVATION

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Though the radiosynthesis of Fluorodopa is well-described, little has been reported on its quality assurance. All radiopharmaceuticals administered to patients must have the safety, quality and efficacy required for their intended use. The employment of short lived radionuclides in radiopharmaceuticals poses problems in quality control management, since it is not possible to complete all the necessary quality control testing before the product's expiry time. For this reason it is policy and practice of production laboratories to perform a rapid and independent analysis on each product before it is released for human administration. This makes it imperative to employ a range of quick validation techniques in order to test the final product. The design and implementation of an individualised quality assurance programme is the most effective way to ensure that all aspects of preparation as well as the final product are of the desired quality for human administration.

### 3.3-MATERIALS AND METHODS

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#### *Determining chemical purity*

After purification of the Fluorodopa, the products were filtered through a sterile 0.22 $\mu$ m membrane filter and injected onto the HPLC column(C-18, 150 x 4.6mm, 5 $\mu$ m particle size). Elution was by an isocratic method using 1% acetic, 0.1% ascorbic acid and 5% methanol in water as a mobile phase, at a flow rate of 0.8ml/min. The peaks were monitored with a UV detector at 282nm. The injection volumes were 20 $\mu$ L. Use of a radiochemical detector in addition to the UV detector would enable simultaneous radioisotope purity evaluation.

#### *Determining enantiomeric purity*

Enantiomeric purity evaluation was performed on the ChiralPlate TLC plates of 10 x 10cm (Macherey-Nagel, Duren, FRG). The plates were developed using varying methanol/water/acetonitrile ratios to achieve well resolved enantiomers and visualised using ninhydrin reagent. Authentic Fluoro-DL-dopa and Fluoro-L-Dopa (>99%) standards were used as controls.

#### *Determining pH*

The pH was measured using a Renford 2300 pH meter according to manufacturer's instructions. The pH reading was taken alongside the temperature it was measured at for each sample. Instrumental calibrations were performed on known standards (pH 3, pH 5, pH 7) prior to measuring experimental samples.

#### *Determining endotoxin count*

The electronic LAL Endosafe PTS Cartridge was used for endotoxin detection according to manufacturer's instructions. The Endosafe cartridge was inserted into the PTS reader prior to experimentation to reach incubation temperature. Once incubation temperature had been reached ( $\pm$ 4 minutes) 10 $\mu$ L of Fluorodopa product was injected into the sample channel of the Endosafe cartridge. The assay was conducted according to the prompts on the automated PTS instrument. The results were reported in EU/ml.

### *Sterility testing*

The incubator was set at 34°C. Three tubes containing thioglycollate medium (8mls), were boiled for 10 minutes prior to use to rid the media of oxygen. The tubes were then put in a flow cabinet and allowed to cool to room temperature after which the following was added: 0.5mls of bacteria contaminated solution standard to Tube One (positive control), 0.5ml of bacteriostatic water to Tube Two (negative control) and 0.5ml of Fluorodopa to Tube Three. The tubes were then incubated at 34°C for 7 days after which they were analysed for evidence of bacterial growth. Growth was reported as positive for an increasingly turbid (cloudy or hazy) solution or negative for a clear solution. Each Fluorodopa sample was tested in triplicate.

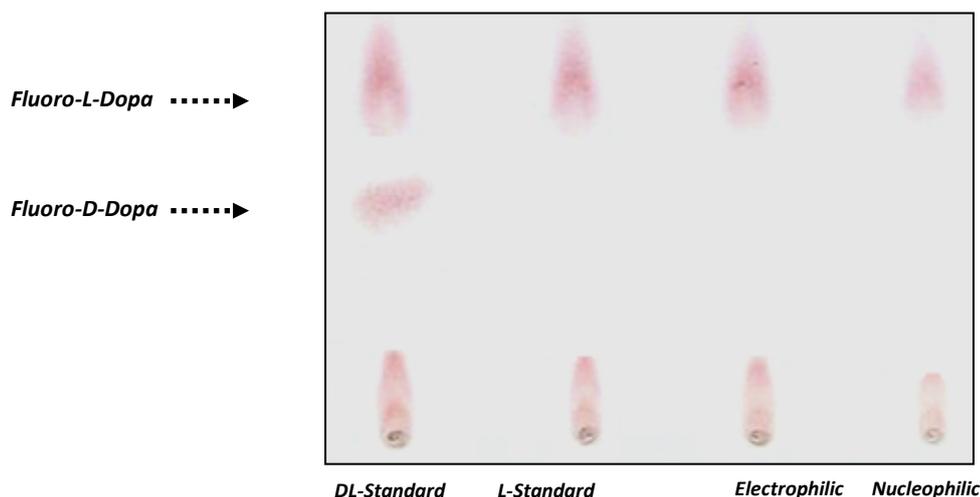
### *Documentation*

Quality control documents were designed to define the specifications and procedures for the methods of manufacture and control of Fluorodopa and to provide individualised guiding principles for the safe and efficacious preparation of Fluorodopa within the environment of a radiopharmaceutical production facility. Production Quality Control Sheets were designed to include the necessary parameters for Fluorodopa production. Standard operating procedures (SOP) included the step by step methodology for testing these parameters: chemical purity with High Performance Liquid Chromatography (HPLC), enantiomeric purity with chiral thin layer chromatography (TLC), endotoxin count with the limulus lysate (LAL) test and sterility with the thioglycollate incubation method.

## 3.4-RESULTS

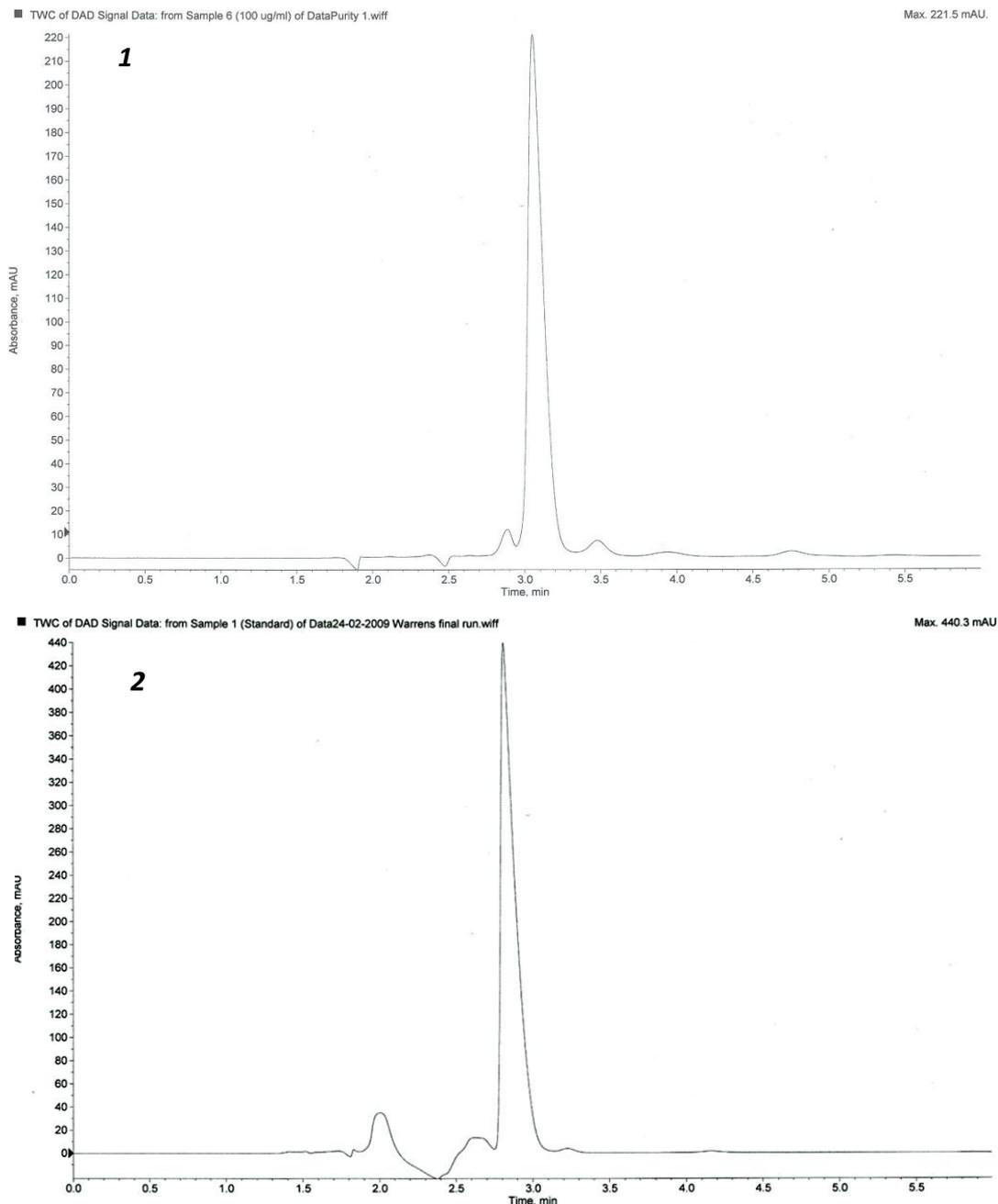
### *Quality control results*

A typical enantiomeric separation of Fluorodopa synthesised by the electrophilic and nucleophilic synthesis routes are shown in Figure 3.1: the products from the electrophilic and nucleophilic synthesis were found to be essentially enantiomerically pure, exhibiting a single L-isomer of Fluorodopa with the same R<sub>f</sub> value as the L isomer of an authenticated Fluoro-L-Dopa standard. The products from both the electrophilic and nucleophilic synthesis were found to be chemically pure (>90%), however below the purity required for radiopharmaceutical production (>95%).



**Figure 3.1- Chemical purity of Fluorodopa:** This figure illustrates a typical ChiralPlate identification of Fluorodopa for the electrophilic (lane three) and nucleophilic (lane four) synthesis. Both the electrophilic and nucleophilic products display only the L isomer, but do not display the D isomer, confirmed against a Fluoro-DL-Dopa (lane one) and a Fluoro-L-Dopa standard (lane two).

Figure 3.2 illustrates a typical HPLC chromatogram obtained for the analysis of in house synthesised Fluorodopa. The HPLC chromatogram shows some interference just before the main Fluorodopa peak in both the electrophilic and nucleophilic synthesis and the chromatogram for the nucleophilic synthesis exhibits several other small additional contaminants (<10%). Detection was performed at 282nm for the electrophilic and nucleophilic synthesis, however with a broader bandwidth or different wavelength, further



**Figure 3.2- Chemical purity of Fluorodopa:** This figure illustrates a typical HPLC chromatogram obtained from the **1-** electrophilic and **2-** nucleophilic synthesis of Fluorodopa. The chromatographic retention times obtained from the Fluorodopa products were in agreement with the Fluorodopa reference standard (retention time 2.6- 3.1 minutes) although interference prior to the Fluorodopa peak is visible in both samples.

contaminants are expected to be found. Both the electrophilic and nucleophilic pharmaceutical preparations of Fluorodopa were confirmed sterile via the thioglycollate method after an incubation time of seven days; however neither the electrophilic nor the

nucleophilic Fluorodopa products were pyrogen free, both samples with endotoxin levels in excess of 15 EU/ml (Table 3.1).

