

**POLYCYCLIC CAGE COMPOUNDS AS
CARRIER MOLECULES FOR
NEUROPROTECTIVE NON-STEROIDAL ANTI-
INFLAMMATORY DRUGS**

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Dedicated to my parents, Hennie and Annelie Prins

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ABBREVIATIONS

6-OHDA	-	6-hydroxydopamine
AZT	-	zidovudine
CNS	-	central nervous system
COX	-	cyclooxygenase
<i>D</i>	-	diffusion coefficient
LC-MS/MS	-	liquid chromatograph tandem mass spectrometry
Log <i>P_{oct}</i>	-	logarithm of the <i>n</i> -octanol-water partition coefficient
MAO	-	monoamine oxidase
MDA	-	malondialdehyde
MPTP	-	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
<i>M_r</i>	-	molecular weight
MRP1	-	multidrug resistance associated protein-1
NF-κB	-	nuclear factor kappa B
NMDA	-	N-methyl-D-aspartate
NO	-	nitric oxide
NOS	-	nitric oxide synthase
NSAIDs	-	non-steroidal anti-inflammatory drugs
OATP2	-	organic anion transporting polypeptide 2
OATP-A	-	human brain organic anion transporter
<i>P_c</i>	-	permeability coefficient
P-gp	-	P-glycoprotein
<i>pK_a</i>	-	ionisation constant
PPARγ	-	peroxisome proliferator-activated receptor gamma
PSA	-	polar surface area
QSPR	-	quantitative structure-property relationship
ROS	-	reactive oxygen species
SEM	-	standard error of the mean
TLC	-	thin layer chromatography
γ-GTP	-	γ-glutamyl transpeptidase

ABSTRACT

The blood-brain barrier is formed by the brain capillary endothelium and plays the predominant role in controlling the passage of substances between the blood and the brain. For a compound with low blood-brain barrier permeability to be able to cross this barrier, certain physicochemical characteristics thereof need to be altered. Recent studies on polycyclic structures, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine, indicated favourable distribution thereof to the brain and it was concluded that these polycyclic structures and their derivatives penetrate the blood-brain barrier readily.

Neuroprotection can be afforded by a variety of mechanisms, including the inhibition of oxidative stress. The neuroprotective activity of certain non-steroidal anti-inflammatory drugs (NSAIDs) can be attributed to their antioxidant properties. Acetylsalicylic acid and ibuprofen used in this study, are both weak organic acids with low blood-brain barrier permeation.

No known transport mechanisms are present at the blood-brain barrier that are able to transport these drugs into the central nervous system (CNS). If these drugs could be delivered into the brain, they would demonstrate potent neuroprotective activity as a result of their anti-inflammatory and antioxidant properties.

It was therefore hypothesised that the polycyclic cage compounds described above could be used as carrier molecules to deliver neuroprotective drugs 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 Cookson's diketone, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione, was reacted with 3-aminopropanol to obtain the rearranged oxazinane cage structure. Acetylsalicylic acid and ibuprofen were subsequently conjugated to this cage structure by esterification, yielding the respective ester prodrugs. Amide prodrugs were synthesised by conjugating the NSAIDs to amantadine. The structures were confirmed by ¹H and ¹³C NMR, MS and IR.

An *in vivo* blood-brain barrier permeability assay employing HPLC and LC-MS/MS analytical procedures was used to compare brain and blood concentrations of the relevant drugs 1 hour after administration to male C57BL/6 mice. The antioxidant activities of the compounds were assessed with an *in vitro* lipid peroxidation assay.

Both NSAIDs were detected in the brain tissue of test mice after administering the synthesised prodrugs, indicating blood-brain barrier permeation. The ester prodrugs were found to be very labile and significant amounts of it were hydrolysed within the 1 hour time period. This was evident as neither of the intact ester prodrugs was detected in quantifiable amounts 1 hour after administration to the test mice. When administering these ester prodrugs (compounds **22** and **23**), the free NSAIDs were detected at higher concentrations compared to when the free drugs were administered. The amide prodrugs (compounds **25** and **26**), exhibited lower free drug concentrations than that obtained after the NSAIDs were administered on their own. This observation could be attributed to incomplete hydrolysis of the amide bond at 1 hour after administration and might also explain the lower brain concentration of the free drug observed for both prodrugs. However, the novel prodrugs synthesised exhibited favourable blood-brain barrier penetration, and the more efficient delivery of the drug to the brain would afford higher concentrations of the neuroprotectant and thus better neuroprotection.

Lipid peroxidation results indicated that the ester and amide prodrugs, significantly increased the ability of the free drugs to attenuate lipid peroxidation. This was especially true for compounds **22** and **26**, with **22** being in the range of the model antioxidant, Trolox.

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

UITTREKSEL

Die bloedsreinskans word gevorm deur die breinskappilêre endoteelselle en is verantwoordelik vir die beheer van die beweging van stowwe tussen die bloed en die brein. Die swak bloedsreinskansdeurlaatbaarheid van sekere verbindings kan verbeter word deur die fisies-chemiese eienskappe daarvan te verander. Onlangse studies het aangedui dat die bloedsreinskans geredelik deurlaatbaar is vir die polisikliese strukture, pentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undekaan en amantadien.

Neurobeskerming kan verkry word deur verskillende meganismes wat onder andere, die inhibisie van oksidatiewe skade insluit. Die neurobeskermende eienskappe van sekere nie-steroidale anti-inflammatoriese middels (NSAIDs) kan toegeskryf word aan hul anti-oksidatiewe werking. Asetiëlsalisiesuur en ibuprofeen, gebruik in hierdie studie, is beide swak organiese sure en toon lae bloedsreinskansdeurlaatbaarheid.

Transportmeganismes by die bloedsreinskans vir die vervoer van hierdie verbindings na die sentrale senuweestelsel is nie bekend nie.

Effektiewe aflewering van hierdie geneesmiddels kan moontlik kragtige neurobeskermende effekte tot gevolg hê as gevolg van die anti-inflammatoriese en anti-oksidatiewe eienskappe daarvan.

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

Die welbekende Cooksondiketoon, pentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undekaan-8,11-dioon, is gereageer met 3-aminopropanol om die herrangskikte oksazinaanhokstruktuur te verkry. Asetiëlsalisiesuur en ibuprofeen is gekonjugeer met die herrangskikte hokstruktuur deur middel van esterifikasie, om die onderskeie esterprogeneesmiddels te lewer. Amiede is verkry deur die NSAIDs te konjugeer met amantadien.

Die strukture van al die verbindings is bevestig met behulp van ^1H en ^{13}C KMR, MS en IR.

'n *In vivo* evalueringsmetode, wat gebruik maak van HPLC en LC-MS/MS tegnieke, is aangewend om die brein- en bloedkonsentrasies van die spesifieke geneesmiddels te vergelyk 1 uur na toediening aan manlike C57BL/6-muise. Die pro-geneesmiddels is ook geanaliseer vir anti-oksidatiewe werking deur middel van 'n *in vitro* lipiedperoksidasiestudie.

Beide NSAIMs is waargeneem in breinweefsel nadat die gesintetiseerde pro-geneesmiddels aan die muise toegedien is, wat aanduidend is van bloedsbreinskansdeurdringing. Geen intakte esterprogeneesmiddels is in kwantifiseerbare hoeveelhede waargeneem 1 uur na toediening aan die muise nie. Die esterprogeneesmiddels is dus baie labiel en substansiële hoeveelhede daarvan hidroliseer reeds tydens die uur periode na toediening. Die vry NSAIMs is in hoër konsentrasies waargeneem na toediening van die esterprogeneesmiddels (verbinding 22 en 23), in vergelyking met konsentrasies verkry nadat slegs die NSAIMs toegedien is. Die teenoorgestelde is egter waar vir die amiedprogeneesmiddels (verbinding 25 en 26) en kan waarskynlik toegeskryf word aan onvolledige hidrolise van die amiedbinding binne 1 uur na toediening aan die muise. Dit kan ook die laer breinkonsentrasie van die vry geneesmiddel wat waargeneem is vir beide hierdie progeneesmiddels verklaar. Die nuutgesintetiseerde progeneesmiddels vertoon nietemin goeie bloedsbreinskansdeurlaatbaarheid en die effektiewer geneesmiddel- aflewering na die brein sal hoër konsentrasies neurobeskermer en dus verbeterde neurobeskerming tot gevolg hê.

Die lipiedperoksidasiestudie het getoon dat ester- en amiedprogeneesmiddels die vermoë van die vry geneesmiddels om lipiedperoksidase te verminder, aansienlik verhoog. Dit is veral waar vir verbinding 22 en 26, met die aktiwiteit van 22 vergelykbaar met dié van die model anti-oksidant, Trolox.

Die nuutgesintetiseerde progeneesmiddels optimiseer dus die geteikende aflewering van die middels op meer as een wyse, aangesien dit die bloedsbreinskansdeurlaatbaarheid en die anti-oksidatiewe aktiwiteit van die ongekonjugeerde NSAIMs verhoog.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Neurodegenerative disorders have a negative influence on the quality of life of millions of people around the world. In the quest to successfully prevent these disorders, the blood-brain barrier presents as a major obstacle by preventing the entrance of certain substances into the central nervous system (CNS).

1.1.1 NEURODEGENERATION

Neurodegeneration can be described as the process in which certain neurons in the CNS and especially the brain are damaged by a variety of mechanisms. Neurodegenerative disorders include several diseases such as Parkinson's disease and Alzheimer's disease, and are characterised by a progressive and permanent loss of neurons from specific regions of the brain. Parkinson's disease is observed in more than 1% of persons over the age of 65 whereas Alzheimer's disease affects as much as 10% of the same population (Standaert & Young, 1996).

There are three key mechanisms of neuronal cell death, which may act separately, or cooperatively to cause neurodegeneration. The "lethal triplet" of metabolic compromise, excitotoxicity and oxidative stress causes neuronal cell death that is both necrotic and apoptotic in nature (fig. 1.1). Aspects of these three mechanisms are believed to play a role in the neurodegeneration that occurs in both acute conditions such as stroke and epilepsy and in chronic neurodegenerative disorders like Parkinson's disease and Alzheimer's disease. (Alexi *et al.*, 2000; Greene & Greenamyre, 1996).

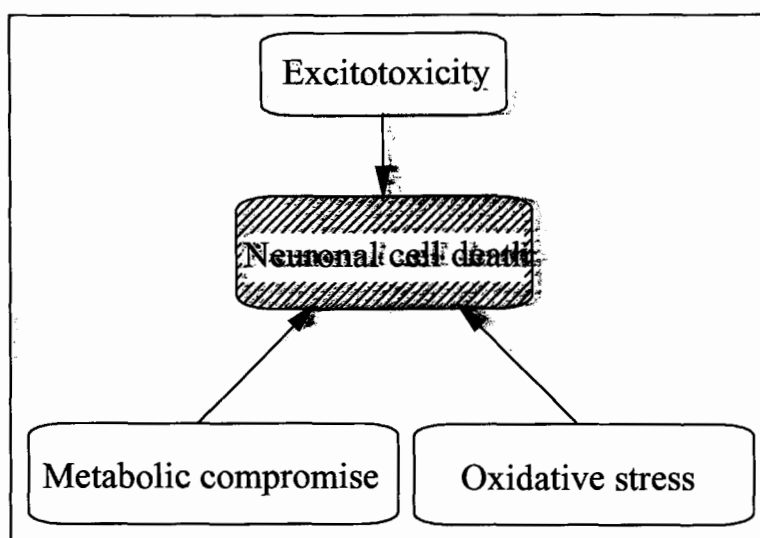


Figure 1.1: Lethal triplet in neuronal cell death.

