# Novel polycyclic analogues of non-steroidal anti-inflammatory drugs for increased blood-brain barrier permeability

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*Dedicated to my parents, Ben and Marie van den Berg.*

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# ABSTRACT

Various recent studies have confirmed that inflammation plays a key role in the pathogenesis of neurodegenerative disorders like Alzheimer's disease and Parkinson's disease. In this study the focus was on delivery to the brain of certain non-steroidal anti-inflammatory drugs (NSAIDs), as a possible treatment for neurodegeneration, due to their anti-inflammatory and antioxidant activity.

The blood-brain barrier (BBB) plays the predominant role in controlling the passage of substances between the blood and the brain. There are certain physicochemical characteristics necessary for a compound to cross the BBB. NSAIDs, for example, ibuprofen and acetylsalicylic acid are relatively hydrophilic and does not cross the blood-brain barrier in sufficient amounts to reach therapeutic concentrations in the central nervous system (CNS). Studies done on polycyclic cage structures, for example pentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$0.0$^{5,9}$]undecane, indicated favourable distribution to the brain and it was concluded that these polycyclic structures penetrate the BBB readily.

It was therefore hypothesised that the polycyclic cage compounds could be used as carrier molecules to enhance the delivery of neuroprotective compounds into the CNS and the aim of this study was to design novel polycyclic structures incorporating selected NSAIDs in order to improve their blood-brain barrier permeability.

The well-described Cooksen's diketone, pentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$0.0$^{5,9}$]undecane-8,11-dione, was conjugated to 2-aminoethanol by means of reductive amination. This gave a pentacycloundecylamine with a sterically free linker section. In the relevant compounds, the hydroxyl group on the linker was then conjugated to the benzoic acid moiety to yield the respective ester prodrugs. This esterfication was done using the activation agent, 1-ethyl-3-(3'-dimethylamino) carbodiimide (EDC) to activate the benzoic acid group on ibuprofen and by using the commercially available acid chloride derivative of acetylsalicylic acid. Amide prodrug syntheses were done by conjugating the NSAIDs to 1, 2-diaminoethane by means of Fischer-esterfication and aminolysis reactions. Structure elucidation was done using one dimensional nuclear magnetic resonance (NMR), infrared (IR) absorption spectroscopy and mass spectrometry (MS).

An *in vivo* BBB permeability assay employing HPLC analytical procedures was used to compare blood and brain concentrations of the relevant drugs 15 min, 30 min and 60 min after administration to the male C57BL/6 mice. The antioxidant activities of the compounds were assessed with the *in vitro* Thiobarbituric Acid (TBA) assay.

Both NSAIDs were detected in the brain tissue of test mice, indicating blood-brain barrier permeation. The ester prodrugs were found to be very labile and significant amounts of it were hydrolysed. When administering these ester prodrugs (compound **11** and **12**), the free NSAIDs were detected at higher concentrations compared to when the free drugs were administered. The amide compounds (compounds **15** and **17**) were found to be toxic during administering of the prodrugs to the mice and were not further investigated.

Lipid peroxidation results indicated that the ester compounds marginally increased the ability of the free drugs to attenuate lipid peroxidation, but not to the level of the model antioxidant Trolox and is therefore not significant.

The novel synthesised prodrugs therefore present with a possible multiple drug targeting action as the blood-brain barrier permeability and the antioxidant activity of the free NSAIDs were increased.

# UITTREKSEL

Verskeie onlangse studies het bewys dat inflammasie 'n baie belangrike rol in die patogenese van neurodegeneratiewe siektes speel. In hierdie studie is daar op sekere nie-steroidale anti-inflammatoriese middels (NSAIMs) gefokus vir die behandeling van neurodegenerasie na aanleiding van hul anti-inflammatoriese en anti-oksidatiewe eienskappe.

Die bloedbreinskans is verantwoordelik vir die beheer van die beweging van verbindings tussen die bloed en die brein. Om die bloedbreinskans binne te dring, moet 'n geneesmiddel aan sekere fisies-chemiese eienskappe voldoen. Sekere nie-steroidale anti-inflammatoriese middels soos byvoorbeeld ibuprofeen en asetielsalisielsuur is relatief hidrofilies en sal nie die bloedbreinskans in genoegsame hoeveelhede binnedring, om sodoende terapeutiese konsentrasies in die sentrale senuweestelsel te bereik nie. Onlangse studies het aangedui dat die bloedbreinskans geredelik deurlaatbaar is vir die polisikliese struktuur, pentasiklo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undekaan.

Die hipotese vir hierdie studie was dat die polisikliese hokstrukture as draermolekules gebruik sou kon word om neurobeskermde geneesmiddels in die sentrale senuweestelsel af te lewer. Die doel van die studie was dus om nuwe polisikliese verbindings te ontwerp wat die geselekteerde NSAIMs inkorporeer ten einde die bloedbreinskansdeurlaatbaarheid daarvan te verbeter.

Die welbekende Cooksondiketoon, pentasiklo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undekaan-8,11-dioon, is met 2-aminoetanol deur reduktiewe aminering gereageer om die pentasikloundekaan-amien te verkry. Esterifikasie is bewerkstellig deur gebruik te maak van 1-etiel-3-(3'-dimetielamino)karbodiimied (EDC), om die groep bensoësuur op ibuprofeen te aktiveer en deur die kommersieel beskikbare derivaat suurchloried van asetielsalisielsuur te gebruik. Die amiede is verkry deur die NSAIMs met 1,2-diaminoetaan te reageer deur van die Fischer-esterfikasie en aminolise reaksies gebruik te maak. Die strukture van al die verbindings is bevestig met behulp van $^{1}$H en $^{13}$C KMR, MS en IR.

'n *In vivo* evalueringsmetode, wat gebruik maak van HPLC tegnieke, is aangewend om die brein- en bloedkonsentrasies van die spesifieke geneesmiddels te vergelyk 15 minute, 30 minute en 60 minute na toediening aan manlike C57BL/6-muise. Die progeneesmiddels is ook geanaliseer vir anti-oksidatiewe werking deur middel van 'n *in vitro* lipiedperoksidasie-studie.

Beide NSAIMs is waargeneem in breinweefsel nadat die gesintetiseerde progeneesmiddels aan die muise toegedien is, wat aanduidend is van bloedbreinskansdeurdringing. Die esterprogeneesmiddels is baie labiel en substansiële hoeveelhede daarvan hidroliseer. Die vry NSAIMs is in hoër konsentrasies waargeneem na toediening van esterprogeneesmiddels (verbindings **11** en **12**). Daar is tydens toediening gevind, dat die amiedprogeneesmiddels (verbindings **15** en **17**) hoë toksisiteit toon en hulle is nie verder geëvalueer nie.

Uit die lipiedperoksidasiestudie is dit duidelik dat die esterprogeneesmiddels die vermoë van die vry geneesmiddels om lipiedperoksidasie te verminder, matig verhoog maar dit is nie vergelykbaar met die waargenome vermidering van die model antioksidant, trolox nie.

Die nuutgesintetiseerde progeneesmiddels blyk dus moontlikhede te bied vir geteikende aflewering van die neurobeskermende geneesmiddels, eerstens op grond van verhoogde bloedbreinskansdeurlaatbaarheid en tweedens as gevolg van die verhoging in die anti-oksidatiewe aktiwiteit van die ongekonjugeerde NSAIMs.

# CHAPTER 1

# INTRODUCTION

## 1.1 BACKGROUND

Alzheimer's and Parkinson's diseases are affecting the lives of millions of people around the world. Various drug candidates to treat these diseases are under investigation and studies are being done to increase drug permeability across the blood-brain barrier for drug delivery into the central nervous system (CNS). Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the groups of drugs receiving much attention in the search for neuroprotective compounds. Most of these compounds however, show unfavourable blood-brain barrier (BBB) permeation and various strategies to overcome this shortcoming are being investigated.

### 1.1.1 NEURODEGENERATIVE DISEASES AND NEURODEGENERATION

Alzheimer's disease stands out among the neurodegenerative diseases as the fourth leading cause of death in Western countries and the most common cause of dementia in the elderly population (Mayeux, 2003). It is characterised by widespread cognitive impairments that begin with episodic memory declines. As the neurodegeneration progresses, Alzheimer's disease is characterised by a progressive cognitive impairment that extends to the domains of language (aphasia), skilled movements (apraxia), recognition (agnosia) and executive functions such as decision making and planning (Conejero-Goldberg *et al.*, 2008).

The pathologic features of Alzheimer's disease are the presence of senile plaques, which are aggregations of amyloid beta protein and neurofibrillary tangles, which are aggregations of tau (Akiyama et *al.*, 2000). Roney *et al.* (2005) demonstrated that a pathologically, ventricular enlargement and atrophy of the hippocampus and the cerebral cortex is characteristic. At present, the definitive diagnosis of Alzheimer's disease is made upon histological verification of the amyloid beta plaques during autopsy.

In Western countries, Parkinson's disease is the second most common neurodegenerative disorder. Like Alzheimer's disease, it is currently an incurable disease. It is characterised by the progressive loss of dopaminergic neurons in the substantia nigra and by the presence of intraneuronal aggregates known as lewy bodies. Depletion of dopamine causes dysregulation of the motor circuits that project through the basal ganglia, resulting in the

clinical manifestations of Parkinson's disease, which include tremor, bradykinesia, rigidity and postural instability (Cavalli *et al.*, 2009).

Neurodegeneration is the progressive loss of neuronal function and structure by a variety of mechanisms that lead to cell death in the form of apoptosis and/or necrosis. In general necrosis is a form of cell death that involves an injury from which the cell cannot recover, while apoptosis is an active program of cell suicide, whereby the cell activates a self-destruct mechanism which causes it to shrink and ultimately be phagocytosed by microglia (Dragunow *et al.*, 1998; Pettmann & Henderson, 1998; Huges *et al.*, 1999).

There are three main mechanisms of neuronal cell death which may act separately or cooperatively to cause neurodegeneration. For example, metabolic impairment may elicit secondary excitotoxicity. This lethal triplet of metabolic compromise, excitotoxicity and oxidative stress causes neuronal cell death that is both necrotic and apoptotic in nature (Greene & Greenamyre, 1999). The depolarization of neurons and loss of ionic integrity caused by bioenergetic impairment releases the voltage block on the N-methyl-D-asparte (NMDA) receptor thus activating it and causing secondary excitotoxity in neurons that possess these receptors (Zeevalk & Nicklas, 1990). Biological insults that cause neuronal cell death, thus generally do so *via* one or more mechanism of the lethal triplet.

Metabolic compromise may also cause oxidative stress by inducing the production of free radicals, both from the electron transport chain and due to the burden of increased intracellular $Ca^{2+}$ on the mitochondrial function. The reverse interaction may also occur, as oxidative stress may cause metabolic impairment and initiate excitotoxic pathways. For example, oxidative stress can cause lipid peroxidation which yields the by product 4-hydroxynonenal. 4-Hydroxynonenal impairs glucose transport which can lead to energetic failure (Morel et *al.*, 1999).

Excitotoxicity can also lead to oxidative stress. Intrastriatal injection of N-methyl-D-aspartate ligands results in increases in markers of reactive oxygen species while oxidative stress attenuate the neuropathology (Morel *et al.*, 1999; Pederson *et al.*, 1999). Glutamate and related excitatory amino acids account for most of the excitatory synaptic activity in the mammalian CNS and are released by an estimated 40% of all synapses, by activating ionotropic receptors, including N-methyl-D-aspartate and kainic acid (KA) receptors. The calcuim-mediated effect of glutamate receptor over-activation leads to excitotoxicity (Gilgun-Sherki *et al.,* 2002).

Inflammation plays a key role in the pathogenesis of age-related neurological disorders such as Parkinson's disease and Alzheimer's disease. The accumulation of amyloid and extra

cellular tangles in Alzheimer's disease, or Lewy bodies in Parkinson's disease, apparently act as irritants, causing activation of the complement system (Mcreer *et al.*, 1993).

The majority of factors produced by the activated microglia are pro-inflammatory and neurotoxic. These include the cytokines, tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1 (IL-1), which has been shown to cause degeneration of human fetal brain cells (Chao *et al.,* 1995). Recent studies confirmed inflammatory changes in the Alzheimer's disease brain, including increased concentrations of pro-inflammatory cytokines and complement proteins, together with the presence of large numbers of activated microglia (Akiyama *et al., 2000).*

It is well known that inflammation might raise Reactive Oxygen Species (ROS) levels leading to oxidative stress, which is one of the major factors contributing to the pathology of neurodegenerative diseases (Gilgun-Sherki *et al.,* 2001).

A free radical is any chemical species that contains one or more unpaired electrons. Unpaired electrons act as electron acceptors from other molecules, leading to their oxidation. The most common cellular free radicals are hydroxyl, superoxide and nitric oxide (NO) radicals. Free radicals and related molecules are classified as reactive oxygen species (ROS) for their ability to lead to oxidative changes within the cell. The cells possess an intricate network of defence mechanisms to neutralise excessive ROS accumulation, including antioxidant compounds for example glutathione (GSH), arginine, citrulline, taurine, zinc, vitamin E, vitamin C and vitamin A. Therefore, under physiological conditions, cells are able to cope with the flux of ROS. Oxidative stress describes a condition in which cellular antioxidant defences are insufficient to keep the levels of ROS below a toxic threshold (Schulz *et al.*, 2000).

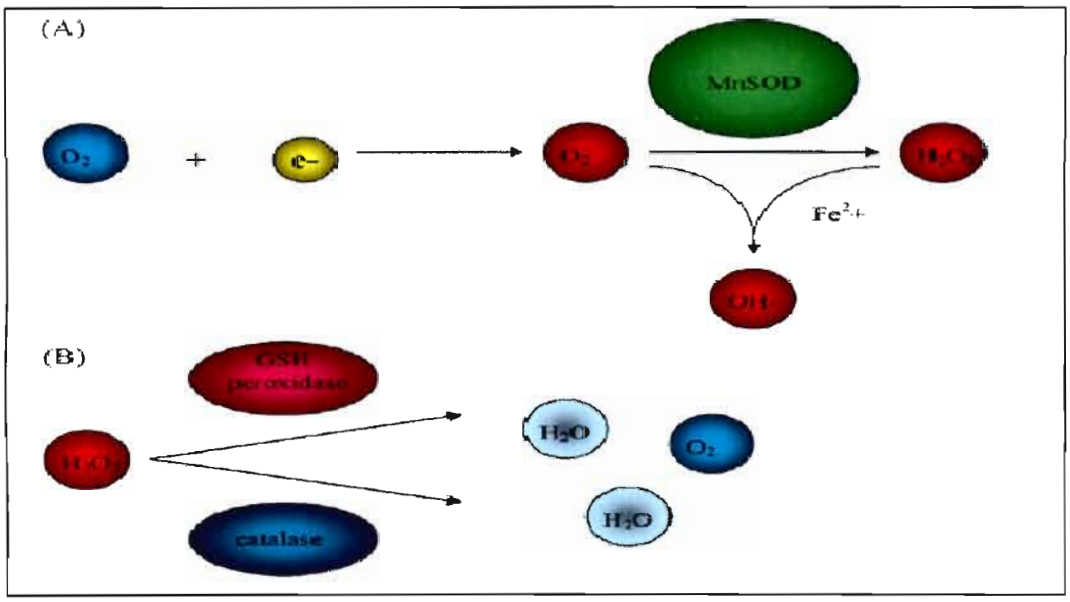



**Figure 1.1:** Schematic representation of the formation of ROS in the cell (Migliore *et al.*, 2009).

A growing body of evidence indicates the crucial involvement of oxidative stress in several steps of the pathogenesis of many neurodegenerative diseases (Migliore *et al.*, 2005; Keller *et al.*, 2005; Moreira *et al.*, 2007; Mancuso *et al.*, 2006). Significant biological changes related to a condition of oxidative stress have been found not only in brain tissues but also in peripheral tissues of individuals affected by AD and PD (Mancuso *et al.*, 2006).

NSAIDs possess, amongst others, neuroprotective properties due to their antioxidant activity. Maharaj (2004) indicated that both acetylsalicylic acid and acetaminophen exhibit neuroprotective activity because of their ability to inhibit oxidative stress. This was deduced using *in vitro* as well as *in vivo* biological assays and the antioxidant activity of these drugs were at its highest when used in combination with each other. Ibuprofen also has protective properties as a result of its direct nitric oxide radical scavenging activities in neuronal cells (Asanuma *et al.*, 2001). The potential use of certain NSAIDs as neuroprotectants is therefore apparent.

Several recent studies using lipopolysaccharide provide evidence that inflammation and excitotoxicity are interrelated either directly or by an indirect mechanism involving oxidative stress (Bal-Price *et al.*, 2001; Morimoto *et al.*, 2002).

The role of inflammation in neurologial and neurodegenerative conditions such as Parkinson's disease and Alzheimer's disease is less well understood, primarily because the events triggering the inflammatory response are still obscure. Nevertheless, increasing evidence suggests that inflammation contributes to the pathogenesis of these disorders,

which was previously thought to be purely neuronal (Allan & Rothwell, 2001; Hartmann *et al.*, 2003; Hirsch *et al.*, 2003; Hunnot & Hirsch, 2003; Matyszak,1998; Van Gool *et al.*, 2003).

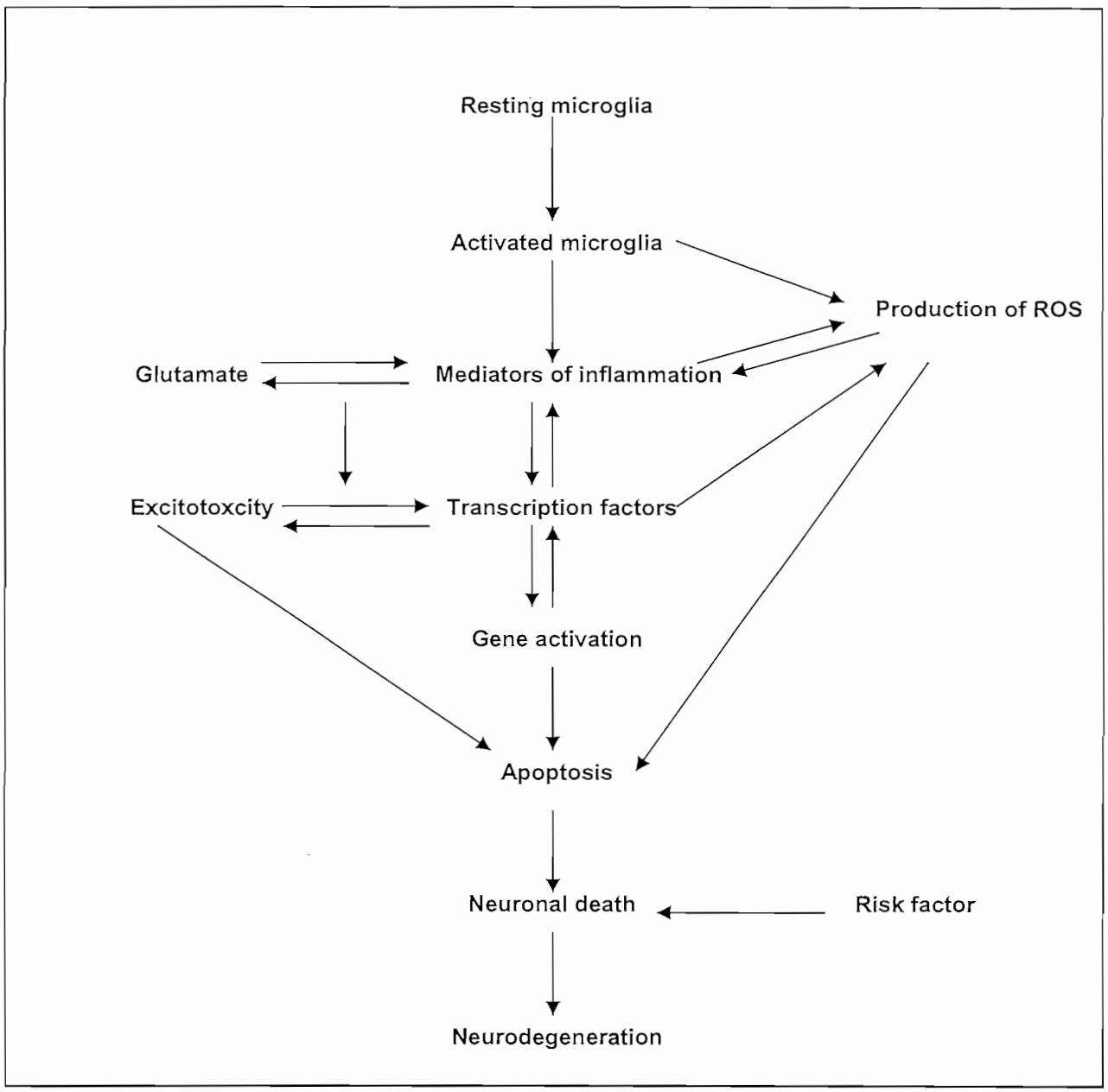



**Figure 1.2:** Inflammation-induced neurodegenerative diseases (Gilgun-Sherki *et al.*, 2002).

### 1.1.2 NEUROPROTECTION

Neuroprotective strategies aim to protect vulnerable, surviving neurons from whatever neurotoxic processes are responsible for the observed neuropathological changes, thus preserving their function (Foley *et al.*, 2000).

Mandel *et al.* (2003) demonstrated that neroprotection can be afforded by a variety of mechanisms including $Ca^{2+}$ channel antagonists, glutamate antagonist, nitric oxide synthase inhibitors and antioxidants.

## 1.2 AIM OF THE STUDY

As mentioned earlier, the blood-brain barrier is the primary barrier that has to be crossed in order for a specific drug to be delivered into the CNS. Neurodegenerative mechanisms as observed in Alzheimer's and Parkinson's disease are focused in the brain and the CNS. These mechanisms include oxidative stress as discussed above and in order for a drug to elicit its antioxidant activity it has to be able to cross the blood-brain barrier.

Based on the above mentioned evidence that inflammation plays a role in the pathogenenisis of Parkinson's disease as well as Alzheimer's disease, scientists have investigated the use of anti-inflammatory drugs as neuroprotective agents. Most of these compounds however, show unfavourable blood-brain barrier permeability. Therefore the aim of this study was to enhance CNS delivery of the NSAIDs, ibuprofen (**2**) and acetylsalicylic acid (**3**) by conjugating the drugs by means of a chemical linker to the polycyclic moiety, pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**1**), by amination, amidation and esterification.

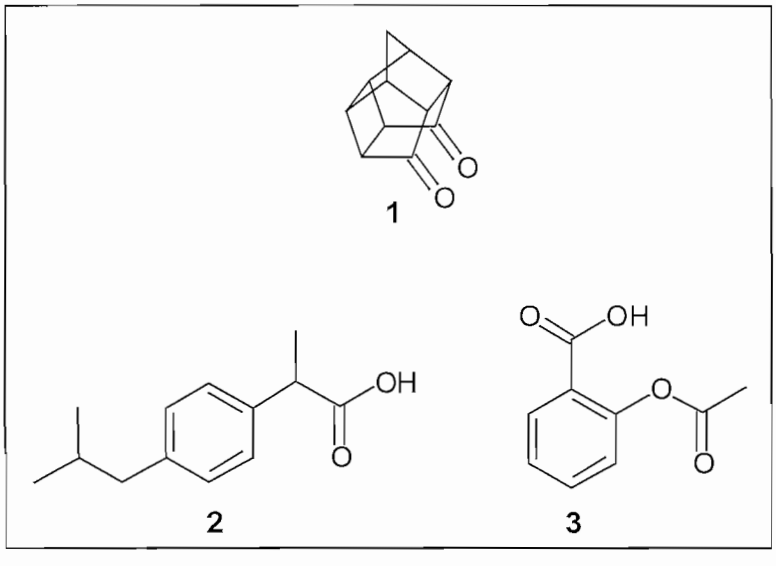
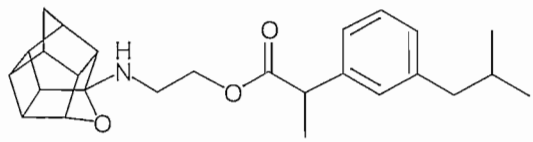
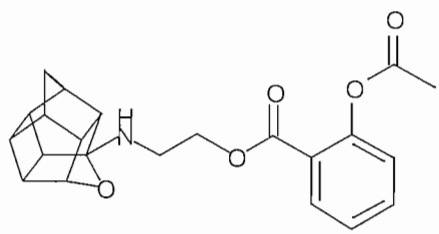
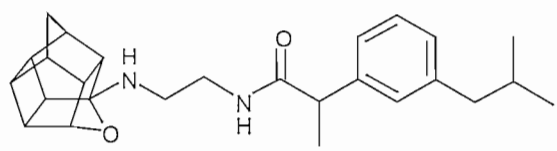
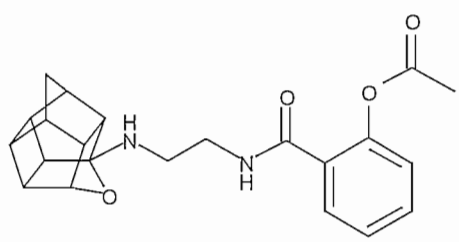



**Figure 1.3:** Structures relevant to the study.

**Tabel 1.1**: Compounds evaluated and synthesised in this study.

| Compound | Structure |
|---|---|
| 8,11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8-(2-aminoethyl)-(4-isobutylphenyl)-propanoate (**11**) |  |
| 8,11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8-(2-aminoethyl)-2-acetyloxybenzoate (**12**) |  |
| 8,11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8-(2-aminoethyl)-(4-isobutylphenyl)-propanamide (**15**) |  |
| 8,11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8-(2-aminoethyl)-2-acetyloxybenzamide (**17**) |  |

The hypothesis is that these novel synthesised compounds have the potential to be used in the prevention and treatment of neuronal inflammation and oxidative stress as observed in Alzheimer's disease and Parkinson's disease. The hypothesis was tested by subjecting the synthesised compounds to *in vivo* blood-brain barrier permeability and *in vitro* lipid peroxidation assays.

To reach the aim of the study the following must be conducted:

- Synthesis of the selected compounds through the conjugation of the NSAIDs to the polycyclic cage moiety.
- Structure elucidation using one dimensional nuclear magnetic resonance (NMR), infrared (IR) absorption spectroscopy and mass spectrometry (MS).
- Blood-brain barrier (*in vivo*) assay to determine the blood-brain barrier permeability of the prodrugs.
- Lipid peroxidation (in vitro) assay to determine the anti-oxidant activity of the prodrugs.
- Theoretically determination of the physicochemical characteristics needed for good blood-brain barrier permeability.

# CHAPTER 2

# THE BLOOD-BRAIN BARRIER

## 2.1 INTRODUCTION

The existence of the blood-brain barrier was described by the studies of Ehrlich (1885) in the late 19th century, showing that brain tissue remained unstained after injection of a vital dye into the systemic blood circulation of rats. In contrast, the brain tissue was stained after direct injection of trypan blue into the brain ventricular system, indicating the existence of some kind of barrier at the site of brain microvessels (Goldmann, 1909).