**Table 3.1- Sterility and pyrogenicity of Fluorodopa:**

| <b>Thioglycollate - Sterility Test</b> |                 |                           | <b>LAL Endotoxin Tests- Pyrogenicity</b> |                       |                           |
|--|-----------------|---------------------------|--|-----------------------|---------------------------|
|  | <u>Reading</u>  | <u>Result</u>             |  | <u>Reading</u>        | <u>Result</u>             |
| <b>Sample One</b>                      | <i>Negative</i> | <i>Sterile</i>            | <b>Sample One</b>                        | <i>&gt; 15 EU/ml</i>  | <b><i>FAILED</i></b>      |
| <b>Sample Two</b>                      | <i>Negative</i> | <i>Sterile</i>            | <b>Sample Two</b>                        | <i>&gt; 15 EU/ml</i>  | <b><i>FAILED</i></b>      |
| <b>Positive Control</b>                | <i>Positive</i> | <u><i>Not Sterile</i></u> | <b>Positive Control</b>                  | <i>&gt; 175 EU/ml</i> | <u><i>Pyrogen +ve</i></u> |
| <b>Negative Control</b>                | <i>Negative</i> | <i>Sterile</i>            | <b>Negative Control</b>                  | <i>&lt; 15 EU/ml</i>  | <i>Pyrogen -ve</i>        |

Quality Control Sheet for Fluorodopa production

## FDOPA QUALITY CONTROL SHEET

PRODUCT:

DATE:

LOT NUMBER:

FORMULATION:

PURPOSE:

| Test                | Sampling Requirements | Limits                                  | Results |
|---------------------|-----------------------|---|---------|
| Chemical Purity     | 1ml                   | Chemically Pure (>95% (HPLC)            |         |
| Enantiomeric Purity | 1ml                   | Chiral HPLC (>95%)<br>Chiral Plate Pure |         |
| pH                  | 0.1ml                 | 4-6                                     |         |
| Appearance          | stock                 | Colourless and Clear                    |         |
| Bacterial Endotoxin | 0.5ml                 | <175 EU/ml                              |         |
| Osmolarity          | 1ml                   | 250-350 mOSM                            |         |
| Sterility           | 1ml                   | Sterile                                 |         |
| <b>Signature</b>    | <b>Date</b>           |   |         |

**Figure 3.3-Fluorodopa Quality Control documentation:** including the necessary regulatory parameters for Fluorodopa production. Osmolarity is recommended, but not required for radiopharmaceutical quality control (British Pharmacopoeia, 2007)

Standard operating procedures (SOP's) for the *preparative separation and quality control procedure of Fluorodopa*

**NOTE:** -Radionuclide purity has not been included

-The manual LAL test has been included as opposed to an automated LAL testing system

## STANDARD OPERATING PROCEDURE FOR TESTING THE CHEMICAL PURITY OF FLUORODOPA

### *Introduction*

The following protocol is used to check the purity of a sample of Fluorodopa, utilizing a HPLC UV system (282nm).

### *Materials*

- 1- Freshly made vial of Fluorodopa
- 2- C-18 HPLC column (15cm by 0.46cm) - Alltech Apollo 5µm particle size, dedicated to analysis
- 3- Methanol, Water, Ascorbic Acid, Acetic Acid
- 4- HPLC system housing a 20µL loop

### *Preparation of HPLC mobile phase*

The mobile phase is a 1% Acetic acid, 0.1% Ascorbic Acid and 5% methanol solution in water. This solution should be made freshly for each analysis and not stored for longer than 8 hours.

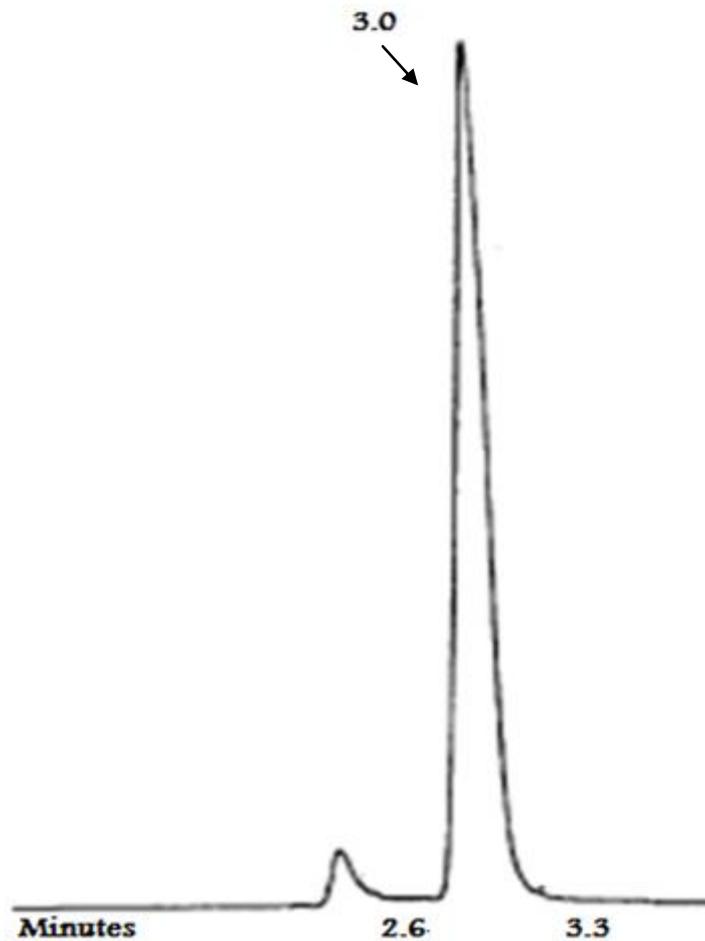
- 1- In 80ml of water dissolve
  - 5ml Methanol
  - 1ml Acetic Acid
  - 0.1g Ascorbic Acid
- 2- Make up to 100ml with water and mix all the ingredients thoroughly
- 3- Filter the solution through a 0.45 micron filter
- 4- Vacuum degas the solution with a vacuum chamber connected to a vacuum pump for removing trapped gasses from compounds.
- 5- Pass 20ml of 5% aqueous Methanol solution through the HPLC system at 1ml/minute to flush out the system
- 6- Pass 20ml of the mobile phase through the HPLC system at 0.8ml/minute. Ensure constant pressure and flow

### *Analysis of results*

Read the peaks with a UV detector at 282nm. Fluorodopa must elute between 2.6 and 3.3 minutes. Compare the retention time to an authentic Fluorodopa standard and ensure a retention time shift of less than 20 seconds. Fluorodopa must have a peak area of >95% of the total peak area to be confirmed chemically pure for human administration.

## STANDARD OPERATING PROCEDURE FOR TESTING THE CHEMICAL PURITY OF FLUORODOPA

Column: Alltech Apollo C-18 HPLC column 5 $\mu$ m 15cm by 0.46cm  
Eluent: Freshly made 5% Methanol, 1% Acetic Acid, 0.1% Ascorbic Acid  
Flow Rate: 0.8ml/min  
Injection Volume: 20 $\mu$ L  
UV Wave Length: 282nm  
Elution time: 2.6- 3.3 minutes



## STANDARD OPERATING PROCEDURE FOR STERILITY TESTING OF FLUORODOPA

### *Introduction*

The following protocol is used to check the sterility of a sample of Fluorodopa, utilizing the Thioglycollate incubation method.

### *Materials*

- 1- Incubator set at 34°C
- 2- Thioglycollate medium (Sigma Aldrich, Cat # 90404)
- 3- 0,1 ml of sterile water (as negative control)
- 4- Bacteria contaminated solution (as positive standard)
- 5- Test products

### *Procedure*

- 1- Boil 3 thioglycollate tubes for 10 minutes to rid media of oxygen with tubes caps slightly open to prevent cracking
- 2- Allow the tubes to cool to room temperature in a sterile environment
- 3- Add 0.1ml of sterile water to test tube one (negative control)
- 4- Add 0.1ml of bacteria contaminated solution to test tube two (positive control)
- 5- Add 0.1ml of test product to test tube three
- 6- Close tubes and incubate for seven days in 34°C

### *Analysis of results*

Examine cultures after seven days for evidence of bacterial growth. Bacterial growth will result in the solution becoming increasingly turbid (cloudy or hazy). Report results as no growth (-ve)(clear solution) or growth (+ve) (hazy solution).

## STANDARD OPERATING PROCEDURE FOR TESTING ENDOTOXIN LEVELS

### *Introduction*

The following protocol is used to check the endotoxin levels for a given product. (*Adapted from the British pharmacopoeia*).

### *Materials*

- 1- Limulus amoebocyte lysate (LAL) lyophilised
- 2- E-coli endotoxin 50mg lyophilised standard
- 3- Pyrogen free water
- 4- 10 (75ml) glass reaction tubes, pyrogen free

### *Procedure*

- 1- Set up pyrogen free reaction tubes for standards and unknown samples in triplicate
- 2- Reconstitute control standard endotoxins with required amount of pyrogen free water as stated on the vial and dilute to a concentration of 0.5EU/ml
- 3- Reconstitute LAL lyophilised according to the manufacturer's instructions
- 4- Make a series of dilutions of 0.25, 0.125, 0.06 and 0.03 EU/ml for standards
- 5- Use pyrogen free water as the negative control
- 6- Carefully transfer 100µL of water, standards and test samples into appropriate reaction tubes, using separate pipettes for each transfer to avoid cross contamination
- 7- Add 100µl of reconstituted LAL to each reaction tube, immediately mix and place at 37° in a water bath. After exactly 30 minutes incubation, remove the tubes from bath and examine for signs of gelation (coagulation of the sample to form a gel).

### *Analysis of results*

Record results as either positive (+ve) or negative (-ve) for gelation. The endpoint dilution is determined as the last dilution of endotoxin which still yields a positive result)

## STANDARD OPERATING PROCEDURE FOR TESTING ENANTIOMERIC PURITY OF FLUORODOPA

### *Introduction*

The following protocol is used to check the enantiomeric purity of a sample of Fluorodopa.

### *Materials*

- 1- Freshly made vial of Fluorodopa
- 2- Macherey and Nagel ChiralPlate (10cm by 5cm)(Cat # 08598)
- 3- Fluoro-DL-Dopa Standard
- 4- Fluoro-L-Dopa Standard
- 5- Water, methanol, acetonitrile

### *Method*

Use methanol/water/acetonitrile 30:30:100 (v/v/v) as the mobile phase. Use the ChiralPlate in ascending one dimensional development in a TLC chamber ensuring chamber vapour saturation.

- 1- Activate plates for 15 min at 110°C in a drying cabinet.
- 2- Spot 2µl of synthesised Fluorodopa sample 1cm from the base of the plate
- 3- Spot 2µl of Fluoro-DL-Dopa standard (10µM in 5% ETOH) adjacent to the test product
- 4- Allow plates to dry in a well ventilated area
- 5- Place the TLC plate in the development chamber
- 6- Remove the plate once the mobile phase has run 80-90% of the plate length
- 7- Place a pencil mark on the TLC plate indicating the mobile phase front
- 8- Leave the plate to dry in a well ventilated area

### *Detection*

Spray the plates in a 0.3% ninhydrin solution (in acetone) until wet, and then place in a drying cabinet for 10 minutes at 100°C.

### *Analysis of results*

Purple spots will develop for any primary amine compound. Two purple spots will form for the Fluoro-DL-Dopa. The test sample product must form only a single purple spot at the same R<sub>f</sub> value as the Fluoro-L-dopa standard.