It has been shown that the prolonged availability of extracellular glutamate during hypoxia-ischemia is transduced by neuronal membrane receptors into potentially lethal intracellular ionic disturbances, in particular, intracellular Na^+ and Ca^{2+} overload. Excitotoxic neuronal cell death correlates well with measures of total Ca^{2+} influx, while removal of extracellular Ca^{2+} attenuates glutamate-induced neuronal death (Dugan & Choi, 1999). Sustained elevations in intracellular Ca^{2+} can initiate “toxic cascades”, which are capable of ultimately killing the cell. These cascades include activation of catabolic enzymes, such as proteases, phospholipases and endonucleases (Choi, 1995). Elevated concentrations of intracellular Ca^{2+} can further lead to initiation of protein-kinase and lipid-kinase cascades, impairment of metabolism and generation of reactive oxygen species (ROS) (Dugan & Choi, 1999).

Mandel *et al.* (2003) demonstrated that, at the time of death, patients suffering from Parkinson’s disease presented with a cascade of lethal events. These events may contribute to the demise of the melanin-containing nigro-striatal dopamine neurons and include increased levels of iron and monoamine oxidase B (MAO-B) activity, oxidative stress, inflammatory processes, glutamatergic excitotoxicity, nitric oxide synthesis, abnormal protein folding and aggregation, reduced expression of trophic factors, depletion of endogenous antioxidants such as reduced glutathione, and altered calcium homeostasis. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-

hydroxydopamine (6-OHDA) animal models of Parkinson's disease utilised by the same authors, confirmed these findings to a large extent. Their studies indicated that neuroprotection could be afforded in these models with iron chelators, radical scavenger antioxidants, MAO-B inhibitors, glutamate antagonists, nitric oxide synthase inhibitors, calcium channel antagonists and trophic factors.

In this study the main focus fell on the CNS delivery of NSAIDs with possible anti-oxidative properties. With this in mind oxidative stress as a cause of neuronal cell death is discussed in further detail.

An important deleterious consequence of Ca^{2+} overload following excitotoxic glutamate receptor activation is the formation of ROS. Free radical production is linked to elevated $[\text{Ca}^{2+}]$ in several ways:

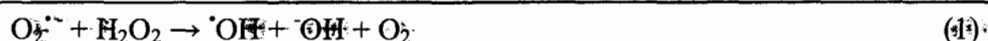
- Ca^{2+} -dependent activation of phospholipase A_2 , with liberation of arachidonic acid and further metabolism thereof, leading to "by-product" free radical production and lipid peroxidation (Dugan & Choi, 1999).
- Stimulation of N-methyl-D-aspartate (NMDA) receptors and increased Ca^{2+} influx, leading to activation of nitric oxide synthase (NOS) and the release of nitric oxide (NO), which can then interact with ROS from other sources to generate highly reactive peroxynitrite (Beckman & Koppenol, 1996).
- Uncoupling of mitochondrial electron transport, enhancing mitochondrial production of free radicals (Dykens, 1994).

Oxidative stress is the result of an increase in the concentration of highly reactive, free radicals such as the super oxide anion ($\text{O}_2^{\cdot -}$), hydroxyl radical ($\cdot\text{OH}$) and nitric oxide radical ($\text{NO}^{\cdot}$). The oxidising action of these reactive species destroys membrane lipids, proteins and DNA and can be damaging to cells if they accumulate at high levels or if deficits in cellular antioxidant defence systems occur (Gilgun-Sherki *et al.*, 2001).

Hydroxyl free radicals are the primary means by which oxidation reactions can become toxic. The Haber-Weiss and the Fenton reaction are proposed to be responsible for the generation of these radicals.

The Haber-Weiss reaction (eq. 1) comprises a single electron transfer from superoxide to hydrogen peroxide, while in the Fenton reaction (eq. 2) a single electron is transferred

from iron to hydrogen peroxide converting iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state (Gilgun-Sherki *et al.*, 2001; Bester, 2000).



Hydroxyl radicals are more likely to be formed by the Fenton reaction, especially since the Haber-Weiss reaction is very slow under physiologic conditions. The substantia nigra also has a high concentration of transition metals, specifically Fe^{2+} , in comparison to other brain regions, which leads to higher radical formation (Gilgun-Sherki *et al.*, 2001; Bester, 2000).

The notion that oxidative stress increases in the aging brain was examined in a study done by Floyd and Hensley (2002). Their studies showed a logarithmic age-dependant increase in oxidised proteins and oxidised DNA lesions. The increase in oxidised protein is indicative that oxidative stress as well as neuronal cell damage does become more profound with old age. Oxidative damage to DNA produces strand breaks as well as many oxidised bases. Typically, strand breaks are repaired and oxidised bases are removed by specific enzymes. It is considered that oxidative damage to RNA and proteins is not repaired, but that these damaged macromolecules are degraded into products, some of which are recycled for use in the synthesis of new proteins or RNA molecules.

Oxidative inactivation of enzymes is another index of age-dependent oxidative damage to proteins. In a study conducted it was clear that glutamine synthase activity is selectively lost in the diseased brain when comparisons are made between subjects suffering from Alzheimer's disease and age-matched controls (Smith *et al.*, 1991). Experimental data obtained also indicated that lipid peroxidation increased in diseased brains when compared to age-matched controls (Floyd & Hensley, 2002).

Inflammation is a defence mechanism against diverse insults, designed to remove lethal agents and to inhibit their detrimental effects. In neurodegenerative disorders, inflammation may be triggered by the accumulation of proteins with abnormal conformations or by signals emanating from injured neurons (Wyss-Coray & Mucke,

2002). Recent evidence has indicated that inflammation in the brain is closely associated with the pathogenesis of several degenerative neurological disorders including, among others, Parkinson's and Alzheimer's diseases (Hald & Lotharius, 2005; Akiyama *et al.*, 2000). Cytokines and chemokines are key regulators of inflammatory processes and have been implicated in the pathogenesis of neurodegenerative diseases (Mennicken *et al.*, 1999; Allan & Rothwell, 2001). They regulate the activity and survival of inflammatory cells and mediate the communication of immune cells with each other and with other cells of the body. At the centre of brain inflammation is the activation of microglia, which may be a general consequence of neuronal death or may reflect the active participation of microglia in the neurodegenerative process (McGeer *et al.*, 1988). Herrera *et al.* (2000) provided evidence that microglial activation can trigger cell loss. This was done by intranigral injection of lipopolysaccharide in rats, which resulted in the activation of microglia and degeneration of the dopaminergic system. The cytokines and chemokines presumably play similar intercellular and intracellular signalling roles in microglia and astrocytes as they do in the periphery. Virtually all the cytokines and chemokines that have been studied in Alzheimer's disease, including interleukin (IL)-1 β , IL-6, IL-8, tumour necrosis factor (TNF)- α , transforming growth factor (TGF)- β and macrophage inflammatory protein (MIP)-1 α , seem to be upregulated in patients with Alzheimer's disease compared to control values (Akiyama *et al.*, 2000). An array of human and experimental data also exists indicating the role that inflammation plays in Parkinson's disease. Data indicating an increased number of activated microglia in the brains of patients with Parkinson's disease has been published by McGeer *et al.* (1988). The increased levels of cytokines in the cerebrospinal fluid and striatum in the brains of patients with Parkinson's disease (Muller *et al.*, 1998) and the decreased risk of developing Parkinson's disease in regular NSAID users (Chen *et al.*, 2003) has also been indicated.

Keeping the above discussion in mind it can be seen that compounds with antioxidant and anti-inflammatory activity may be useful in the prevention of oxidative and inflammatory damage to neuronal cells and thus be neuroprotective.

1.1.2 NEUROPROTECTION AND NEURORESCUE

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). As described earlier, neuroprotection can be afforded by a variety of mechanisms including the use of Ca^{2+} channel antagonists, glutamate antagonists, nitric oxide synthase inhibitors and antioxidants. In this study we specifically focused on free radical scavenging antioxidants and their ability to afford neuroprotection. More specifically the focus was on the antioxidant activity of certain NSAIDs.

Neurorescue, on the other hand, is a phenomenon that describes a decrease in neuronal cell death by a compound, when it would be expected that neurodegeneration had already commenced. To date, only two compounds are known to be “neurorescuers” namely deprenyl and acetylsalicylic acid (aspirin) (Foley *et al.*, 2000). The mechanisms by which these two compounds appear to “rescue” are still controversial. The MAO inhibiting activity of deprenyl and the antioxidant effect of acetylsalicylic acid are regarded as the main mechanisms (Obata & Chiueh, 1992). Acetylsalicylic acid also affords protection by way of nuclear factor kappa B (NF- κ B) blockade (Grilli *et al.*, 1996).

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 associated with disorders such as Parkinson’s and Alzheimer’s diseases are focused in the brain and 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 permeate the blood-brain barrier.

Several studies have indicated that NSAIDs play a vital role as neuroprotective agents (Bisaglia *et al.*, 2002; Combs *et al.*, 2000) and may therefore be applied in the treatment of neurodegenerative disorders. This is prevented by the fact that most NSAIDs do not penetrate the blood-brain barrier in significant amounts (Mannila *et al.*, 2005). The cage structures pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (1) and amantadine (2) have been indicated to permeate the blood-brain barrier readily (Zah *et al.*, 2003; Tsuzuki *et al.*,

1994). It is thus hypothesised that these cage structures can be applied as carrier molecules for neuroprotective drugs with low blood-brain barrier permeability, in this case specifically the NSAIDs acetylsalicylic acid (3) and ibuprofen (4).

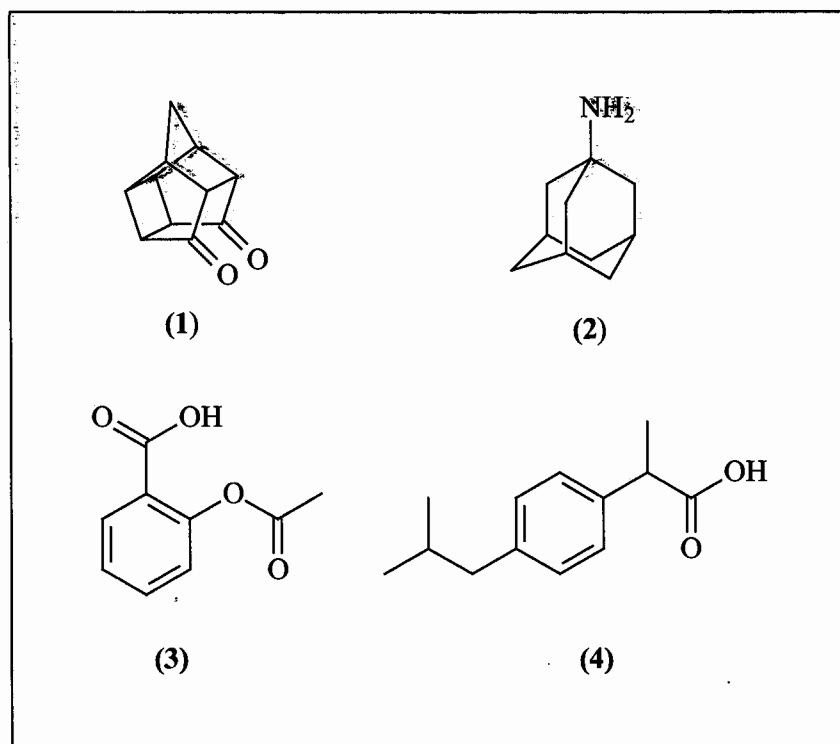


Figure 1.2: Structures relevant to the study.