The most important factor limiting the development of new drugs for the central nervous system is the blood-brain barrier as it limits the brain penetration of most CNS drug candidates (Pardridge, 2007). The global market for drugs for the central nervous system is greatly underpenetrated and would have to grow by over 500% just to be comparable to the global market for cardiovascular drugs (Pardridge, 2002). The principle reason for this under-development of the global brain drug market is that the great majority of drugs do not cross the brain capillary wall, which forms the blood brain barrier *in vivo*. Only a relatively small quantity of drug molecules with high lipid solubility (log P and a low molecular mass of <400-500 Daltons (Da), cross the blood brain barrier passively (Pardridge, 2001). The recommendation by Pardridge (2007) is that brain drug development programs need to be adjusted so that compounds are designed for active brain uptake. Thus for the development of new central nervous system (CNS) drugs it is important to have an understanding of the morphology and function of the blood-brain barrier for effective drug delivery in the CNS.

## 2.2 MORPHOLOGY AND FUNCTION

Neural signalling requires precise homeostatic regulation of the interstitial fluid of the brain. Three barrier sites contribute to this regulation: The blood-brain barrier (BBB) formed by the brain endothelial cells lining cerebral capillaries, the arachniod membrane of the meninges, and the choroid plexus epithelium that secretes cerebrospinal fluid (Abbott, 2004). Of these, the BBB is the most important site for regulating drug access to the brain, given its large surface area and the short diffusion distance from capillaries to neurons (Schlageter *et al.*, 1999). The BBB also supplies key nutrients and protects the brain from neuroactive and

potentially toxic compounds circulating in the plasma. The barrier function of the BBB is a combination of physical restriction (tight junctions reduce paracellular permeability of hydrophilic molecules), transport regulation (uptake and efflux carriers and selective transcytosis regulate transcellular molecular flux) and metabolic activity (enzymes metabolise many potentially harmful agents) (Abbott, 1996; Begley & Brightman, 2003). This multi-tasking BBB poses problems for therapeutic approaches that require delivery of drugs and other molecules to the brain for the treatment of CNS disorders (Abbott, 2005; Begley, 2004).

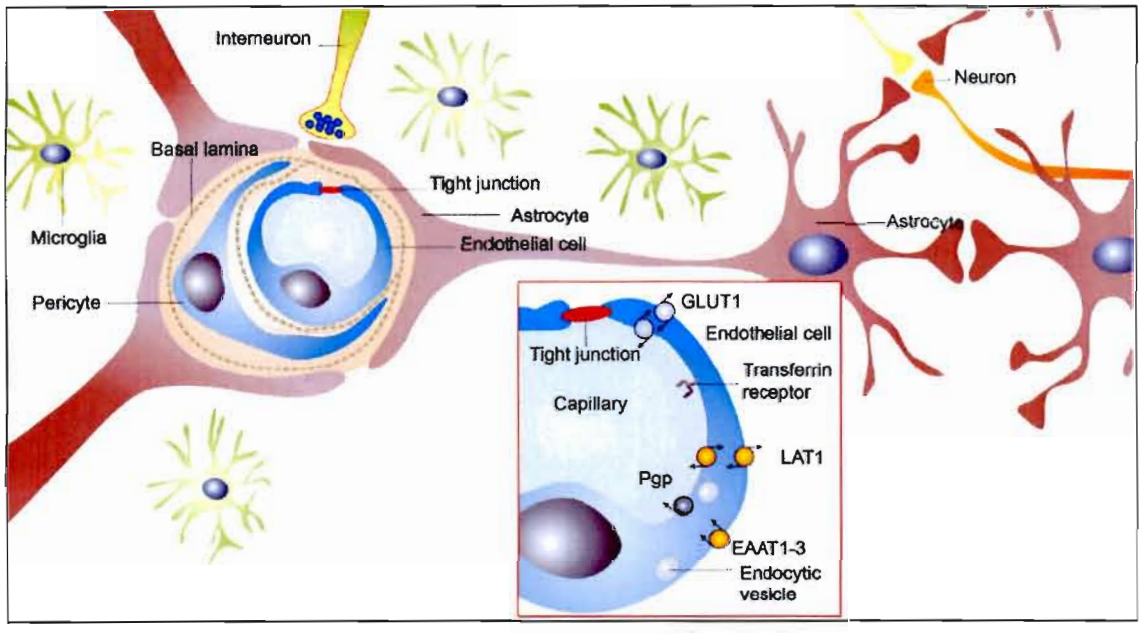



**Figure 2.1**: The blood-brain barrier is formed by brain microvessel endothelial cells that form tight junctions and express different transport systems (Pardridge, 2005).

An understanding the physiological features of these barriers is necessary for discovery of the means towards effective delivery of drugs and imaging agents. The blood-brain barrier consists of walls of capillaries that separate the brain from circulating blood (Kabanov & Gendelman, 2007). It is formed by a complex cellular system of endothelial cells, astroglia, pericytes, perivascular macrophages and a basal lamina (Bradbury, 1985). Astrocytes project their end feet tightly to the cerebral endothelial cells, influencing and conserving the barrier function of these cells. Cerebral endothelial cells are embedded in the basal lamina together with pericytes and perivascular macrophages (Graeber *et al.*, 1989).

Pericytes are characterised as contractile cells that surround the brain capillaries with long processes, and are believed to play a role in controlling the growth of endothelial cells. Due

to their close contact with the endothelial cells, pericytes may influence the integrity of the capillaries and conserve the barrier function. Pericytes additionally limit the transport by the ability to phagocytose compounds which have crossed the endothelial barrier. Finally the lumen of the cerebral capillaries is covered by cerebral endothelial cells in which the functional and morphological basis of the BBB resides (Broadwell & Salcman, 1981). The cerebral endothelial cells possess narrow intercellular tight junction structures. These tight junctions hinder transport of hydrophilic compounds across the cerebral endothelium. The absence of fenestrae in the endothelial plasma membrane and the presence of high densities of mitochondria in the cytosol are other prominent morphological features of the cerebral endothelial cells (Cervos-Navarro *et al.*, 1988).

Certain enzymes which reside selectively in the cerebral endothelial cells constitute a metabolic barrier, which also contributes to the protective function of the BBB. For instance, enzymes like monoamine oxidase A and B, catechol O-methyltransferase or pseudocholinesterase are involved in the degradation of neurotransmitters present in the CNS (Maxwell *et al.*, 1987).

A large number of lipophilic compounds are rapidly effluxed from the brain into the blood by extremely effective drug efflux systems expressed in the BBB. These efflux systems include P-glycoprotein, multi-drug resistance proteins, breast cancer resistance and the multi-specific organic anion transporter (Begly, 1996; Tamai & Tsuji, 2000; Fromm, 2000; Loscher *et al.*, 2005).

## 2.3 PHYSICOCHEMICAL CHARACTERISTICS NEEDED FOR BBB PERMEATION

The diffusion of compounds across the plasma membranes of endothelial cells of the BBB is dependent on the physicochemical properties of these compounds, such as lipid solubility, molecular weight and electrical charge or extent of ionisation (De Vries *et al.*, 1997).

Several 'rules of thumb' have emerged from studies of brain permeation data that have been conducted in recent years that is more or less the same as Lipinski's 'rule of 5' for intestinal absorption (Clark, 2003).

Lipinski's rule of five states that poor absorption or permeation is more likely when:

- There are more than five hydrogen-bond donors ( expressed as the sum of OH's and NH's);
- The molecular weight is greater than 500 Da;
- The logarithm of the n-octanol-water partition coefficient (log D) is greater than five;
- There are more than ten H-bond acceptors (expressed as the sum of N's and O's);

Exceptions to Lipinski's rule are substrates of biological transporters (Lipinski *et al.*, 1997).

Rules of thumb that favour brain permeation according to Clark (2003):

- The sum of the nitrogen and oxygen molecules in a compound is less than five
- The polar surface area (PSA) should be less than 90 $Å^2$
- The molecular weight (MW) should be less than 450
- The log D value should be between one and three
- A decrease in the hydrogen-bonding capacity of a molecule leads to an increase in blood-brain barrier permeability.

The properties of the drug molecule that may thus have a bearing on membrane penetration include molecular weight and size, solubility, partition coefficient, degree of ionization, surface activity, structural isomerism, intramolecular forces, oxidation-reduction potentials, interatomic distances between functional groups and stereochemistry (Malan *et al.*, 2002).

### 2.3.1 LIPOPHILICITY

The lipophilicity of a molecule is related to its free energy of partitioning and partition coefficients are directly related to the free energy of transfer of a substance between two immiscible phases (Chien, 1992). The relative lipophilicity of a solute is often expressed by its octanol/buffer partitioning (log $D_{oct}$) which is assumed to be related to membrane partitioning. The term log D is the log of the ratio of the amount in the organic phase compared to that in the buffer at a given pH (usually 7.4). The log P is the ratio when the compound is in a unionised state. The log $D_{oct}$ is relatively easy to measure and is the most commonly used descriptor of lipophilicity (Atkinson *et al.*, 2002).

A number of groups have shown a relationship between lipophilicity and some measure of brain penetration. Hansch *et al.* (1986) correlated log $D_{oct}$ with hypnotic activity of a series of CNS depressants and showed a correlation with increasing lipophilicity, suggesting the now well-known rule of thumb that for CNS activity the optimal log D is around 2.

The correlation between BBB permeability and log $D_{oct}$ values (representing lipophilicity) is well described in the literature and includes diverse structures (Levin, 1980; Sawada *et al.*, 1999; Van Bree *et al.*, 1988). Increasing lipophilicity tends to increase brain permeation. This is in keeping with the fact that lipophilic compounds tend to traverse membranes more easily than do hydrophilic ligands (Ooms *et al.*, 2002).

### 2.3.2 HYDROGEN BONDING CAPACITY

The importance of hydrogen bonding as a determinant of drug permeation across absorbing membranes has been recognised in several studies (Potts & Guy, 1995; Abraham *et al.*, 1997). Hydrogen-bond donor ($\alpha$) and acceptor ($\beta$) parameters are generally derived from substructure summation and have been successfully used in relation to steroid drugs to provide predictive algorithms (r = 0.96 for this series of steroid drugs) for transdermal permeability (Abraham *et al.*, 1997). In some cases correlation between a measure for brain uptake with Δlog D, specified as the difference between log $D_{oct}$ and log D in an alternative solvent such as cyclohexane, appear to be better than those seen with lipophilicity expressed as log D alone ( Ter Laak *et al.*, 1994; Young *et al.*, 1988). It was later found that Δlog D encodes mainly for hydrogen bonding (Young *et al.*, 1988). This descriptor thus reflects the water shell which forms around the molecules and which has to be de-solvated before the molecules can cross membranes such as the BBB (Chickhale, 1994; Burton & Borchardt, 1996). Brain penetration decreases with increasing Δlog D suggesting that the greater the level of hydrogen bonding, the less membrane permeation is observed (Ter Laak *et al.*, 1994). This parameter is particularly important for peptides due to their high hydrogen bonding potential (Chickhale *et al.*, 1994).

### 2.3.3 MOLECULAR WEIGHT AND SIZE

Lipophilicity as measured by the partion coefficient is obviously an important determinant of brain penetration. However, the diffusion coefficient (D) has also been shown to be important (eq. 1):

$$\text{Perm} = \text{Pm}.\ D/h \qquad (1)$$

where Perm is the permeability coefficient, D the diffusion coefficient, Pm the partition coefficient between membrane tissue and the aqueous environment and h the thickness of the membrane (Atkinson *et al.*, 2002). Looking at the above equation it is important to see that molecular size must play an important role as there is a clear relationship between the diffusion coefficient (D) and molecular weight (Mr) (eq. 2):

$$D(Mr) = \text{constant} \qquad (2)$$

Molecular weight can be used in combination with lipophilicity to derive better correlation with BBB penetration (Shah *et al.*, 1989). Below a weight of 400, increasing lipophilicity improves permeability, whereas a molecular weight of 660 or so was once considered to be the cut-off point for BBB permeation (Levin, 1980). Recent studies however indicated penetration of the BBB by molecules with molecular weights of up to 809 (Hirohashi *et al.*, 1997). The

maximum molecular size for permeability was described as 70-90 Å with the upper limit at 100 Å (Gaillard & De Boer, 2000). Exceptions to this have however been demonstrated as described for the penetration of the blood-brain barrier by lipid-coated nanoparticles; 60 nm in size (Fenart *et al.*, 1999).

There appears to be an inverse relationship between the absorption rate and molecular weight (Pauletti *et al.,* 1997). Generally, an increase in the molecular volume is associated with the hydrophobic surface area and this leads to enhanced permeability through a lipid membrane (Fenart et *al.*, 1999). Conversely, larger molecules diffuse more slowly because they require more space to be created in the medium and this in turn leads to diminished permeability. Small molecules penetrate more rapidly than large ones, but within a narrow range of molecular size, there is little correlation between size and penetration rate (Liron & Cohen, 1984).

### 2.3.4 SOLUBILITY

The aqueous solubility of a drug molecule is partly dependent on other physiochemical properties, for example, partition coefficient and molecular surface features that are relevant to drug absorption (Pagliara *et al.*, 1999). As a result, a correlation between poor solubility and poor absorption can be expected and has been demonstrated (Burton *et al.*, 1996). The solubility of a substance thus greatly influences its ability to penetrate biological membranes. In essence, the aqueous solubility of a drug determines the concentration presented to the absorption site and the partition coefficient strongly influences the rate of transport across the absorption barrier (Idson, 1975).

### 2.3.5 STATE OF IONISATION

Most drugs are weak acids or bases and according to pH-partition theory, may exist in an ionised or non-ionised form, depending upon the pH of the vehicle. The drug's activity coefficient changes significantly as a function of pH for pH values greater than pKa for acidic compounds and less than pKw-pKb for basic drugs (Barr, 1962). The pH of the vehicle in which the penetrant is administered, in combination with the drug molecule's ionisation constant, pKa, determines the actual concentrations of the ionised and non-ionised species (Wiechers,1989). The importance of degree of dissociation or ionisation as a factor determining permeation through the BBB is also well documented (Bates, 1984). This is especially so for drugs with pKa values close to the physiological pH of 7.4; the effect is incorporated in the log D (log $P_{oct}$ at pH = x) term, which in many cases dominates the contributions of other physicochemical factors (Chou *et al.*, 1995).

### 2.3.6 STEREOCHEMISTRY AND STERIC INTERACTIONS

Studies have been conducted on the effects of the stereochemistry of substituent groups on the permeation of 11$\alpha$-and 11$\beta$-hydroxyprogesterone, 20-$\alpha$-hydroxyprogesterone (20$\alpha$-dihydroprogesterone), 20$\beta$-hydroxyprogesterone and 17$\alpha$-estradiol and estradiol (17$\beta$-estradiol) across biological membranes (Bates, 1984). The orientation of the hydroxy group played a pivotal role in determining the permeation rate of the isomers. Depending on the orientation, the hydroxyl groups may be sterically hindered and the relative steric hindrance of the hydroxyl groups then determines aqueous solubility, which in turn affects the permeation rate. For delivery of compounds across the blood-brain barrier, stereochemical considerations are of further importance as specific transporter systems are described for large neutral, small neutral, basic and acidic L-amino acids (Teraski & Tsuji, 1994).

### 2.3.7 MOLECULAR CONFORMATIONAL AND SURFACE PROPERTIES

As the conformation of a molecule changes, physiochemical properties, such as hydrogen bonding activity, are also affected and may influence membrane permeability (Leo *et al.*, 1971). The surface area of drug molecule accessible to a solvent is another potential influence on their permeation (EL Tayar *et al.*, 1991b). The dynamic polar surface area (PSAd) accounts for the shape and flexibility of the drug molecule and is also related to its hydrogen-bonding capacity. The polar surface area (PSA) is defined as the sum of the surfaces of polar atoms in a molecule. Time requirements to calculate the PSA are generally high and calculations require specialised software to generate 3-D molecular structures and to determine the surface area itself, for this reason a new and fast protocol to calculate the PSA is developed and it is based on the topological information only. The new methodology for the calculation of PSA termed TPSA (topological PSA) is based simply on the summation of tabulated surface contributions of polar fragments (Ertl *et al.*, 2000). According the findings of Ertl *et al.* (2000), it was demonstrated that PSA = TPSA and that compounds with values ranging between 60-90 $Å^2$ showed favourable BBB penetration.

## 2.4 MECHANISMS OF TRANSPORT ACROSS THE BBB

There are both chemistry-based and biology-based approaches for developing BBB drug-targeting strategies. The chemistry-based strategies are the conventional approaches that rely on lipid-mediated drug transport across the BBB. The biology-based approaches require prior knowledge of the endogenous transport systems within the brain capillary endothelium, which forms the BBB *in vivo*. The biology-based strategies for brain drug delivery are founded on the principle that there are numerous endogenous transport systems within the BBB and these transporters are conduits to the brain (section 2.4.2). The endogenous BBB transport systems may be broadly classified as carrier-mediated transport, active efflux

transport and receptor-mediated transport. These three BBB transport systems are situated on the luminal and abluminal membranes of the capillary endothelium. Drug delivery to the brain through the many endogenous transport systems within the BBB requires redesign of the drug so that the drug can access the BBB transport system and enter the brain (Pardridge, 2003).

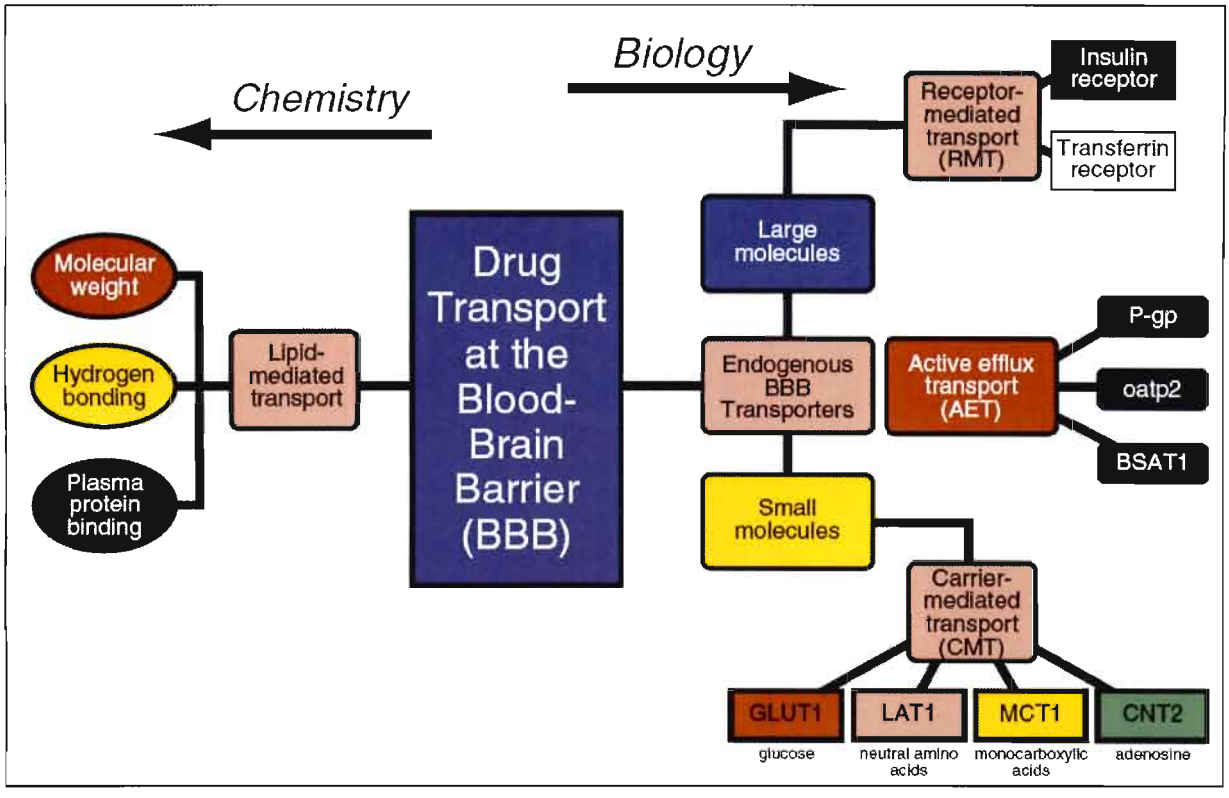



**Figure 2.2**: BBB drug targeting strategies (Pardridge, 2003).

### 2.4.1 CHEMISTRY-BASED APPROACH: BBB LIPID-MEDIATED TRANSPORT

There are two ways that a drug can be made lipophilic. Firstly, the polar functional groups on the water-soluble drug can be masked by conjugating them with lipid-soluble moieties. Secondly the water soluble drug can be conjugated to a lipid-soluble drug carrier. Either redesign of the drug leads to the production of a prodrug, which is lipid soluble and can cross the BBB. Ideally, the prodrug is metabolised within the brain and converted to the parent drug. There have been few examples wherein the prodrug approach has been used to successfully solve the BBB drug-delivery problem in clinical practice. Two limitations of the

prodrug approach are the adverse pharmacokinetics and the increase in molecular weight of the drug that follow from lipidation (Pardridge, 2003).

- **The pharmacokinetic rule**

The percentage of injected dose of a drug that is delivered per gram brain is directly proportional to the BBB permeability surface area and the area under the plasma consentration curve (Pardridge, 2003).

When a drug is lipidated, the BBB permeability surface area product increase. However, the penetration of the lipidated drug is also increased in all organs of the body, which alters the plasma clearance of the drug. Following lipidation, the blood half-time of a drug may decrease from several hours to only a few minutes. Thus, there is a reduction in the plasma AUC in parallel with the increase in membrane permeation caused by lipidation. The increased permeability surface area product and the decreased plasma AUC have offsetting effects leading to a nominal increase in the % ID/g of brain, which is not increased in proportion to the increase in BBB permeability surface area product or lipid solubility (Pardridge, 2003).

- **Molecular Weight Threshold**

The conversion of a water-soluble drug into a lipid-soluble prodrug leads to an increase in the molecular weight of the drug. This increase in molecular weight can be substantial depending on the strategy used to lipidate the drug (Pardridge, 2003). The molecular weight of virtually all CNS-directed drugs in present day clinical practice are under 400 – 500 Da (Ajay, Bemis & Murcko, 1999; Ghose *et al.*, 1999; Lipinski, 2000). Lipid soluble drugs with masses above the 400 – 500 Da threshold, with some exceptions, do not cross the BBB in significant amounts (Trauble, 1971).

## 2.4.2 BIOLOGY-BASED APPROACH: BBB ENDOGENOUS TRANSPORTERS

The transporters are grouped into three categories: carrier-mediated transport (CMT), active efflux transport (AET) and receptor-mediated transport (RMT).

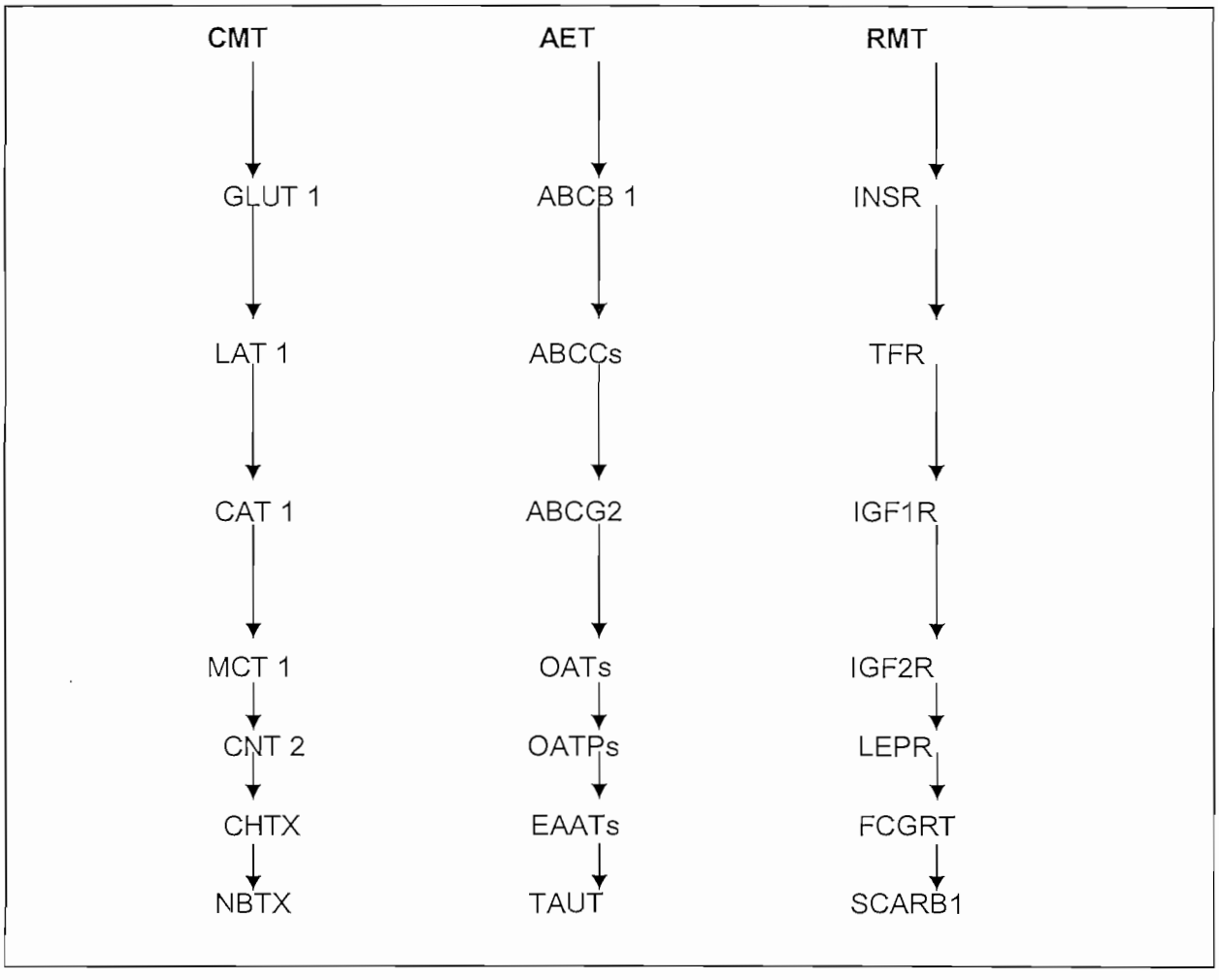



**Figure 2.3**: Endogenous blood-brain barrier transporters (Pardridge, 2007).

### *2.4.2.1 CARRIER-MEDIATED TRANSPORT (CMT)*

CMT systems are responsible for the transport of small molecules between blood and brain (Tsamai & Tsuji, 2000; Kushuhara & Sugiyama, 2001). The GLUT1 glucose transporter transports glucose and other hexoses. LAT1 transports large and small neutral amino acids, as well as certain amino acids drugs including L-Dopa, $\alpha$-methyl-dopa, $\alpha$-methyl-paratyrosine or $\alpha$-gabapentin. Gabapentin is also recognised by the LAT1 transporter, which transports mainly $\alpha$-amino acids. The cationic amino acid transporter CAT1 transports basic amino acids, such as arginine or lysine. The monocarboxylic acid transporters MCT1 transports lactate, pyruvate, ketone bodies and certain monocarboxylic acid drugs such as probenecid (Pardridge, 2007). The concentrative nucleoside transporter CNT2 transports purine nucleosides and certain pyrimidine nucleosides such as uridine. The purine bases, but not

the pyrimidine bases, undergo carrier-mediated transport across the BBB (Cornford & Olendorf, 1975). Choline is a ubiquitous molecule, found throughout almost every tissue in the body. Given it is a charged cation, nearly every cellular membrane has a transport mechanism to meet the intracellular and membrane need for choline. The blood-brain barrier is no exception in that a carrier-mediated transport mechanism is present to deliver choline from plasma to brain. Future work is being completed to determine if other cationic or positively charged therapeutics can be effectively delivered to brain via this carrier (Allan & Lockman, 2003). Choline undergoes carrier-mediated transport across the BBB *via* a sodium-independent process and although sodium-dependent choline transporters (CHT) have been cloned, the sodium-independent CHT at the BBB has not been cloned (Conford et *al.*, 1978). The various CMT systems provide a diverse space of molecular structure that could be mimicked with medicinal modifications of drugs that normally do not cross the BBB. Additionally, certain medicinal chemistry strategies could be employed to render the molecule susceptible to enzymes in the brain that convert the modified drug back to the parent drug once it crosses the BBB. Although the blood-brain barrier CMT systems are saturable at high substrate concentration, the transporters are not effectively saturated by endogenous drug *in vivo* (Pardridge, 2007).