## Discussion

The results and experience described in this chapter exemplify the importance of establishing good manufacturing practice that incorporates a good method of preparative separation and an independent quality control procedure for the production of Fluorodopa. After completion of the synthesis the radiopharmaceutical preparation was captured in a sterile vial after passage through a 0.22 $\mu$ m Millipore filter. This system was shown to be free from bacteria and microorganisms in both the electrophilic and nucleophilic Fluorodopa products. The LAL test however revealed that both the nucleophilic and electrophilic products contained in excess of 15 EU/ml (endotoxin). Although attempts were made to synthesise the Fluorodopa products free from pyrogens, the production facility used for the cold synthesis was not within a controlled environment, and environmental pollutants such as dust, aerosol particles and chemical vapours could not be avoided. However, it is required that for the commercial production of Fluorodopa a controlled environment designed to exclude dust and contaminants through high efficiency particulate air (HEPA), and ultra low penetration air (ULPA) filters be used (*British Pharmacopoeia*). In conjunction with other control factors this should enable the production of a pyrogen free product. The chemical purity of the nucleophilic and electrophilic synthesis was confirmed quickly and accurately with the HPLC system in which precursors and chemical by-products were shown to have been efficiently separated from the final product, however there were still signs of contamination (<10%) in both the Fluorodopa products. The enantiomeric purity was confirmed on the ChiralPlate, however there was concern about the quantitative aspect of this process: when concentrating the same Fluorodopa solutions as well as the authenticated Fluoro-L-Dopa standard, both L and D isomers were identified in solution, emphasising the importance of drug concentration when identifying enantiomeric purity on the ChiralPlate. This raised quality control concerns: the ability to classify an enantiomerically impure solution as pure, if diluted down to a concentration where only the major enantiomer would be visualised due to the limit of sensitivity of the ninhydrin reagent. Enantiomeric separation and/or identification with chiral HPLC would provide a method that is quicker, more accurate and would define the enantiomeric purity quantitatively (*Ward T, 1986; Wainer W, 1987*). The SOPs for the radiopharmaceutical

quality control were adapted from the guidelines set out in the British Pharmacopoeia, 2007, and although the parameters for Fluorodopa production were left unchanged, the methods themselves were adapted and in most cases simplified to ensure a quick, accurate and rigorous technique for QC testing of Fluorodopa in any facility. In conjunction with the SOPs, quality control data sheets were designed to include the necessary regulatory parameters for Fluorodopa production: chemical purity (including chiral purity), sterility, pH, physical appearance and pyrogenicity.

### 3.5- CONCLUSION

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Without assurance that Fluorodopa meets acceptable standards of quality, safety and efficacy, any use in man, is evidently compromised. As this radiopharmaceutical is to be administered by intravenous injection there are several parameters that needed to be addressed during the quality assurance process. This chapter illustrates an “*in-house*” quality control schedule to take into account the local factors that might affect product quality. Pharmaceutical quality control was achieved by setting test parameters, test methods and limits for these control parameters. Following the quality control guidelines as set out in this chapter should allow for the successful implementation of a synthetic protocol of a reproducible and documented procedure that has a demonstrated ability to deliver a pharmaceutical with satisfactory standards of chemical purity and sterility.



## **CHAPTER FOUR- TOXICITY STUDY**

### **AN IN VITRO TOXICITY ASSAY OF FLUORODOPA ON RESTING AND PHA STIMULATED HUMAN LYMPHOCYTES**

## 4.1-INTRODUCTION

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### *General introduction, in vitro toxicity testing*

In general there is no single method that can cover the complexity of general toxicity in humans, however, data can be obtained from several sources including *in vivo* and *in vitro* toxicological studies. Application of cell culture techniques in toxicological studies is referred to as *in vitro* toxicology, which describes a field of study that applies technology using isolated organ tissues or cell cultures to study the toxic effect of chemicals (Hayes & Markovic, 1999). *In vitro* models represent an important tool for the investigation of molecular and biochemical mechanisms involved in cellular differentiation, developmental regulation and the toxic process (Guillouza et al, 1987; Atterwil et al, 1987; Chenery et al, 1987; Haugland P, 1996).

### *Biological assays for cellular viability*

Basic cell culture techniques are designed to provide a proper support of cells or tissues for their normal survival, growth, and function in vitreous media outside the body. Many of the biological assays for toxicological studies *in vitro* require the measurement of surviving and/or proliferating mammalian cells (Cosma et al, 1984; Atterwil et al, 1987; Chenery et al, 1987). This can be achieved by several methods that include: the inhibition of incorporation of labelled RNA or DNA precursors, amino acids or other metabolites, exclusion of dyes or enzymes, decrease of intracellular ATP content or the formation of coloured products (Mossman T, 1983; Ramos et al, 1984; Guillouza et al, 1987; Atterwil et al, 1987; Chenery et al, 1987; Haugland P, 1996).

### *The MTT assay for assessing cellular proliferation*

One of the most widely recognised colorimetric methods for assessing cellular proliferation is the MTT assay (Mollinari et al, 2003; Plumb J, 2004; Burton J, 2005). The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a highly coloured formazan product that is

insoluble (Mossman T, 1983; Cory et al, 1991). The conversion of MTT to a formazan product is accomplished by mitochondrial dehydrogenase enzymes found in metabolically active cells. The MTT is reduced to its formazan product by metabolically active cells primarily due to the action of dehydrogenase enzymes generating reducing compounds such as NADH or NADPH, but also intracellular molecules like glutathione, which in turn reduces MTT to a deeply coloured purple product. The intensity of the formazan product, which is measured spectrophotometrically, has been shown to correlate directly to the number of living cells in culture (Mossman T, 1983; Cory et al, 1991; Riss & Moravec, 1996; Wilson et al, 2000; Mollinari et al, 2003).

#### *Human lymphocytes as a model for neuronal cells*

There are only a few cell lines that can serve as accurate *in vitro* models of neurons, more specifically human central nervous system (CNS) neurons (Gladkevich A et al, 2004; Jimenez Del Rio M et al, 2006). Normal mature neurons do not generally divide and are thus not readily maintained *in vitro*. Human neurons therefore present great challenges for the development of adequate *in vitro* model systems. These difficulties have led to the use of substitute cell types that are able to mimic or express neuronal properties. There is increasing evidence pointing to a close integration between the central nervous system (CNS) and human lymphocytes: lymphocytes express multiple biological systems previously considered to pertain exclusively to neuronal cells (Carr et al, 1989; Blaylock J, 1994; Ilani et al, 2001; Amenta et al, 2001; Gladkevich A et al, 2004; Jimenez Del Rio M et al, 2006) and because the use of healthy brain biopsies from living patients is unrealistic for biochemical investigation, lymphocytes have been demonstrated to be a convenient and accessible alternative model (Gladkevich A et al, 2004; Jimenez Del Rio M et al, 2006).

#### *Fluorodopa toxicity in vitro*

Although high doses of Fluorodopa have been found to be toxic to neurons in pure neuronal cultures *in vitro*, toxicity of L-Dopa and its derivatives has been shown to reduce significantly when cell culture conditions are chosen to more accurately reflect *in vivo* conditions (Olanow et al, 1996; Trodec et al, 2001; Pardo et al, 2005). Most *in vitro* studies of dopamine

signalling cells in culture subjected to dopamine and its derivatives have demonstrated toxicity at 100-250 $\mu$ M after 1-5 days culture (*Mytilineou et al, 1993; Ziv et al, 1994*). However, under certain conditions, the opposite effect has been observed: at 30-50 $\mu$ M in the presence of ascorbic acid, increased branching of dopamine processes and increased cell survival has been reported (*Kratzing et al, 1985; Kalire et al, 1991; Mytilineou C, 1993*).

## 4.2-MOTIVATION FOR RESEARCH

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In general, fluorinated aromatic substances are not highly toxic and Fluorodopa has been shown to exert characteristics much like its non-fluorinated parent molecule (L-Dopa) both *in vitro* and *in vivo*. The research work reported here comprises the first pharmaceutical synthesis of Fluorodopa within South Africa's borders, and as such it is required under the conditions of its intended use that its toxicological and pharmacological risk be determined. Confirming a safety profile *in vitro* is the first requirement in preclinical toxicological evaluation of any drug intended for human use.

## 4.3-MATERIALS AND METHODS

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### *Materials*

Trypsin/EDTA solution (0.25%) was purchased from Highveld Biologicals (Kempton Park, RSA). Sodium bicarbonate, ammonium chloride and crystal violet solution were purchased from Merck Chemicals Pty Ltd. Dimethyl sulphoxide (DMSO), EDTA, Histopaque 1077, Heparin and 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Foetal calf serum (FCS) was purchased from Adcock Ingram (Johannesburg, RSA).

### *Experimental*

All plastic tips and tubes for freezing drugs were autoclaved and kept in closed containers in the laminar flow cabinet. Bottles containing culture medium were kept sterile and stored at 4°C. All drugs used for the experiment were filter-sterilised and all work was performed under sterile conditions.

### *Preparation of drugs and drug concentrations*

The experimental drugs were dissolved in sterile water and diluted to reach 16 individual concentrations ranging from 0,04µM-5000µM. The individual Fluorodopa concentrations were then diluted with medium (10 fold dilution) when added to the culture plates to obtain final concentrations in the wells of:

|                  |                 |                 |                 |                 |
|------------------|-----------------|-----------------|-----------------|-----------------|
| Conc 1- 500µM    | Conc 2- 400µM   | Conc 3- 300µM   | Conc 4- 200µM   | Conc 5- 100µM   |
| Conc 6- 50µM     | Conc 7- 25µM    | Conc 8- 12.5µM  | Conc 9- 6.25µM  | Conc 10- 3.1µM  |
| Conc 11- 1.5µ    | Conc 12- 0.75µM | Conc 13- 0.32µM | Conc 14- 0.16µM | Conc 15- 0.08µM |
| Conc 16- 0.004µM |                 |                 |                 |                 |