The aim of this study was therefore to conjugate two NSAIDs with known antioxidant properties, namely acetylsalicylic acid and ibuprofen, to the pentacyclo [5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine scaffolds in order to improve their blood-brain barrier permeability. The hypothesis was tested by subjecting the synthesised compounds to *in vivo* blood-brain barrier permeability and *in vitro* lipid peroxidation assays.

CHAPTER 2

THE BLOOD-BRAIN BARRIER AND PERMEATION THEREOF

2.1 INTRODUCTION

Studies in the late 19th century indicated the existence of the blood-brain barrier, describing 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 presence of some kind of barrier at the site of the brain microvessels (Goldmann, 1909). Originally it was believed that the glial sheets covering the brain capillaries formed the blood-brain barrier (Dempsey & Wislocki, 1955). It was however shown that the anatomical localisation of the blood-brain barrier could in fact be detected at the level of the cerebral capillary endothelial cells. This was done by administration of the electron-dense marker horseradish peroxidase into the brain ventricular system directly as well as through intravenous (IV) injection. No horseradish peroxidase was found in the extracellular space surrounding the brain capillaries after IV administration, whereas horseradish peroxidase was retained at the cerebral endothelial plasma membrane after direct injection into the brain ventricular system (Brightman & Reese, 1969). The blood-brain barrier is thus formed by the brain capillary endothelium (Pardridge, 2005) and plays the predominant role in controlling the passage of endogenous and xenobiotic substances between the circulating blood and the extracellular fluid environment of the brain (Eisenblätter *et al.*, 2003). The transfer of compounds across this barrier is strictly regulated by brain capillary endothelial cells, which are interconnected by well-developed tight junctions, without fenestrations (Eisenblätter *et al.*, 2003).

Development of drugs intended for the brain is limited by blood-brain barrier permeability and one approach to solving this problem is to target endogenous blood-brain barrier transport systems. This must be done by developing CNS drug delivery strategies that take advantage of these natural portals of entry into the brain (Pardridge,

1999a). Several other mechanisms have also been developed to enhance the CNS delivery of drugs. They include prodrugs, chemical delivery systems, carrier-mediated transport and vector-mediated drug delivery. These approaches will be discussed in section 2.5 of this chapter.

2.2 MORPHOLOGY AND FUNCTION

2.2.1 STRUCTURAL ASPECTS

Besides a minor contribution from the choroid plexus epithelium, 95% of the blood-brain barrier comprises a complex network of cerebral microvessels and its unique physiological and biochemical fingerprint is expressed in the endothelial cells that form these brain capillaries (Smith, 1989). It has been established that perivascular elements such as astrocytes and pericytes play a key role in inducing and sustaining certain distinctive endothelial properties. It furthermore seems that a barrier is present only in animals with a complex level of higher neural function, and qualitative observations suggest that “higher” functions of neural tissue are protected by a tighter barrier (Abbott *et al.*, 1986).

Figure 2.1 illustrates how the cerebral capillary endothelium differs from peripheral tissue endothelia.

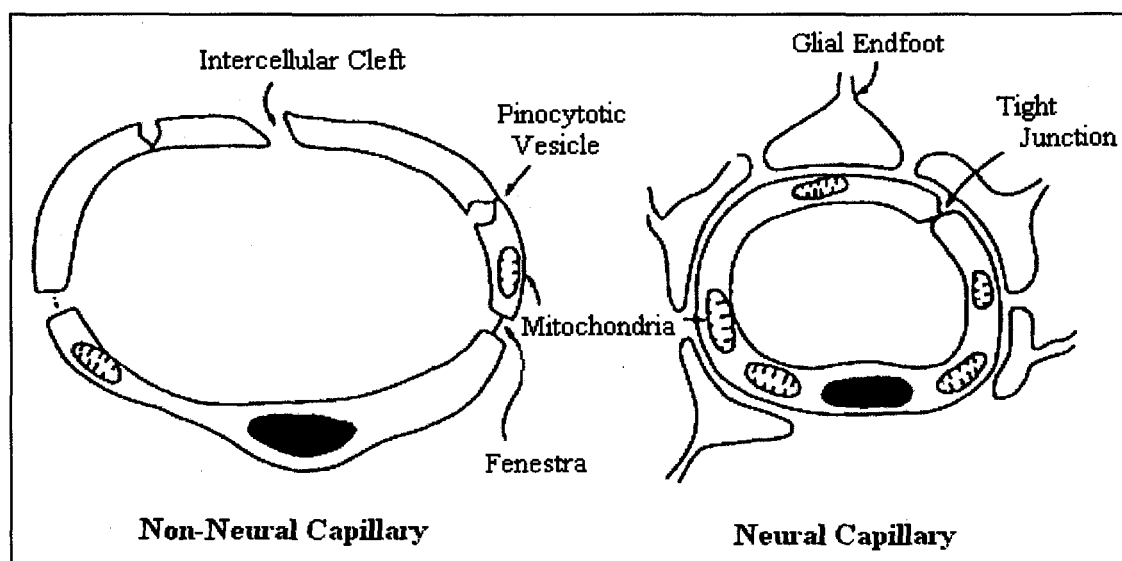


Figure 2.1. Structural differences between peripheral and cerebral endothelial cells (De Vries *et al.*, 1997).

There are approximately 644 kilometres (400 miles) of capillaries in the brain, with a surface area of approximately 20 m². The brain capillaries are approximately 40 µm apart and it takes about one second for a drug to diffuse 40 µm. Therefore, a drug is immediately distributed to the entire brain volume upon transportation of the molecule through the blood-brain barrier from blood (Pardridge, 1999a).

The identity of the cerebral endothelium emerges mainly in the following distinguishing characteristics:

- A uniform basal lamina surrounding the endothelial and pericyte cells; endothelial cytoplasm of consistent thickness, lack of fenestrations, and little pinocytotic activity. Blood-brain barrier transport thus involves translocation through (1) the luminal (apical) capillary membrane, (2) the endothelial cytoplasm, and (3) the abluminal (basolateral) membrane and basal lamina (Cornford & Hyman, 1999).
- Tight junctions (*zonulae occludentes*) sealing the intercellular contacts between adjacent cells. Formation of some capillaries from completely tubular, or seamless, endothelial cells.
- Greater densities of mitochondria, the increase in density which is not attributed to larger numbers of, or bigger mitochondria, but rather to the effect caused by the small dimensions of the brain microvessels and the consequently smaller cytoplasmic area (Stewart & Tuor, 1994).
- An increase in electrical resistance characteristic of tight epithelia, and consistent with a low permeability to ionic materials.
- Elevation of enzymes such as γ -glutamyl transpeptidase (γ -GTP), alkaline phosphatase, and aromatic acid decarboxylase in cerebral microvessels, but a reduction or absence thereof in non-neural capillaries, forming an enzymatic barrier in mammalian brain endothelial cells (Minn *et al.*, 1991). Aldehyde dehydrogenase in brain capillaries may reduce ethanol delivery to the brain, and drugs such as L-dopa and codeine, as well as neurotoxins such as parathion and paraquat, are metabolised by this enzymatic barrier.
- A potential difference (polarity) between the luminal and abluminal membrane surfaces of brain capillaries. Enzymes such as Na⁺/K⁺-ATPase, and the sodium dependent (A-system) neutral amino acid transporter are localised in the

abluminal membrane, whereas γ -GTP is present only in the luminal endothelium (Gottesman & Pastan, 1993; Abbott & Romero, 1996). The P-glycoprotein (P-gp) active drug efflux transporter is restricted to the astrocyte foot processes in brain microvasculature (Pardridge *et al.*, 1997).

2.2.2 FUNCTIONAL PROPERTIES

Establishing guidelines for *in vitro* cerebral capillary endothelial cell cultures, Janigro *et al.* (1999) summarised the functional properties of the blood-brain barrier as follows:

- Expression of tight junctions.
- Selective (and asymmetric) permeability to physiologically relevant ions, such as Na^+ and K^+ , implying the expression of site-specific pumps and ion-carriers. The importance of strict electrophysiological control within the brain is evident.
- Selective permeability to molecules, based on molecular weight, oil/water partition behaviour and other physicochemical attributes.
- Expression of blood-brain barrier specific transporters for metabolic substrates necessary for neuronal and glial cell physiology. The optimal functioning of the neural environment depends on the constant availability of essential nutrients that cannot reach the brain *via* lipophilicity-enhanced diffusion through the blood-brain barrier. Obvious examples are D-glucose and charge-bearing L-amino acids.
- Functional expression of mechanisms responsible for the active extrusion of otherwise permeable substances. The main function of these mechanisms is the protection of the neural parenchyma against permeable, but noxious substances.

2.3 PHYSICOCHEMICAL CHARACTERISTICS NECESSARY FOR BLOOD-BRAIN BARRIER PERMEATION

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

Research has indicated the following guidelines for compounds intended to enter the brain:

- For a compound to exhibit good blood-brain barrier permeation, the polar surface area (PSA) thereof should be below a certain limit. The PSA is a measure of a molecule's hydrogen-bonding capacity and is commonly calculated by summing the contributions to the molecular surface area from oxygen and nitrogen atoms. Two differing limits have been proposed; a limit of $90 \text{ \AA}^2$ was suggested by Van de Waterbeemd *et al.* (1998); whereas Kelder *et al.* (1999) indicated a lower limit of $60\text{--}70 \text{ \AA}^2$.
- Molecular weight should be kept below 450 Da to facilitate brain permeation (Van de Waterbeemd *et al.*, 1998).
- For optimum diffusion into the CNS, the $\log P_{oct}$ value should be in the region of 2 (Levin, 1980).
- A $\log D_{oct}$ value in the range 1-3 is recommended (Van de Waterbeemd *et al.*, 1998). By analogy to $\log P_{oct}$, $\log D$ is the logarithm of the distribution coefficient (D) of a compound. D is the ratio of the sum of the concentrations of all species of the compound in octanol to the sum of the concentrations of all species of the compound in water. For neutral compounds, $\log D$ is equal to $\log P_{oct}$.

Recent studies of brain permeation data have revealed several 'rules of thumb'. These rules give a guideline concerning the molecular properties that favour blood-brain barrier permeation. Two very simple rules were proposed by Norinder and Haeblerlein (2002):

- A molecule has a high chance of entering the brain, if the sum of the nitrogen and the oxygen (N + O) atoms in the molecule is five or less.
- If $\text{Clog } P_{oct} - (\text{N} + \text{O}) > 0$, then the ratio of the steady-state concentrations of the compound in the brain to those in the blood, denoted as $\log BB$, is likely to be positive. In this rule, $\text{Clog } P_{oct}$ denotes the logarithm of the *n*-octanol-water partition coefficient (P_{oct}) of a compound, as computed by the Daylight $\text{Clog } P_{oct}$ program (<http://www.daylight.com>). P_{oct} is the ratio of the concentration of the compound in octanol to the concentration of the compound in water.

From a comparative study, it has been indicated that, overall, compared to non CNS drugs, CNS drugs tend to be more lipophilic, more rigid, have fewer hydrogen bond

donors, fewer formal charges (particularly negative charges) and lower PSA ($< 80 \text{ \AA}^2$) (Mahar-Doan *et al.*, 2002).

In a review Malan *et al.* (2002) stated that the properties of the drug molecule that may have a bearing on membrane penetration include lipophilicity, hydrogen-bonding capacity, molecular weight and size, solubility, state of ionisation, stereochemistry and steric interactions and molecular conformational and surface properties.