#### *2.4.2.2 ACTIVE EFFLUX TRANSPORT (AET)*

A large number of lipophilic compounds are rapidly effluxed from the brain into the blood by extremely effective drug efflux systems expressed in the BBB. These efflux systems include P-glycoprotein, multi-drug resistant proteins (MRP's), breast cancer resistance protein (BCRP) and the multi-specific organic anion transporter (MOAT) (Begly, 1996; Tamai & Tsui, 2000; Fromm, 2000; Loscher & Potschka, 2005). The most studied AET system at the BBB is P-glycoprotein, which is a product of the ABCB1 gene. However, blood-brain barrier AET transport biology extends beyond P-glycoprotein. There are multiple other members of the ABC gene family that present energy-dependent active efflux transporters at the BBB, including members of the ABCC and the ABCG2 gene family (Pardridge, 2007). In addition, active efflux transport of drug from brain to blood is a process mediated by two transporters in series, including an energy-dependent transporter (Pardridge, 2005). The energy-dependent transporter from the ABC gene family can be expressed at the luminal membrane, whereas the energy-independent transporter can be expressed at the abluminal membrane of the brain capillary endothelial cell. The energy-independent transporters are members of the solute carrier (SLC) gene families and include members of the organic anion transporters, acidic amino acid transporters such as the tautine transporter. It is also possible that the energy-or sodium-dependent transporter is present at the abluminal membrane, whereas the energy-independent transporter is present at the luminal

membrane. The challenge in understanding AET's at the BBB is to identify the pair of energy-dependent and energy-independent transporters that work in concert to mediate the active efflux of a given pharmaceutical, and to localise these transporters to the respective luminal or abluminal endothelial membrane (Pardridge, 2007).

#### *2.4.2.3 RECEPTOR-MEDIATED TRANSPORT (RMT)*

The RMT systems are responsible for the transport of certain endogenous large molecules across the BBB (Pardridge, 2007). Certain large-molecule peptides in the blood undergo RMT across the BBB *via* the endogenous peptide receptors (Pardridge, 2001). Insulin in the blood undergoes RMT across the BBB *via* endogenous BBB insulin receptor (INSR). Brain iron derived from circulating transferrin is translated *via* RMT across the BBB transferrin receptor (TFR) (Pardridge, 2007). The insulin-like growth factors (IGF) IGF-1 and IGF-2 undergo RMT across the BBB *via* a separate type 1 and type 2 IGF (IGF1R and IGF2R) in rodents. The IGF2R also transports protein conjugated with mannose-6-phosphate. However, the IGF receptor at the human BBB differs from the IGFR at the animal BBB, in that both IGF-1 and IGF-2 bind with affinity to a single variant IGFR (Duffy, 1988). Unlike insulin or transferrin, the circulating IGF's are >99,9% bound by IGF-binding proteins. (Pardridge, 2007). The BBB expresses the short form of the leptin receptor (LEPR) (Boado, 1998). This receptor might participate in the RMT of leptin across the BBB *in vivo*, although definite evidence for this has yet to be reported (Pardridge, 2007). IgG present in the brain is rapidly exported to blood *via* reverse transcytosis on the BBB neonatal Fc receptor (Yhang & Pardridge, 2001; Schlachetzki *et al.*, 2002), also called FcRN or FCGRT. This is an asymmetric RMT system because the blood-brain barrier FcRN does not mediate the transport IgG from blood to brain. Modified lipoproteins such as acetylated low density lipoprotein (LDL), undergo receptor-mediated endocytosis into the brain capillary endothelial cell via the scavenger receptor type B1 (SCARB1) (Pardridge, 2007). However this is not a transcytosis system because acetylated LDL in blood is completely sequestered by the brain capillary endothelium and does not transcytose through the endothelium to enter the brain interstitial fluid from blood (Pardridge, 2001).

## 2.5 ROUTES ACROSS THE BLOOD-BRAIN BARRIER: DRUG DELIVERY

From the physiology outlined above, it is clear that there are several routes for drug delivery to the brain. Under normal conditions the tight junctions severely restrict penetration of polar (hydrophilic) molecules, but they may act as a route for leukocyte traffic. Such cells may have the ability to unzip the junction locally as they transmigrate with minimal leakage, or to adhere in the region of the junction then migrate through the cell (Lennington & Yang, 2003; Wolburg, Wolburg-Buchholz & Engelbrecht, 2004). However, given the low volume of

leucocyte traffic across the BBB, these cells do not make a suitable drug delivery vehicle. Many lipid soluble drugs can diffuse through the endothelial cell membranes, although the nature of these membranes may place limits on permeation (Bodor & Buchwald, 2003). Nevertheless, drug lipidisation and pro-drug strategies remain favoured modes of increasing drug delivery to the brain (Begely, 2004), and a number of *in silico* modelling approaches are able to give a reasonably good prediction of passive permeation *via* this route (Abbott, 2004). Carrier-mediated entry on uptake transporters has been exploited for delivery of a number of drugs, including L-Dopa, gabapentin and the L-system amino acid carrier is particularly suitable given its ability to accept a relatively large hydrophobic moiety on the drug (Begley, 2004).

Many of the efflux transporters are located on the luminal membrane of the brain endothelium, causing reduced entry of drug substances for these transporters. The proportion of marketed drugs that are P-gp substrates is lower for CNS drugs than for non-CNS drugs (Mahar Doan *et al.*, 2002), hence there is a great interest in the three strategies reducing affinity for efflux transporters, blocking P-gp function and the passing of P-gp by the use of vehicles that is not detected by the transporters (Begley, 2004; Sherrmann & Temsammani, 2005; Kreuter, 2005). Considerable progress has been made in understanding vesicular mechanisms (RMT and AMT) as a route for large molecular delivery, with potential clinical applications (Pardridge, 2005). Modulation of the permeability of tight junctions may allow transient barrier opening; this occurs in some pathologies, but may also be important physiologically. A promising method for transient opening following controlled nerve stimulation may prove to be of clinical value, particulary for delivery of complex molecules too large or polar to use other routes (Yarnitsky *et al.*, 2004).

## 2.6. CONCLUSION

The passage of a variety of drugs into the CNS is prevented by the blood-brain barrier. Utilising endogenous blood-brain barrier transport system or applying certain prodrug strategies to enhance the BBB permeability of certain drugs will open a whole new field of drug therapy. Enhancing the BBB permeability of the NSAIDs, could have a profound influence on the way we treat neurodegenerative disorders.

# CHAPTER 3

# NEUROPROTECTION

## 3.1 INTRODUCTION

### 3.1.1 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND NEUROPROTECTION

Based on the evidence that inflammation plays a key role in the pathogenenisis of Parkinson's disease as well as Alzheimer's disease, research has focussed on the use of anti-inflammatory drugs as neuroprotective agents in the use of Parkinson's disease and Alzheimer's disease.

Non-steroidal anti-inflammatory drugs is a family of drugs that include the salicylate, propionic acid, acetic acid, fenamate, oxicam and the cyclooxygenase-2 (COX-2) inhibitor classes. They have antipyretic and anti-inflammatory properties and function by inhibiting the cyclooxygenase (COX) enzyme that catalysis the initial step in the conversion of arachidonic acid to several eicosanoids including thromboxane, leukotrienes and prostaglandins (Tuppo *et al.*, 2005).

There are two COX isoforms: COX-1, which is a constitutive enzyme expressed in most tissues and involved in cell-cell signalling and tissue homeostasis; and COX 2, which is a highly inducible enzyme that is particularly related to inflammation, specifically in the brain since it is known to be produced in neurons, astrocytes and endothelial cells (Chandrasekharan *et al.*, 2002).

Inhibition of COX by NSAIDs might repress its oxidation, which is associated with calcium-dependent glutamate release. In this way, NSAIDs indirectly reduce the glutamate-induced neurodegeneration (Breitner *et al., 1996).*

Another property of NSAIDs is the activation of the proliferator-activated receptor-y (PPAR-gamma) resulting in transcriptional regulatory actions causing suppression of a wide range of pro-inflammatory molecules and microglial activity. Moreover, some NSAIDs show antioxidant activity. Therefore as inflammation is involved in neurodegenerative diseases and considering the other properties of NSAIDs, it is reasonable to suggest that COX inhibitors, especially COX-2 inhibitors are possible candidates for the treatment of neurodegenerative diseases. (Maffei *et al.*, 1993; Kataoka *et al.*, 1997; Mohanakumar *et al.*, 2000 & Grilli *et al.,* 1996.)

Since patients with rheumatoid arthritis and osteoarthritis are typically treated with and are exposed to NSAIDs for a long period of time, epidemiological studies have looked into the association of these diseases and Alzheimer's disease. Many of those studies showed an inverse relationship between having arthritis and being treated with NSAIDs and Alzheimer's disease (Zandi & Breitner, 2001). Mckenzie (2001), reported that post-mortem studies have also shown the ability of NSAIDs to reduce the inflammation that is consistently seen in Alzheimer's disease brain tissue. Deletion of COX-2 in mice resulted in protection against MPTP-induced dopaminergic cell loss, suggesting that NSAIDs do in fact exert their neuroprotective effects *via* inhibition of COX-2 (Feng *et al.*, 2002).

Investigating the intake of NSAIDs by a large cohort of Americans (n = 50,000) showed that the risk for developing Parkinson's disease in persons regularly taking NSAIDs was decreased by 45% (Chen *et al.*, 2003). Thus the use of NSAIDs may result in neuroprotection in Parkinson's disease and mechanisms other than modulation of the inflammatory state cannot be ruled out. For instance, the ability of NSAIDs to scavenge OH• and NO could result in protection of dopaminergic neurons exposed to oxidative stress (Chen et *al.*, 2003).

## 3.2 BBB-PERMEABILITY OF THE NSAIDs USED IN STUDY

NSAID therapy must be fairly long in duration, at least two years, in order to reduce the risk of Alzheimer's disease (Stewart *et al.*, 1997). In order to use NSAIDs effectively as a medication against Alzheimer's disease or other neurodegenerative diseases, sufficiently high concentrations of these drugs must enter the CNS, particularly the brain parenchyma. Studies suggest that NSAIDs are only marginally distributed into the CNS (Deguchi *et al.*, 2000; Matoga *et al.*, 2002; Eriksen *et al.*, 2003; Smith *et al.*, 2003). The CNS penetrations of NSAIDs has not been a major driving force in previous studies, and in some studies CNS delivery was determined by comparing plasma concentration with cerebrospinal fluid (CSF) concentration. CSF levels do not, however, always correspond with levels in the brain parenchyma, which is the actual target of many CNS drugs (Pardridge, 1995).

### 3.2.1 IBUPROFEN AND ACETYLSALICYLIC ACID

Ideally, an effective drug must be both lipophilic and small enough to pass through the BBB and the physiochemical characteristics of ibuprofen and acetylsalicylic acid restricts its brain delivery. Most NSAIDs are weak organic acids (Furst & Munster, 2001) and the pKa value for ibuprofen is 5.2 and that for acetylsalicylic acid is 3.5 (Katzung, 2001). Ibuprofen is fairly lipophilic in its unionised form (log P 3.5). However, when the pH is above its pKa value (5.2), ibuprofen becomes ionised leading to a substantial decrease in its lipophilicity. In the

systemic circulation, the pH is approximately 7.4, where ibuprofen and acetylsalicylic acid are totally ionised. Thus ibuprofen as well as acetylsalicylic acid are too hydrophillic to be efficiently absorbed by passive diffusion through the BBB. This is supported by the findings of Deguchi *et al.* (2000), who reported that the brain penetration of ibuprofen can be enhanced by making the molecule more lipophilic through prodrug technology. To determine the distribution of ibuprofen in the brain parenchyma of rats, the ibuprofen solution was infused to conscious rats for 1-6 h through the femoral vein and blood samples were drawn before and during infusion. Brains were removed from the cranium 1-2 min after decapitation, washed with saline and immediately frozen on dry ice. The brain penetration was found to be extremely low and the brain to plasma ratio at the steady state was only 0.02 (Manilla *et al.*, 2000).

## 3.3 CONCLUSION

The anti-inflammatory as well as antioxidant activity of NSAIDS give them the potential to be used in the treatment of neurodegenerative diseases. The need for an effective CNS delivery mechanism for these drugs is thus clear. For these reasons, a prodrug strategy for these hydrophilic drugs were pursued by conjugation with pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane to increase the lipophilicity of these drugs.

# CHAPTER 4

# POLYCYCLIC STRUCTURES AS CARRIERS TO ENHANCE BBB-PERMEABILITY

## 4.1 INTRODUCTION

The chemistry as well as the pharmacological properties of polycyclic cage compounds have intrigued many researchers over the past few decades. Many of these compounds have important pharmaceutical applications, including the symptomatic and proposed curative treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Malan *et al.*, 2003; Geldenhuys *et al.*, 2005). Interest in the pharmacology of polycyclic cage amines was stimulated by the early findings of Davis *et al.* (1964) that 1-aminoadamantane, commonly known as amantadine (**4**), had antiviral activity against a range of viruses causing influenza, hepatitis C and herpes zoster neuralgia. Later Schwab et *al.* (1986) reported that amantadine was beneficial to patients with Parkinson's disease and that hydrophobicity of the hydrocarbon cage enabled this molecule to cross the blood-brain barrier and enter the central nervous system. Memantine (**5**) was found to be a clinically well-tolerated NMDA receptor antagonist (Danyzs *et al.*, 1997). These compounds show promise as neuroprotective drugs by preventing excessive influx of calcium ions into neuronal cells (Brouillet *et al.*, 1993). Oliver et al. (1991) reported that amino-(D3)-trishomocubanes and petacycloundecyl amines possessed anti-viral activity against herpes simplex 1 and 2, influenza A2/taiwan and Rhino 1A virus. NGP-101 and related compounds were found to be potential lead structures for the development of neuroprotective compounds having anti-parkinsonian activity (Oliver *et al.*, 1991; Van der Schyf *et al.*, 1986). Moreover, derivates of polycyclic compounds have been used as anti-depressant, analgesics, anti-inflammatory and anti-tumor agents as well as in the inhibition of proteases in a number of different diseases (Van der Schyf *et al.*, 1986).

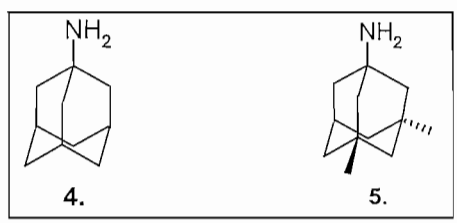



**Figure 4.1**: Amantadine (**4**) and memantine (**5**)

## 4.2 ROLE OF PENTACYCLOUNDECYLAMINES IN NEUROPROTECTION

Amongst several drug therapies used in the symptomatic treatment of Parkinson's disease patients is the polycyclic cage amine, amantadine. Amantadine expresses its antiparkinsonian activity, at least in part, by increasing extracellular dopamine levels *via* re-uptake inhibition or dopamine release (Mizoguchi *et al.*, 1994; Danyz *et al.*, 1997). Electrophysiological studies further indicated that amantadine binds to the phencyclidine (PCP) or MK-801 (dizocilpine) binding site located within the NMDA receptor/ion channel complex (Parsons *et al.*, 1999). In addition, amantadine and its dimethyl derivative, memantine have been shown to be neuroprotective (Parsons *et al.*, 1999; Wenk *et al.*, 1995). Oliver (1991) thus suggested that the pentacyclic cage amines might be a new class of anti-parkinsonian agents due to anticataleptic and mild to weak anticholinergic activities.

L-type calcium channel antagonism has been reported by our laboratory for pentacycloundecylamines, in particular for the prototypical compound 8-benzy-amino-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (NGP1-01) (**6**), (Van der Schyf *et al.*, 1986; Liebenberg *et al.*, 1996; Malan *et al.*, 1998 & Malan *et al.*, 2000). Recently an *in vivo* pilot evaluation of a small series of pentacycloundecylamines in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) parkinsonian mouse model was reported and it was found that one of the compounds, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane, tested positive as a neuroprotective agent (Geldenhuys *et al.*, 2003). MPTP is a protoxin that requires activation by means of monoamine oxidase (MAO-B) to form the neurotoxic pyridinium species, 1-methyl-4-phenyl-pyridinium ($MPP^+$) (Castagnoli *et al.*, 2001). $MPP^+$ is then transported into dopaminergic neurons *via* the dopamine transporter, where it inhibits mitochondrial complex 1 (Gerlach *et al.*, 1991; Speciale, 2002). Excitotoxicity is also implicated in the MPTP model of neuronal cell death and involves the over-stimulation of the (NMDA) receptor by glutamate, ultimately resulting in excess intracellular calcium and cell death (Trist, 2000). The putative protective activity of the cage compounds can be hypothesized to be initiated by a multiple mechanism of action including attenuation of NMDA receptor activation, thereby preventing excessive influx of $Ca^{2+}$ into neuronal cells, as well as the direct blockade of L-type $Ca^{2+}$ channels and dopamine transport inhibition (Geldenhuys *et al.*, 2003).

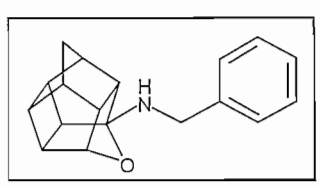



**Figure 4.2**: NGP 1-01 (**6**)

## 4.3 PHYSICOCHEMICAL PREDICTION OF A BRAIN-BLOOD DISTRIBUTION PROFILE IN POLYCYCLIC AMINES

Since the postulated target systems for these polycyclic amines are within the brain and CNS, the need exists for generating concrete data and guidelines on their BBB permeability (Zah *et al.*, 2003). A previous project entailed the synthesis and characterisation of a series of pentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecyl amines in terms of lipophilicity, minimum energy conformation, molecular size, electronic character, hydrogen bonding behaviour and brain-blood distribution data. The study unambiguously proved that the N-substituted 8-amino-8,11-oxapentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecanes significantly partition into the brain after systemic administration independent of the mode of entry or whether specific transport systems or efflux mechanisms are involved. Regression models established the significance of lipophilicity, solvent accessible molecular volume and molar refractivity to the BBB permeability of the polycyclic amines. The good linear regression obtained by including an energy term also stresses the importance of conformational aspects in the transport of these compounds across the BBB (Zah *et al.*, 2003). This study clearly indicates that pentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecanes can be applied as carrier molecules to deliver drugs into the brain.

## 4.4 CONCLUSION

Polycyclic structures, specifically pentacyclo-[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane, posses the ability to cross the BBB and can be used as carrier molecules for the CNS delivery of drugs. They may therefore be applied as carrier vectors for the delivery of neuroprotective drugs with poor blood-brain barrier permeability, into the CNS.

# CHAPTER 5

# EXPERIMENTAL

## 5.1 STANDARD EXPERIMENTAL PROCEDURES

### 5.1.1 INSTRUMENTATION

#### *5.1.1.1 NUCLEAR MAGNETIC RESONANCE (NMR)*

$^{1}H$ and $^{13}C$ NMR spectra were obtained using a Varian Gemini 300 spectrometer at a frequency of 300.075 MHz and 75.462 MHz, for compound **9.** $^{1}H$ and $^{13}C$ NMR spectra were obtained using a Varian Gemini 600 spectrometer at a frequency of 600.14 MHz and 150.92 MHz, for compounds **11, 12, 15** and **16**. This was done in a 7 Tesla magnetic field and tetramethylsilane (TMS) was used as internal standard. A bandwidth of 1000 MHz at 24 kG was applied for $^{1}H$-$^{13}C$-decoupling. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS (d=0) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s – singlet, bs – broad singulet, d- doublet, dd – doublet of doublets, t – triplet, q – quartet and m – multiplet. Distorsionless Enhancement by Polarisation Transfer (DEPT) experiments were also conducted for the novel synthesised compounds.

#### *5.1.1.2 MASS SPECTROMETRY (MS)*

An analytical VG 7070E mass spectrometer was used to record mass spectra with fast atom bombardment (FAB) or electron impact (EI) at 70 eV as ionization techniques.

#### *5.1.1.3 INFRARED SPECTROSCOPY (IR)*

IR spectra were recorded on a Nicolet Magna – IR 550 spectrometer. Samples were applied as a film or incorporated in KBr pellets.

### 5.1.2 CHROMATOGRAPHIC TECHNIQUES

#### *5.1.2.1 THIN LAYER CHROMATOGRAPHY (TLC)*

Analytical TLC was performed on 0.20 mm thick aluminium silica gel sheets (Alugram® SIL G/$UV_{254}$, Kieselgel 60, Macherey-Nagel, Düren, Germany). Visualisation was achieved using UV light (254 nm), a spray reagent containing ninhydrin in ethanol or iodine vapours, with mobile phases as indicated for each compound.

### *5.1.2.2 COLUMN CHROMATOGRAPHY*

Compounds were purified using a standard glass column 71 cm in length and with an inner diameter of 4 cm. The stationary phase used was silica gel (0.063-0.200 mm/70-230 mesh ASTM, Machery-Nagel, Düren, Germany) with mobile phases as indicated for each compound. Flash column chromatography was also applied when necessary.

## 5.2 SYNTHESIS OF SELECTED COMPOUNDS

### 5.2.1 GENERAL APPROACH

The well-described Cookson's diketone, pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8,11-dione (**8**), was synthesised according to the published method (Cooksen et al., 1958, 1964). This structure served as primary basis for the synthesis of the proposed compounds. The reaction involved Diels-Alder adduct (**7**) formation and subsequent photocyclisation to yield the polycyclic cage structure (fig 5.1).

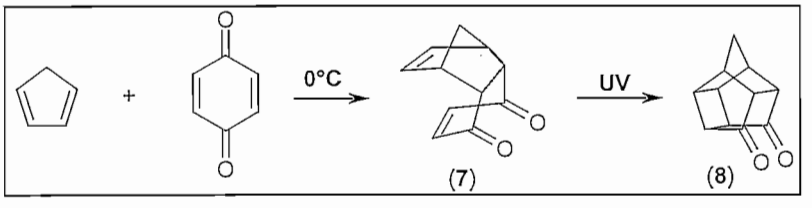



**Figure 5.1**: Synthesis of pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane-8-11-dione.

### 5.2.2 SYNTHESIS OF THE ESTER PRODRUGS

Pentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane (**8**) was linked to 2-aminoethanol (**9**) to yield compound (**10**). The OH group was used for esterification to the carboxylic acid groups of ibuprofen and acetylsalicylic acid respectively. This was achieved by activating the carboxylic acid group of ibuprofen with a carbodiimide, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCL), where after this activated structure was reacted with the OH group on compound **10** to yield the ibuprofen (**11**) ester prodrug. For acetylsalicylic acid the commercially available acid chloride derivative was reacted directly with the OH group on compound **10** to yield the acetylsalicylate (**12**) prodrug (fig 5.2).

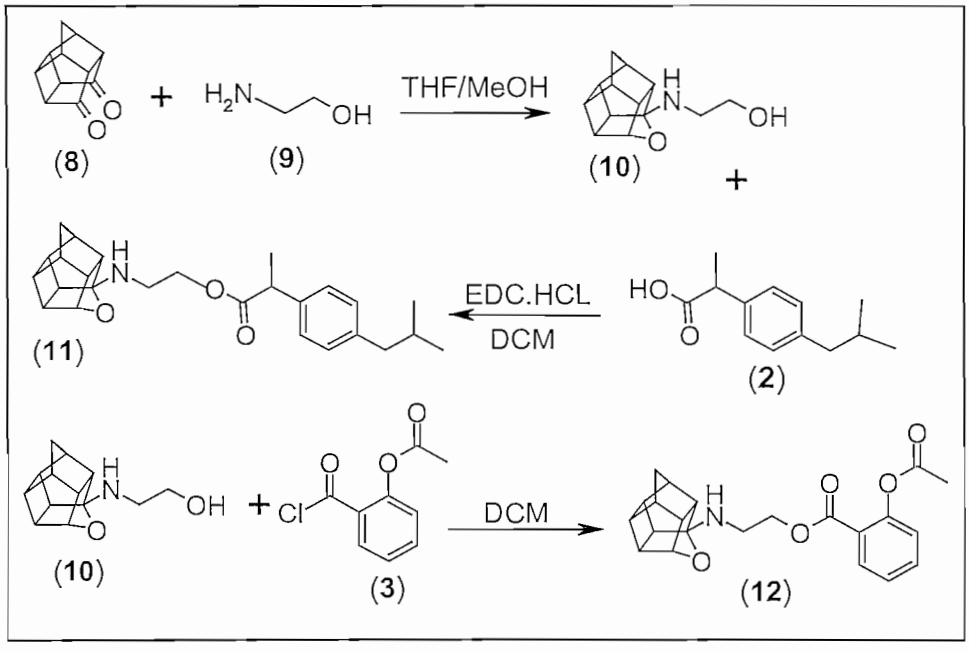



**Figure 5.2**: Synthetic pathway for ester prodrugs (**11**, **12**) of the selected NSAIDs (**2**, **3**)

### 5.2.3 SYNTHESIS OF AMIDE PRODRUGS

Ibuprofen (**2**) was converted to a methyl ester by a Fischer-esterification reaction. 1,2-Diaminoethane (**13**) was used as a linker to form the ibuprofen amide derivative (**14**). This product was conjugated to pentacyclo- [5.4.0.$0^{2,6}$ .$0^{3,10}$.$0^{5,9}$]undecane (**8**), dehydrated to form the imine and then reduced to yield the product (**15**). O-Acetylsalicyloyl chloride (**3**) was reacted with an excess of 1,2-diaminoethane (**13**) to form the amide derivate (**16**). This product was conjugated to pentacyclo- [5.4.0.$0^{2,6}$ .$0^{3,10}$.$0^{5,9}$]undecane (**8**), dehydrated to form the imine and reduced to yield the acetylsalicylic (**17**) amide prodrug (fig. 5.3).