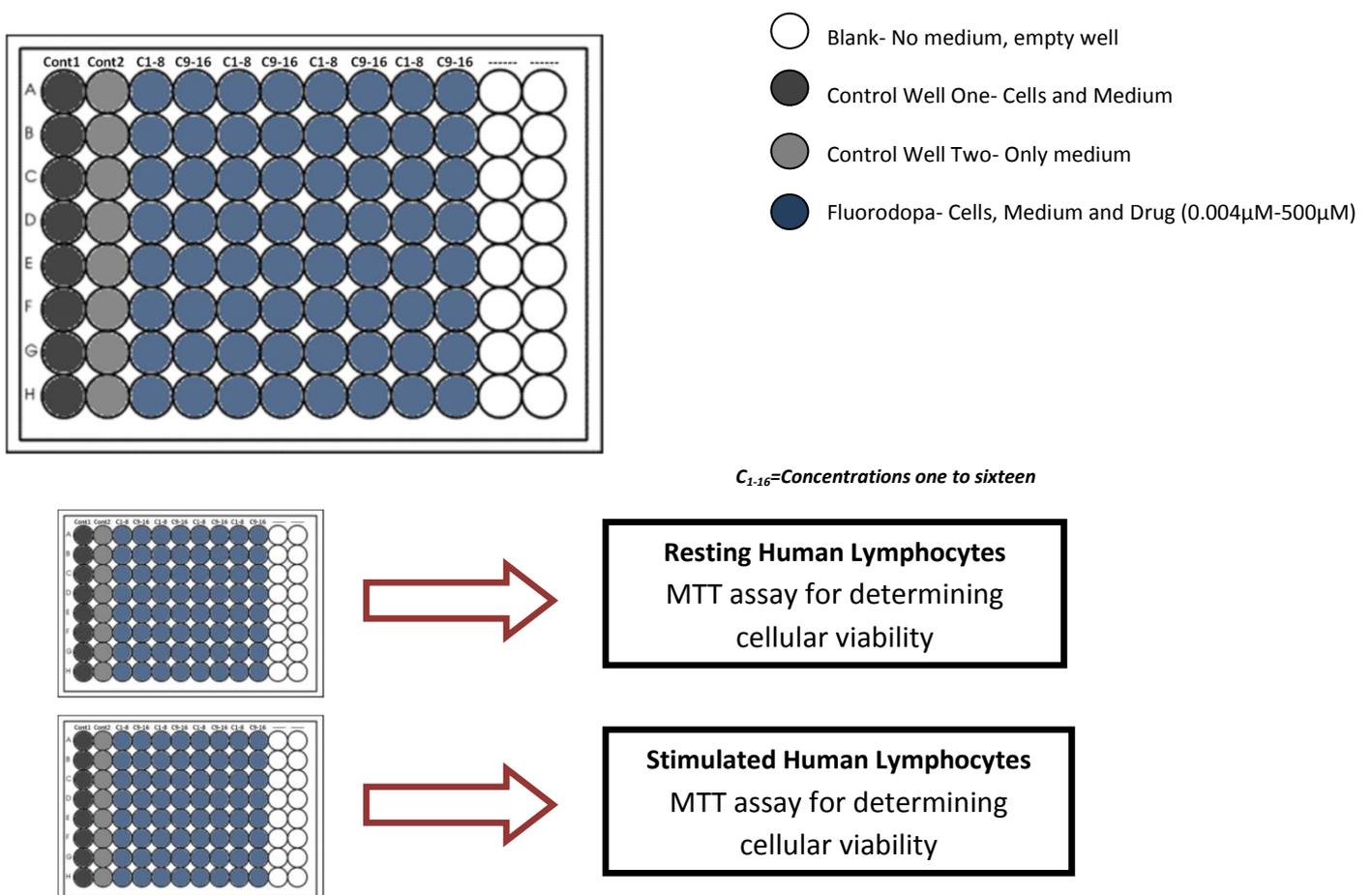
### *Lymphocyte proliferation*

Thirty millilitres (ml) of freshly drawn preservative free heparinised blood was layered onto 15ml of Histopaque 1077 and then centrifuged at 600g's for 25 minutes at 21°C. The top plasma layer was carefully removed, and the lymphocyte band was transferred into sterile

50ml tubes. These tubes were then filled with RPMI(-) medium and centrifuged again for 15 minutes at 450g's to remove the contaminating platelets. After discarding the supernatant, the tubes were then filled up with filter-sterilised, cold-buffered 0.83% ammonium chloride and left to stand on ice for 10 minutes in order to lyse the contaminating red blood cells. The tubes were then re-centrifuged for 10 minutes at 400g's, the supernatant discarded, and the tubes refilled with RPMI(-). Centrifugation was then repeated and the pellet that formed was suspended with 1ml of RPMI supplemented with 10% Foetal Calf Serum (FCS). Cell concentration was then determined, and the cells were diluted with RPMI (+) medium to  $2 \times 10^6$ /ml for the experiment.

*96 well plate layout*

Figure 4.1 illustrates the set-up of the 96 well plates for the toxicity and MTT assay. Table 4.1 illustrates the procedure used for the complete toxicity and MTT assay.



**Figure 4.1-In vitro toxicity and MTT assay plate layout for determining cellular viability: on stimulated and resting human lymphocytes in vitro (Mossman T, 1983).**

**Table 4.1- procedure for the MTT assay:**

|                                | CONTROL WELLS ONE<br>( MEDIUM ) | CONTROL WELLS TWO<br>(MEDIUM AND CELLS) | EXPERIMENTAL WELLS<br>(ACTIVE DRUGS) |
|--------------------------------|---------------------------------|---|--------------------------------------|
| CELLS (2 x10 <sup>6</sup> /ml) | 0µL                             | 100µL                                   | 100µL                                |
| RPMI(+)                        | 160µL                           | 60µL                                    | 60µL                                 |

***Incubate for 1 hour***

|                 | PLATES WITH RESTING CELLS | PLATES WITH STIMULATED CELLS |
|-----------------|---------------------------|------------------------------|
| RPMI(+)         | 20µL                      | -                            |
| PHA (2.5 µg/ml) | -                         | 20µL                         |

|                 | CONTROL WELLS ONE<br>(MEDIUM) | CONTROL WELLS TWO<br>(MEDIUM AND CELLS) | EXPERIMENTAL WELLS<br>(ACTIVE DRUGS) |
|-----------------|-------------------------------|---|--------------------------------------|
| RPMI+           | 20µL                          | 20µL                                    | -                                    |
| DRUG @ [C] TEST | -                             | -                                       | 20µL                                 |

***Incubate for 72 hours at 37°C (5% CO<sub>2</sub>) 99% RH***

***Perform MTT assay for determining cellular viability***

### *The MTT assay for determining cellular viability*

An aliquot of 20 $\mu$ l of a sterile MTT solution (5mg/ml PBS) was added to each well, and the plates were re-incubated for 3-4 hours at 37°C in a 5% CO<sub>2</sub> incubator. The cells were then centrifuged at 400g's for 10 minutes and the supernatant was carefully removed so as not to disturb the pellet. The pellets were then washed with medium and centrifuged again. The supernatant was carefully removed and the pelleted cells were then left to air dry for 2 hours. An aliquot of 100 $\mu$ l of DMSO was then added to solubilise the cell membranes and the formozan. The plate was then placed on a shaker until the pellets had fully dissolved and then the absorbance of the wells were measured spectrophotometrically (570nm with a reference 630nm).

### *Statistics*

Dose response curves were plotted for Fluorodopa on resting and PHA stimulated human lymphocytes using GraphPad Prism 4 software. Inhibitory concentrations at 10%, 50% and 90% were calculated from best fit linear regression analysis using the same software.

Results are expressed as mean % of untreated controls  $\pm$  SEM.

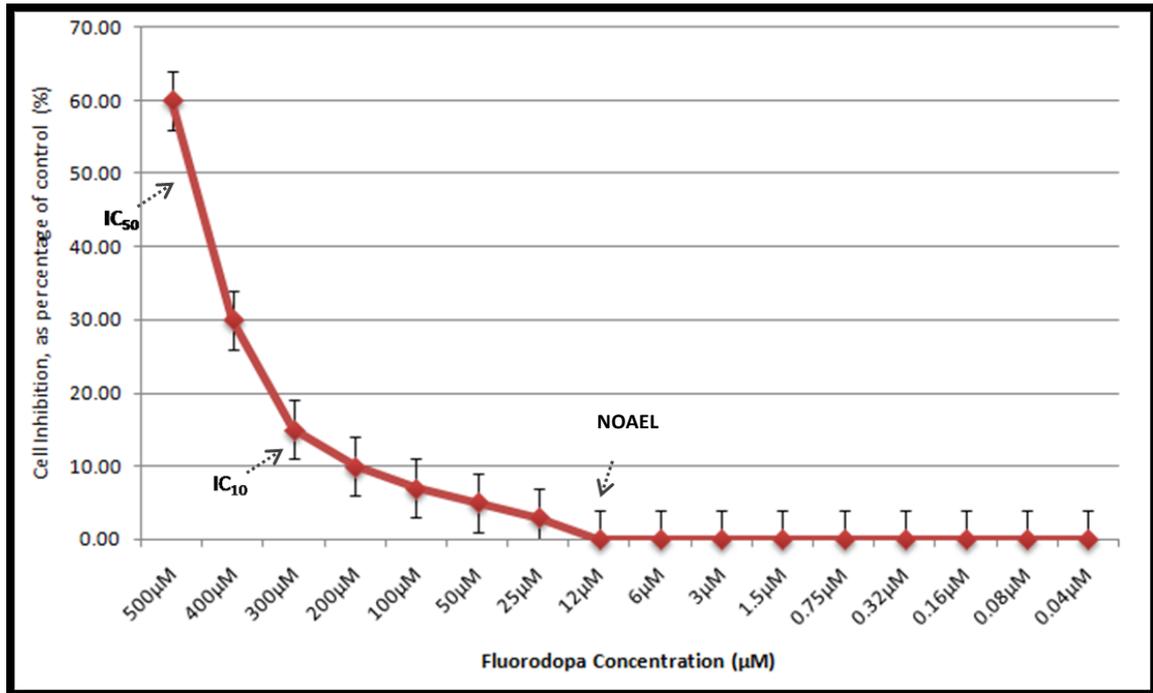
## 4.4-RESULTS

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The results from the MTT cytotoxicity assay are illustrated in Figure 4.2 (resting lymphocytes) and Figure 4.3 (PHA stimulated lymphocytes). The maximum drug concentration tested (500 $\mu$ M) was not sufficient to cause total cell inhibition in either the resting or stimulated lymphocytes *in vitro* and therefore Total Lethal Concentration (LD<sub>100</sub>) could not be determined. The maximum cell inhibition achieved was 90% in the stimulated lymphocytes and 60% in the resting lymphocytes at a drug concentration of 500 $\mu$ M. The stimulated lymphocytes demonstrated a steeper dose response curve compared to the resting lymphocytes, indicative of less resistance to Fluorodopa toxicity at low concentrations and an increased sensitivity at the highest concentrations within the tested concentration range. A slight plateau in cytotoxic effect was seen between 270 and 420 $\mu$ M Fluorodopa after which the slope of the cytotoxic effect became very steep again. At the maximum drug concentration (500 $\mu$ M) stimulated lymphocytes demonstrated 90% inhibition (IC<sub>90</sub>). Extrapolative prediction would suggest a dose of 660-700 $\mu$ M of Fluorodopa would result in 90% inhibition on the resting lymphocytes. Inhibitory concentration (50%) (IC<sub>50</sub>) was calculated to be 320 $\mu$ M for the stimulated lymphocytes and 440 $\mu$ M for the resting lymphocytes. At lower dosages, that would be more relevant to the physiological situation during diagnostic procedures, the resting lymphocytes and stimulated lymphocytes demonstrated no cytotoxic effects and no observed adverse effect limit (NOAEL) concentrations of  $\pm 12\mu$ M and  $\pm 6\mu$ M respectively.