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 most common expression of lipophilicity is the logarithm of the *n*-octanol-water partition coefficient ($\log P_{oct}$). The correlation between blood-brain barrier permeability and $\log P_{oct}$ values (representing lipophilicity) is well described in the literature and includes correlations for diverse structures (De Vries *et al.*, 1997; Levin, 1980), pyrrolopyrimidines (Sawada *et al.*, 1999), beta-blocking agents and non-steroidal anti-inflammatory drugs (Van Bree *et al.*, 1988).

In general, the correlation is described as parabolic with an optimum $\log P_{oct}$ value of about 2 as stated earlier (Burton *et al.*, 1996; Young *et al.*, 1988). The influence of electronic properties, and resonance and steric effects like conformational crowding and molecular folding on lipophilicity, should also be considered (Leo *et al.*, 1971).

2.3.2 HYDROGEN-BONDING CAPACITY

Ordered lipid layers (fig. 2.2) provide an infinite amount of hydrogen bonding groups. These groups, the majority of which are hydrogen bond acceptors, are located exclusively in the head group region of lipids. In order to partition into the hydrocarbon region of the bilayer, the solute must be lipophilic enough to overcome the energy losses that occur in breaking the hydrogen bonds with water or the lipid head groups. This step can thus present a considerable energy barrier for solutes that exhibit strong hydrogen bonding (donor) tendencies. Accordingly, biological permeation can be expected to markedly depend on the hydrogen bonding capacity of the solute (El Tayar *et al.*, 1991a; Conradi *et al.*, 1991).

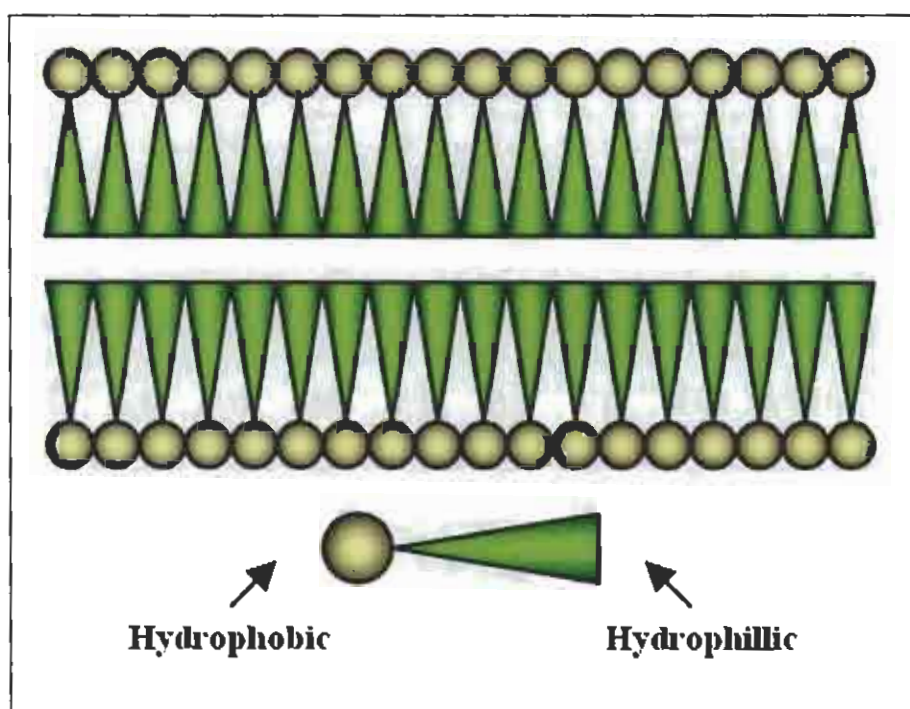


Figure 2.2: Schematic representation of a lipid bilayer.

Hydrogen bond donor (α) and acceptor (β) parameters are generally derived from substructure summation and have been successfully used in relation to steroid drugs to provide predictive algorithms ($r^2 = 0.96$ for this series of steroid drugs) for transdermal permeability (Abraham *et al.*, 1997).

An intensive study on the effect of hydrogen bonding potential was done by Chikhale *et al.* (1994), investigating a series of model peptides consisting of D-phenylalanine residues. The chain length was gradually increased across the series and the corresponding brain capillary permeability coefficient (P_c) determined. The progressive decrease in hydrogen bonding potential by the consecutive methylation of the amide nitrogen was also evaluated. They reported poor correlation between permeability and lipophilicity, but described an excellent correlation between P_c and the number of potential hydrogen bonds in the molecule. The rationale behind this observation is best described by Burton *et al.* (1996) in that the energy required for desolvation of the polar amide bonds in the molecule is the principle determinant of transport and diffusion across the blood-brain barrier. Therefore, the effect of N-methylation is to remove a hydrogen

bond donor group from the peptide and effectively reduce the transfer of energy from water into the cell membrane.

The importance of hydrogen bonding potential as a determinant of blood-brain barrier penetration is well recognised and in many cases a better correlation was found for this parameter than for lipophilicity (Reichel & Begley, 1998; Sawada *et al.*, 1999; Young *et al.*, 1988). The energy required for desolvation of hydrogen bond forming groups is the principal determinant in this case (Burton *et al.*, 1996).

2.3.3 MOLECULAR WEIGHT AND SIZE

Levin (1980) concluded that there is a clear relationship between the diffusion coefficient (D) and molecular weight (M_r):

$$D(M_r)^{-1/2} \approx \text{constant}$$

Based on the above relationship it follows that, for molecules with $M_r < 1000$ Da:

$$P_c \propto K(M_r)^{-1/2}$$

Two conclusions were drawn from his study. Firstly, for compounds with molecular weights below 400 Da, increasing lipophilicity will improve P_c . Secondly, the range within which a cut-off point will be reached regarding blood-brain barrier penetration lies between 400 and 657 Da. In terms of angstrom (Å), the range for blood-brain barrier penetration is estimated between 70-90 Å, with a cut-off point around 100 Å. 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). Hirohashi *et al.* (1997) proved that the blood-brain barrier does not always limit the influx of all molecules larger than 600 Da, demonstrating the non-restricted passive diffusion of 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-tetraacetoxymethyl ester, a molecule with $M_r = 809$ Da, across the blood-brain barrier.

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 physicochemical properties, for example, the 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

The pH of the vehicle in which the penetrant is administered, in combination with the drug molecule's ionisation constant, pK_a , determines the actual concentrations of the ionised and non-ionised species (Wiechers, 1989). For many weak acids and bases, only the non-ionised form possesses appreciable lipid solubility. The mucosal absorption of many compounds is maximal at the pH at which they are mostly non-ionised and decreases as the degree of ionisation increases (Hicks, 1973). Membranes are more permeable to the non-ionised forms, because of their greater lipid solubility (Abdou, 1989). A change in solution pH alters the state of ionisation of a drug and hence its hydrophilicity/lipophilicity as it crosses a membrane (Chou *et al.*, 1995).

Most drugs are weak acids or bases and therefore their activity coefficients change significantly as a function of pH. Physiological pH-values are usually greater than the pK_a for acidic compounds, and less than the pK_a for basic drugs (Barr, 1962). The importance of the degree of dissociation or ionisation as a factor determining permeation through the blood-brain barrier is well documented (Bates, 1984). This is especially so for drugs with pK_a -values close to the physiological pH of 7.4 and the effect is incorporated in the $\log D_x$ ($\log P_{oct}$ 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 α - and 11 β -hydroxyprogesterone, 20 α -hydroxyprogesterone (20 α -dihydroprogesterone), 20 β -hydroxyprogesterone, and 17 α -estradiol and estradiol (17 β -estradiol) across biological membranes (Bates, 1984). The orientation of the hydroxyl group played a pivotal role in determining the permeation rate of the isomers. Depending on the orientation, the hydroxyl groups may be sterically masked determining the 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 (with stereospecific activity) are described for large neutral, small neutral, basic and acidic L-amino acids (Terasaki & Tsuji, 1994).

2.3.7 MOLECULAR CONFORMATIONAL AND SURFACE PROPERTIES

As the conformation of a molecule changes, physicochemical properties, such as hydrogen bonding activity are also affected and may influence membrane permeability (Leo *et al.*, 1971).

The surface area of drug molecules accessible to a solvent is another potential influence on their permeation (El Tayar *et al.*, 1991b). The polar surface area (PSA) accounts for the shape and flexibility of the drug molecule and is related to its hydrogen-bonding capacity. The PSA is calculated as a statistical average in which the surface area of each low-energy conformer is weighed by its probability of existence (Palm *et al.*, 1997).

2.4 MECHANISMS OF TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER

In general, two distinct pathways exist for the movement of solutes across continuous endothelium. The paracellular pathway is an aqueous route involving diffusion of the solute between adjacent endothelial cells, a process that, in the case of the blood-brain barrier, is restricted by the presence of tight junctions. The alternative transcellular pathway requires movement of the solute across or through the cell. For such a process to occur, the solute must interact with components of the cell membrane. In the case of a

polarised endothelial cell, the apical membrane consists of an array of peripherally and integrally associated proteins within an organised lipid matrix. These proteins may serve as specific transporters or receptors for uptake of selected solutes into the cell (fig. 2.3). However, for the vast majority of drug molecules, no such specific mechanisms exist and diffusion remains the only available option (Burton *et al.*, 1996).

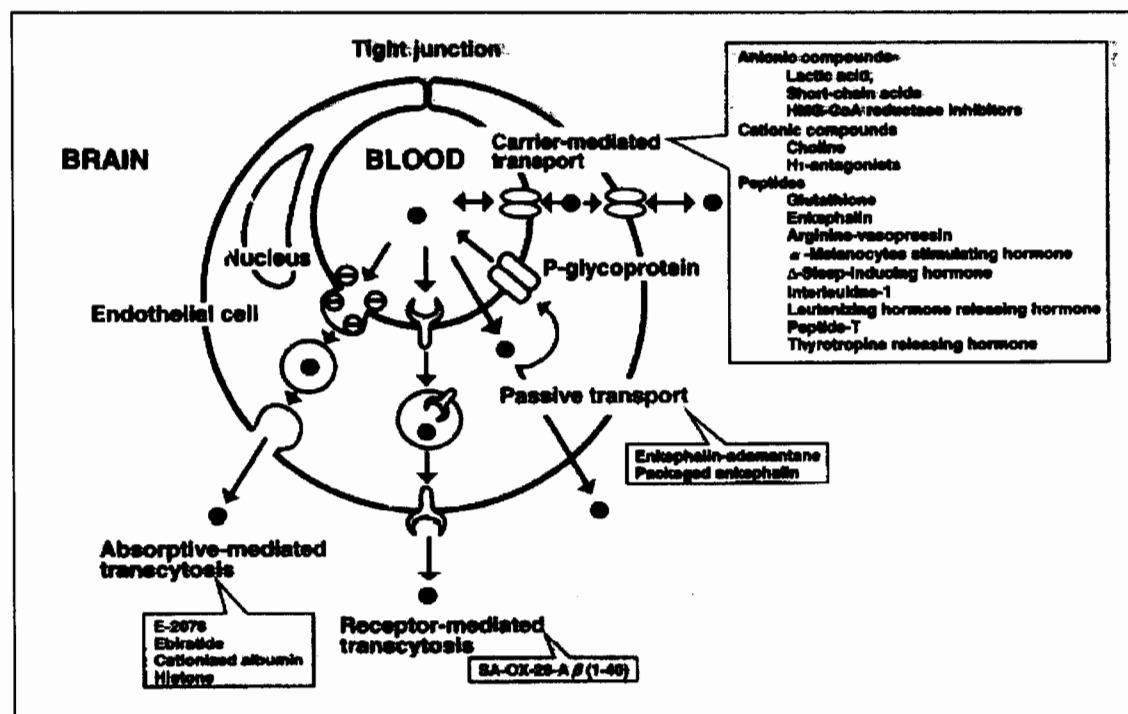


Figure 2.3: Summary of blood-brain barrier transport mechanisms (Tamai & Tsuji, 1996).