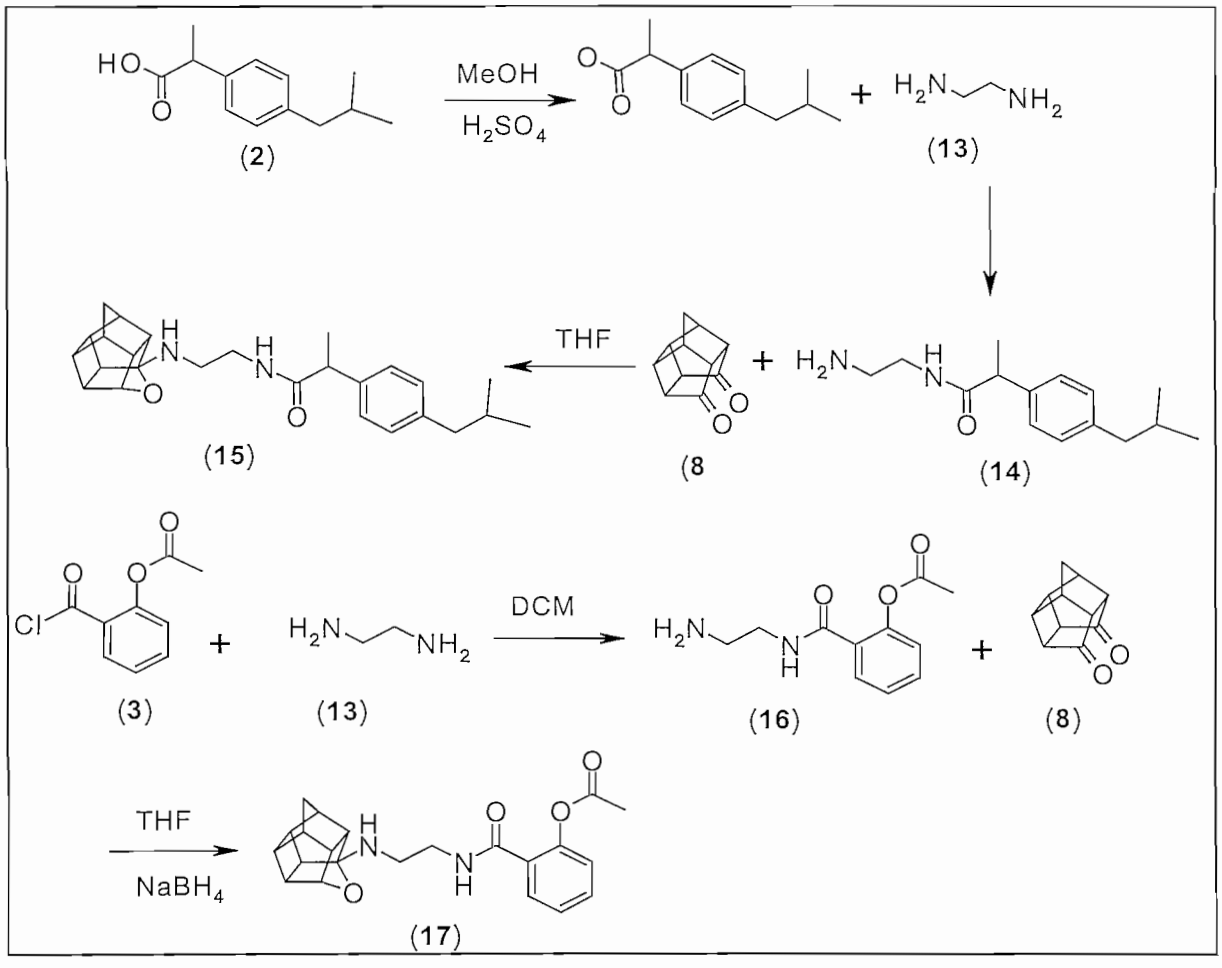
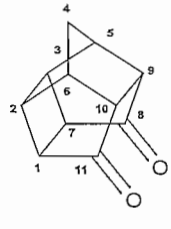



**Figure 5.3:** Synthesis of the amide prodrugs (**15**, **17**) of the selected NSAIDs (**2**, **3**)

## 5.3 SYNTHESIS

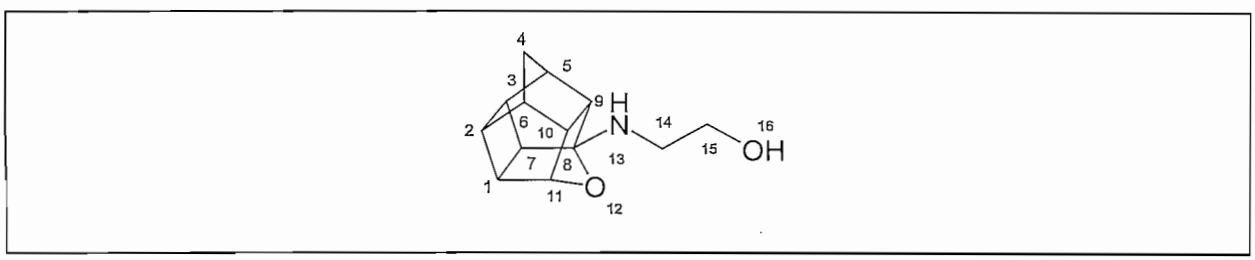
### 5.3.1 PENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE-8,11-DIONE (8)




***Synthetic method:*** *p*-Benzoquinone (10 g, 9.251 mmol) was dissolved in dried benzene (400 ml) and oxidized with $MnO_2$ (50 mg) by refluxing the mixture for 20 minutes. The reaction mixture was protected from light by covering the reaction flask with aluminium foil. Five spatula measures of activated charcoal were added and the mixture was allowed to

reflux for a further 5 minutes. The hot mixture was vacuum-filtered through Celite® to produce a clear yellow solution. Crystallisation of the freshly oxidised *p*-benzoquinone was prevented by flushing the same filtering system with dried methanol (100 ml). This solution was cooled down to 5°C by placing it on an external ice-bath. Freshly monomerised cyclopentadiene (12.229 g, 9.251 mmol) was slowly and stoichiometrically added, keeping the reaction temperature between 5 and 18°C. The reaction was monitored by means of TLC and presumed complete when the *p*-benzoquinone spot was no longer visible on the TLC plate. The photo labile reaction mixture was removed from the external ice-bath, protected from light and stirred for 1 hour at room temperature. Thereafter 3 spatula measures of activated charcoal were added and the mixture was stirred at room temperature for a further 30 minutes. Removal of the activated charcoal, followed by *in vacuo* evaporation of benzene resulted in the formation of intensely coloured amber oil. Excess solvent was allowed to fully evaporate in a dark fume hood to afford the yellow Diels-Alder adduct crystals. The crystals were dissolved in ethyl acetate (4 g per 100 ml) and irradiated with UV light for 6 hours, using a photochemical reactor. Decolouration of the solution afforded a light yellow residue, which was purified by Soxhlet extraction in cyclohexane to produce the cage compound as fine white crystals (Yield: 14 g, 8.037 mmol, 86.88%). The physical characteristics of these crystals correlated with that reported by Cooksen et *al.* (1985, 1964) and therefore no spectral data is presented. The melting point (MP) was found to be 245°C.

### 5.3.2 8-(2-AMINOETHANOL)-8,11-OXAPENTACYCLO[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]UNDECANE (10)

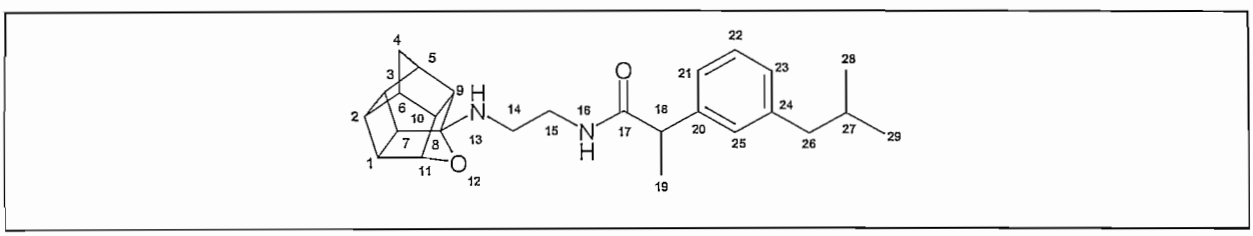



***Synthetic method:*** The pentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8,11-dione (5.1 g, 29 mmol) was dissolved in dry tetrahydrofurane (50 ml) and cooled down to 5°C, while stirring on an external ice-bath. 2-Aminoethanol (1.8 g, 29 mmol) was added drop wise, while stirring at lowered temperature (± 5°C). The carbinolamine started to precipitate after 10 minutes and the mixture was stirred for another 20 minutes. The carbinolamine was filtered and dissolved in dry methanol (30 ml). A spatula point (± 1.5 g) of sodium borohydride was slowly added

and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated *in vacuo*, washed with water (100 ml), and extracted with dichloromethane (3 x 50 ml). The organic fraction was dried overnight with $MgSO_4$ and evaporated to give a light yellow oil. The mixture was purified by column chromatography (mobile phase, ethyl acetate: ethanol (2:1)) and the desired amine was obtained as a light yellow oil ($R_f$ 0.40, 1.5 g, 6.85 mmol, 30%).

***Physical data:*** $C_{13}H_{17}NO_2$; **$^{13}$C NMR** (75 MHz, $CDCl_3$) $\delta_C$ (Spectrum 1): 82.34 (C-11), 77.43 (C-14), 76.58 (C-8), 63.332 (C-15), 55.09 (C-10), 54.65 (C-9), 46.28 (C-7), 44.80 (C-5), 44.48 (C-3), 44.30 (C-1), 43.01 (C-4), 41.82 (C-6), 41.43 (C-2); **$^1$H NMR** (300 MHz, $CDCl_3$) $\delta_H$ (Spectrum 2): 4.6 (t, 1H, J = 5.22, H-11), 3.6 (m, 2H, H-15a,b), 3.1 (m, 2H, H-14a,b), 2.91-2.63 (m, 4H, H-1, 7, 9,10), 2.62 – 2.43 (m, 4H, H-2, 3, 5, 6), 1.95 (AB-q, 2H, J = 10.5 Hz, H-4a,b).

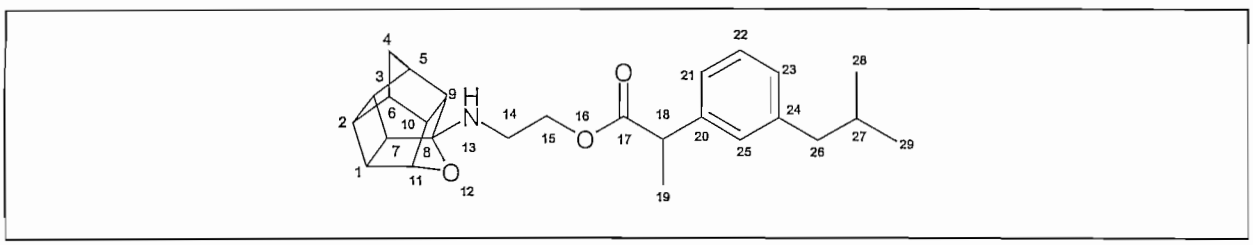
### 5.3.3 8, 11-OXAPENTACYCLO [5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]UNDECANE-8-(2-AMINOETHYL)-(4-ISOBUTYLPHENYL)-PROPANAMIDE (15)



***Synthetic method:*** Ibuprofen (5 g, 24.24 mmol) was dissolved in dry methanol (125 ml) and $H_2SO_4$ (12.5 ml) was added drop wise as a catalyst. The reaction mixture was refluxed for one hour and concentrated *in vacuo* to yield *a* colourless oil. The mixture was washed with water (100rnl) and extracted with DCM (3 x 50 ml). The organic fraction was dried overnight with $MgSO_4$. The reaction mixture was concentrated *in vacuo* to yield the methyl ester as a colourless oil (4 g, 19.39 mmol, 80%). 4 g (19.39 mmol) of the synthesised ester was added drop wise to ethylenediamine (11.65 g, 193.90 mmol) and refluxed overnight. The reaction mixture was washed with saturated NaCl solution (100 ml) and extracted with DCM (3 x 50 ml). The organic fraction was dried overnight with $MgSO_4$ and concentrated *in vacuo* to yield the ibuprofen amine derivative. Pentacyclo[5.4.0.0$^{2,6}$ .0$^{3.10}$.0$^{5,9}$]undecane-8,11-dione (0.8 g, 4.54 mmol) was dissolved in tetrahydrofuran (30 ml) and cooled down to 5°C while stirring in an ice bath. Ibuprofen amine (1 g, 4.54 mmol) was added slowly, with continued stirring of the reaction mixture at 5°C. The reaction was allowed to reach completion for 30 min. Water was azeotropically removed from the reaction mixture by refluxing it in 60 ml dry benzene using a Dean-Stark apparatus for 1 h or until no water collected in the trap. The excess

benzene was removed under reduced pressure and the dark brown oil was dissolved in a mixture of anhydrous methanol (30 ml) and anhydrous THF (150 ml). A reduction was carried out by adding sodium borohydride (1.5 g) and stirring overnight at room temperature. The solvent was removed *in vacuo*, the residue was suspended in water (50 ml) and extracted with dichloromethane. The combined organic fractions were washed with water (2 x 100 ml), dried over anhydrous magnesium sulphate and evaporated *in vacuo* to yield a dark brown oil. Resolution of the product mixture could not be accomplished completely and yielded a mixture of amino and imino alcohols (Spectra 3-6, Yield: 300 mg, 0.73 mmol, 16%).

### 5.3.4 8,11-OXAPENTACYCLO[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]UNDECANE-8-(2-AMINOETHYL)-(4-ISOBUTYLPHENYL)-PROPANOATE (11)

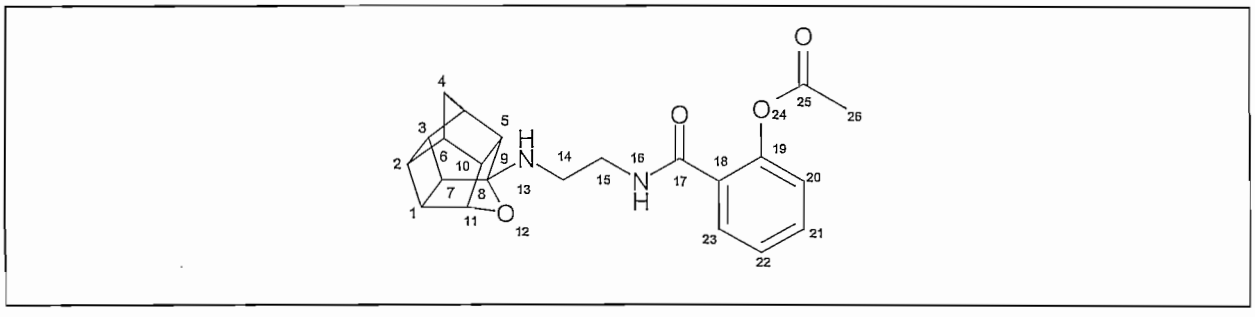


***Synthetic method:*** Ibuprofen (2 g, 9.69 mmol) was dissolved in dry dichloromethane (80 ml) and excess EDC.HCL ( 3.72 g, 19.39 mmol) was added. The reaction mixture was stirred at room tempertaure for 48 hours. TLC indicated that ibuprofen had been completely converted to its activated form. 8-(2-Aminoethanol)-8,11-oxapentacyclo[5.4.0.$0^{2,6}$.$0^{3,10}$.$0^{5,9}$]undecane; (0.7 g, 3.19 mmol) was dissolved in dry dichloromethane (20 ml). The activated complex (0.65 g, 3.19 mmol) was dissolved in dry dichloromethane (10 ml) and added drop wise to the reaction mixture. The reaction mixture was stirred at room temperature for 48 hours and concentrated *in vacuo* to yield a colourless oil. The mixture was purified using column chromatography to yield a colourless oil (EtOAc:ETOH, 2:1, $R_f$ = 0.87, Yield: 150 mg, 0.37 mmol, 12%).

***Physical data:*** $C_{26}H_{33}NO_3$; **$^{13}$C NMR** (150 MHz, $CDCl_3$) $\delta_C$ (Spectrum 7): 175.22 (C-17), 140.53 (C-20), 137.85 (C-24), 129.58 (C-22), 129.33 (C-23), 129.26 (C-25), 127.34 (C-21), 54.64 (C-18), 44.68 (C-26), 44.46 (C-14), 43,19 (C-4), 42.69 (C-1), 41.30 (C-27). 22.37 (C-28/29), 18.68 (C-19); **$^{1}$H NMR** (600 MHz, $CDCl_3$) $\delta_H$ (Spectrum 8): 6.9-7.29 (m, 4H, H-21, 22, 23, 25), 4.57 (t, 1H, J = 5.28 Hz, H-11), 4.5 (t, 2H, H-15), .4.05 (q, 1H, H-18), 3.0 (t, 2H, H-14), 2.79-2.41 (3 x m, 9H, 1, 2, 3, 5, 6, 7, 9, 10), 2.5 (d, 2H, H-26), 1.42 (AB-q, 2H, J = 10.5 Hz, H-4a,b), 1.33 (d, 3H, H-19), 1.7-1.9 (m, 1H, H-27),1.19 (2xd, 6H, H-28, 29); **MS** (EI, 70

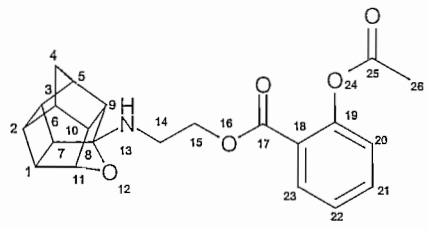
eV) *m/z* (spectrum 9): 408.03 [($M^+$)+1], 338.12, 250.17, 219.77; . **IR** (KBr) $\gamma_{max}$ (Spectrum 10): 3220, 2950, 2800, 1730, 1510, 1390, 1100, 820 $cm^{-1}$.

### 5.3.5 8,11-OXAPENTACYCLO[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]UNDECANE-8-(2-AMINOETHYL)-2-ACETYLOXY- BENZAMIDE (17)



***Synthetic method:*** O-Acetylsalicyloyl chloride (2 g, 10.06 mmol), was dissolved in dry dichloromethane (20 ml) and added drop wise to an excess 1,2 diaminoethane (7 ml, 100.69 mmol). The reaction mixture was stirred for 24 hours at room temperature. The solvent was removed under reduced pressure to yield a yellow oil. This was washed with saturated NaCl water (100 ml) and extracted with DCM (3 x 50 ml). The organic fraction was dried overnight with $MgSO_4$ and concentrated *in vacuo* to yield the amine derivative. Pentacyclo[5.4.0.0$^{2,6}$.0$^{3.10}$.0$^{5,9}$]undecane-8,11-dione (0.4 g, 2.25 mmol) was dissolved in tetrahydrofuran (30 ml) and cooled down to 5°C while stirring in an ice bath. The acetylsalicyl amine (0.5 g, 2.25 mmol) was added slowly, with continued stirring of the reaction mixture at 5°C. The reaction was allowed to reach completion for 30 min. Water was azeotropically removed from the reaction mixture by refluxing it in 60 ml dry benzene using a Dean-Stark apparatus for 1 h or until no more water collected in the trap. The excess benzene was removed under reduced pressure and the dark brown oil was dissolved in a mixture of anhydrous methanol (30 ml) and anhydrous THF (150 ml). A reduction was carried out by adding sodium borohydride (1.5 g) and stirring overnight at room temperature. The solvent was removed *in vacuo*, the residue was suspended in water (50 ml) and extracted with dichloromethane. The combined organic fractions were washed with water (2 x 100 ml), dried over anhydrous magnesium sulphate and evaporated *in vacuo* to yield a dark brown oil. Resolution of the product mixture by column chromatography (EtOAc:ETOH, 1:1) was incomplete and a mixture of amides were obtained (Yield: 100 mg,0.26 mmol,11.69%) and could not be purified further (spectra 11 – 14).

### 5.3.6 8,11-OXAPENTACYCLO[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]UNDECANE-8-(2-AMINOETHYL)-2-ACETYLOXYBENZOATE (12)



***Synthetic method***: 8-(2-Aminoethanol)-8,11-oxapentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane (**9**) (0.8 g, 4 mmol) was dissolved in dry dichloromethane ( 20 ml). A mixture of O-acetylsalicyloyl chloride (0.7 g, 4 mmol) and dry dichloromethane (10 ml) was added drop wise and the reaction mixture was stirred at room temperature for 48 hours. The solvent was removed *in vacou* to yield *a* light yellow wax. Resolution of the product mixture was accomplished by column chromatography (EtOAc: ETOH, 1:1, $R_f$ = 0.83, Yield: 450 mg, 1.18 mmol, 29.5%)

***Physical data:*** $C_{22}H_{23}NO_5$; **$^{13}$C NMR** (150 MHz, DMSO) $\delta_C$ (Spectrum 15): 172.01 (C-25), 162,49 (C-17), 130.18 (C-23/21), 126.22 (C-21/23), 119.90 (C-22), 115.89 (C-20), 66.21 (C-8), 64.95 (C-15), 54.29 (C-7/9), 46.24 (C-14), 44.82 (1C), 44.60 (C-4), 44.09 (1C), 42.42 (1C), 39.50 (1C), 39.22 (1C), 18.95 (C-26); **$^{1}$H NMR** (600 MHz, DMSO) $\delta_H$ (Spectrum 16): 7.0-8.1 (m, 4H, H-21, 22, 23, 25), 5.24 (s, 1H, H-13), 4.7 (t, 1H, J = 5.32, H-11), 4.5 (d, 2H, H-15), 3.01 (d, 2H, H-14), 2.5 (s, 3H, H-26), 2.24-2.8 (m, 8H, H-1, 2, 3, 5, 6, 7, 9, 10), 1.84 (AB-q, 2H, J = 9.42, H-4a,b); **MS** (EI, 70 eV) *m/z* (Spectrum 17): 381.89 ($M^+$), 339.82, 303.80, 261.78, 219.67, 207.73, 140.42; **IR** (KBr) $\gamma_{max}$ (Spectrum 18): 3200, 2980, 2900, 1740, 1680, 1495, 850, 700 cm$^{-1}$.

## 5.4 ACQUISITION OF BLOOD-BRAIN BARRIER PERMEABILITY DATA *(IN VIVO)*

The test model developed by Zah *et al.* 2003 was applied to determine the BBB permeability of the synthesised compounds (**11**, **12**, **15**, and **17**). Adult male C57BL/6 laboratory mice (25-30 g) were used to compare the CNS delivery of the prodrugs to that of the free drugs. This involved the intraperitoneal injection of the test compounds, followed by decapitation at selective time intervals: 0, 15, 30 and 60 min, where after whole blood and brain samples were collected, frozen with liquid nitrogen and kept at -77°C.

### 5.4.1 CHEMICALS

Analytical grade acetonitrile (multisolvent) was purchased from Merck (Darmstadt, Germany) and orthophosphoric acid from Saarchem (Muldersdrift, South-Africa). Diethyleter for analysis and hydrochloric acid (32%) were purchased from Saarchem (Muldersdrift, South-Africa). Throughout sample preparation, double-distilled, de-ionised water was used.

### 5.4.2 ANIMALS

Three month old C57BL/6 laboratory mice (25-30 g) were used to conduct the biological evaluation. All animals were maintained under controlled laboratory conditions at a temperature of 21 ± 0.5°C, and a relative humidity of 50 ± 5%. Free access to food and water was allowed at all times. Approval for the performance of this study was obtained from the Ethics Committee for Research on Experimental Animals of the North-West University (Potchefstroom campus). Evaluation considerations were based on "Guidelines on Ethics for Medical Research" Revised Edition, 1993, South African Medical Research Council.

### 5.4.3 ADMINISTRATION AND TISSUE HARVESTING

As a result of toxicity problems encountered for the amide compounds **15** and **17,** only the ester compounds **11** and **12** were used. Acetylsalicylic acid, ibuprofen and compounds **11** and **12** were dissolved in a solvent of 40% (v/v) ethanol in water for injection. Each animal's body mass was noted where after the freshly constituted test preparations (38 mg/ml; ± 0.3ml) were administered intraperitoneally. To obtain similar molar ratios of free NSAID in relation to the relevant cage compounds, the following doses were administered: 150 mg/kg of acetylsalicylic acid and ibuprofen, 377 mg/kg (0.925 mmol) of compound **11** and 386 mg/kg (1.012 mmol) of compound **12**. After administration of the relevant compound, the test animals were allowed free movement and *ad lib* access to water and food.

Animals were promptly sacrificed at selective time intervals by means of decapitation. Whole brain samples were collected by cutting open the craniums and removing the brains. Blood samples were collected from the exposed aorta. Both brain and blood samples were collected in pre-weighed open-topped 15 ml Pyrex® vials and 4 ml 7.2 mg K3E anticoagulant–containing BD Vacutainer™ vials respectively. Samples were frozen with liquid nitrogen and kept at -77°C until analysed.

### 5.4.4 STANDARISATION AND ANALYTE RECOVERY

Each drug/test compound was administered in four time groups: 0 min, 15 min, 30 min and 60 min, with each time group consisting of 8 mice. In order to determine the extraction efficiency, control samples were prepared. Stock solutions were prepared containing 10 mg

of ibuprofen and salicylic acid in 100 ml ethanol. The stock solutions were diluted where after 100 $\mu$l freshly obtained plasma was spiked with 50 $\mu$l of the different dilutions, giving concentrations of 10 $\mu$g/ml, 20 $\mu$g/ml, 30 $\mu$g/ml, 40 $\mu$g/ml and 50 $\mu$g/ml. The normal extraction procedure, as for the injected groups, was followed and the tests were repeated three times. The given standard concentration solution of the free drugs were injected onto the HPLC column (section 5.4.6). This enabled cross reference of experimental curves with standard curves when test compounds were administered to the mice, thus giving the ability to determine if the test compound or metabolites permeated the blood-brain barrier. The percentage recovery was determined by comparing the peak area obtained from an extracted sample to that of an equivalent pure sample concentration (unextracted) representing 100% recovery. The percentage recovery was found to be approximately 51%.