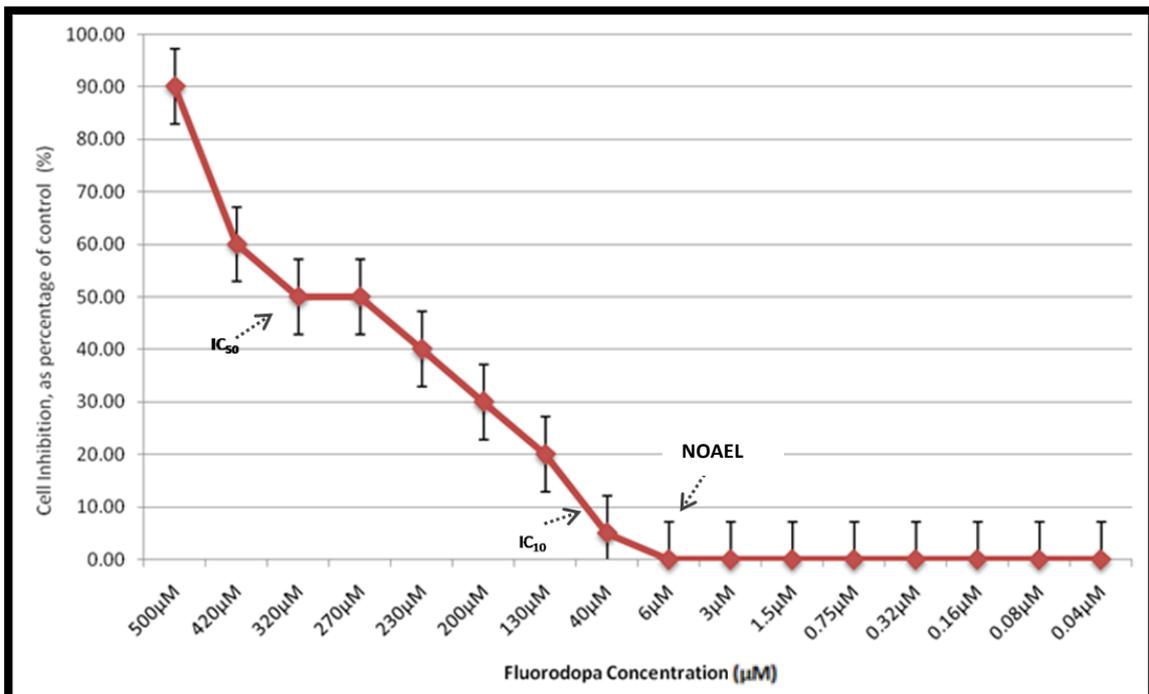
### *Discussion*

By monitoring the cellular toxicity of the Fluorodopa over a broad concentration gradient (0,004 $\mu$ M-500 $\mu$ M) *in vitro*, high, intermediate and low concentrations of Fluorodopa on cell viability were assessed using both resting and PHA stimulated cells. By simulating a typical healthy state (resting lymphocytes) versus PHA stimulated lymphocytes, the effects of Fluorodopa on two contrasting cellular states could be assessed. The concentration of Fluorodopa used in the cell culture experiments exceeded by a large margin the estimated



**Figure 4.2- In vitro toxicity of Fluorodopa on resting human lymphocytes:** The results from the cytotoxicity assay are illustrated above. Fluorodopa inhibitory concentrations  $IC_{50}$ ,  $IC_{10}$ , and no observed effect concentration (NOAEL) resulted from 460µM, 320µM and 12µM for Fluorodopa respectively.

**NOTE:** Graph is not on a linear scale to emphasize the response, Results are expressed as mean % of untreated controls ± SEM



**Figure 4.3- In vitro inhibition toxicity curve of Fluorodopa on stimulated human lymphocytes:** The results of the cytotoxicity assay are illustrated above. Fluorodopa inhibitory concentrations  $IC_{90}$ ,  $IC_{50}$ ,  $IC_{10}$ , and no observed effect concentration (NOAEL) resulted from 500µM, 320 µM, 45µM and 6µM for Fluorodopa.

**NOTE:** Graphs are not on a linear scale to emphasize the response, Results are expressed as mean % of untreated controls ± SEM

maximal concentrations that one could expect after a single bolus IV administration of L-Dopa in patients with PD (5 to 50 $\mu$ M) (Cedarbaum J, 1987). In view of the fact that the molar quantities of Fluorodopa used diagnostically are a hundredth of the dose of L-Dopa administered therapeutically, one could expect maximal Fluorodopa plasma concentrations, as a very broad approximation, of 0.05 $\mu$ M to 0.5 $\mu$ M after the administration of a single dose of Fluorodopa. For resting and stimulated lymphocytes the no observed adverse effect limit (NOAEL) was  $\pm$  12 $\mu$ M and  $\pm$ 6 $\mu$ M respectively, notably higher than estimated *in vivo* concentrations expected during diagnostic procedures. The resting cells were also more resistant to toxicity at doses < 20 $\mu$ M.

Research has shown that catecholamines can regulate immune function at systematic, cellular and molecular levels and play an inhibitory role in the modulation of T lymphocytes and the cellular immune response (Heilig *et al*, 1993; Madden *et al*, 1995, 2000; Delrue-Perollet *et al*, 1995; del Rey *et al*, 2002). L-Dopa and its derivatives have been shown to modulate these immune responses by influencing the cytokine networks negatively (Maden *et al*, 1995; Madden *et al*, 1995, 2000; Qui Y, 1996), leading to inhibition of expression of adhesion molecules, inhibition of cytokine and chemokine production, inhibition of neutrophil chemotaxis, disturbed T-cell proliferation and down regulation of delayed type hypersensitivity responses. This could explain the increased toxic effects of the PHA stimulated T lymphocytes to Fluorodopa as demonstrated in the *in vitro* cell culture experiments. Most literature has demonstrated that L-dopa toxicity *in vitro* ranges from 50 - 250 $\mu$ M (Mytilineou *et al*, 1993; Ziv *et al*, 1994) after one to five days incubation, however Fluorodopa toxicity has been known to vary significantly depending on the type of cell, the cell culture conditions and the ability to accurately reproduce the *in vivo* environment. Based on the *in vitro* data reported in this study, *in vivo* toxicity at the maximum administered diagnostic dose of Fluorodopa (1 $\mu$ mole/kg)(body mass) would not be expected, however, *in vivo* toxicity still had to be assessed.

## 4.5-CONCLUSION

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By assessing the cytotoxicity of Fluorodopa over a concentration gradient (0,004 $\mu$ M - 500 $\mu$ M) on resting and PHA stimulated human lymphocytes in *in vitro* culture for three days, it was possible to demonstrate a dose response curve for the risk assessment of Fluorodopa on both resting and PHA stimulated human lymphocytes *in vitro*. Stimulated lymphocytes were found to be more vulnerable than resting cells to the toxic effects of Fluorodopa at medium and high concentrations, however at low concentrations (<20 $\mu$ M) resting cells were found to be more resistant. Fluorodopa was only found to exert its toxicity above 12 $\mu$ M for resting cells and above 6 $\mu$ M for stimulated cells, which are both concentrations far above the extrapolated *in vivo* concentration that would be expected after the administration of Fluorodopa for diagnostic applications.

# **CHAPTER FIVE – IN VIVO TOXICITY**

## **A SEVEN DAY ACUTE TOXICITY STUDY OF INTRAVENOUSLY ADMINISTERED FLUORODOPA IN VIVO**

## 5.1-INTRODUCTION

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### *General introduction, in vivo toxicology*

Conventional methods of toxicological assessment are based on whole animal studies. A basic assumption of *in vivo* or animal toxicity studies is that chemicals that cause adverse effects in animals will cause similar effects in humans. The evaluation of human chemical exposure requires toxicity information for determining the magnitude of the potential adverse health effects before human experiences occur (Zurlo *et al*, 1994; Thorne, 2001; Greenberg & Philips, 2003).

### *Chemical toxicity of Fluorodopa in vivo*

In general, fluorinated aromatic substances are not highly toxic and literature demonstrates that in many other pharmaceutical parameters such as uptake, storage, and metabolism, Fluorodopa behaves similarly to that of its non-fluorinated analogue (Adam M, 1988; Patricia L, 1994; Cooper L, 1996; Kirk *et al*, 1996). It appears that like the situation in cell cultures, dopamine and its derivatives can either be toxic or neuroprotective, depending on the dose, time of administration and presence of other compounds (Perry *et al*, 1984; Olanow *et al*, 1996; Melemed *et al*, 1998; Trodec *et al*, 2001; Pardo *et al*, 2005): high concentrations of dopamine have been shown to be pro-apoptotic whereas low concentrations of dopamine are able to prevent cell death, possibly due to the ability of dopamine to affect intracellular oxidative processes (Fornstead *et al*, 1989). Studies on dopamine have failed to demonstrate a decreased rate of disease progression or any negative effect on survival of substantia nigra pars dopamine neurons in normal rodents, non-human primates, or humans after its administration at therapeutic doses (Hefti *et al*, 1981; Perry *et al*, 1984; Quinn *et al*, 1986; Diamond & Markham, 1990; Uitti *et al*, 1993; Delta *et al*, 2005).

### *Radiation toxicity of Fluorodopa in vivo*

Although a Fluorodopa PET scan is “non-invasive”, it does however involve exposure to ionizing radiation. The total dose of radiation is usually around 7 mSv which is expected to

be far below the level of non-stochastic effects (*Int Rad, 1977; Jones S, 1982; Harvey et al, 1985*). The radiation dose which is similar to that of FDG administration (*Int Rad, 1977; Jones S, 1982*) can be compared to 2.2 mSv average annual background radiation in the United Kingdom (*Hughes et al, 2005*). When interpreting Fluorodopa kinetics, the ratio of radioactivity in the striatum compared to that of the surrounding brain tissues has been shown to increase steadily over time for at least 120 minutes after injection. With a radioactive half life of 109 minutes, by the time Fluorodopa has reached peak concentrations in the brain, the absolute radiation dose has diminished considerably because of radioisotope decay (*Jones S, 1982; Harvey et al, 1985; Mejia A, 2001*).

## 5.2-MOTIVATION

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International regulations relating to human health require that all new pharmaceutical drugs are tested for their safety, prior to their use in human volunteers and patients. Fluorodopa is being produced for the first time in South Africa for pharmaceutical application. A key stage in ensuring the safety of Fluorodopa is to conduct toxicity tests in appropriate animal models. This will allow the assessment of the safety of Fluorodopa before it can be made commercially available for testing in humans.

## 5.3-MATERIALS AND METHODS

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### *Seven day acute toxicity study in mice*

Studies took place at the University of Pretoria Biomedical Research Centre (UPBRC) following ethics approval from The Animal Use and Care Committee (AUCC). All experimental procedures including dose administration, blood collection, the caring for and the handling of animals were performed by senior animal technicians at the UPBRC. The project was performed under the supervision of Dr Roland Auer, head of the UPBRC. All signs of ill health, together with any behavioural change or reaction to treatment were recorded during the observation period. On completion of the trial, blood cell counts (red blood cell, haematocrit, haemoglobin, white blood cell and platelets) were determined on a fully automated analyser and the wet organs (kidneys, liver, heart, spleen and brain) of each mouse were isolated, weighed, dissected and then inspected for any histopathological changes.