2.4.1 PASSIVE TRANSPORT (DIFFUSION)

Diffusion is an energy-independent process characterised by solute movement in response to a chemical potential gradient. Thus flux is independent of direction – the rate of diffusion from apical to basolateral is the same as that in the opposite direction (Burton *et al.*, 1996).

For a molecule to enter the brain, it must escape from the polar environment of the blood plasma and penetrate the non-polar lipid membrane of the cerebral capillary endothelial cells. The diffusion of compounds across the endothelial cells of the blood-brain barrier is therefore dependent on the physicochemical properties of these compounds. Biological complexity however, causes regular contradictions to the expected behaviour of drugs at

the blood-brain interface. An explanation for this may be found in the complex nature of both inter- and intramolecular forces that govern the membrane penetration kinetics. Steric, electrostatic and hydrophobic interactions have to be considered, as well as the influence of molecular structure on the physical equilibrium between these forces (Gaillard *et al.*, 1994).

2.4.2 CARRIER-MEDIATED TRANSPORT

Several specific carrier systems exist at the blood-brain barrier. Mediating systems for hexoses, neutral amino acids, acidic amino acids, basic amino acids, β -amino acids, monocarboxylic acids, choline, amines, nucleosides and peptides have been identified. An overview of these transporters and their relevance in brain-specific drug targeting was done by Terasaki and Tsuji (1994). However, Tsuji and Tamai (1999) also pointed out the fact that the blood-brain barrier cannot be regarded as a static lipid membrane barrier across which transport is only diffusion-limited, but rather has to be viewed as a dynamic interface that has specific physiological functions of selective transport from blood to brain and *vice versa*.

2.4.3 TRANSCYTOSIS (ENDOCYTOSIS)

Transcytosis refers to plasmalemma vesiculates enclosing extracellular fluid at one border of the cell, shuttling through the cytosol and, after fusion with the cell membrane at the opposite border of the cell, delivering the vesicle payload to the extracellular fluid. Several endogenous peptides, insulin, insulin-like growth factor and transferrin in the plasma are transported through the blood-brain barrier *via* receptor-mediated endocytosis, while other peptide analogues enter by means of absorptive-mediated endocytosis. Since these mechanisms are investigated mainly for peptide drugs, receptor-mediated endocytosis refers to the affinity of the peptide for the transport receptor, while absorptive-mediated endocytosis involves an electrostatic interaction between a positively charged moiety of the peptide and a negatively charged plasma membrane surface region (Terasaki & Tsuji, 1994).

2.4.4 EFFLUX

Efflux transport at the blood-brain barrier limits the brain tissue exposure to a variety of potential therapeutic agents, including compounds that are relatively lipophilic and would be predicted to permeate the endothelial lining of the brain microvasculature.

Recent advances in molecular and cell biology have led to the identification of several specific transport systems at the blood-brain interface (Golden & Pollack, 2003).

- **ORGANIC ANION TRANSPORTING POLYPEPTIDE TRANSPORTERS**

The Na⁺-independent organic anion transporting polypeptide 2 (OATP2) has been cloned from rat brain (Noé *et al.*, 1997) and has been detected in rat brain capillary endothelial cells and choroid plexus (Gao *et al.*, 1999; Abe *et al.*, 1998). The substrate specificity of OATP2 appears to be broad, with evidence that it transports anionic compounds and may have both influx and efflux capabilities at the blood-brain and blood-cerebrospinal fluid barriers (Asaba *et al.*, 2000). The human brain organic anion transporter, designated OATP-A, has been localised at the human blood-brain barrier by visualisation with confocal immunofluorescence microscopy (Gao *et al.*, 2000).

- **ORGANIC CATION TRANSPORTERS**

Far less is known about the localisation and significance of organic cation transporters at the blood-brain barrier and blood-cerebrospinal fluid barrier. To date, organic cation transporter-systems in the choroid plexus have been localised at the apical membrane in rat choroid plexus epithelial cell cultures and appear to serve as efflux transporters, transferring organic cations from the cerebrospinal fluid into the epithelial cell (Villalobos *et al.*, 1997).

- **ABC-TRANSPORTERS**

ABC-transporters can be subdivided into two separate classes:

- **P-glycoprotein (P-gp)** is a 170-kDa transmembrane glycoprotein, ATP-dependent efflux transporter that confers multidrug resistance in tumours. P-gp also functions to exclude substrates from cells in a number of normal tissues, including the brain and demonstrates a broad affinity for a number of dissimilar

lipophilic and amphiphilic substrates (Chen *et al.*, 1993; Zamora *et al.*, 1988). Contributors to substrate recognition by P-gp include the presence of electron donor groups that will bind with putative reactive hydrogen bonding sites in the interior of channels formed by the 12 transmembrane helices of P-gp (Seelig, 1998). Given the presence of such donor groups, hydrophobicity governs the degree of interaction with the transporter, thereby differentiating between competing substrates (Seelig & Landwojtowicz, 2000):

- The **multidrug resistance associated protein-1 (MRP1)** is a primary active transporter that transports conjugated metabolites such as glutathione- and glucuronide-conjugates (Kusuhara *et al.*, 1998). MRP1 confers multidrug resistance to anticancer drugs such as doxorubicin, daunorubicin, epirubicin, vincristine, vinblastine and etoposide. In contrast to conjugated metabolites, the reduced form of glutathione is required for any ATP-dependent uptake of vincristine in membrane vesicles from cells expressing MRP1. Correspondingly, reduced-glutathione transport *via* MRP1 is also stimulated by vincristine and verapamil, suggesting that MRP1 co-transporters vincristine and reduced-glutathione (Mao *et al.*, 2000).

2.5 DRUG DELIVERY INTO THE CENTRAL NERVOUS SYSTEM (BARRIER CIRCUMVENTION)

Several mechanisms have been developed for the enhanced delivery of drugs into the CNS. They include prodrugs, chemical delivery systems, carrier-mediated transport and vector-mediated drug delivery.

2.5.1 PRODRUGS

The term prodrug refers to a pharmacologically inactive compound that is converted to an active drug by metabolic biotransformation (Albert, 1951). In most cases of prodrug-design, specific modifications in the drug are made on the basis of known metabolic transformations. It is expected that after administration it will be appropriately metabolised to the active form. This has been termed drug latention to signify the rational design approach (Harper, 1959). The term drug latention was refined even further by Wermuth (1983) into two classes which he called carrier-linked prodrugs and

bioprecursors. A carrier-linked prodrug is a compound that contains an active drug linked to a carrier group that can be removed enzymatically, such as an ester, which is hydrolysed to an active carboxylic acid-containing drug. Carrier-linked prodrugs can be subdivided even further into bipartate, tripartate, and mutual prodrugs. A bipartate prodrug is a prodrug comprised of one carrier attached to the drug. When a carrier is connected to a linker that is connected to the drug, the term tripartate prodrug is used. A mutual prodrug consists of two, usually synergistic, drugs attached to each other (one drug is the carrier for the other and *vice versa*).

2.5.2 CHEMICAL DELIVERY SYSTEMS

Chemical delivery systems represent novel and systematic ways of targeting active biological molecules to specific target sites or organs based on predictable enzymatic activation. They are inactive chemical derivatives of a drug obtained by one or more chemical modifications so that the newly attached moieties are monomolecular units and provide a site-specific or site-enhanced delivery of the drug through multistep enzymatic and/or chemical transformations. During the chemical manipulations, a targetor moiety is responsible for targeting, site-specificity, and lock-in. This idea is essentially different from the prodrug concept by the introduction of multistep activation and targetor moieties. Within the present formalism, one can say that prodrugs contain one or more moieties for protected or enhanced overall delivery, but they do not contain targetor moieties (Bodor & Buchwald, 1999).

The application of this system is exemplified in the zidovudine (AZT)-chemical delivery system. Chu *et al.* (1990) demonstrated a 9.32-fold increase in the relative brain exposure to AZT in mice after intravenous administration of an AZT-chemical delivery system.

2.5.3 CARRIER-MEDIATED TRANSPORT

As described earlier, there are a variety of carrier mechanisms for essential nutrients that exist at the blood-brain barrier. Utilisation of differences in the affinity and the maximal transport activity among these transport systems expressed at the blood-brain barrier is an attractive strategy for controlling the delivery and retardation of drugs into the brain (Tsuji & Tamai, 1999). Pseudo-nutrients are small molecules that have a molecular structure that mimics a nutrient such that it has an affinity for one of several blood-brain

barrier nutrient carrier systems. For example, L-dopa is an effective CNS pharmaceutical because this small molecule has a neutral amino acid structure and has high affinity for the blood-brain barrier neutral amino acid carrier (Pardridge, 1995).

2.5.4 VECTOR-MEDIATED DRUG DELIVERY

Vector-mediated drug delivery to the brain employs chimeric peptide technology, wherein a non-transportable drug is conjugated to a blood-brain barrier transport vector. The latter is a modified protein or receptor-specific monoclonal antibody that undergoes receptor-mediated transcytosis through the blood-brain barrier. The conjugation of a drug to a transport vector is facilitated with chemical linkers, avidin-biotin technology, polyethylene glycol linkers, or liposomes. Multiple classes of therapeutics have been delivered to the brain with the chimeric peptide technology, including peptide-based pharmaceuticals, such as a vasoactive intestinal peptide analog or neurotrophins such as brain-derived neurotrophic factor, antisense therapeutics including peptide nucleic acids, and small molecules incorporated within liposomes (Pardridge, 1999b).

2.6 CONCLUSION

As described above, the blood-brain barrier prevents the passage of a variety of drugs into the CNS. It has been estimated that the blood-brain barrier prevents the uptake of more than 98% of all potential neurotherapeutics by the brain – and make the blood-brain barrier much less ‘leaky’ than the intestinal epithelium. Consequently, paracellular transport of compounds across the barrier is effectively hindered (Clark, 2003). Utilising endogenous blood-brain barrier transport systems or applying other CNS delivery mechanisms will open a whole new field of drug therapy. The CNS delivery of NSAIDs will, for example, have a profound influence on the way we treat neurodegenerative disorders.

CHAPTER 3

POLYCYCLIC CARRIERS TO ENHANCE BLOOD-BRAIN BARRIER PERMEABILITY

3.1 INTRODUCTION

Drugs with limited transport over the blood-brain barrier have been conjugated with lipophilic carriers to vastly improve their blood-brain barrier permeability (Pardridge, 2001). The polycyclic cage appears to be a useful scaffold to yield drugs with a wide scope of applications, and can be used also to modify and improve the pharmacokinetic and pharmacodynamic properties of drugs in current use (Geldenhuys *et al.*, 2005). The adamantane nucleus has already been used as a lipid carrier for blood-brain barrier drug transport and a conjugate of the antiretroviral drug zidovudine (AZT) linked with adamantane through an ester bond substantially improved the transport of AZT into the CNS (Tsuzuki *et al.*, 1994). The polycyclic cage of pentacycloundecane could be used in a similar way to sequester drugs in the CNS.

3.2 ADAMANTANE STRUCTURES

As described above, the adamantane nucleus has been applied by Tsuzuki *et al.* (1994) to improve drug delivery into the CNS. In their study, five derivatives of AZT conjugated via an ester link to a lipophilic adamantane-derived moiety were synthesised (5-9, fig. 3.1). When these prodrugs were administered intravenously to laboratory rats, the prodrugs were detected at a 7-18 fold higher concentration in brain tissue than unconjugated AZT. The mechanism for the uptake of these prodrugs (5-9) into the CNS, is however still not clear. The introduction of the 1-adamantane moiety to AZT thus resulted in the enhancement of the blood-brain barrier penetration of the drug and this pharmaceutical approach would be beneficial for the effective treatment of the CNS infection by human immunodeficiency virus.