#### *5.4.4.1 BRAIN TISSUE EXTRACTION*

Sample weight was recorded, where after HCl (10 %; 100 $\mu$l) was added to each vial and the tissue homogenised using a Polytron® tissumiser. Cross-contamination was prevented by rinsing the rotator head with ethanol followed by water between applications. The acidic homogenates were then transferred to 2 ml Eppendorf® vials, using 8 vials for each of the four groups, resulting in a total of 32 vials, each consisting of homogenate in their own specific time interval: 0 min, 15 min, 30 min and 60 min. 1000 $\mu$l diethyleter was added to each vial and the mixtures were vortexed for 20 s. Following centrifugation (10 min. at 10 000 x g; 4°C), the supernatants were frozen with liquid nitrogen and the organic fractions brought to dryness under a mild stream of nitrogen gas. A double extraction was performed on the supernatants using 1000 $\mu$l diethyleter, followed by the same vortex and centrifugation method used in the first extraction. The organic residue was re-dissolved in a solvent (200 $\mu$l) composed of ethanol:water; 60:40 and HPLC analyses was carried out. For the HPLC analysis the mobile phase consisted of 65% acetonitrile and 35% of 0.1% $H_3PO_4$ in water at a flow rate of 1ml/min.

#### *5.4.4.2 BLOOD EXTRACTION*

After recording sample weight, HCl (10%; 100 $\mu$l) was added to each vial in the four selected time intervals, containing blood samples of the corresponding brain samples. 1000 $\mu$l diethyleter was added to each vial and the mixtures were vortexed for 20 s. Following centrifugation (10 min. at 10 000 x g; 4°C), the supernatants were frozen with liquid nitrogen and the organic fractions brought to dryness under a mild stream of nitrogen gas. A double extraction was performed on the supernatants using 1000 $\mu$l diethyleter followed by the same vortex and centrifugation method used in the first extraction. The organic residue was re-dissolved in a solvent (200 $\mu$l) composed of ethanol:water; 60:40 and HPLC analyses

was carried out. The mobile phase was exactly the same as that for the brain tissue extraction.

### 5.4.5 INSTRUMENTATION AND ANALYSIS

An Agilent 1100 series, HPLC (Agilent Technologies, Palo Alto, <A), equipped with a vacuum degasser, gradient pump, auto sampler and diode array UV detector was used to analyse the samples. ChemStation software (Rev. A.08.03) was used for data acquisition and analysis. Chromatograms were registered at 210 nm with 4 nm bandwidth. The samples were separated on a reversed-phase Eclipse XDB C18 column (150 mm x 4.6 mm, 5 $\mu$m particle size, Agilent Technologies, Palo Alto, United States of America ) using gradient elution. The method was based on a gradient (Table 5.1); solvent A consisted of milliQ water, with 0.1 % $H_3PO_4$ and solvent B consisted of acetonitrile, flow rate was 1 ml/min, the injection volume was 20 $\mu$l and the run time was 15 minutes.

**Table 5.1**: The solvent gradient that was applied for chromatography

| TIME (min) | %A<br>$H_3PO_4$ | %B<br>Acetonitrile |
|---|---|---|
| 0 | 65 | 35 |
| 5 | 0 | 100 |
| 10 | 0 | 100 |
| 10.10 | 65 | 35 |

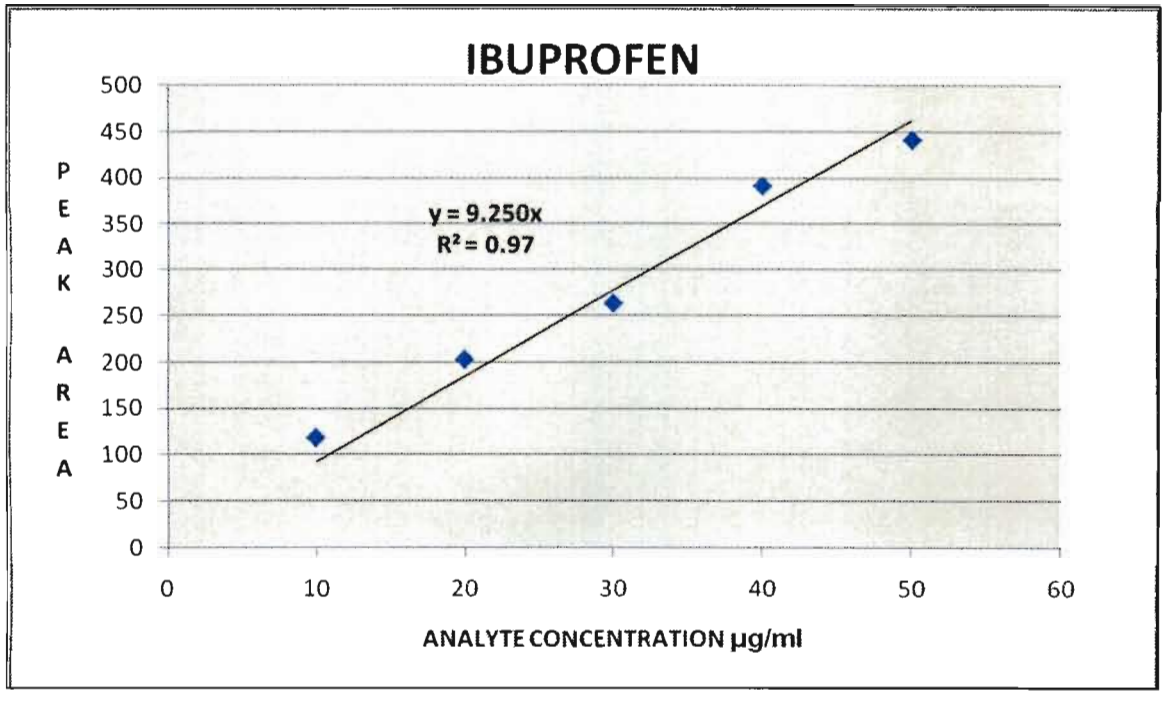
Statistical analysis of the obtained data was done using the Student-Newman-Keuls multiple range test. GraphPad InStat 2.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Office Excel 2003, SP3 were used. Dunnet's one-way analysis of variance (ANOVA) with post-test was performed on all the tested concentrations, to indicate significant differences ( $^{*}$ $p < 0.05$ was considered to be statistically significant, $^{**}$ $p < 0.01$ was considered to be statistically very significant and $^{***}$ $p < 0.001$ was considered as statistically extremely significant).

### 5.4.6 RESULTS

As described in the previous chapter, HPLC analytical procedures were applied to determine the blood-brain barrier permeability of the novel synthesised prodrugs. The single most important parameter that was measured was therefore the brain to blood concentration ratio for the ester compounds. HPLC results were primarily used to determine the amount of salicylic acid (the main acetylsalicylic acid metabolite) and ibuprofen observed within blood and brain samples of mice injected with the test compounds.

#### *5.4.6.1 IBUPROFEN AND IBUPROFEN PRODRUG (11)*

Stock solutions of the test compound were prepared in analytical grade ethanol and were used to draw a calibration curve of ibuprofen. This was achieved by preparing five different concentrations, by diluting the relevant stock solution, resulting in concentrations of 10, 20, 30, 40 and 50 $\mu$g/ml. In figure 5.4 the calibration curve for the HPLC analysis of ibuprofen, is illustrated. Ibuprofen prodrug was rapidly metabolised therefore only free ibuprofen peaks were observed in the brain and in the blood (Annexure B: Chromatogram 1, 2 and 3).




| ***ANALYTE CONCENTRATION*** ***(µg/ml)*** | ***ANALYTE PEAK AREA*** |
|---|---|
| 10 | 118.5 |
| 20 | 203.1 |
| 30 | 263.2 |
| 40 | 391.6 |
| 50 | 441.4 |

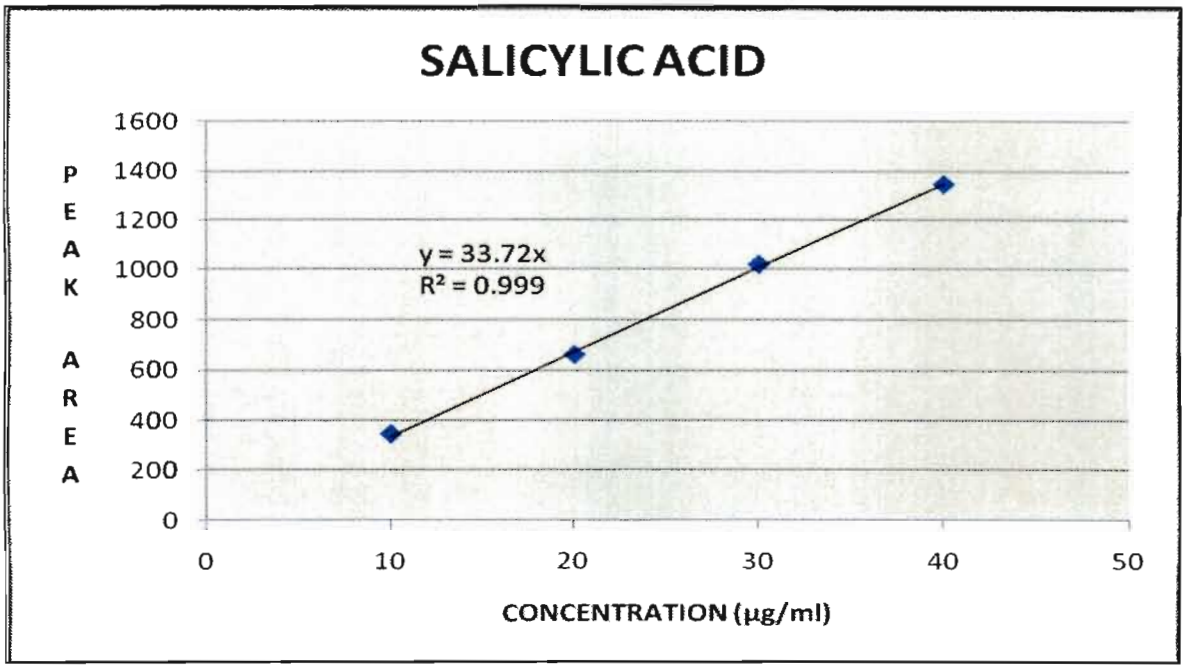
**Figure 5.4**: Calibration curve for ibuprofen.

**Table 5.2:** Ibuprofen in ibuprofen prodrug vs. ibuprofen – BRAIN: BLOOD concentration ratio at selective time intervals.

| IBE *(AUC)* | | | | IB *(AUC)* | | | |
|---|---|---|---|---|---|---|---|
| *Time(min)* | *[BLOOD]* | *[BRAIN]* | *RATIO* | *Time(min)* | *[BLOOD]* | *[BRAIN]* | *RATIO* |
| 15 | 345.6 | 84.92 | **0.25** | 15 | 24.85 | 3.95 | **0.16** |
| 30 | 266.37 | 96.25 | **0.36** | 30 | 13.23 | 2.42 | **0.18** |
| 60 | 168.79 | 110.62 | **0.66** | 60 | 1.82 | 1.72 | **0.95** |

### *5.4.6.2 ACETYLSALICYLIC ACID AND ACETYLSALICYLIC PRODRUG (12)*

Stock solutions of the test compound were prepared in analytical grade ethanol and were used to obtain a calibration curve of salicylic acid. This was achieved by preparing four different concentrations, by diluting the relevant stock solution, resulting in concentrations of 10, 20, 30, 40 $\mu$g/ml. In figure 5.5 the calibration curve for the HPLC analysis of salicylic acid, is illustrated. The acetylsalicylic acid prodrug as well as the free acetylsalicylic acid was rapidly metabolised. Only salicylic acid peaks were observed in the brain and in the blood (Annexure B: Chromatogram 4, 5 and 6).




| ***ANALYTE CONCENTRATION (µg/ml)*** | ***ANALYTE PEAK AREA*** |
|---|---|
| 10 | 346.52 |
| 20 | 661.5 |
| 30 | 1021.29 |
| 40 | 1346.1 |

**Figure 5.5**: Calibration curve for salicylic acid.

**Table 5.3:** Salicylic acid in acetylsalicylic acid prodrug VS salicylic acid – BRAIN:BLOOD concentration ratio in selective time intervals.

| ASE (AUC) | | | | SALI (AUC) | | | |
|---|---|---|---|---|---|---|---|
| *Time(min)* | *[BLOOD]* | *[BRAIN]* | *RATIO* | *Time(min)* | *[BLOOD]* | *[BRAIN]* | *RATIO* |
| 15 | 183.42 | 44.14 | **0.24** | 15 | 113.54 | 24.55 | **0.22** |
| 30 | 102.34 | 42.54 | **0.42** | 30 | 103.9 | 25.72 | **0.25** |
| 60 | 66.49 | 54.52 | **0.82** | 60 | 80.77 | 23.21 | **0.29** |

## 5.5 LIPID PEROXIDATION STUDY (*IN VITRO*)

### 5.5.1 ASSAY PROCEDURE

The thiobarbituric acid assay (TBA test) is based on the formation of a red adduct during a reaction between two molecules of TBA and one molecule of malondialdehyde (MDA), a colourless end product of lipid peroxide decomposition. This red adduct (MDA-TBA complex) has an absorption maximum at 532 nm (Janero, 1990).

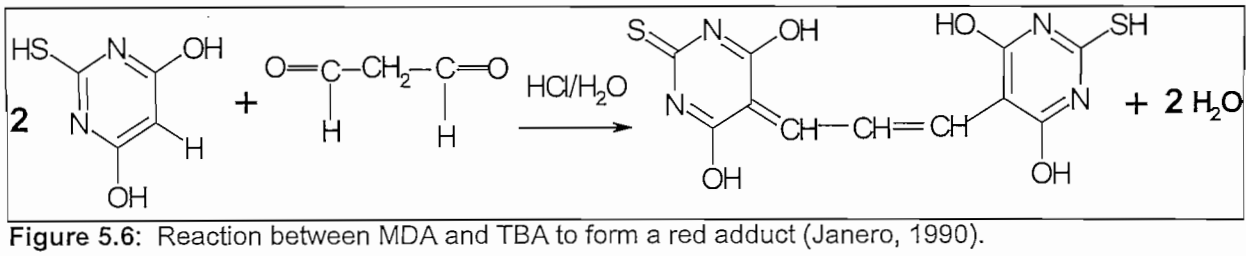



**Figure 5.6:** Reaction between MDA and TBA to form a red adduct (Janero, 1990).

The TBA test has however been widely criticised because of low efficiency of fatty acid hyperoxide breakdown to MDA (Janero & Bughardt, 1988). Keeping this in mind, the TBA test was performed with a few modifications to improve its efficacy: Male Sprague-Dawley rats, weighing approximately 200-250 g, were decapitated and the brains rapidly excised and homogenised with 0.1 M phosphate-buffered saline (PBS), pH 7.4, so as to give a final concentration of 10% w/v. This is necessary to prevent lysosomal damage of the tissue.

Generally, lipid peroxidation proceeds through the reduction of iron (Minotti & Aust, 1989) and therefore a system containing $H_2O_2$, $FeCl_3$ and ascorbic acid (vitamin C) was used to produce the necessary hydroxyl radicals through the Fenton reaction. This system allowed the constant regeneration of the ferrous ($Fe^{2+}$) ions in a reaction in which the ferric ($Fe^{3+}$) ions are reduced by ascorbic acid. Although ascorbic acid can act as a scavenging antioxidant and, in this capacity, is known to protect plasma lipids and regenerate vitamin E from its free radical form (Frei, 1991), it acts as a pro-oxidant in the presence of free iron, as is the case here (Auroma,1994).

Animal material usually contains large amounts of protein, to which MDA may be bound (Draper & Hadley, 1990). It is therefore very important to release the protein-bound MDA, which was accomplished by hot acid hydrolysis (pH 2-3) using trichloroacetic acid (TCA). This allowed for the formation of the MDA-TBA complex as well as the release of protein bound MDA. Butylated hydroxytoluene (BHT) was used in order to prevent artificial lipid peroxidation by tissue processing and lipid oxidation in the TBA test. Reaction temperatures were kept at 37 °C to minimise the decomposition of lipid hydroperoxides.

The final modified method used, was as follows: Rats were decapitated and their brains homogenised. The test tubes were then prepared as described in table 5.2 (**A** = toxin, **B** = positive control (Trolox + toxin) and **C** = negative control (ethanol + toxin)), where after every test tube was vortexed for approximately 60 seconds. Incubation was carried out at 37°C for 1 hour in an oscillating water bath. The test tubes were centrifuged for 20 minutes at 2000 x g and the supernatants decanted into new tubes. 125 $\mu$l BHT (to stop the reaction), 250 $\mu$l TCA (precipitation of macromolecules such as proteins, DNA) and 125 $\mu$l of a 33% TBA solution (colour reaction), were then added to every test tube and vortexed for 60 seconds. The test tubes were incubated for 1 hour at 60 °C in an oscillating water bath and thereafter they were cooled down using an ice bath. 500 $\mu$l butanol was then added to every test tube and vortexed for 60 seconds. Centrifugation of the samples for 10 minutes at 2000 x g was followed by measuring the absorbancies at 532 nm with butanol as blank.

Statistical analysis of the obtained data was employed using the Student-Newman-Keuls multiple range test.

**Table 5.4**: Sample preparation data.

| Test Tubes (μl) | 10 % Homogenate (μl) | PBS (μl) | $H_2O_2$ (μl) | $FeCl_3$ (μl) | Ascorbic acid (μl) | Trolox 10mM (μl) | EtOH (μl) | Drug (μl) |
|---|---|---|---|---|---|---|---|---|
| Control | 160 | 40 | | | | | | |
| **A** | 160 | 20 | 10 | 5 | 5 | | | |
| **B** | 160 | | 10 | 5 | 5 | 20 | | |
| **C** | 160 | | 10 | 5 | 5 | | 20 | |
| **D** (Acetylsalicylic acid) | 160 | | 10 | 5 | 5 | | | |
| **E** ( Ibuprofen) | 160 | | 10 | 5 | 5 | | | |
| **F** (Compound **11**) | | | | | | | | |
| IBE 0.4 mM | 160 | | 10 | 5 | 5 | | | 20 |
| IBE 0.2 mM | 160 | | 10 | 5 | 5 | | | 20 |
| IBE 0.1 mM | 160 | | 10 | 5 | 5 | | | 20 |
| **G** (Compound **12**) | | | | | | | | |
| ASE 0.4 mM | 160 | | 10 | 5 | 5 | | | 20 |
| ASE 0.2 mM | 160 | | 10 | 5 | 5 | | | 20 |
| ASE 0.1 mM | 160 | | 10 | 5 | 5 | | | 20 |

## 5.5.2 RESULTS

### *5.5.2.1 STATISTICAL ANALYSIS*

All the data are presented as means ± SEM. Data analysis was carried out using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple range test. The level of significance was accepted a $p < 0.05$.

The final results are expressed as nmoles MDA/mg tissue, which gives an indication of the concentration MDA produced in the presence (and absence) of the test compounds. From the results it is clearly evident that compounds **11** and **12** both marginally exhibit antioxidant capacity in the presence of the toxin ($H_2O_2$ + $FeCl_3$ + ascorbic acid) (fig 5.7), but is not significant.

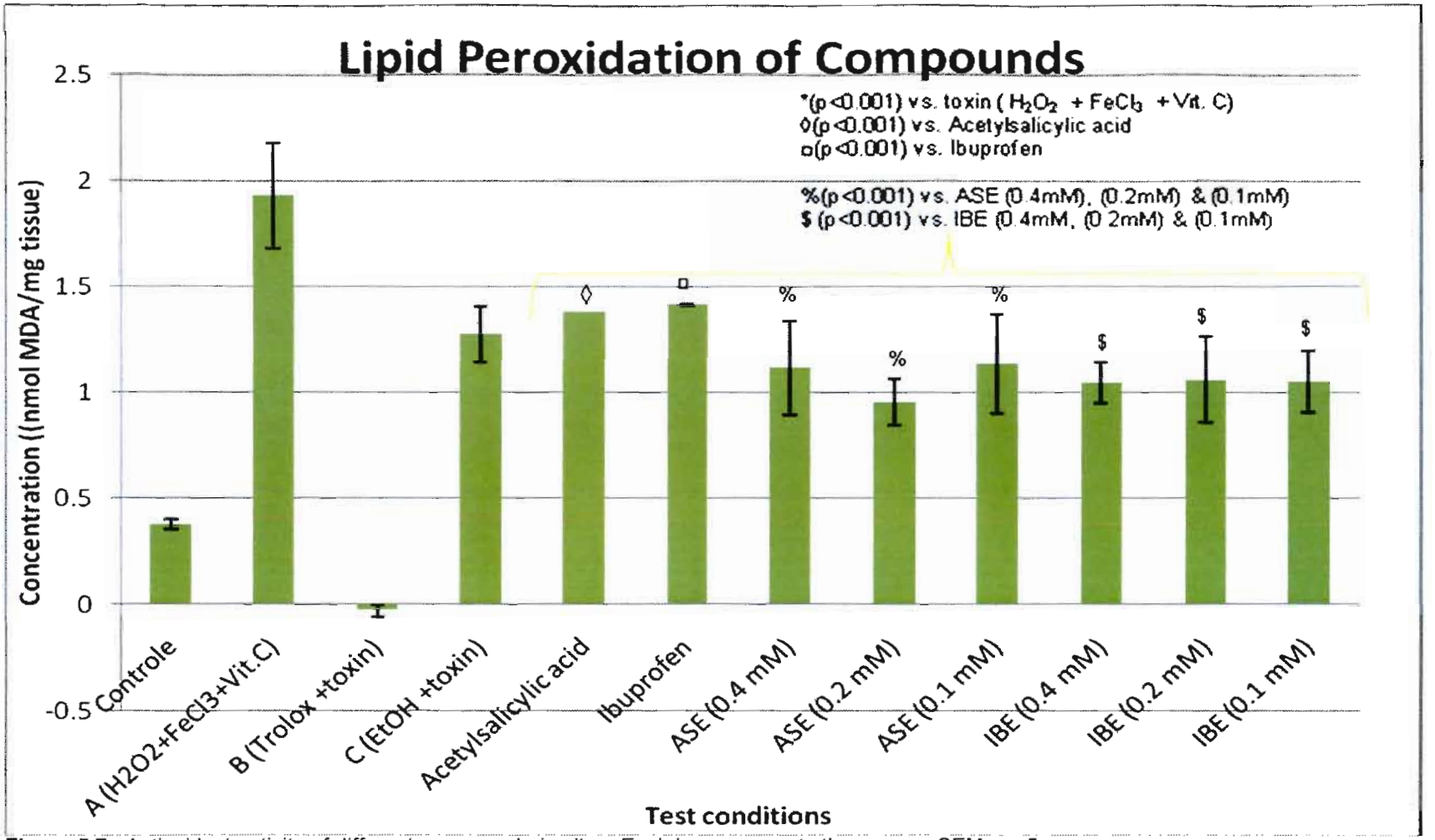



**Figure 5.7:** Antioxidant activity of different compounds *in vitro*. Each bar represents the mean ± SEM: n=5.

## 5.6 PHYSICOCHEMICAL ATTRIBUTES GOVERNING BBB PERMEABILITY

### 5.6.1 METHOD TO DETERMINE PHYSICOCHEMICAL CHARACTERISTICS

Theoretically determination of the physicochemical characteristics was computed using the LogD, Log P and Pka suite of ACD® version 4.0 software for Microsoft Windows®.

### 5.6.2 RESULTS

The theoretically determined physicochemical characteristics of compounds **11**, **12**, **15** and **17**.

**Table 5.5**: Physicochemical characteristics needed for BBB permeation (section 2.3).

| Compound | Molecular weight (Da) | Log p | Log D (pH 7.4) | Pka |
|---|---|---|---|---|
| 11 | 435.61 | 3.96 ± 0.40 | 4.07 | 5.47 ± 0.20 |
| 12 | 381.43 | 1.24 ± 0.41 | 1.24 | 5.37 ± 0.20 |
| 15 | 378.52 | 2.62 ±0.47 | 2.80 | 15.35 ± 0.46 |
| 17 | 380.45 | -0.09 ± 0.48 | -0.12 | 13.94 ±0.46 |

# CHAPTER 6

# DISCUSSION AND CONCLUSION

## 6.1 SYNTHESIS

Pentacyclo[5.4.0.0$^{2,6}$.0$^{3,10}$.0$^{5,9}$]undecane-8,11-dione, was conjugated to 2-aminoethanol by means of reductive amination. This gave a pentacycloundecylamine with a sterically free linker section. In the relevant compounds, the hydroxyl group on the linker was conjugated to the benzoic acid moiety to yield the respective ester prodrugs. This esterfication was done using the activation agent, 1-ethyl-3-(3'-dimethylamino)carbodiimide (EDC) to activate the benzoic acid group on ibuprofen and by using the commercially available acid chloride derivative of acetylsalicylic acid. Amide prodrug syntheses were done by conjugating the NSAIDs to 1,2-diaminoethane by means of Fischer-esterfication and aminolysis reactions. Since the compounds are reported for the first time, thorough structure elucidation was necessary. This was done using one dimensional nuclear magnetic resonance (NMR), infrared (IR) absorption spectroscopy and mass spectrometry (MS).