### *Animal care and housing*

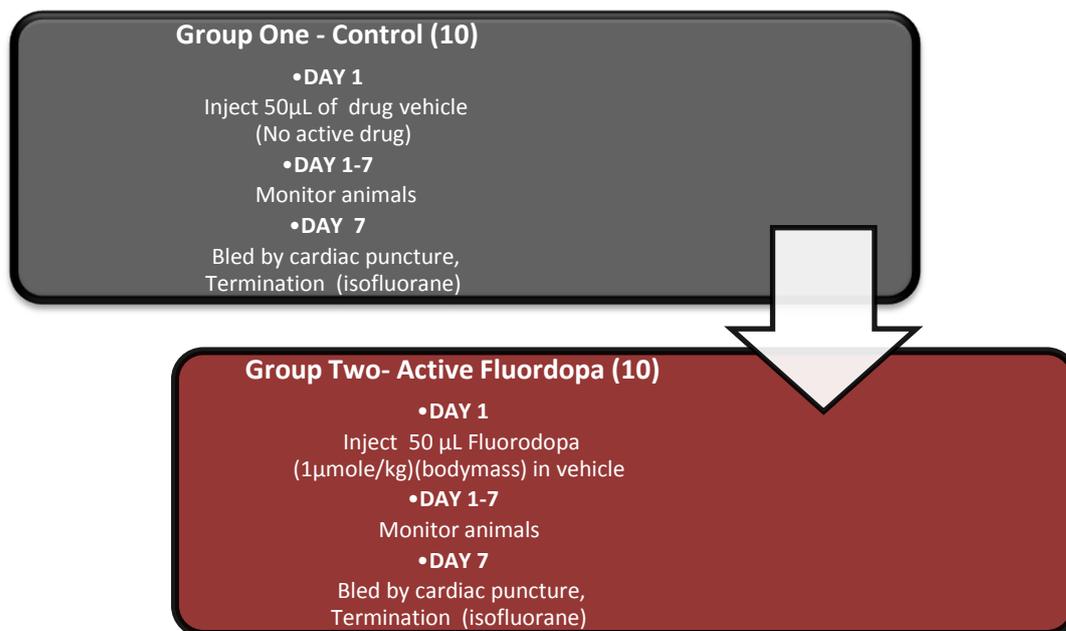
The animals were all housed at the UPBRC for 7 days prior to the study to allow for acclimatisation. They were housed three mice per cage in well ventilated rooms with 12 hour cycles of day and night light. Temperature was maintained at 25°C. Food and water was provided *ad libitum*.

### *Monitoring of animals and early withdrawal*

The animals were monitored by qualified UPBRC personnel throughout the study and morbidity was determined on a daily basis. Evaluation was made via a humane endpoint score sheet which consisted of reduced food and water intake, observation of movement (particularly as it pertained to the ability of the animal to obtain food and water), hypoactivity, crouching, excessive grooming and ease of breathing. (See supporting documents for humane endpoint score sheet).

*Study Design, seven day acute toxicity study*

Healthy BALB/c female mice, weighing 20–25 g, were divided into 2 groups of 10 animals matched for weight and size. 50µL of 0.9% NaCl saline was administered to the control group (Group One). Fifty microlitres equivalent to 1µmole/kg (body mass) of Fluorodopa (in 0.9% NaCl saline) was administered to the active group (Group Two). The Fluorodopa was aseptically dissolved in normal saline (0.9% NaCl solution) and administered as a single intravenous injection of 1µmole/kg (body mass) in the tail vein. The general behaviour of the test animals was monitored carefully for 1 hour after dosing, periodically during the first 24 hours (with special attention given during the first 4 hours), and then twice daily thereafter, for a total of 7 days. Changes in the normal activity of mice and their weights were monitored and signs of toxicity were recorded.



**Figure 5.1- Seven day acute toxicity study in mice:** after the administration of 1µmole/kg(body mass) of Fluorodopa, animals were monitored for a period of seven days. Parameters that were measured include: general behaviour, food and water intake, haematological values and histopathological changes.

On day 7 the animals were bled by cardiac puncture and were terminated with isofluorane overdose. Tissue specimens were collected from the brain, liver, spleen, thymus, small and large intestines, gonad (female genitalia), stomach and spleen. After 48 hours tissue

fixation, specimen blocks were selected from the above mentioned organs and processed in an automated tissue processor for histological slide preparation, after which wax blocks were produced, sections were cut at 5 $\mu$ m and the slides were stained in an automatic stainer using routine Haematoxylin and Eosin staining.

### *Statistics*

Data is expressed as mean  $\pm$  standard deviation. The student *t*-test was used to assess the statistical significance between the active and control group

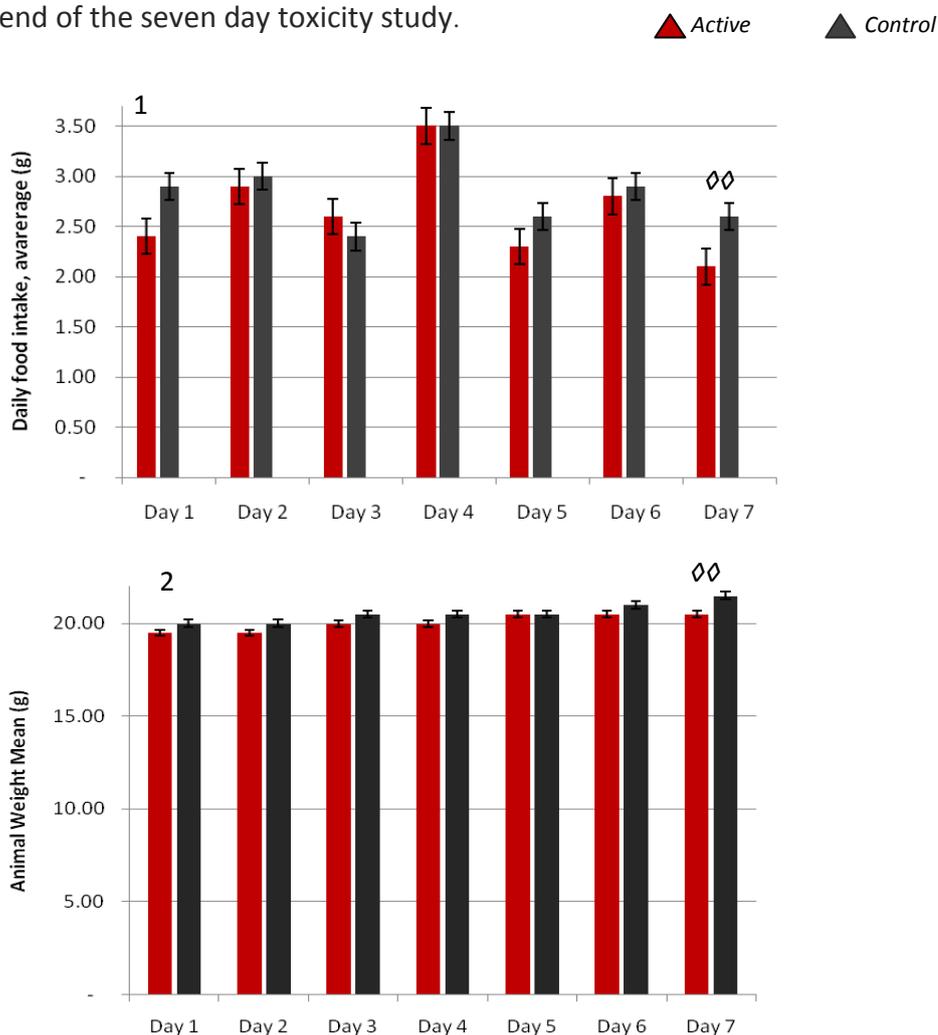
**Table 5.1- Seven day acute toxicity study in mice, summary of study parameters:**

|   | GROUP ONE- CONTROL (10)   | GROUP TWO- FLUORDOPA (10)   |
|---|---|---|
| <b>DAY ONE- DRUG ADMINISTRATION</b>         |   |   |
| <b>DRUG</b>                                 | None  | Fluorodopa  |
| <b>CONCENTRATION</b>                        | N/A   | Fluorodopa 1.0µmole/kg body mass  |
| <b>DILUENT</b>                              | Saline (0.9% NaCl)  | Saline (0.9% NaCl)  |
| <b>VOLUME</b>                               | 50µl  | 50µl  |
| <b>ADMINISTRATION ROUTE</b>                 | Intravenous (IV) via the tail vein  | Intravenous (IV) via the tail vein  |
| <b>PARAMETERS MEASURED DURING TRIAL</b>     |   |   |
| <b>OBSERVATIONS</b>                         | First four hours and then twice daily   | First four hours and then twice daily   |
| <b>FOOD CONSUMPTION</b>                     | Twice Daily   | Twice Daily   |
| <b>BODY WEIGHTS</b>                         | Once Daily  | Once Daily  |
| <b>DAY SEVEN- POST MORTEM INVESTIGATION</b> |   |   |
| <b>HAEMATOLOGY</b>                          | Full Blood Count  | Full Blood Count  |
| <b>HISTOPATHOLOGY</b>                       | Brain, heart, kidneys, liver, spleen, pancreas, stomach, thymus, intestine (small), intestine (large), gonad (female genitalia), lung | Brain, heart, kidneys, liver, spleen, pancreas, stomach, thymus, intestine (small), intestine (large), gonad (female genitalia), lung |
| <b>BLOOD CHEMISTRY</b>                      | ALT, AST, ALB, GGT, LDH   | ALT, AST, ALB, GGT, LDH   |
| <b>ORGAN WEIGHTS</b>                        | Brain, heart, kidneys, liver, spleen  | Brain, heart, kidneys, liver, spleen  |

## 5.4-RESULTS

### General behaviour of test animals

The administration of Fluorodopa caused no noticeable change in the general behaviour of the mice at any time after dosing compared to the control group. There were no significant changes in average weight gain and final mean body weight ( $p < 0.05$ ) (Figure 5.2). Food and water intake of the mice exposed to Fluorodopa were closely comparable to the vehicle controlled mice (Figure 5.2). Both the control and treated mice appeared uniformly healthy at the end of the seven day toxicity study.