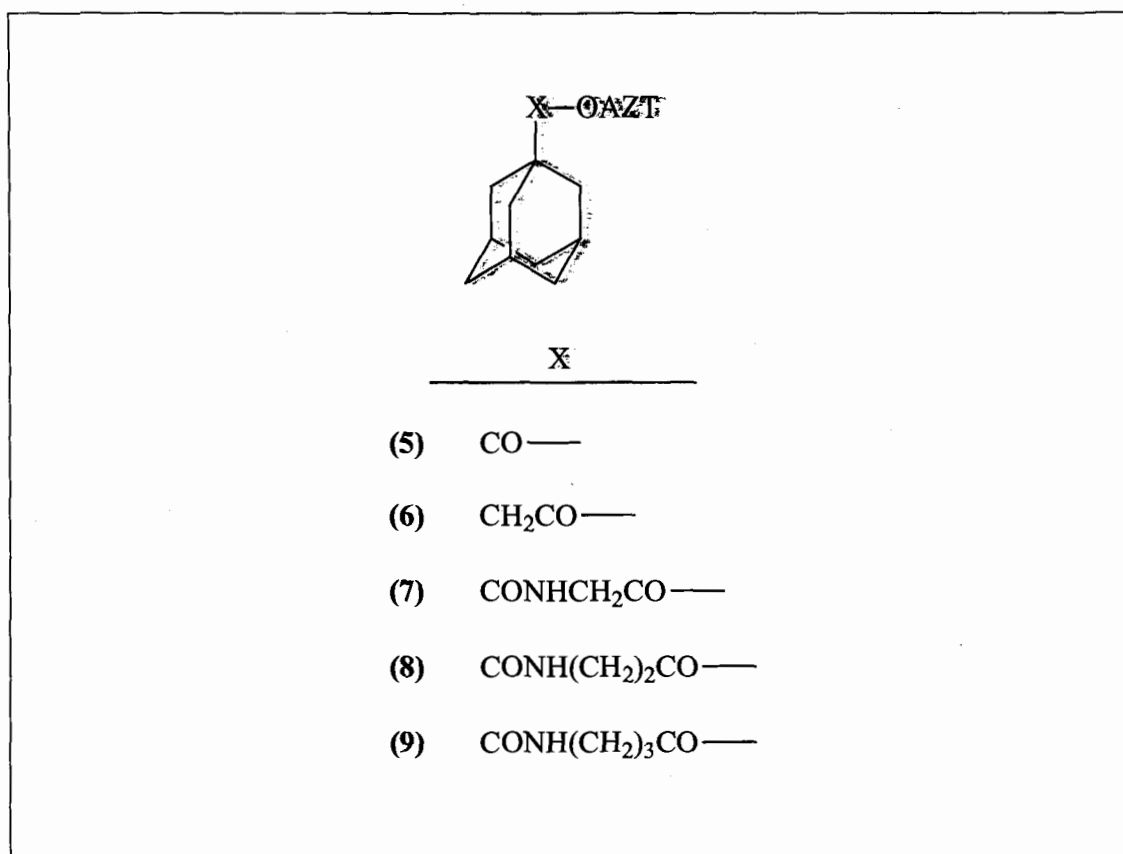


Figure 3.1: Derivatives of AZT conjugated to a lipophilic adamantane-derived moiety (Tsuzuki *et al.*, 1994).

Based on the physicochemical and pharmacological properties of drugs having an adamantane skeleton, an adamantane-based moiety was also evaluated as a drug carrier for poorly absorbed compounds by Kitagawa *et al.* (1997). These compounds included peptides that are active in the CNS. Seven [D-Ala²]Leu-enkephalin derivatives conjugated with an adamantane-based moiety at the C-terminus or N-terminus were prepared by the solution-phase method and their biological activities were examined. The compounds derivatised at the C-terminus through an ester or amide linkage were much more lipophilic than the parent peptide and exhibited moderate *in vitro* opioid activity (guinea-pig ileum assay). This result suggested that the introduction of the lipophilic adamantane moiety to [D-Ala²]Leu-enkephalin would improve the permeation of the poorly absorbed parent peptide through the blood-brain barrier without loss of antinociceptive effect.

3.3 PENTACYCLO[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]UNDECANE

Until very recently, no data had been reported on the ability of the pentacycloundecylamines to cross the blood-brain barrier. This consideration is especially important for compounds where drug targets are located in the CNS (Geldenhuys *et al.*, 2005). Zah *et al.* (2003) developed a quantitative structure-property relationship (QSPR) model to predict blood-brain barrier permeability for a small series of pentacycloundecylamines. Regression analysis was used to examine the correlation of *in vivo* blood-brain distribution data, with a series of physicochemical properties. A highly sensitive electrospray ionisation tandem mass spectrometry (ESI-MS/MS) analytical procedure was developed for the detection of the test compounds in biological preparations. Using this technique, it was indicated that significant drug concentrations were reached in the brain after intraperitoneal administration of the compounds to C57BL/6 mice. This is independent of the mode of entry or whether specific transport systems or efflux mechanisms are involved.

Van der Merwe (2003) also synthesised a number of 8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane derivatives (**10-16**, fig. 3.2) and measured the blood-brain barrier permeability thereof. This was done using an *in vitro* method employing bovine brain microvessel endothelial cells. The results obtained from this study indicated that the synthesised compounds do penetrate the blood-brain barrier readily over a period of five hours.

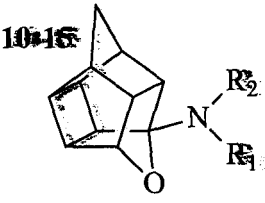
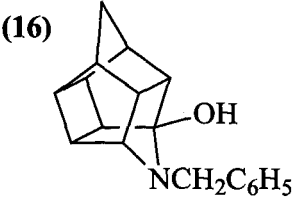
 (16) 	(10) $R_1 = (\text{CH}_2)_3\text{CH}_3$: $R_2 = \text{H}$ (11) $R_1 = (\text{CH}_2)_4\text{CH}_3$: $R_2 = \text{H}$ (12) $R_1 = \text{C}_6\text{H}_5$: $R_2 = \text{H}$ (13) $R_1 = \text{CH}_2\text{C}_6\text{H}_5$: $R_2 = \text{H}$ (14) $R_1 = \text{CH}_2\text{C}_6\text{H}_5$: $R_2 = \text{CH}_3$ (15) $R_1 = \text{CH}_2\text{C}_6\text{H}_5\text{Cl}$: $R_2 = \text{H}$
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Figure 3.2: Synthesised compounds by Van der Merwe (2003).

3.4 CONCLUSION

As discussed above polycyclic structures, specifically adamantane and pentacyclo [5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, possess the ability to permeate the blood-brain barrier. This common property makes these structures very useful as carrier molecules for 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 4

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN NEUROPROTECTION

It has only recently been discovered that non-steroidal anti-inflammatory drugs (NSAIDs) may have neuroprotective effects and may therefore be used in the treatment and even prevention of neurodegenerative diseases. In this section the neuroprotective effects as well as the inability of these drugs to cross the blood-brain barrier, are discussed.

4.1 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS IN NEUROPROTECTION

Several pathways have been proposed for the neuroprotective activity of NSAIDs:

- (1) Decreasing prostaglandin synthesis by inhibition of cyclooxygenase (Vane *et al.*, 1998).
- (2) Inhibition of nuclear factor kappa B (NF- κ B), a transcription factor associated with inflammation (Grilli *et al.*, 1996).
- (3) Inhibition of peroxisome proliferator-activated receptor gamma (PPAR γ), a retinoic acid receptor-like molecule that mediates inflammation and beta-amyloid-induced neurotoxicity (Combs *et al.*, 2000).
- (4) Inhibition of oxidative stress (Bisaglia *et al.*, 2002).

Cyclooxygenase (COX) catalyses the first committed step in the synthesis of prostanoids, a large family of arachidonic acid metabolites comprising prostaglandines, prostacyclin and thromboxanes, and is a major target of NSAIDs. COX exists as constitutive and inducible isoforms. COX-2 is the inducible isoform, rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules. By extension, COX-2 expression in the brain has been associated with pro-inflammatory activities, thought to be instrumental in neurodegenerative processes of several acute and chronic diseases such as Parkinson's disease and Alzheimer's disease. COX remains

central to the development of anti-inflammatory treatments for a variety of pathologies, including neuroinflammatory and neurodegenerative diseases (Minghetti, 2004).

It is well known that acetylsalicylic acid (aspirin, 3) and ibuprofen (4) are non-selective COX-inhibitors (inhibits both isoforms). Therefore the neuroprotective activity of these drugs may, at least in part, be as a result of the inhibition of this enzyme. In spite of the intense research of the last decade, evidence of a direct role of COX-2 in neurodegenerative events is however still controversial (Minghetti, 2004).

In a study done by Bisaglia *et al.* (2002) on the neuroprotective activity of acetaminophen (paracetamol) it was concluded that this drug reduces the increased NF- κ B binding activity, which occurs in degenerative conditions. Moreover, activation of NF- κ B by amyloid-derived peptides was greatly impaired by acetaminophen pre-treatment in hippocampal cells.

A recently described target of NSAIDs is the ligand-activated nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR γ). PPAR γ is a DNA-binding transcription factor of which the transcriptional regulatory actions are activated after agonist binding. Combs *et al.* (2000) reported that NSAIDs, drugs of the thiazolidinedione class, and the natural ligand prostaglandin J2 act as agonists for PPAR γ and inhibit the β -amyloid-stimulated secretion of proinflammatory products by microglia and monocytes responsible for neurotoxicity and astrocyte activation. The activation of PPAR γ also arrested the differentiation of monocytes into activated macrophages. PPAR γ agonists were shown to inhibit the β -amyloid stimulated expression of the cytokine genes interleukin-6 and tumour necrosis factor α . Furthermore, PPAR γ agonists inhibited the expression of COX-2. The efficacy of these agents in inhibiting a broad range of inflammatory responses suggests PPAR γ agonists may provide a novel therapeutic approach to Alzheimer's disease.

Maharaj (2004) investigated the neuroprotective properties of acetylsalicylic acid and acetaminophen. It was demonstrated that acetylsalicylic acid and acetaminophen alone and in combination significantly attenuated cyanide-induced superoxide anion ($O_2^{\bullet-}$) generation *in vitro*. The combination of these agents afforded significant and complete protection against cyanide-induced $O_2^{\bullet-}$ generation by reducing the levels of $O_2^{\bullet-}$ below

that of the control basal value. Acetylsalicylic acid and acetaminophen was also shown to alleviate the quinolinic acid-induced rise in $O_2^{\bullet}$ generation in the hippocampus *in vivo*. The combination of these agents proved more potent than either agent alone and reduced the $O_2^{\bullet}$ levels below control levels. An *in vivo* study was also done to determine the effects of these agents in protecting against MPP^+ -induced $O_2^{\bullet}$ generation in the striatum. Acetylsalicylic acid and acetaminophen were effective in reducing the MPP^+ -induced rise in $O_2^{\bullet}$ levels. The potent activity exhibited by acetylsalicylic acid could be attributed to possible roles in the enhancement of cellular respiration and superoxide dismutase activity in the presence of MPP^+ .

Asanuma *et al.* (2001) reported that ibuprofen, among other drugs, afforded protective effects against apoptosis mainly due to its direct nitric oxide radical scavenging activities in neuronal cells.