## 6.2 BLOOD-BRAIN BARRIER PERMEABILITY DATA *(IN VIVO)*

### 6.2.1 IBUPROFEN AND IBUPROFEN PRODRUG (11)

From the results obtained the blood-brain barrier permeability of the test compounds and more importantly, the extent to which the prodrug conjugates improved the CNS delivery of the free drugs were calculated. After 60 minutes free ibuprofen presented with a blood concentration of 1.82 $\mu$g/ml and a brain concentration of 1.72 $\mu$g/ml . Once conjugated to the pentacycloundecane structure to produce compound **11**, blood and brain concentrations for ibuprofen, after 60 min, increased to 168.79 $\mu$g/ml and 110.62 ug/ml indicating much higher concentrations of ibuprofen in both the CNS and blood. This is indicative of significantly enhanced absorption from the intestines after intraperitoneal administration. In Figure 6.1 the relative blood and brain concentrations ($\mu$g/ml), in the selected time intervals, are illustrated by comparing free ibuprofen to ibuprofen in compound **11.**

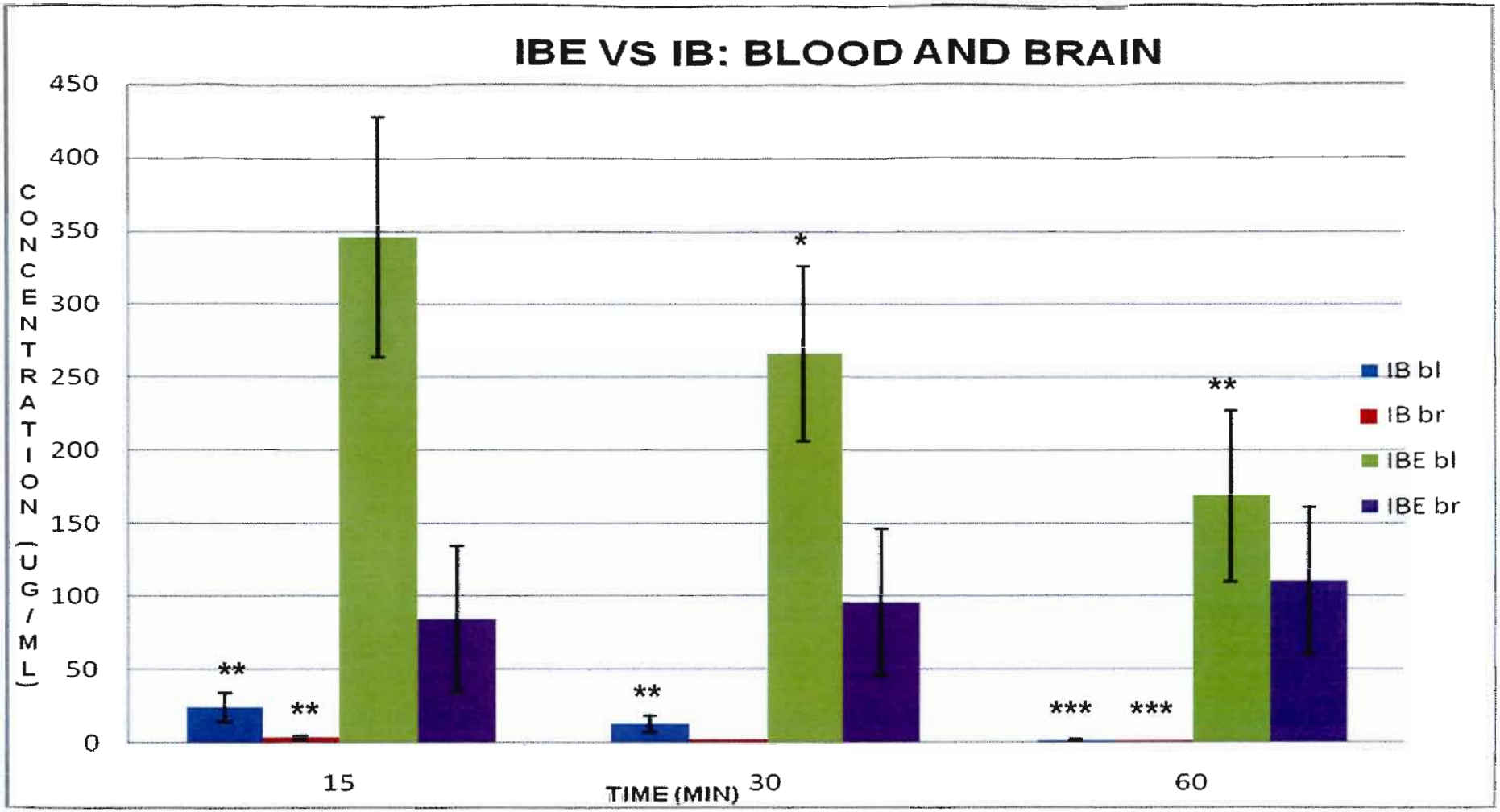



**Figure 6.1:** Histogram of Ibuprofen in Ibuprofen prodrug VS Ibuprofen in blood and brain at selective time intervals (IB = Ibuprofen, IBE = Ibuprofen in Ibuprofen prodrug, bl = blood, br = brain). ($^{*}$ $p < 0.05$ were considered to be statistically significant, $^{**}$ $p < 0.01$ were considered to be statistically very significant and $^{***}$ $p < 0.001$ were considered as statistically extremely significant).

### 6.2.2 ACETYLSALICYLIC ACID AND ACETYLSALICYLIC PRODRUG (12)

After 60 minutes salicylic acid presented with a blood and brain concentration of 80.77 $\mu$g/ml and 23.21 $\mu$g/ml respectively. After administration of compound **12**, the acetylsalicylate ester prodrug, the free drug was observed in concentrations of 66.49 $\mu$g/ml and 54.52 $\mu$g/ml respectively. The salicylate observed after prodrug administration was thus at about twice the concentration in the brain when compared to the free drug administration. This indicated favourable blood-brain barrier permeability for compound **12**. Initial absorption also seems to be enhanced for the prodrug. In Figure 6.2 the relative blood and brain concentrations ($\mu$g/ml), at selected time intervals, are illustrated by comparing free salicylic acid to salicylic acid in compound **12**.

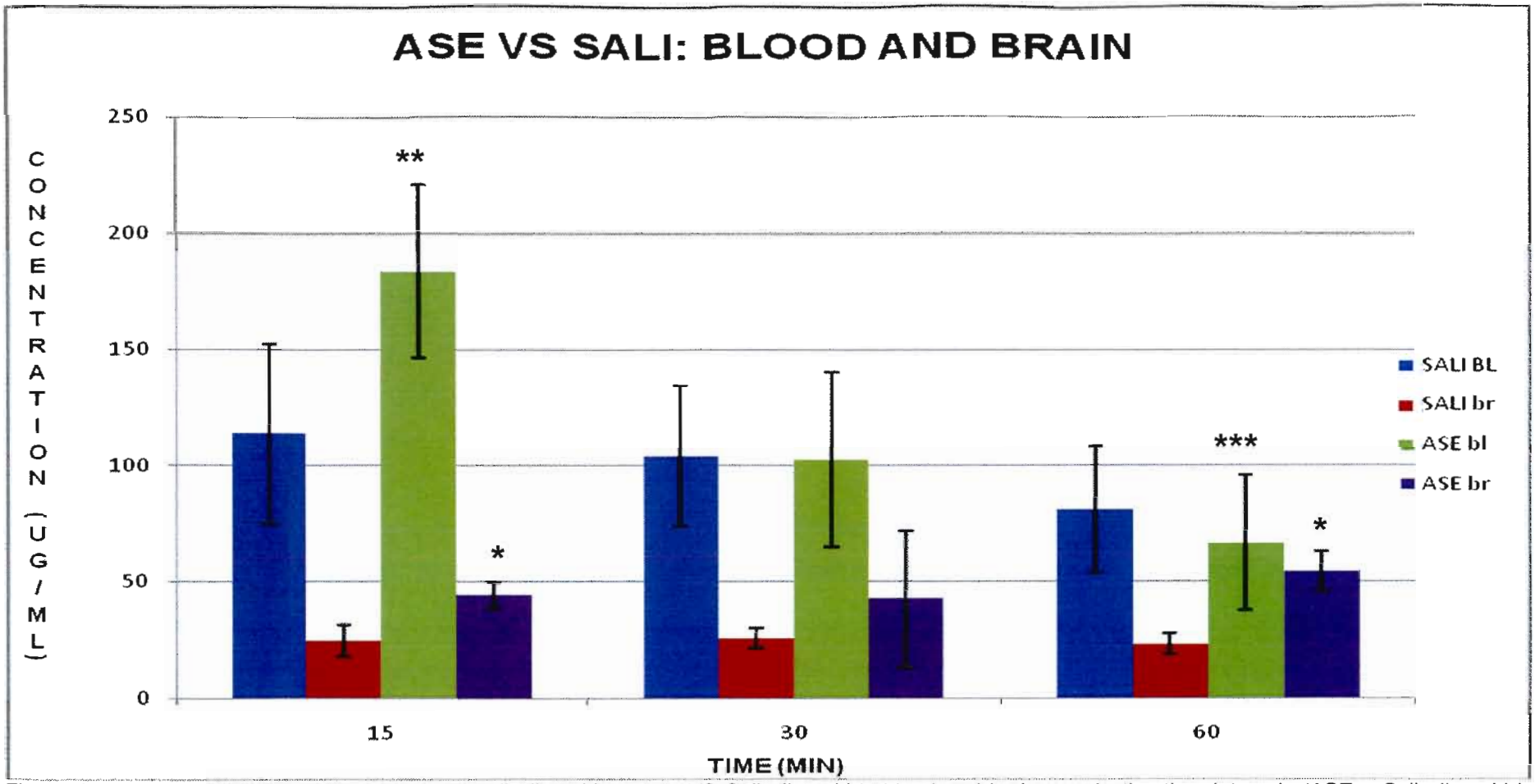



**Figure 6.2**: Histogram of Salicylic acid in Acetylsalicylic acid prodrug VS Salicylic acid in blood and brain at selective time intervals (ASE = Salicylic acid in Acetylsalicylic acid prodrug, SALI = Salicylic acid, bl = blood, br = brain). ($^{*}p < 0.05$ were considered to be statistically significant, $^{**}p < 0.01$ were considered to be statistically very significant and $^{***}p < 0.001$ were considered as statistically extremely significant).

### 6.2.3 FINAL REMARKS

From the obtained data it is thus concluded that the conjugation of ibuprofen and acetylsalicylic acid to the pentacycloundecane structure (compound **11** and **12**), through esterfication, substantially increased their absorption after IP administration. Blood-brain barrier permeability and thus attainable brain concentrations of the free drug was also significantly improved, especially for the acetylsalicylate prodrug. Brain localisation and hydrolysis of the two ester prodrugs probably led to the increased ibuprofen and especially salicylic acid concentrations with time in the brain (fig 6.1 and 6.2). Once in the brain and after hydrolysis of the prodrug, the free acid drugs are too hydrophilic to permeate back to the systemic circulation, thus explaining the increase of the free drugs in the brain over time. The prodrugs thus not only increased the absorption and delivery of the free drugs into the CNS, but also significantly increased the brain to blood concentration ratio, a function attributed to localisation of the drug in the brain (table 5.2 and 5.3). A study done by Prins *et al.*, (2009) with both ibuprofen and acetylsalicylic acid prodrugs, indicated favourable distribution of the prodrugs as well as the free drugs into the brain. The cage moiety and chemical linkers used in this study afforded a slow metabolism of the prodrug resulting in trace amounts of both prodrugs still available in the brain and blood samples.

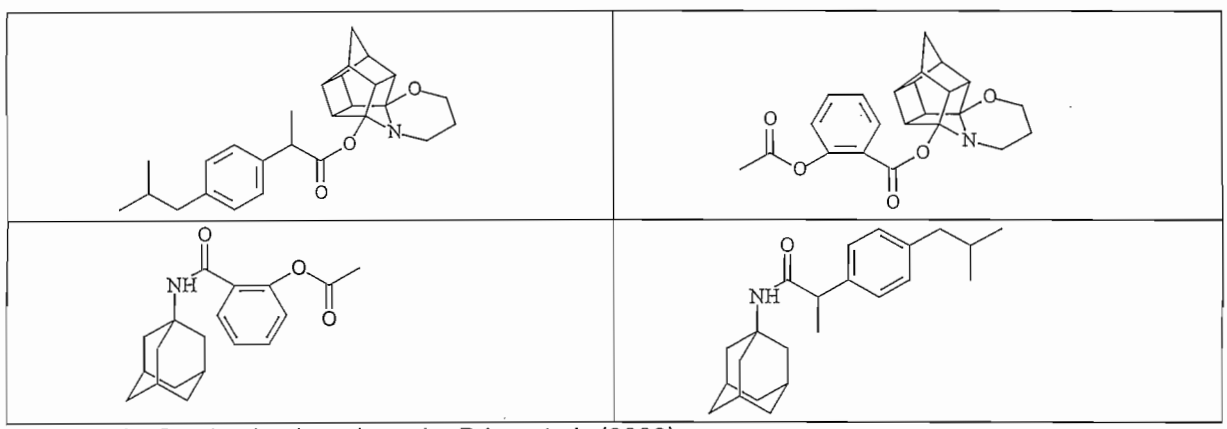


**Figure 6.3**: Synthesised prodrugs by Prins *et al.* (2009).

## 6.3 LIPID PEROXIDATION ASSAY (*IN VITRO*)

Using the results obtained it is concluded that the novel synthesised prodrugs as well as the free drugs, marginally exhibit attenuation of lipid peroxidation when compared to the toxin (all presenting with $p<0.001$). Ibuprofen and acetylsalicylic acid's abilities to attenuate lipid peroxidation are increased by their ester prodrug forms (all having $p$ values of $<0.001$).

From the results of the study the notion that both ibuprofen and acetylsalicylic acid possess certain antioxidant ability, was confirmed. A further conclusion drawn from this study is that the synthesis of ibuprofen and acetylsalicylic acid prodrugs through esterfication with the

pentacylcoundecane cage structure increased the free drug's abilities to marginally attenuate lipid peroxidation (fig 5.7).

The modified TBA assay in this study measured the amount of MDA as an index of lipid peroxidation. From the results it is clear that all of the test compounds possess antioxidant properties in the presence of the Toxin ($H_2O_2$, $FeCl_3$ and Vitamin C) system but shows to be non significant. The study results also indicate that the novel synthesized prodrugs does not positively influenced the antioxidant activity of the free, unconjugated drugs.

## 6.4 PHYSICOCHEMICAL ATTRIBUTES GOVERNING BBB PERMEABILITY

Compounds **11**, **12**, **15** and **17** all present with Molecular weights less than 500 Da as well as log P and Log D (physiologigal pH of 7.4) values less than 5, except for compound **17** that present with a negative Log D value and will thus cross the blood-brain barrier to a lesser extent. All four of the synthesised compounds should show favourable blood-brain barrier permeability according to the physicochemical characteristics needed (section 2.3).

## 6.5 CONCLUSION

Ester and amide prodrugs of ibuprofen and acetylsalicylic acid were synthesised, resulting in four novel polycyclic compounds. The low yields were attributed to the formation of various by-products during the synthetic procedure as well as inadequate purification of product mixture during column chromatography. Yields could be improved through optimisation of synthetic procedures and purification techniques as well as further purification of remaining fractions of product mixture after completion of column chromatography. Characteristic signals observed for each specific compound, gave confirmation of their structure. Only two of the four synthesised prodrugs were subjected to both blood-brain barrier permeability (*in vivo*) and lipid peroxidation (*in vitro*) assays as the two synthesised amide prodrugs were obtained as mixtures and were also observed to be toxic (amide prodrugs) during the *in vivo* assay. The purpose of this study was to enhance the blood-brain barrier permeability of certain neuroprotective NSAIDs. This was done by applying polycyclic cage compounds as carrier molecules for the NSAIDs were after *in vivo* blood-brain barrier permeability as well as *in vitro* lipid peroxidation assays were performed. Results obtained indicated that conjugation of ibuprofen and acetylsalicylic acid to the pentacycloundecane structure was not only possible, but that it led to a significant increase in their absorption and brain concentrations. The formation of the acetylsalicylate conjugate also resulted in a marked increase in blood-brain barrier permeability and thus brain-to-blood ratio. Further conclusions drawn from this study was that the conjugates also marginally increased the ability of the drugs to attenuate lipid peroxidation but shows to be non-significant and that

the compounds should show favourable blood-brain barrier permeability according to their physicochemical characteristics. The novel synthesised prodrugs thus present with higher concentrations in the brain and the antioxidant activity of the free NSAIDs were increased. The amide prodrugs (compounds **15** and **17**) were not obtained in sufficient purity and the mixtures proved to be toxic in the *in vivo* assay. This may be as a result of ethylenediamine (compound **13**) traces in the compounds or from the hydrolysis of the amide. It was therefore decided to leave the amide prodrugs out of consideration when both the *in vivo* and *in vitro* assays were performed. This aspect will have to be looked at when future studies are conducted. A possible solution might be to optimise the purification step during the synthesis of the compounds. The research done in this study gave additional insight into the attenuation of neurodegenerative disorders, such as Parkinson's and Alzheimer's disease, and the compounds synthesised could be useful leads in further drug design. Research may now be focused on improving and optimising the biological test model as well as the analytical detection procedures used.

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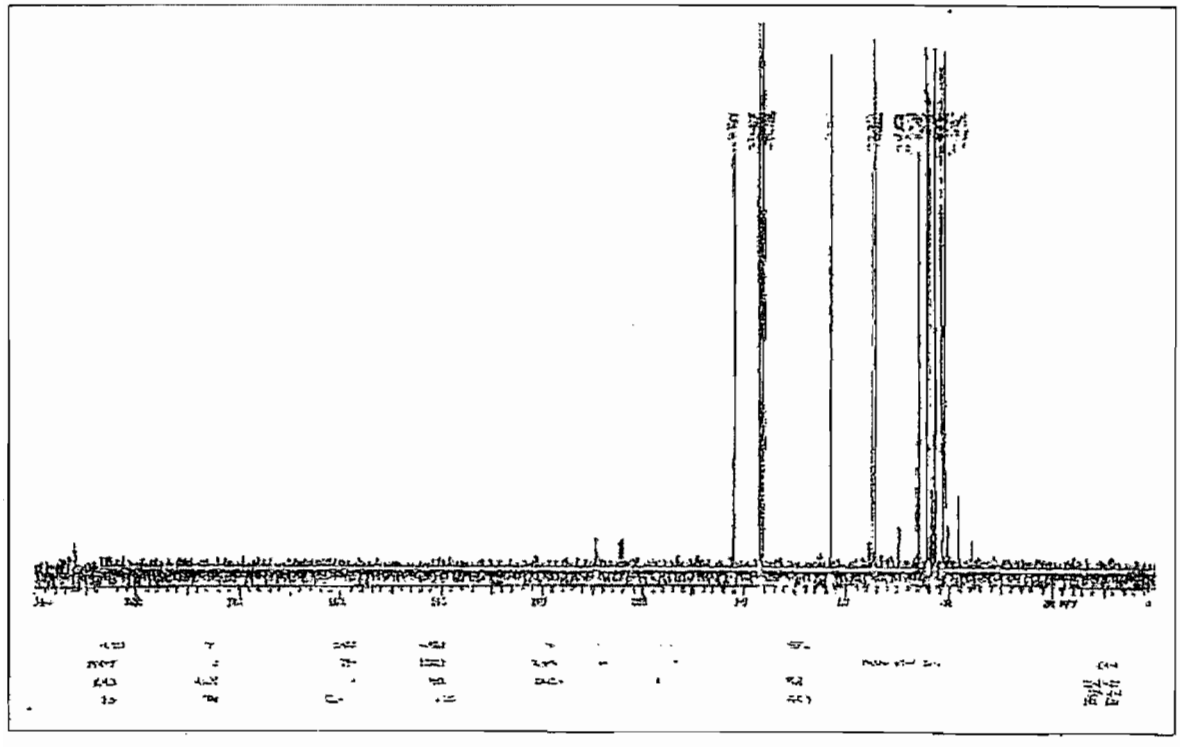
# ANNEXURE A

## SPECTRAL DATA:

*$^{1}H$,$^{13}C$, NMR, DEPT, MS, IR:*

## SPECTRUM 1:



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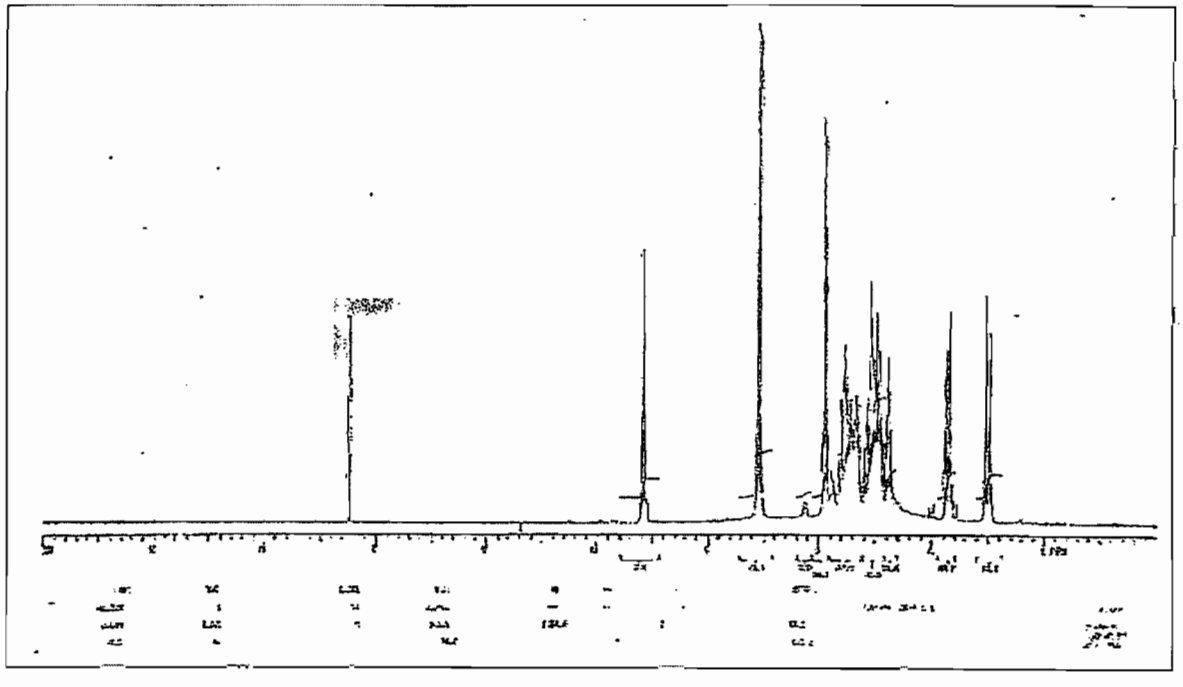

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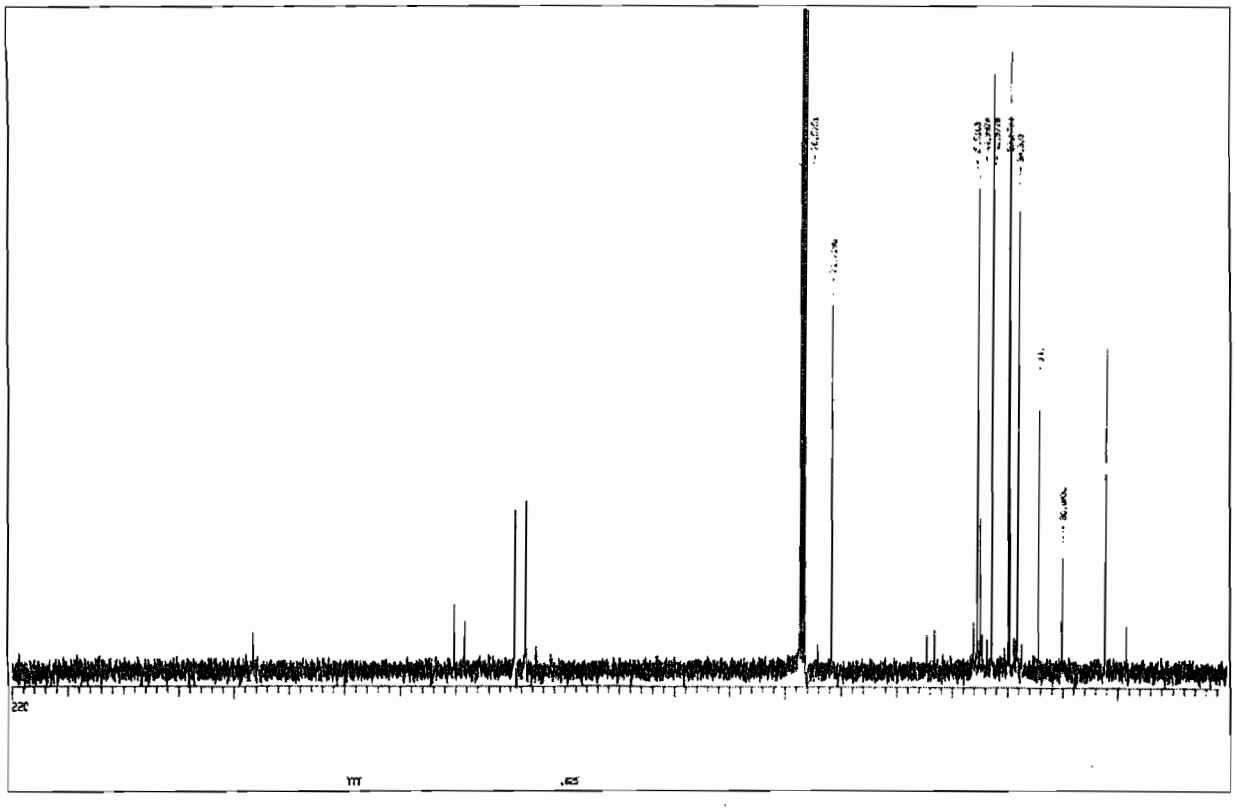

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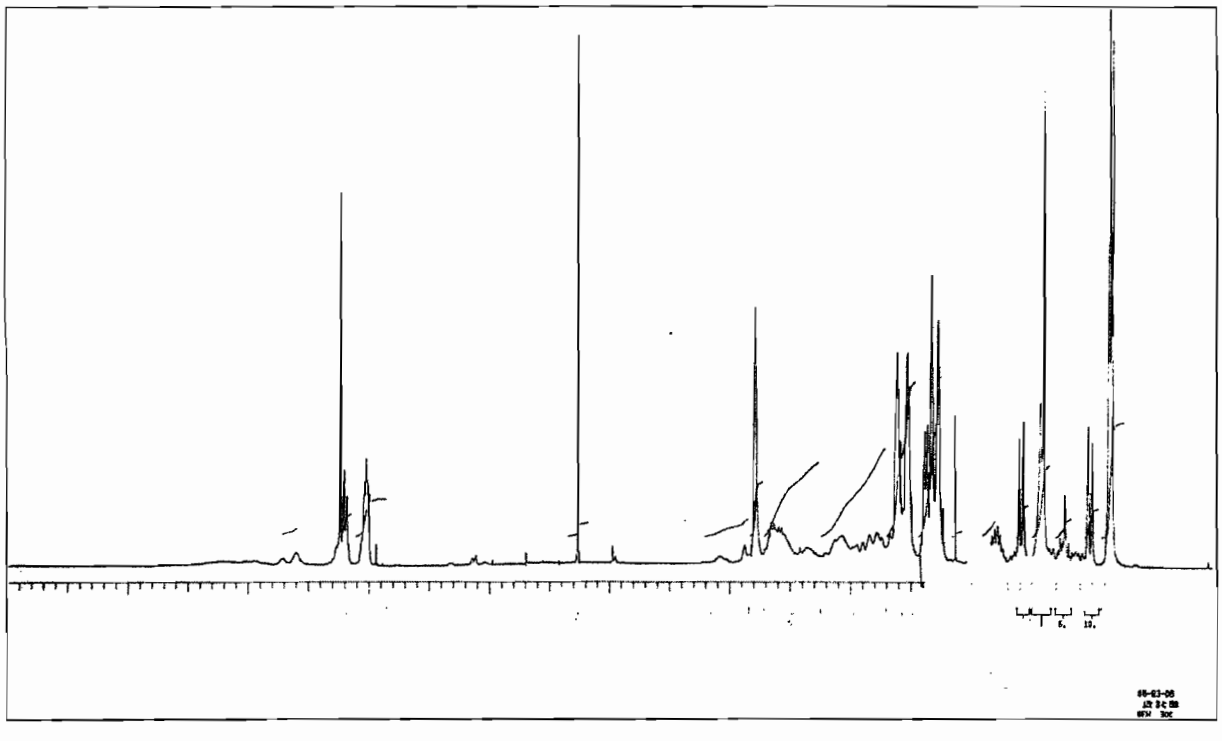