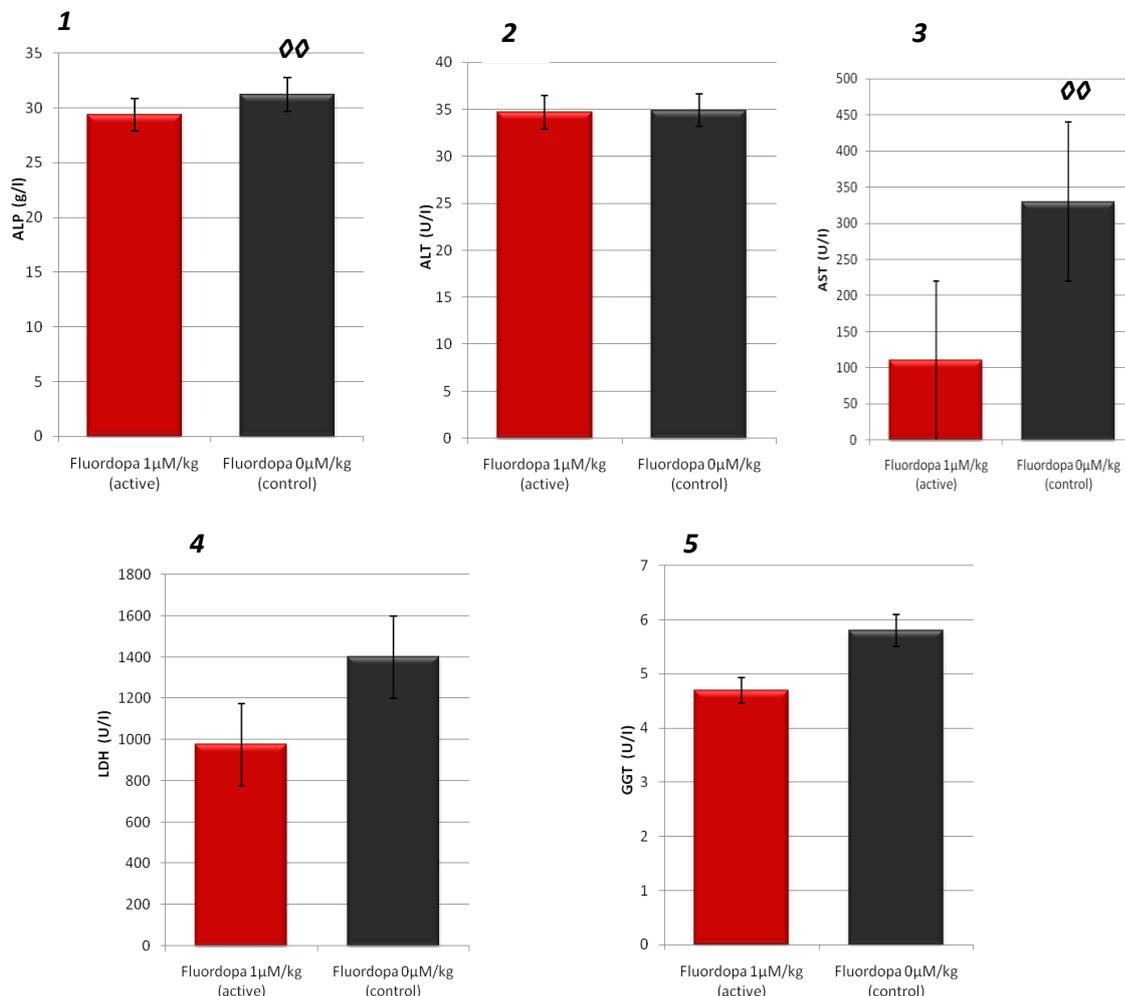


**NOTE:** Values are mean for groups of 10 mice  
 ∅∅ Statistically significant correlation between groups ( $p < 0.05$  (Student T test))

**Figure 5.2- General behaviour of test animals monitored over seven days:** **1-** The combined average food intake of the test animals measured daily for a period of seven days. **2-** The combined average body weights of the test animals measured daily for a period of seven days, revealed a statistically significant correlation between groups ( $p < 0.05$  (Student T test)).

### Clinical biochemistry, liver function tests

The functions of the kidney (urea) and liver (AST, ALT, ALB, CGT and LDH) were within normal limits for the active Fluorodopa dosed group, however the vehicle controlled group revealed some concerning abnormalities: four of the ten control mice had extremely high LDH and AST levels, 400% higher than comparable mice in the active and control groups. This resulted in substantially increased average values for LDH and AST for the vehicle controlled group, however all other enzyme levels within the vehicle controlled group and the active Fluorodopa group remained within normal range.



**NOTE:** Values are mean  $\pm$  SD for groups of 10 mice.

∞∞ Statistically significant correlation between groups ( $p < 0.05$ ) (Student T test)

**Figure 5.3- Results of clinical biochemistry:** 1- Alkaline phosphatase (ALP) 2- Aspartate transaminase (AST) 3- Alanine transaminase (ALT) 4- Lactate dehydrogenase (LDH) 5- Gamma glutamyl transferase (GGT). All values were in normal range for the active Fluorodopa dosed group, however the vehicle controlled group revealed significantly increased AST and LDH levels in four of the mice.

### *Haematological findings*

Four of the control mice did not undergo haematological testing because of procedural blood drawing complications and therefore testing in the control groups consisted of a sample size of six mice as opposed to the normal ten. Complete blood-cell counts in the mice revealed no biologically significant differences between Fluorodopa dosed and vehicle control mice (Table 5.3). The values of haematological parameters for red cells (total count, haemoglobin concentration, haematocrit, mean corpuscular volume, mean cell haemoglobin, mean cell haemoglobin concentration, and red cell distribution width) were within normal limits as were the values of haematological parameters for white cells (monocytes, lymphocytes, basophils and eosinophils). Readings for the platelets (total count and mean platelet volume) could not be quantified accurately due to low blood volume and platelet aggregation. The haematological findings from both the Fluorodopa and the vehicle controlled mice did not reveal any abnormalities that could be attributed to the test drug.

### *Organ weights and histopathological examination*

Table 5.2 summarises the absolute organ weights of the vehicle controlled and Fluorodopa treated groups at the conclusion of the seven day study. There were only minor absolute organ weight variations of brain, liver, kidney and heart between vehicle control and treatment groups and the differences were not statistically significant. One animal in the control group presented with a considerably enlarged spleen, 400% higher than the comparable test animals, which resulted in a heightened average mean and an inflated standard deviation. Closer histological evaluation revealed that this mouse presented with moderate haemosiderosis and extramedullary haemopoiesis. In addition the lymph nodes revealed focal acute bacterial lymphadenitis with numerous cocci bacteria visible within an acute exudative inflammation. These pathological findings suggest lymphadenitis of bacterial origin with secondary hyperplasia of the splenic white pulp and neutrophilic leukostasis in the liver sinusoids. This incidental finding was of bacterial origin and was not attributed to the administration or the drug vehicle. This test animal was therefore omitted from statistical calculations to avoid skewing that would favour the test drug.

**Table 5.3- Haematological Findings of control and experimental mice**

| Parameter   | Unit                  | Fluorodopa Concentration ( $\mu\text{mole/kg}$ )                    |  |
|---|-----------------------|---|--|
|   |                       | 0 $\mu\text{Mole/Kg}$ (Body Mass) Fluorodopa (Control) <sup>A</sup> | 1 $\mu\text{Mole/Kg}$ (Body Mass) Fluorodopa (Active) <sup>B</sup> |
| <b>Haemoglobin<sup>oo</sup></b>                         | g/l                   | 137 +- 11   | 143 +- 11  |
| <b>Red Cell Count</b>                                   | X10e12/l              | 8.94 +- 0.8   | 9.14 +- 0.3  |
| <b>Haematocrit</b>                                      | l/l                   | 0.41 +- 0.033   | 0.42 +- 0.12   |
| <b>Mean Corpuscular Volume<sup>oo</sup></b>             | fl                    | 45 +- 0.2   | 46 +- 0.34   |
| <b>Mean Cell Haemoglobin<sup>oo</sup></b>               | g/dl cells            | 15.4. +- 0.06   | 15.7 +- 0.2  |
| <b>Mean Cell Haemoglobin Concentration<sup>oo</sup></b> | g/dl cells            | 34.1 +- 0.1   | 34.2 +- 0.3  |
| <b>Red Blood Distribution Width</b>                     | %                     | 13.9 +- 0.2   | 12.9 +- 3  |
| <b>White Blood Cell Count</b>                           | X10e9/l               | 4.3 +- 2.1  | 4.67 +- 1.3  |
| <b>Ab N (total)</b>                                     | X10e9/l               | 1.1 +- 1.5  | 0.47 +- 0.4  |
| <b>AbNmat</b>   | X10e9/l               | 1.1 +- 1.3  | 0.46 +- 0.4  |
| <b>AbNimm</b>   | X10e9/l               | 0.06 +- 0.18  | 0.2 +- 0.08  |
| <b>Lymphocytes</b>                                      | X10e9/l               | 2.8 +- 0.6  | 3.92 +- 0.9  |
| <b>Monocytes</b>  | X10e9/l               | 0.2 +- 0.2  | 0.19 +-0.07  |
| <b>Eosinophils</b>                                      | X10e9/l               | 0.15 +- 0.12  | 0.19 +- 0.12   |
| <b>Basophils</b>  | X10e9/l               | 0   | 0.09 +- 0.01   |
| <b>Platelet count<sup>o</sup></b>                       | <b>Not quantified</b> | <b>Not quantified</b>   | <b>Not quantified</b>  |

**NOTE:** <sup>A</sup> Values are mean +- SD for groups of 6 mice.

<sup>B</sup> Values are mean +- SD for groups of 10 mice

<sup>o</sup> Could not be quantified due to low blood volume

<sup>oo</sup> Statistically significant correlation between groups ( $p < 0.05$ )(Student T test)

**Table 5.2- Absolute organ weights of the control and experimental animals seven days after the intravenous administration of control vehicle or active Fluorodopa drug:**

| Absolute Organ Weights of Test Animals |  |                                       |
|--|--|---------------------------------------|
| Organ                                  | 0μMole/Kg (Control Group) <sup>A</sup> | 1μMole/Kg (Active Group) <sup>B</sup> |
|  | Absolute Weight (g)                    | Absolute Weight (g)                   |
| <b>Brain</b>                           | 0.39 +- 0.09                           | 0.41 +-0.08                           |
| <b>Liver</b>                           | 1.13 +- 0.38                           | 1.05 +- 0.41                          |
| <b>Heart</b>                           | 0.096 +- 0.0028                        | 0.102 +- 0.031                        |
| <b>Spleen</b>                          | 0.08 +- 0.01                           | 0.09 +- 0.02                          |
| <b>Kidney</b>                          | 0.24 +-0.06                            | 0.21 +-0.11                           |

**NOTE:** <sup>A</sup> Values are mean +- SD for groups of 9 mice.

<sup>B</sup> Values are mean +- SD for groups of 10 mice

### Discussion

The predominant effects of Fluorodopa are dose-related, with the actual response of an individual patient largely dependent on the clinical status of the patient at the time of drug administration (Perry et al, 1984; Melemed et al, 1998). The toxicity concerns of Fluorodopa are twofold; firstly the radiation dose from the emitting positron and secondly the pharmaceutical toxicity from the drug itself. A single intravenous dose of Fluorodopa at a concentration 1μmole/kg(body mass) (the maximum recommended dose for Fluorodopa PET brain imaging) should be accompanied by a radiation dose of 7 mSv or 2mCi (Int Rad, 1977; Jones S, 1982; Harvey et al, 1985). Considering the molar quantities of Fluorodopa that are associated with 2mCi of <sup>18</sup>Fluorine are made as small as possible to ensure that Fluorodopa is the true tracer in the dopaminergic system, the dose of Fluorodopa is estimated at a mere 100<sup>th</sup> of the therapeutic dose of L-Dopa used to treat Parkinson's patients. Bearing in mind that Parkinson's patients take L-Dopa as a chronic medication without concerns of toxicity, it was not expected that a single administered dose of

Fluorodopa would result in any measurable adverse effects. The macroscopic pathology and histopathology results did not reveal any anatomical or pathological changes that would suggest any form of toxicity and evaluation of behavioural as well as clinical changes between the vehicle controlled and active Fluorodopa groups showed no indication of toxicity. The irregularities in clinical biochemistry parameters in the vehicle controlled group however required further investigation. Four of the ten control mice presented with extremely high LDH and AST levels, 400% higher than comparable mice in either the active or vehicle control groups, however all other enzyme levels remained within normal range. A rise of ALP, GGT and ALT in conjunction with LDH and AST would be expected, however this was not so. These results could suggest that blood haemolysis had taken place because an increase of LDH and ALT has been observed in blood samples that had been recorded as haemolysed in other acute and chronic toxicology studies. The clinical report confirmed that procedural blood drawing complications had taken place in these four mice and that only small volumes of blood had been recovered from these four animals but there was no record of whether haemolysis had taken place or not. Furthermore there were no changes in absolute organ weights or irregularities in histopathological examination that could be attributed in any way to the administration of the drug controlled vehicle. When excluding these four mice from the sample population, the data for the vehicle control in addition to the active group demonstrated comparable values.