Glutamate excitotoxicity has been implicated in Alzheimer's disease, Parkinson's disease as well as other neurodegenerative diseases. Casper *et al.* (2000) evaluated whether acetylsalicylic acid, acetaminophen and ibuprofen protected dopaminergic neurons against excitotoxicity. Cultured primary rat embryonic neurons from mesencephalon, the area primary affected in Parkinson's disease, were used. All three NSAIDs significantly attenuated the decrease in dopamine uptake caused by glutamate, indicating preservation of neuronal integrity. In addition, ibuprofen alone increased the relative number of dopaminergic neurons by 47%.

Due to the fact that oxidative stress plays a profound role in lipid peroxidation, it is clear that NSAIDs may have inhibiting effects on lipid peroxidation. Antioxidants can act at different levels in the oxidative sequence. As far as lipid peroxidation is concerned, antioxidants could act by:

- Decreasing localised O_2 concentrations.
- Preventing first-chain initiation radicals such as $^{\bullet}OH$.
- Binding metal ions in forms that will not generate such initiating species as $^{\bullet}OH$, ferryl, or $Fe^{2+}/Fe^{3+}/O_2$ and/or will not decompose lipid peroxides to peroxy or alkoxy radicals.

- Decomposing peroxides by converting them to non-radical products, such as alcohols.
- Chain breaking, i.e. scavenging intermediate radicals such as peroxy and alkoxy radicals to prevent continued hydrogen abstraction (Halliwell & Gutteridge, 1989).

Because of the brain's high utilisation of oxygen as well as its relatively low concentration of antioxidative enzymes and free radical scavengers, it is particularly vulnerable to oxidative attack by free radicals (Reiter *et al.*, 1995). Furthermore, brain tissues are rich in phospholipids, which can be attacked by highly reactive oxygen species (ROS) for the initiation of lipid peroxidation. Lipid peroxidation is able to cause extensive damage and is known to play a major role in the deterioration of the brain and spinal cord that occurs after traumatic, excitotoxic or ischemic injury (Bautista & Spitzer, 1990). Numerous laboratories have reported studies showing increased accumulation of lipid peroxidation products in diseased regions of patients suffering from Alzheimer's disease (Lovell *et al.*, 1995; Montine *et al.*, 1997; Pappolla *et al.*, 1992).

A study conducted by Kirkova *et al.* (1995) indicated that acetylsalicylic acid slightly affected lipid peroxidation and antioxidant enzyme activity *in vivo*. High doses of the drug decreased the ascorbate-induced lipid peroxidation and the inhibition of malondialdehyde (MDA) production was more pronounced after intraperitoneal injection of the drug than following oral administration. It was suggested that the stronger inhibition of ascorbate-induced MDA production compared to that induced by Fe^{2+} /ascorbate is determined by acetylsalicylic acid's ability to chelate endogenous metal ions. It is known that MDA is one of the products of the enzymatic oxidation of arachidonic acid, particularly of thromboxane biosynthesis (Hamberg *et al.*, 1975), and that acetylsalicylic acid leads to irreversible inactivation of COX (Rang & Dale, 1987). Hence, a decrease of MDA could be expected after treatment with acetylsalicylic acid. The vital role that NSAIDs could play in neuroprotection is thus clear.

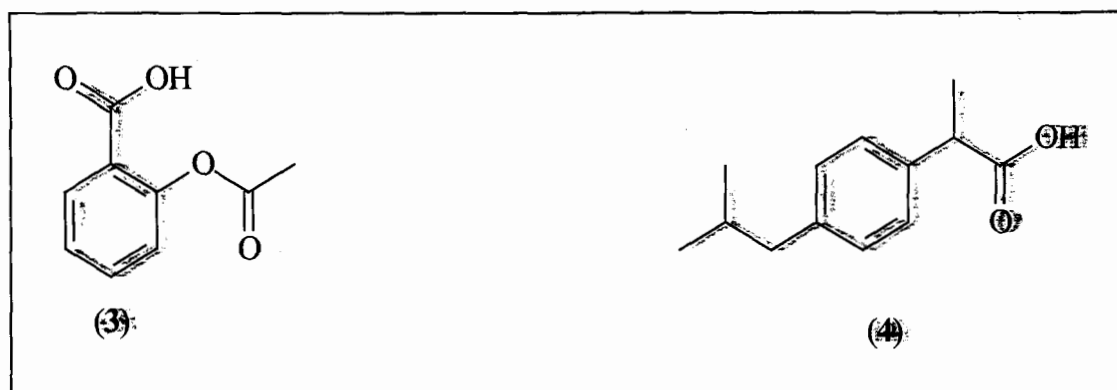


Figure 4.1: Chemical structures of acetylsalicylic acid (3) and ibuprofen (4).

4.2 BLOOD-BRAIN BARRIER PERMEABILITY OF NON- STEROIDAL ANTI-INFLAMMATORY DRUGS

As described earlier, drugs need certain physicochemical characteristics in order to be able to cross the blood-brain barrier, lipophilicity being one of them.

Most NSAIDs are weak organic acids (Furst & Munster, 2001) and the pK_a values for the NSAIDs discussed are as follows:

Acetylsalicylic acid: $pK_a = 3.5$ and

Ibuprofen: $pK_a = 5.2$ (Katzung, 2001).

Taking the physiological pH (7.4) into account it can safely be said that acetylsalicylic acid and ibuprofen would be ionised (relatively hydrophilic) in the human body. This implies that none of these two drugs will penetrate the blood-brain barrier in meaningful quantities.

Mannila *et al.* (2005) reported that inefficient CNS delivery limits the use of ibuprofen in neurodegenerative diseases. Their study results indicated that both plasma and brain concentrations reached steady-state within 6 hours, and that the brain to plasma ratio of ibuprofen was only 0.02. This was indicative of the extremely low brain penetration of the drug. Ibuprofen is fairly lipophilic in its unionised form ($\log P = 3.5$), however, when the pH is above its pK_a value (5.2), ibuprofen becomes ionised, leading to a substantial decrease in its lipophilicity. In the systemic circulation, where the pH is approximately

7.4, ibuprofen is totally ionised and is too hydrophilic to be effectively absorbed by passive diffusion through the blood-brain barrier.

4.3 CONCLUSION

From the discussion it is concluded that NSAIDs have great potential as possible neuroprotective agents. This is as a result of, among others, their anti-inflammatory and antioxidant properties. The need for an effective CNS delivery mechanism for these drugs is thus very clear, and therefore prodrug synthesis of these hydrophilic drugs were pursued by conjugation with pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane and amantadine.

CHAPTER 5

EXPERIMENTAL

5.1 STANDARD EXPERIMENTAL PROCEDURES

5.1.1 INSTRUMENTATION

5.1.1.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

^1H and ^{13}C NMR spectra were obtained using a Varian Gemini 300 spectrometer at a frequency of 300.075 MHz and 75.462 MHz, respectively. 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 ^1H - ^{13}C -decoupling. All chemical shifts are reported in parts per million (ppm) relative to the signal from TMS ($\delta = 0$) added to an appropriate deuterated solvent. The following abbreviations are used to describe the multiplicity of the respective signals: s – singlet, bs – broad singlet, 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 compounds 21, 22, 23, 25 and 26.

5.1.1.2 MASS SPECTROSCOPY (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 ionisation techniques.

5.1.1.3 INFRARED SPECTROSCOPY (IR)

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

5.1.1.4 MELTING POINT (MP) DETERMINATION

Melting points were determined using a Gallenkamp-melting point apparatus and capillary tubes. Recorded melting points are uncorrected.

5.1.2 CHROMATOGRAPHIC TECHNIQUES

Mobile phases used were prepared on a volume-to-volume basis and suitable mobile phases were determined using the Prism model (Nyiredy *et al.*, 1985).

5.1.2.1 THIN LAYER CHROMATOGRAPHY (TLC)

Analytical TLC was performed on 0.20 mm thick aluminium silica gel sheets (Alugram[®] SIL G/UV₂₅₄, 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, Macherey-Nagel, Düren, Germany) with mobile phases as indicated for each compound. Flash column chromatography (Harwood *et al.*, 1999) was also applied when necessary.

5.2 SYNTHESIS OF SELECTED COMPOUNDS

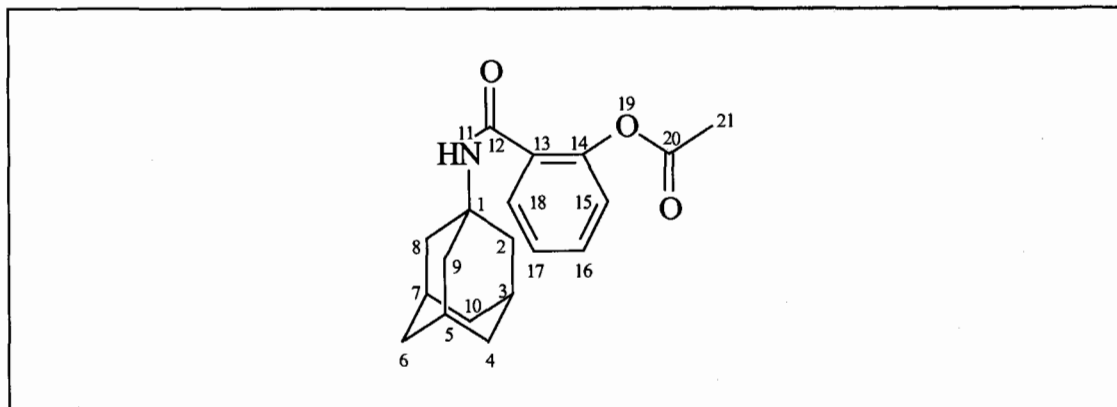
5.2.1 GENERAL APPROACH

The well-described Cookson's diketone, pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (**18**), was synthesised according to the published method (Cookson *et al.*, 1958, 1964). This structure served as primary basis for the synthesis of the proposed compounds. The reaction involved Diels-Alder adduct (**17**) formation and subsequent photocyclisation to yield the polycyclic cage structure. A schematic representation of the synthesis route is given in figure 5.1.

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1C), 41.97 (d, 1C), 40.21 (d, 1C), 38.89 (d, 1C), 30.13 (d, C-28), 23.85 (t, C-15), 22.31 (q, C-20), 22.27 (q, C-29/30), 22.26 (q, C-29/30); **HR-MS**: calc. 419.2460, exp. 419.2447; **MS** (EI, 70 eV) m/z (Spectrum 18): 419 (M^+), 231, 230, 214, 188, 161, 57, 43; **IR** (KBr) $\nu_{\max}$ (Spectrum 23): 2867, 1740, 1510, 1458, 1339, 1269, 1124, 849 cm^{-1} .