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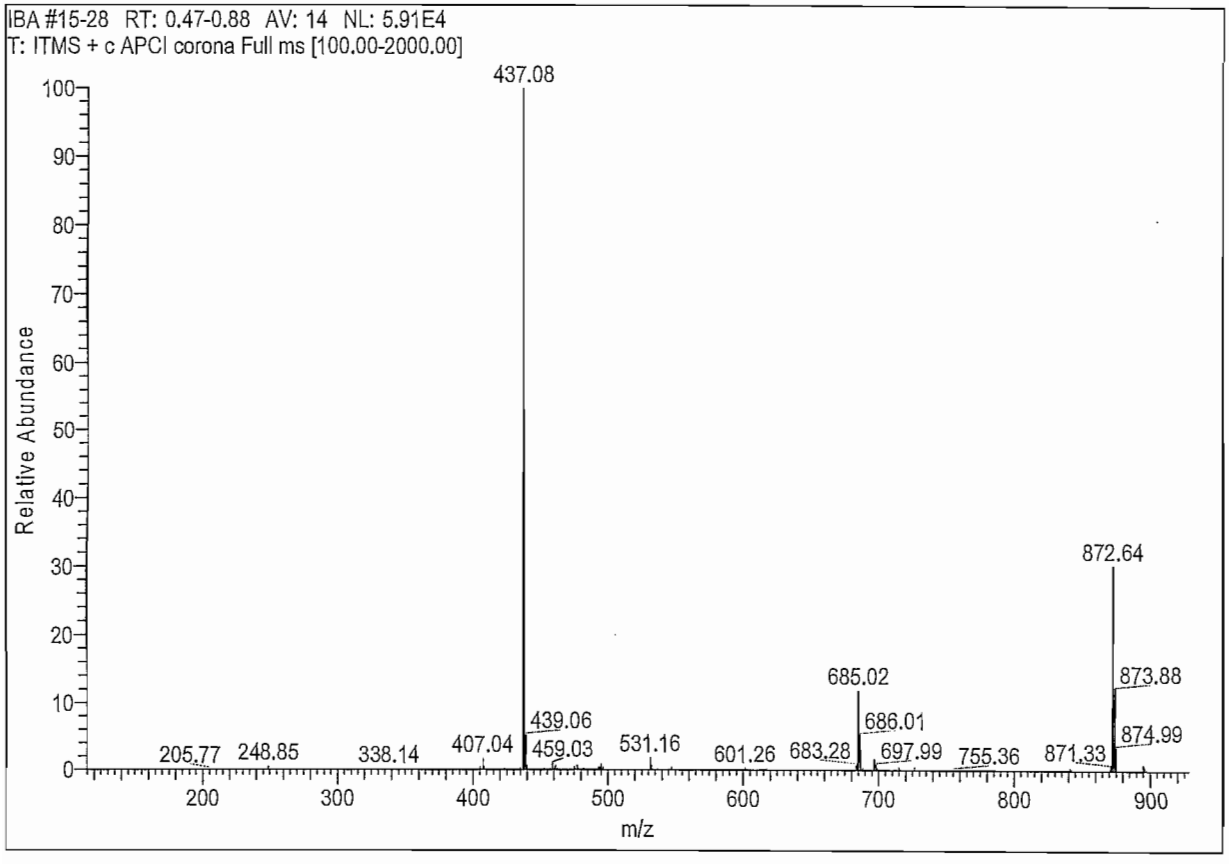


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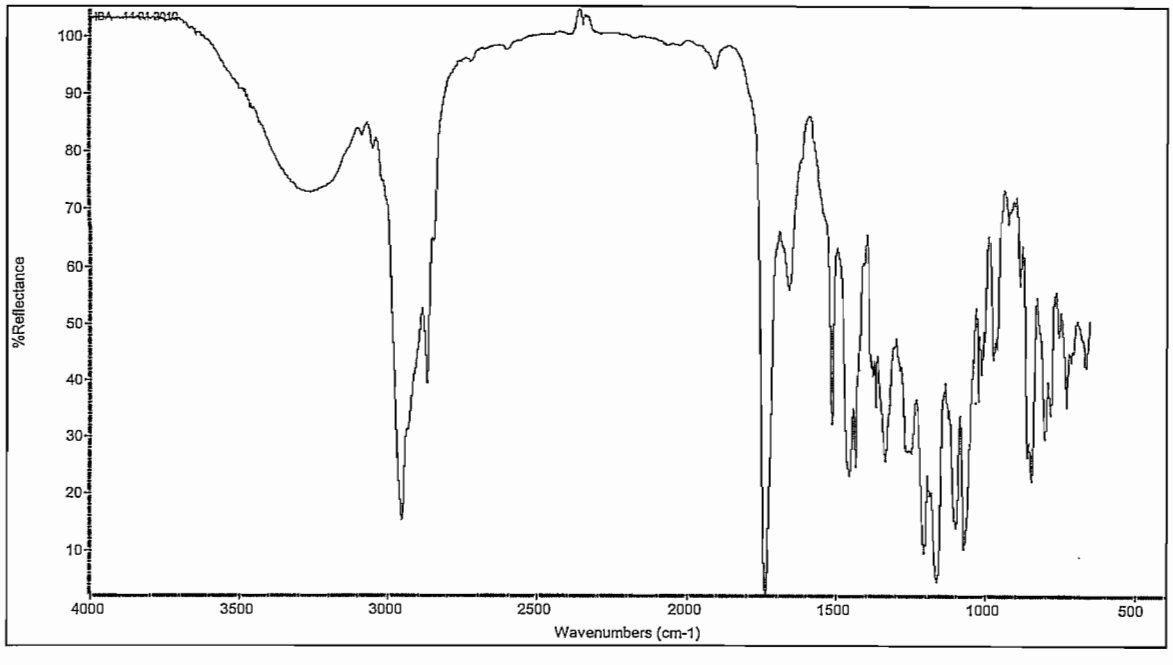

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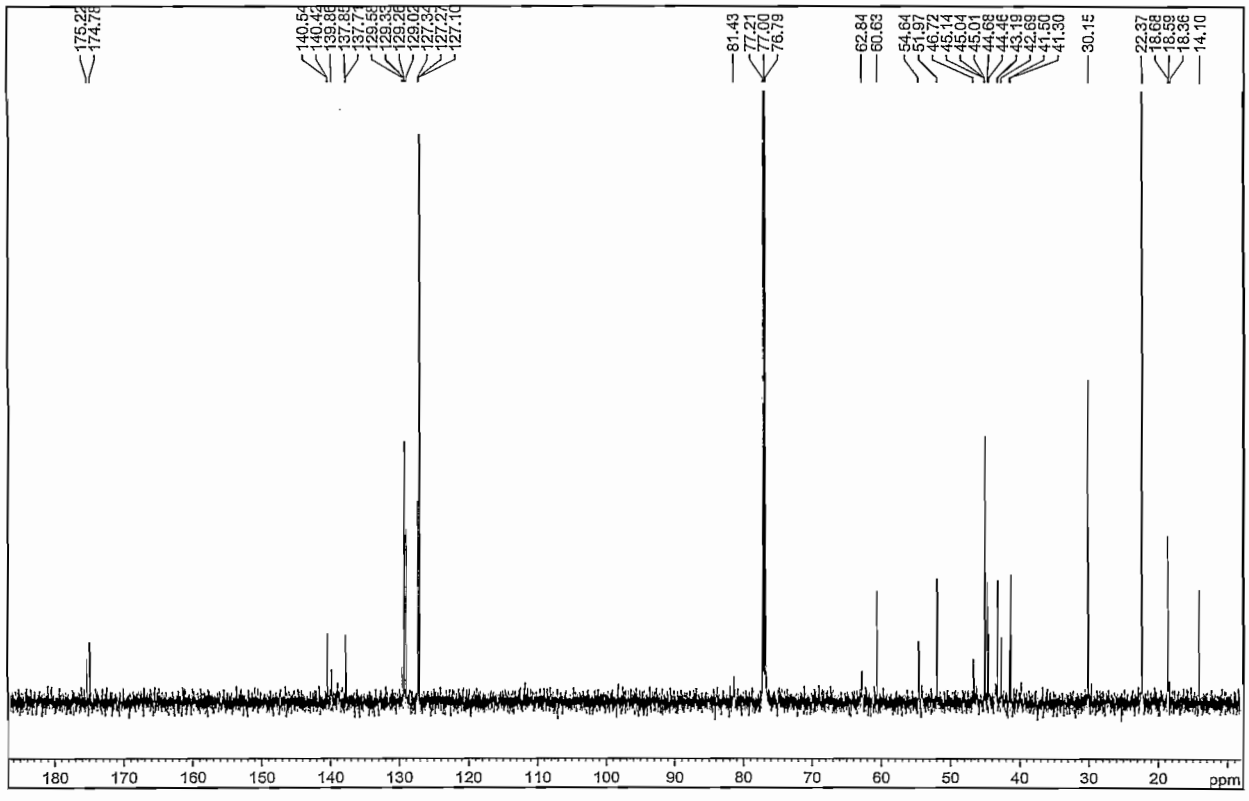


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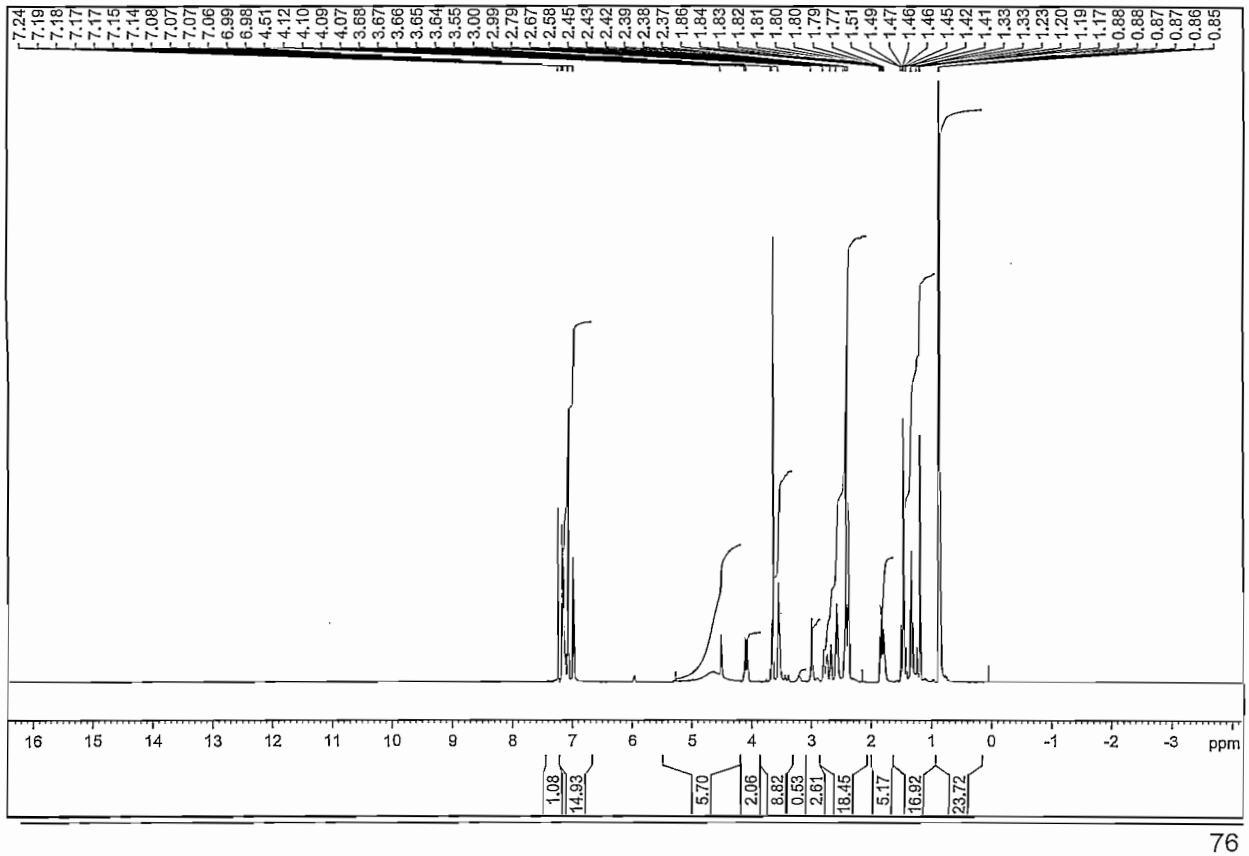

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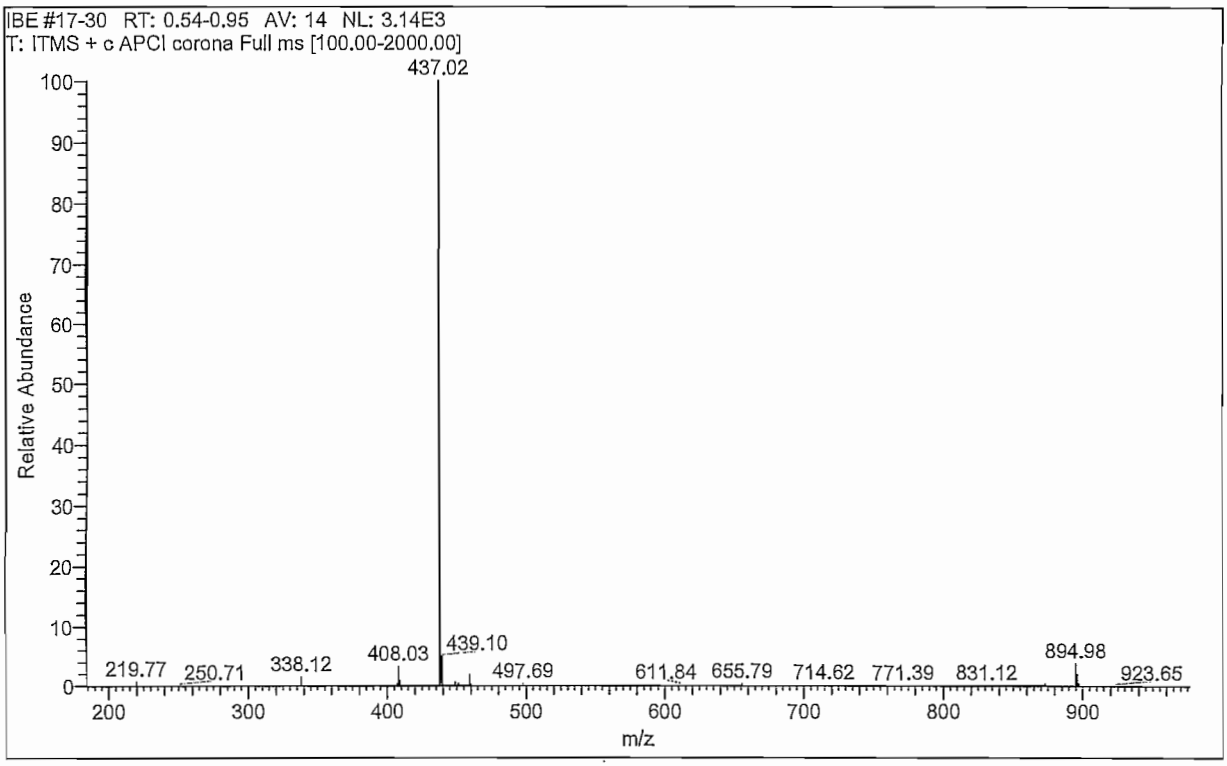


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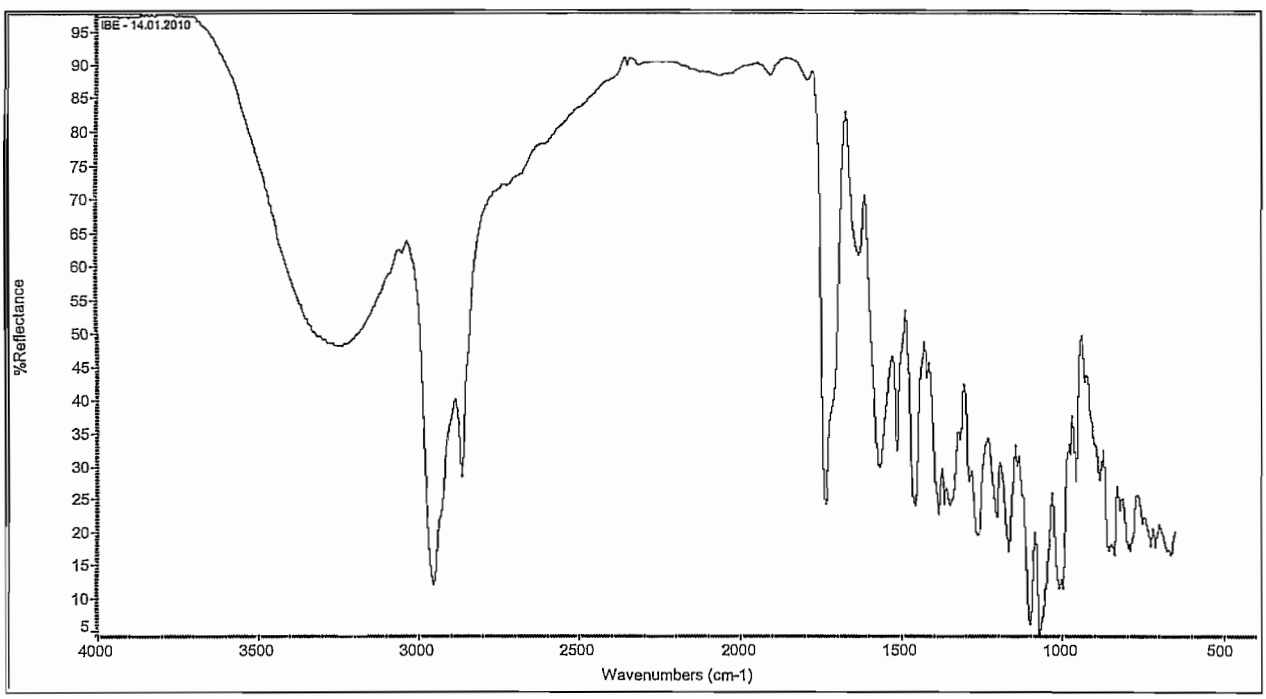

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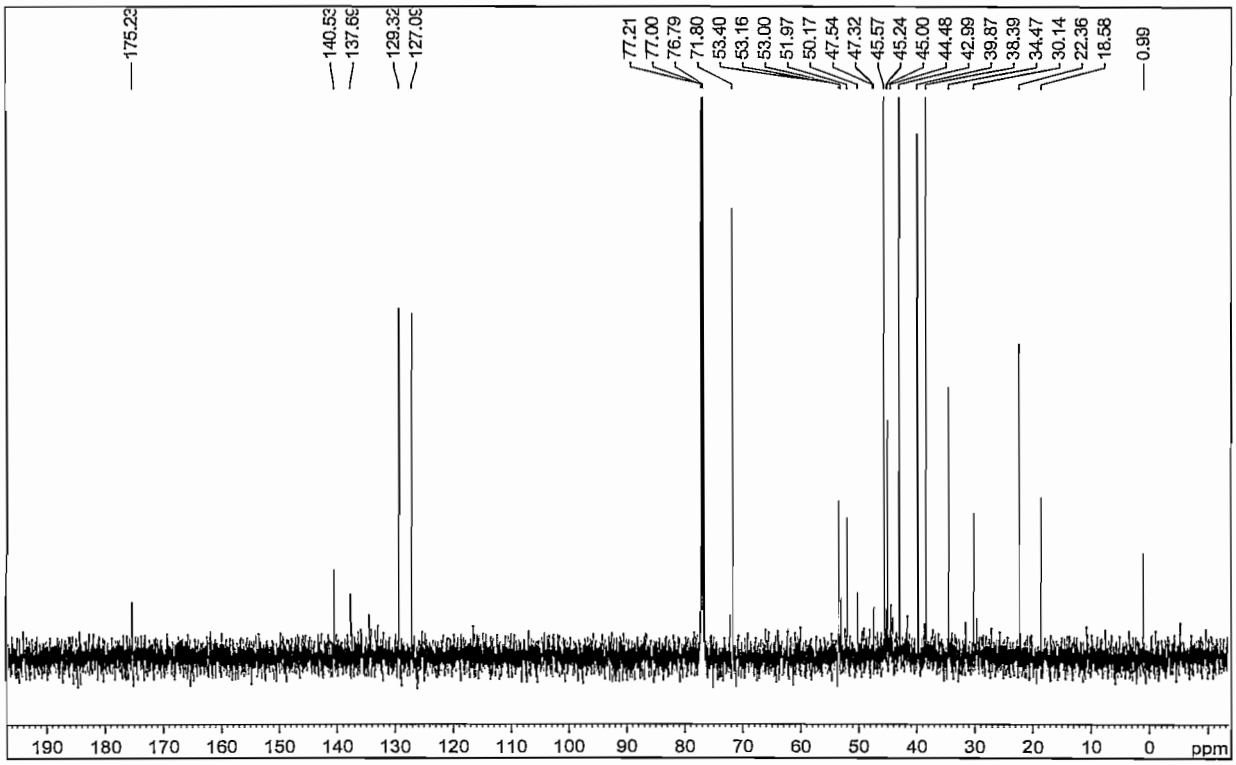


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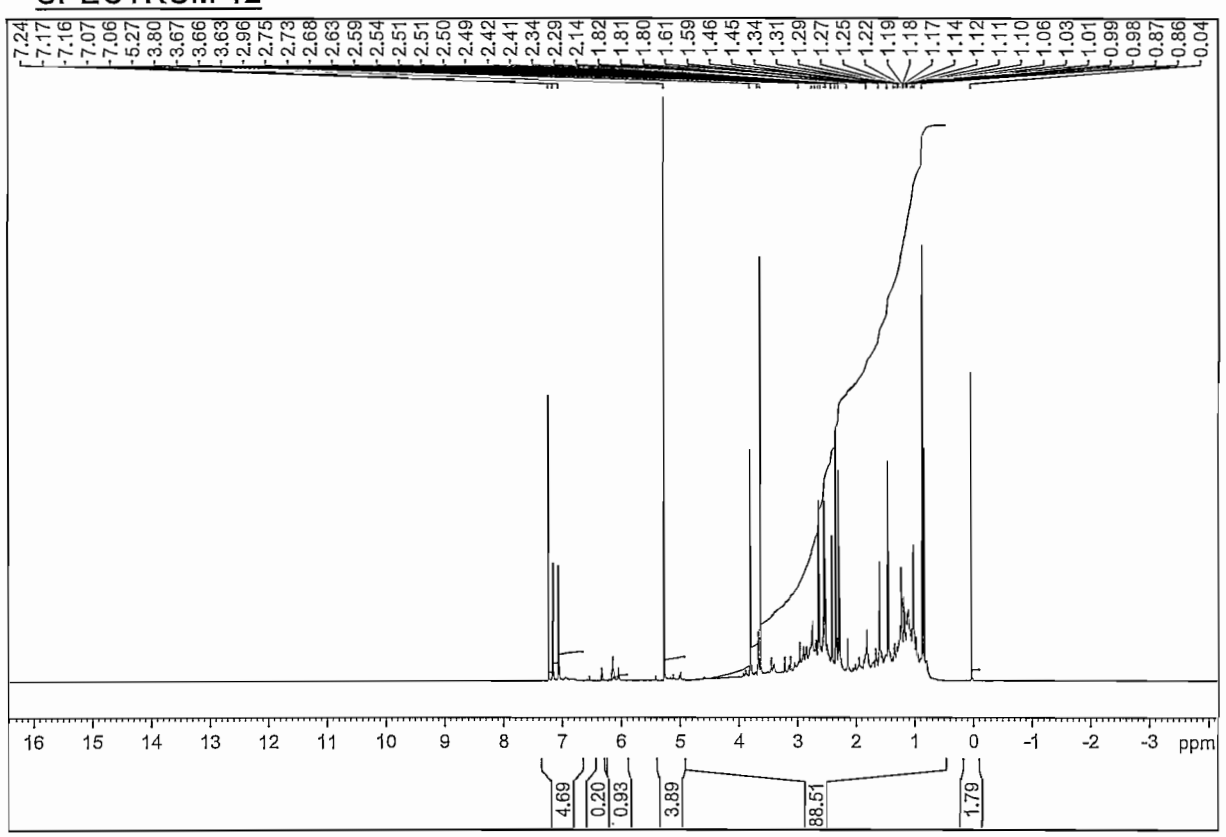

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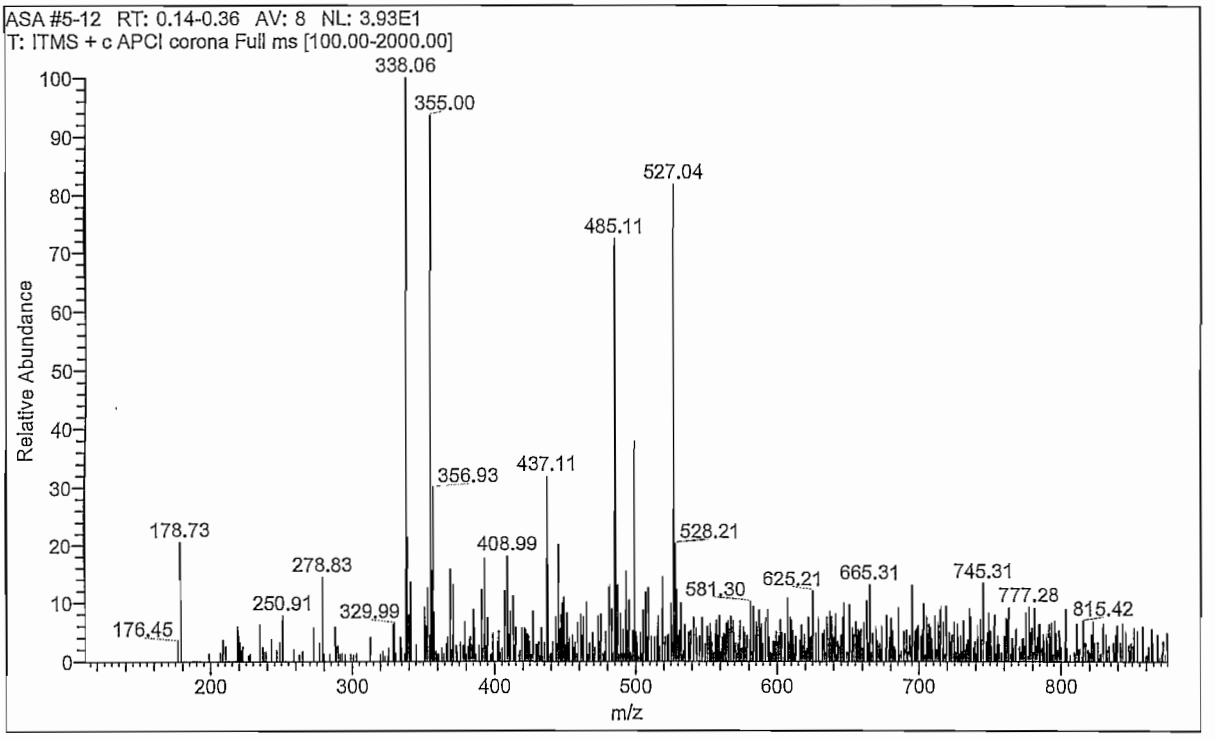