## 5.5- CONCLUSION

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Under the conditions of its intended use, a single intravenous dose of administered Fluorodopa at concentrations up to  $1\mu\text{mole/kg}$  did not result in any measurable form of systemic toxicity. No significant treatment-related effects on survival, body-weight gain, haematological and clinical chemistries, absolute organ weights, or histopathology were noted. The single administered dose of  $1\mu\text{mole/kg}$ (Body Mass) Fluorodopa caused no noticeable change in the general behaviour of the mice and, compared to the control group, no significant changes in body weight, food intake or utilization of food in the treated mice. Both the control and treated mice appeared uniformly healthy at the end of the seven day period study. It can therefore be concluded that Fluorodopa administered intravenously at  $1\mu\text{mole/kg}$  body weight should not result in any toxic or adverse neurological effects.



## **CHAPTER SIX – CONCLUSION**

### **RESEARCH SUMMARY, CONCLUSIVE REMARKS, FUTURE PROSPECTS**

## 6.1- CONCLUSION

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The use of PET imaging has increased rapidly in recent years, as have special requirements in the fields of neurology and oncology for the development of syntheses for new, more specific and selective radiotracers. Fluorine-18 has proved to be an ideal tracer for PET because of its convenient half-life of 109.8 min and ideal emission-energy (max. 0.635 MeV) which allows high resolution and minor radiation effects to the patients. The fluorine and radioactive fluorine can be relatively easily substituted into the chemical structure of target specific molecules by replacement of a reactive hydrogen atom within the molecule. With reference to <sup>18</sup>Fluorodopa several electrophilic and nucleophilic methods for the rapid stereospecific chemical synthesis have been described and reviewed. The increasing demand for <sup>18</sup>Fluorodopa in the research and diagnostic fields of central nervous system conditions has placed a need for pharmaceutical grade <sup>18</sup>Fluorodopa to be produced locally due to the time and distance from production sites in the northern hemisphere. A simple, reliable procedure for routine production of <sup>18</sup>Fluorodopa in South Africa is required to address this problem. Although this study was performed on non radioactive fluorine substitution reactions to obtain Fluorodopa, the main aim was to assess the chemical synthetic routes that could provide the most economically viable route to <sup>18</sup>Fluorodopa using the reported synthetic methods that appeared to best address the potential problems of providing a useful high specific activity radiopharmaceutical.

The electrophilic synthesis provided a reliable and high yielding procedure for preparing a shelf-stable aryltin compound suitable for reacting with radioactive fluorine or fluorine derivatising agents to produce moderate yields of very pure radiolabelled Fluorodopa in an isomerically pure form. The reported electrophilic synthesis should produce <sup>18</sup>Fluorodopa with a greater radiochemical yield than those electrophilic synthesis methods previously described, should take less time to complete, give high regiospecificity and provide modest radiochemical yield. However, electrophilic fluorine is extremely reactive and radiochemical yields can diminish due to the chemical reactivity of fluorine derivatisation compound decomposing or side reactions with the surface of the reaction apparatus, transfer tubing, and components other than the chemical precursor. Another disadvantage is that

electrophilic methods often require the addition of non-radioisotopic carrier fluorine in the labelling process to reduce the specific activity of the final radiotracer. The dilution of the desired radiotracer with the non-radioisotopic form effectively increases the quantity drug in the tracer dose but reduces the radiolabelling efficiency by up to 50%.

The limitations of a newer and less expensive "proton only" medical cyclotron means that many facilities cannot carry out the nuclear reaction most widely used for making electrophilic  $^{18}\text{F}$ . The nucleophilic synthetic approach therefore offers the advantage of accessibility to chemically active fluorine by means of  $^{18}\text{F}^-$  produced from protons from a single particle cyclotron. In addition the nucleophilic synthesis offers an easy availability of low cost starting materials and claims high absolute yields and high specific activity. However the synthetic pathway introduces the radiolabel at a very early stage in the chemical synthesis and then manipulates the radiolabelled precursor through several steps before isolating the final product approximately two hours later. Furthermore, the multistep chemical manipulation results in a loss (over 50%) of radioactivity due to isotope decay, which would contribute to decreased specific activity compared to the quantity of radioactive fluorine produced in the cyclotron in a commercial radiopharmaceutical production setting.

The electrophilic synthesis produces only one structural and enantiomeric isomer which avoids any elaborate purification steps and the mixture can be passed through a semi preparative HPLC column to ensure a high level of both chemical and radio chemical purity. The stable but reactive aryltin intermediate can be chemically purified prior to being introduced into the radioactive facility. The relative simplicity of the reactions after introducing the radioactive fluorine facilitates a remotely operated synthesis requiring minimum handling of radioactivity. There is no requirement for determining contaminating mercury in the final product in contrast to previous methods using regioselective electrophilic fluorination of mercury containing intermediates and the product reacts with equal ease with traditional electrophilic fluorinating agents such as acetyl hypofluorite or OF to give reported isolated yields > 65% Fluorodopa with excellent chemical, radiochemical and enantiomeric purities useful for multiple PET studies.

Direct comparative evaluation of the electrophilic and nucleophilic synthesis methods of Fluorodopa suggest that the electrophilic synthesis method would be preferential for large scale production of  $^{18}\text{F}$ Fluorodopa yielding good chemical, radiochemical, isomeric and enantiomeric purities useful for application as a radiotracer in PET studies.

#### *Future considerations*

Currently there are more than 100 radiopharmaceuticals developed using either reactor or cyclotron produced radioisotopes and which are used for the diagnosis of several common diseases or conditions. In the development of  $^{18}\text{F}$ Fluorodopa, chemical, radiochemical and radiopharmaceutical aspects will need to be taken into consideration. The design and development of automated radiotracer synthesis system will be an important focus in synthesis development especially when high radioactivity is required. The radiotracer will be required not only to be chemically and radiochemically pure but sterile and pyrogen free if it is to be used in PET studies. For radiopharmaceutical production, the labelled end-product will require an effective radioanalytical method to confirm the purity of the radioisotope combined with a radioactivity detection technique to assay the product for specific activity. In addition, high initial specific activity may be required to compensate for radioactive decay and for the sometimes low synthetic yields. High radioactivity would require that the synthesis be carried out in a facility where adequate shielding, remote or robotic operations, and synthesis automation will be of great importance and contribute to the safety of the operators involved in the complete production of the  $^{18}\text{F}$ Fluorodopa. When integrating the experimental design and planning the labelling syntheses with the radioactive isotopes all these requirements which affect the safety would need to be considered. In order to handle radioactivity, special facilities are required to shield the radiation emitted and to prevent contamination of the environment by the radioactive materials released during handling and processing.  $^{18}\text{F}$ Fluorodopa will need to be made under the production conditions of radioisotopes intended for medical use, which requires the establishment of aseptic conditions with clean areas and isolators, as well as by introducing quality assurance as a governing principle in the production of pharmaceutical grade radioactive products.

Automation will need to be put in place to decrease the radiation dose to the operator and environment as well as to ensure more reliable and precise radiochemical processing.

<sup>18</sup>F Fluorodopa products must also be protected from microbiological contamination by their environment and adhere to pharmaceutical good manufacturing practice (GMP). For this, the handling facilities will need to include isolator-like hot cells and clean rooms with HEPA filtered ventilation and air conditioning (HVAC).

With all the above requirements being taken into account it would be the obvious choice to make use of the electrophilic synthetic route for the commercial production of <sup>18</sup>F Fluorodopa as it offers the most advantages. This synthetic route however does not address all the possible problems of synthesising <sup>18</sup>F Fluorodopa but would result in a higher yield relative to the initial activity of the <sup>18</sup>F Fluorine produced and provide a product that would require minimal clean up steps after synthesis.

The next step would be the actual synthesis of the radioactive product to assess the feasibility of carrying out the reaction under the controlled and shielded conditions of an isotope production facility. This would give the production chemist both experience and identify potential problem areas and ways to improve the yields in terms of chemical yield and radiolabelling efficiency. The product would need to be tested to confirm that the same degree of chemical purity, overall yield, isomeric and enantiomeric purity as under the conditions of cold synthesis can be achieved. In addition the specific activities of the <sup>18</sup>F Fluorodopa attainable would need to be confirmed to be confident that the final radioactive product would be usable in both research and diagnostic settings and comply to all the quality specifications of a radiopharmaceutical for use in humans.

# CHAPTER SEVEN- SUPPORTING DOCUMENTS

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## 7.2- SUPPORTING DOCUMENTS

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Animal Welfare Monitoring Sheet

| ANIMAL IDENTIFICATION:.....  | Score  | Date |
|--|--------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|  |        | Time |
| <b>APPEARANCE</b>  |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Normal   | 0      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| General lack of grooming   | 1      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Coat staining, ocular and nasal discharges                           | 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Piloerection, hunched up   | 3      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>FOOD AND WATER INTAKE</b>   |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Normal   | 0      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Uncertain: body weight ↓ <5%   | 1      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| ↓ intake: body weight ↓ 10-15%                                       | 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| No food or water intake  | 3      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>NATURAL BEHAVIOUR</b>   |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Normal   | 0      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Minor changes  | 1      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Less mobile and alert, isolated                                      | 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Vocalization, self-mutilation, restless or very still                | 3      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>PROVOKED BEHAVIOUR</b>  |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Normal   | 0      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Minor depression or exaggerated response                             | 1      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Moderate change in expected behaviour                                | 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Reacts violently, or very weak and prone to stress                   | 3      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>CLINICAL SIGNS</b>  |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Normal cardiac and respiratory rates, hydration                      | 0      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Slight changes, slight dehydration                                   | 1      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| C/R rates ↑ 30%, 10-20% dehydrated                                   | 2      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| C/R rates ↑ 50% or very ↓, >20% dehydrated                           | 3      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>SCORE ADJUSTMENT</b>  |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| If you have scored a 3 more than once, add an extra point for each 3 | 2 - 5  |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| <b>TOTAL</b>   | 0 - 20 |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| Signature (initials)   |        |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

**JUDGEMENT**

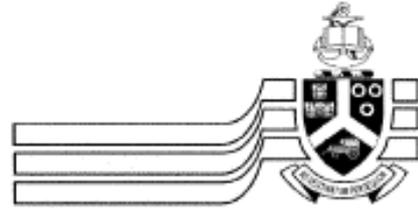
0 - 4 Normal

5 - 9 Monitor carefully, consider analgesics

10 - 14 Suffering; provide relief, observe regularly. Seek second opinion from day-to-day care person and/or veterinary surgeon. Consider termination

15 - 20 Severe pain. Does your experimental protocol need rethinking?

H025-08



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1 July 2008

Prof CE Medlen  
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Faculty of Health Sciences  
University of Pretoria  
([connie.medlen@up.ac.za](mailto:connie.medlen@up.ac.za))

Dear Prof Medlen

**H025-08 - Fluorodopa for Positron Emission Tomography (PET) brain imaging, an acute toxicity study (*W Hochfeld*)**

The above protocol was approved by the Animal Use and Care Committee at its meeting held on 30 June 2008

Please contact this office should you have any questions.

Best regards

Elmarie Mostert  
AUCC Contact Person

Copy: Dr R Auer ([Roland.auer@up.ac.za](mailto:Roland.auer@up.ac.za))

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