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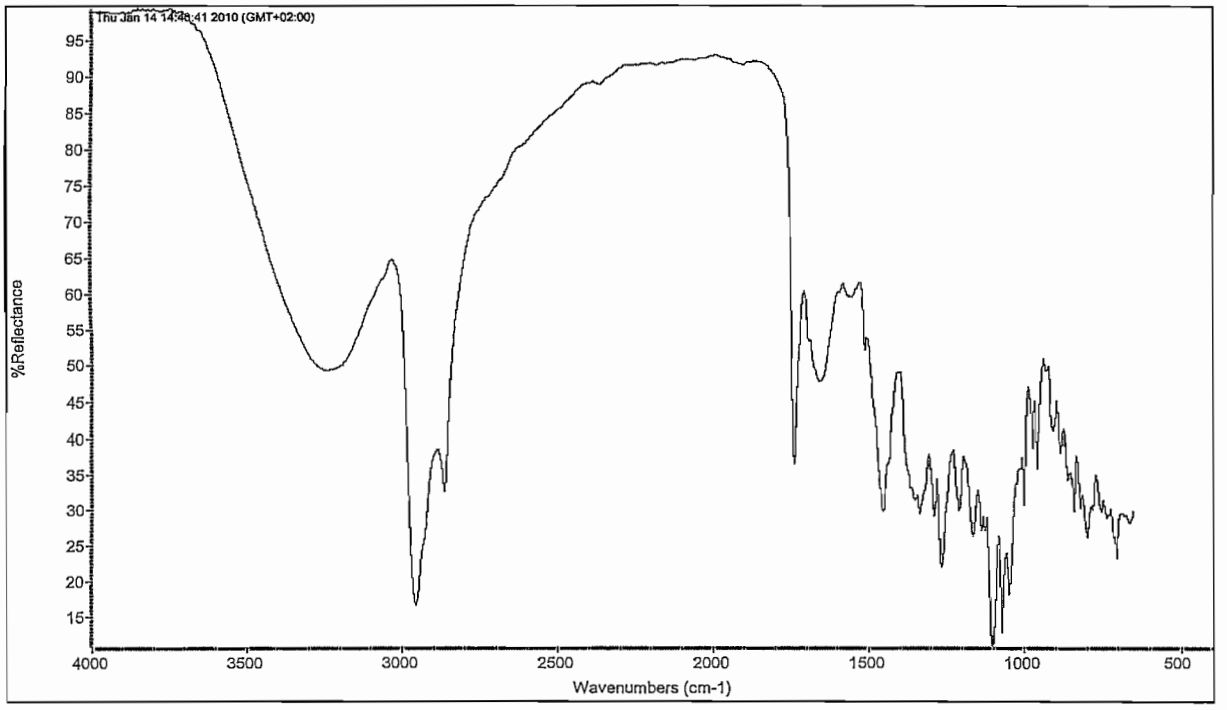

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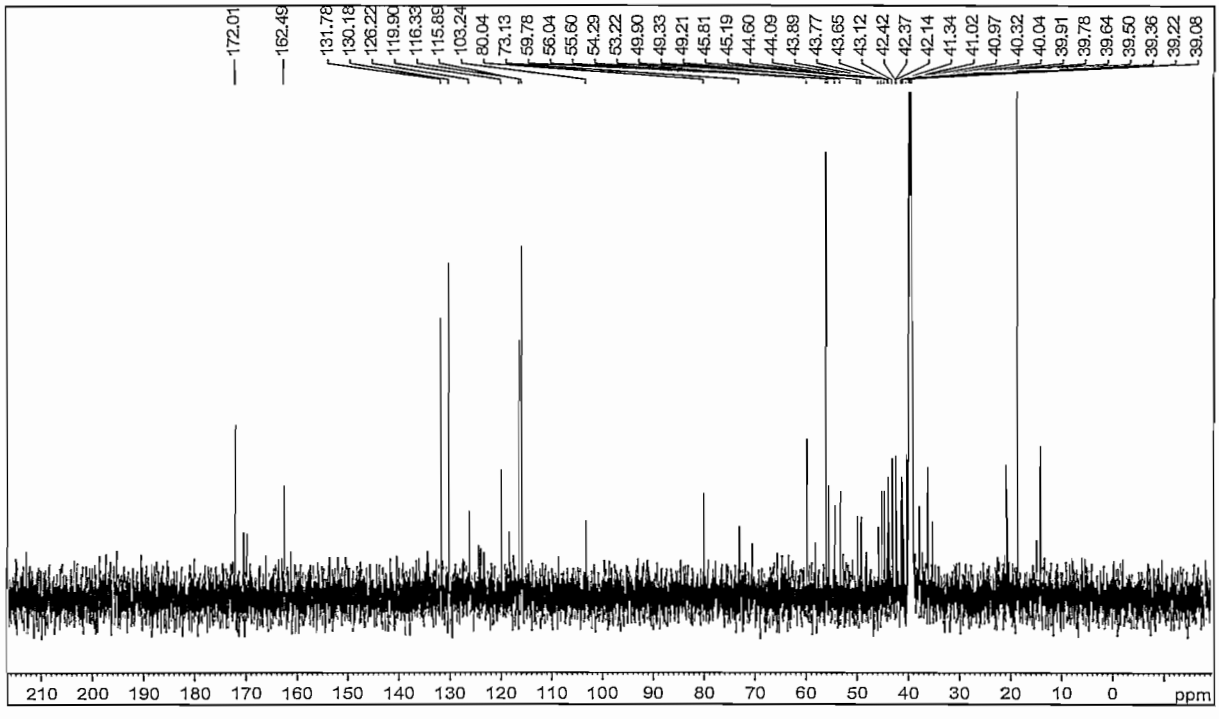


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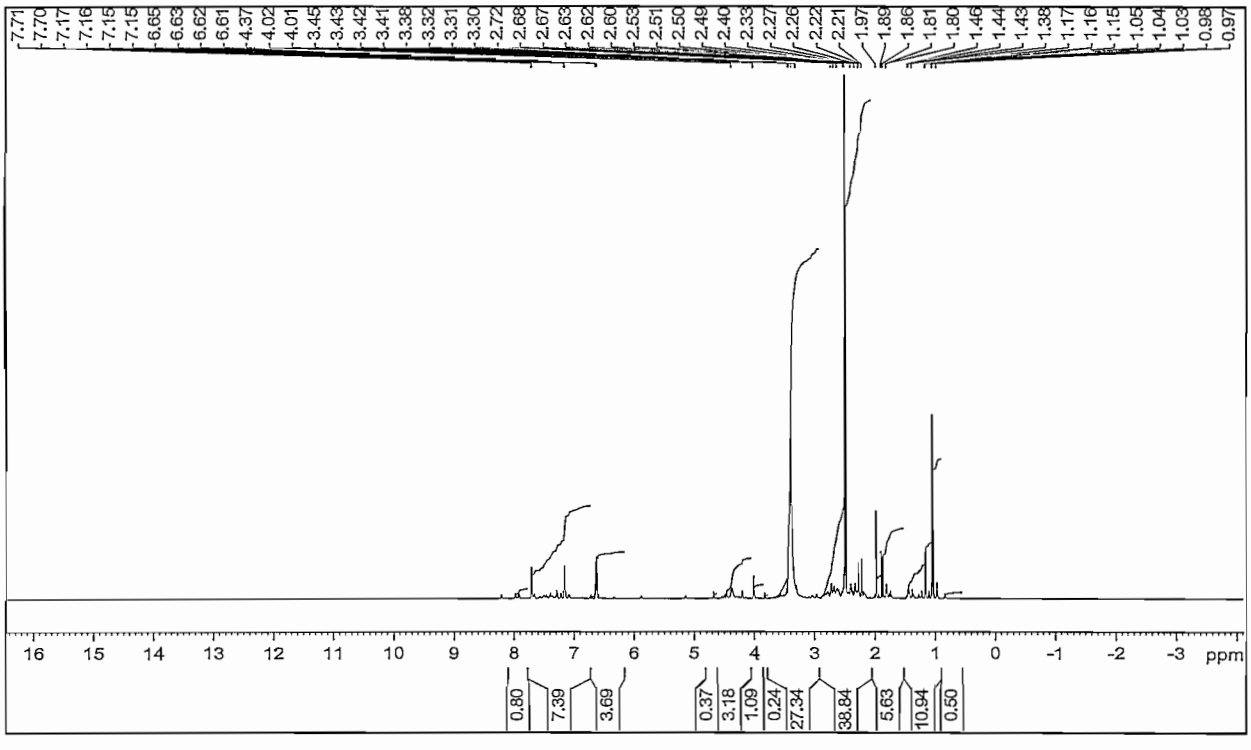

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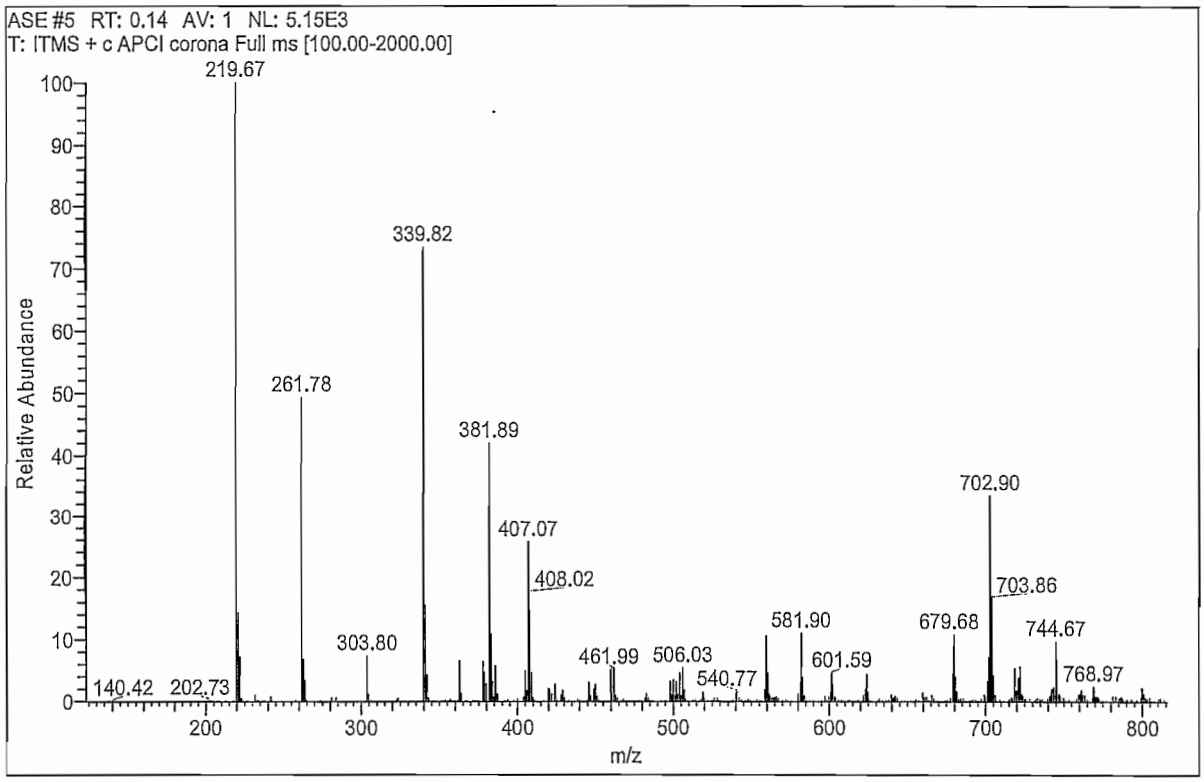


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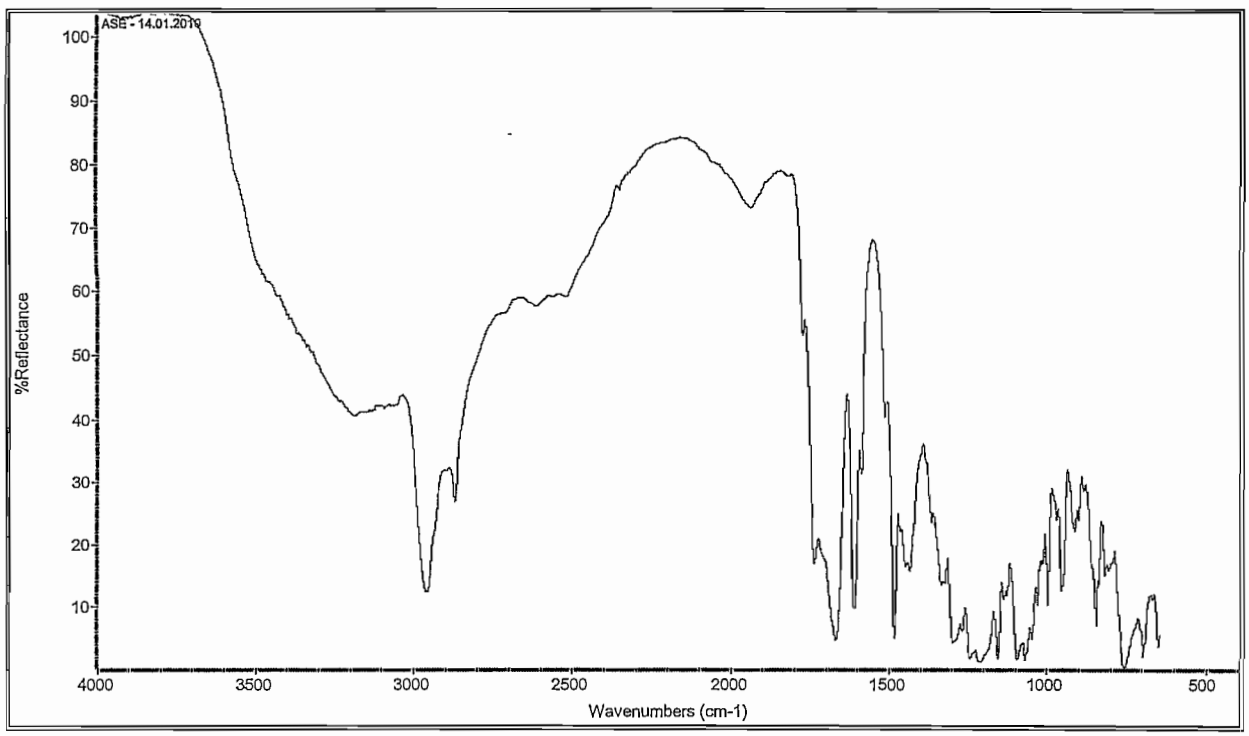
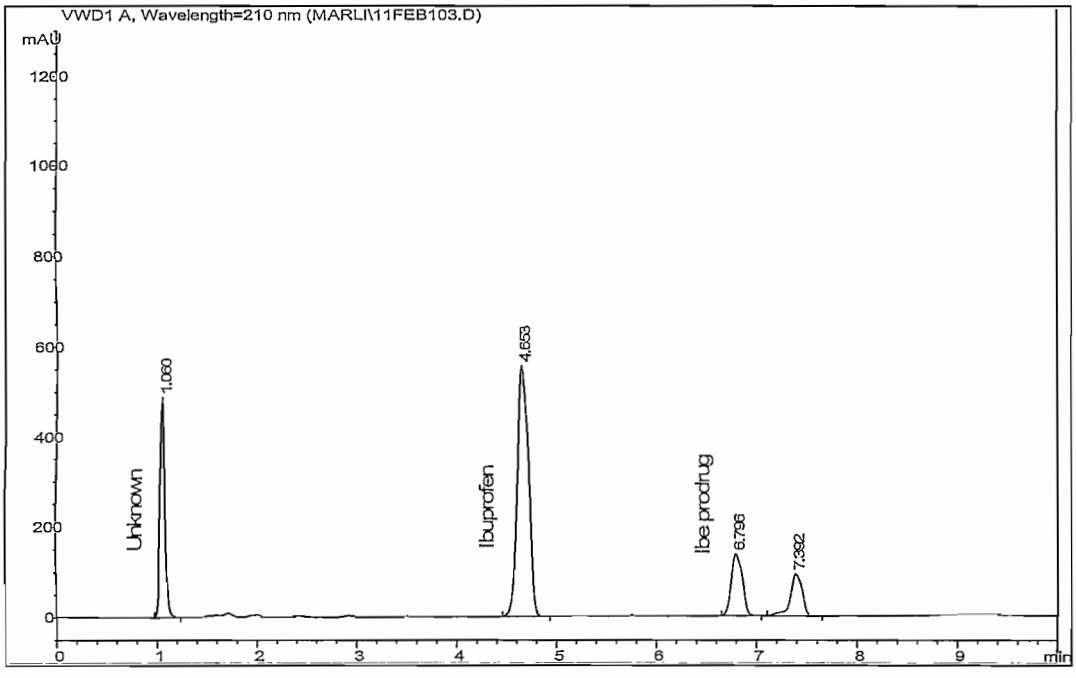


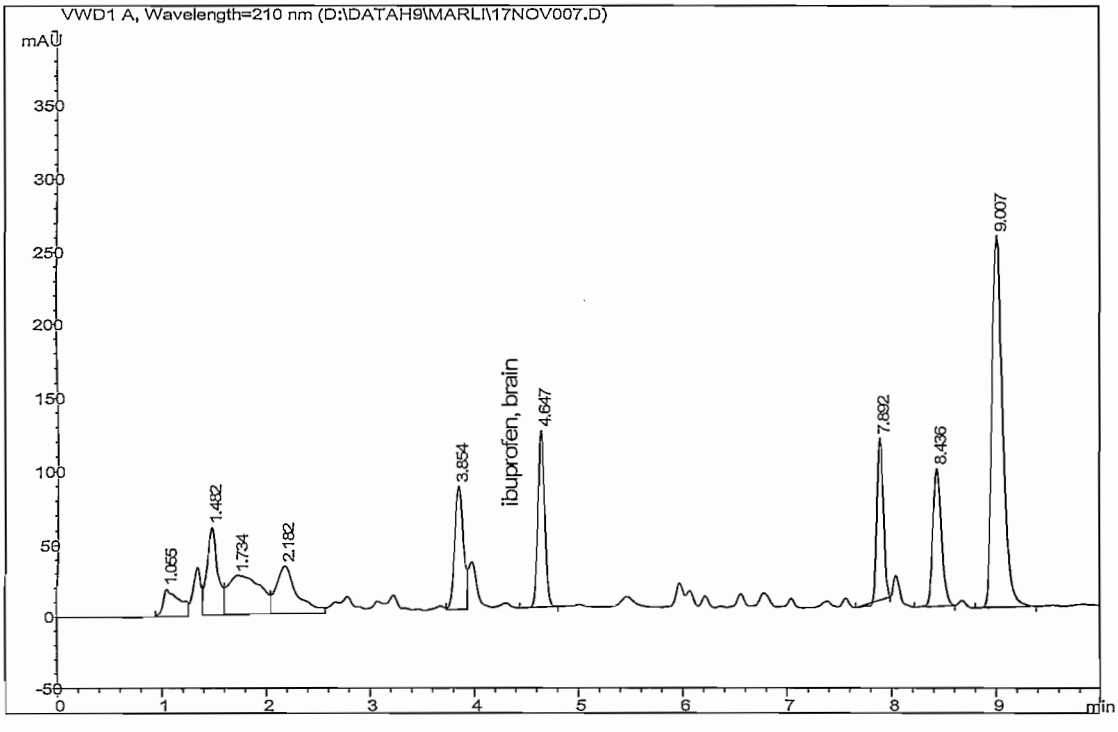
# ANNEXURE B

# HPLC CHROMATOGRAMS

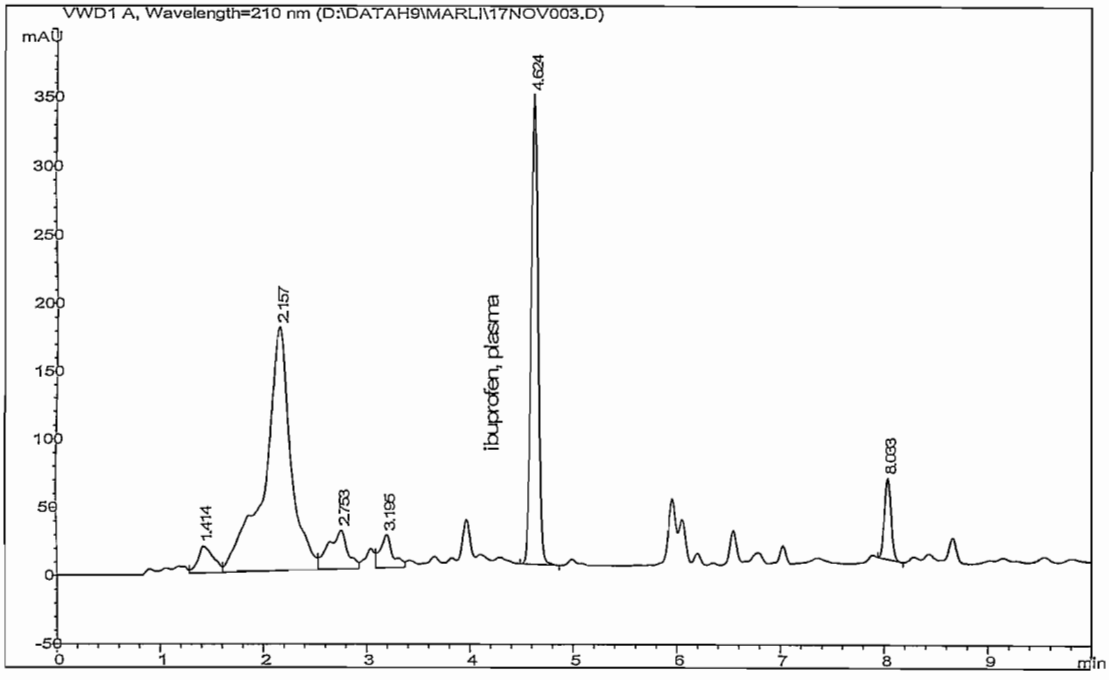
## CHROMATOGRAM 1: IBUPROFEN STANDARD



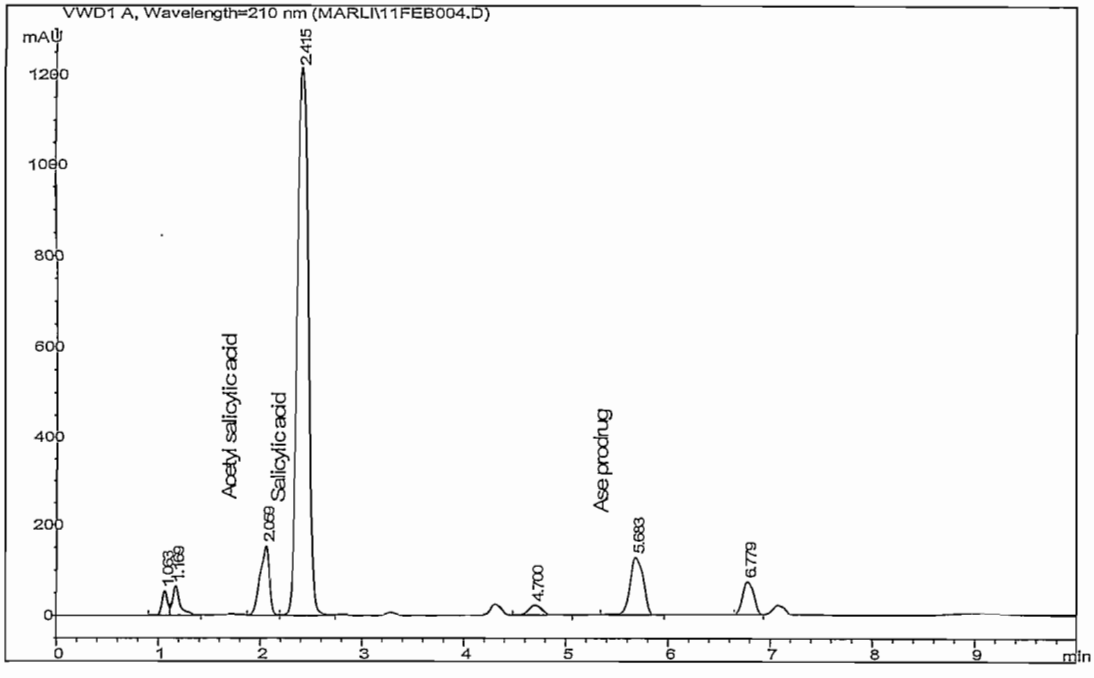

## CHROMATOGRAM 2: IBUPROFEN IN BRAIN



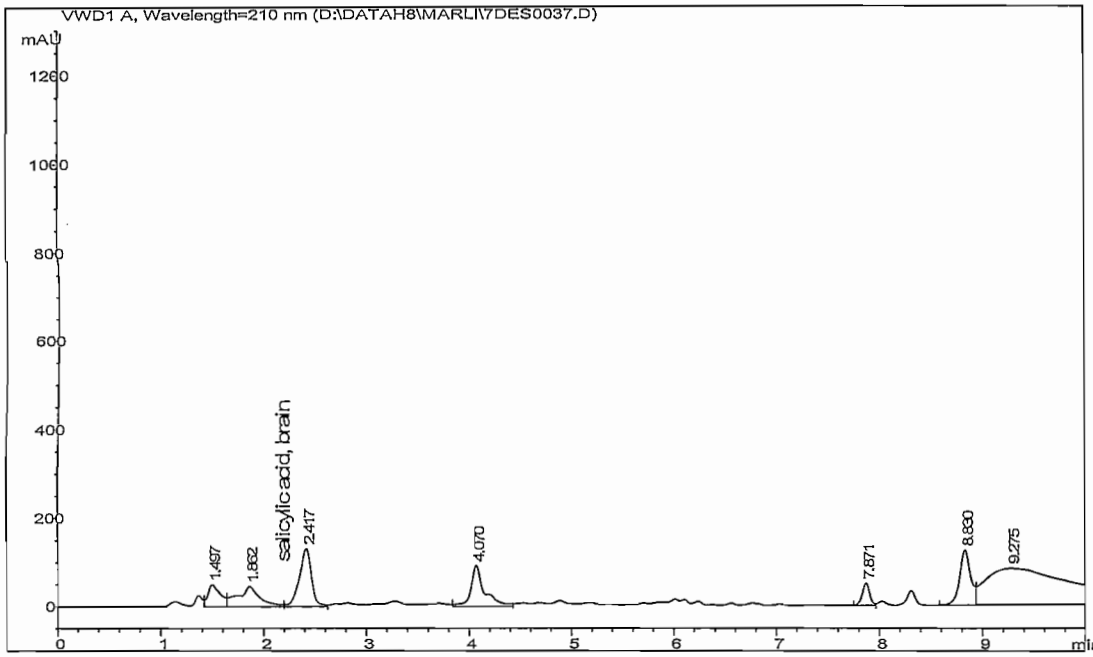
## CHROMATOGRAM 3: IBUPROFEN IN PLASMA




## CHROMATOGRAM 4: ACETYLSALICYLIC STANDARD



## CHROMATOGRAM 5: SALICYLIC ACID IN BRAIN




## CHROMATOGRAM 6: SALICYLIC ACID IN PLASMA