5.2.2.5 1-ADAMANTAN-1-YL-2-[(ADAMANTYLAMINO)CARBOXYL]PHENYLACETATE (25)

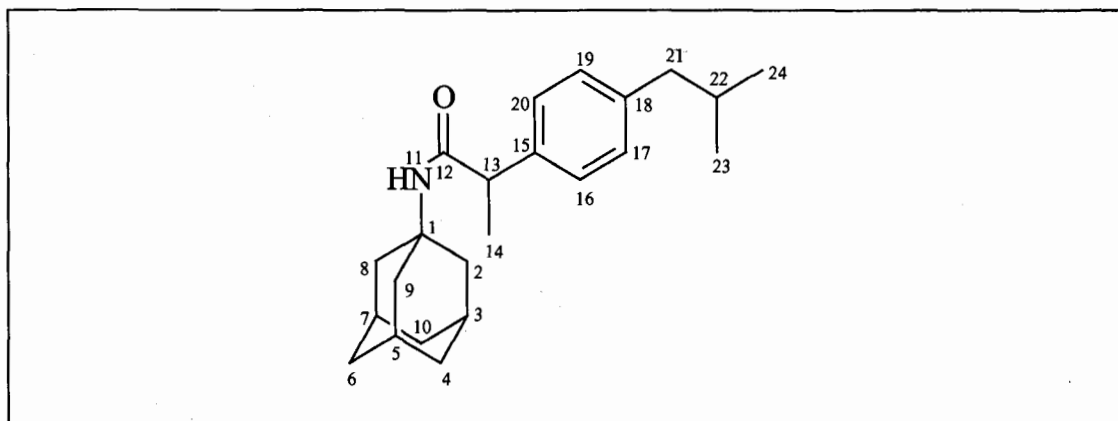


Synthetic method: Amantadine.HCl (1.126 g, 6 mmol) was dissolved in dried dichloromethane (80 ml) with the addition of triethylamine (2.510 ml, 18 mmol). The reaction mixture was stirred for 30 minutes at room temperature to allow all of the amantadine.HCl to be converted into its free base form. *o*-Acetylsalicyloyl chloride (1.192 g, 6 mmol) was added to the above solution and the deep amber coloured mixture stirred at room temperature for 38 hours. After the solvent was removed under reduced pressure tetrahydrofuran:ethyl acetate (2:1) was added to the yellowish compound. A white precipitate formed instantaneously and was filtered. TLC indicated that this was the free amantadine base. Repetition of this procedure afforded a pure solution of the desired amide. *In vacuo* evaporation of the solvent produced the product as a light yellow powder (Yield: 1.263 mg, 4.030 mmol, 67.17 %).

Physical data: $\text{C}_{19}\text{H}_{23}\text{NO}_3$; **mp**: 173 °C; ^1H NMR (300 MHz, CDCl_3) δ_{H} (Spectrum 4): 7.65 (dd, 1H, $J = 7.69, 1.65$ Hz, H-18), 7.44-7.34 (m, 1H, H-16), 7.30-7.20 (m, 1H, H-17), 7.03 (dd, 1H, $J = 8.10, 1.09$ Hz, H-15), 6.06-5.74 (s, 1H, H-11), 2.41-2.27 (s, 3H, H-21a, 21b, 21c), 2.15-2.00 (2 x m, 9H, H-2a, 2b, 3, 5, 7, 8a, 8b, 9a, 9b), 1.76-1.60 (m, 6H, H-4a, 4b, 6a, 6b, 10a, 10b); ^{13}C NMR (75 MHz, CDCl_3) δ_{C} (Spectrum 9): 169.06 (s, C-

20), 164.68 (s, C-12), 147.44 (s, C-14), 131.11 (d, C-16), 130.12 (d, C-18), 129.43 (d, C-17), 126.22 (d, C-15), 122.84 (s, C-13), 52.35 (s, C-1), 41.63 (3 x t, C-2, 8, 9), 36.31 (3 x t, C-4, 6, 10), 29.42 (3 x d, C-3, 5, 7), 21.06 (q, C-21); ~~IR-MS~~ ~~calc~~: 313.1678, ~~exp~~: 313.1668; ~~MS~~ (EI, 70 eV) ~~m/z~~ (Spectrum 19): 313 (M^+), 271, 150, 135, 121, 94, 43; ~~IR~~ (KBr) $\nu_{\max}$ (Spectrum 24): 3422, 1759, 1640, 1194, 909, 805, 753 cm^{-1} .

5.2.2.6 2-(4-ISOBUTYLPHENYL)-N-(ADAMANTYL)PROPANAMIDE (26)



Synthetic method: Ibuprofen (1.238 g, 6 mmol) was dissolved in dried dichloromethane (50 ml) and N,N'-Dicyclohexylcarbodiimide (DCC; 1.362 g, 6.6 mmol) added in molar excess. The reaction mixture was stirred at 5°C on an external ice bath. The hydrated DCC adduct, dicyclohexylurea (DCU), which is produced as a result of the activation reaction, precipitated within 5 minutes. DCU was removed by filtration and the reaction mixture stirred at room temperature for 3 hours, allowing the activation reaction to reach completion. Triethylamine (2.510 ml, 18 mmol) and amantadine.HCl (1.126 g, 6 mmol) was then added and the mixture stirred at room temperature for 38 hours. After the solvent was removed under reduced pressure, tetrahydrofuran:ethyl acetate (2:1) was added to the amber coloured oil. A white precipitate formed immediately and was filtered. TLC again indicated that this was the free amantadine base. The repetition of this procedure afforded a nearly pure solution of the desired amide. Flash column chromatography (silica gel stationary phase, ethyl acetate) was applied yielding the pure product as a white powder (Yield: 878.7 mg, 2.588 mmol, 43.13 %).

Physical data: $\text{C}_{23}\text{H}_{33}\text{NO}$; mp: 145 °C; ^1H NMR (300 MHz, CDCl_3) δ_{H} (Spectrum 5): 7.14 (d, 2H, $J = 8.10$ Hz, H-16, 20), 7.07 (d, 2H, $J = 8.34$ Hz, H-17, 19), 5.11-4.81 (s,

1H, H-11), 4.04-3.80 (q, 1H, H-13), 2.54-2.33 (d, 1H, H-21), 2.06-1.96 (m, 1H, H-22), 1.95-1.71 (m, 7H, H-2a, 2b, 8a, 8b, 9a, 9b, 3/5/7), 1.71-1.49 (m, 6H, H-4a, 4b, 6a, 6b, 10a, 10b), 1.47-1.39 (d, 3H, H-14a, 14b, 14c), 0.94-0.76 (d, 6H, H-23a, 23b, 23c, 24a, 24b, 24c); ¹³C NMR (75 MHz, CDCl₃) δ_C (Spectrum 10): 173.53 (s, C-12), 140.44 (s, C-15), 140.36 (s, C-18), 129.60 (d, C-20), 129.47 (d, C-16), 127.18 (d, C-17), 126.88 (d, C-19), 51.64 (s, C-1), 47.50 (d, C-13), 45.01 (t, C-21), 41.46 (3 x t, C-2, 8, 9), 36.33 (3 x t, C-4, 6, 10), 30.12 (d, C-22), 29.41 (3 x d, C-3, 5, 7), 22.33 (q, C-14), 22.27 (q, C-23/24), 22.24 (q, C-23/24); MS (EI, 70 eV) *m/z* (Spectrum 20): 412 (M⁺), 287, 236, 162, 135, 119, 83, 55, 43; IR (KBr) ν_{max} (Spectrum 25): 3296, 2852, 1655, 1447, 1376, 805 cm⁻¹.

5.3 ACQUISITION OF BLOOD-BRAIN BARRIER PERMEABILITY

DATA (*IN VIVO*)

In order to determine the blood-brain barrier permeability of the novel synthesised compounds (22, 23, 25 & 26), the *in vivo* test model developed in our laboratory was applied (Zah *et al.*, 2003). This involved the intraperitoneal (i.p) injection of the compounds and free NSAIDs to C57BL/6 laboratory mice followed by decapitation 1 hour thereafter. The reason for the administration of free acetylsalicylic acid and ibuprofen was to be able to compare the blood-brain barrier permeability thereof to that of the corresponding synthesised prodrug. The arbitrary time-point (1 hour) was chosen as the result of pilot studies done by the same authors, using 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01). Brain and blood samples were frozen with liquid nitrogen and kept at -77 °C until analysed.

5.3.1 CHEMICALS

Analytical grade acetonitrile (Multisolvant) and methanol were purchased from Sharlau (Barcelona, Spain), and formic acid (99 %) was obtained from Saarchem (Krugersdorp, South Africa). Chloroform (Hipersolv for HPLC) was purchased from BDH (Poole, England) and 25 % ammonia solution for analysis was procured from Merck (Darmstadt, Germany). Hydrochloric acid (32 %) was obtained from Saarchem (Midrand, South Africa). Throughout analyte preparation double-distilled, de-ionised water was used.

5.3.2 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 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.

5.3.3 ADMINISTRATION AND TISSUE HARVESTING

As a result of solubility problems encountered for the ester compounds **22** and **23**, the hydrochloric salt for each one was prepared. This improved their aqueous solubility substantially. The amide compounds **25** and **26** were used in their free synthesised form. The test compounds were not all soluble in the same solvent, necessitating the use of different solvents during administration. The compounds with their specific solvents used are as follows: Acetylsalicylic acid and compounds **21-23** - 40 % (v/v) ethanol diluted with water for injection; Compounds **25-26** - methanol (100 %); Ibuprofen - polyethylene glycol 300 and amantadine.HCl - propylene glycol. Each animal's body mass was noted where after the freshly constituted test preparation (38 mg/ml) was 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 for acetylsalicylic acid, ibuprofen, amantadine.HCl and compound **21**; 327.58 mg/kg for compound **22**; 305.08 mg/kg for compound **23**; 260.935 mg/kg for compound **25** and 246.883 mg/kg for compound **26**. 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 1 hour after administration by means of decapitation. Whole 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.

5.3.4 STANDARDISATION AND ANALYTE RECOVERY

Each drug/test compound was administered to a single mouse, resulting in each test comprising of 8 mice. Two of the test mice received the free NSAIDs, one mouse received amantadine.HCl, another received compound 21, another two mice the ester prodrugs and the final two the amide prodrugs. These tests were repeated three times for statistical purposes. Naproxen was applied as internal standard at 200 µg/ml throughout the test procedure. In order to determine the extraction efficiency, control brain and blood samples were prepared by adding 10 µg naproxen only and 10 µg naproxen together with 10 µg of the relevant test compound to freshly obtained brain and blood tissues. The normal extraction procedure, as for the injected groups, was followed and these tests were also repeated three times. In order to attribute a chromatogram peak to a certain compound or metabolite, standard solutions of the free drugs and compounds under investigation were prepared and injected onto the HPLC column. 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 thereof did permeate through the blood-brain barrier.

5.3.4.1 BRAIN TISSUE EXTRACTION

Sample weight was recorded, where after the brains were rinsed with ice-cold water (2 x 2 ml). HCl (10 %; 2ml) as well as the internal standard naproxen (10 µg in 50 µl methanol) were 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 two vials for each brain homogenate. A total of twelve vials were thus used for the six brain homogenates obtained in each test. Following centrifugation (15 minutes at 11363 x g; 4 °C), the supernatants were decanted and the pH adjusted to approximately 9, using ammonia solution (5 %; 2 ml). The relevant compound was extracted by adding chloroform (3 ml) and vortexing the mixture for 60 seconds. Solvent phases were separated by centrifugation (5 minutes at 5444 x g; 15 °C), the aqueous layer discarded and the organic fraction brought to dryness under a mild stream of nitrogen gas at room temperature (25 °C). The organic residue was re-dissolved

in 250 μ l methanol and HPLC as well as LC-MS/MS analyses carried out. For the HPLC analysis the mobile phase consisted of 75% Milli-Q water (containing 0.2% triethylamine, pH 7) and 25% acetonitrile at a flow rate of 1 ml/min. After 10 minutes of each HPLC run, a solvent gradient program was initiated by decreasing the aqueous mobile phase from 75% to 10% with a concomitant increase of acetonitrile from 25% to 90%. The mobile phase for the LC-MS/MS analysis comprised of 70% Milli-Q water (containing 0.1% formic acid) and 30% acetonitrile (containing 0.1% formic acid) at a flow rate of 200 μ l/min. Here a solvent gradient program was also employed by decreasing the aqueous mobile phase from 70% to 20% with a resultant increase of acetonitrile from 30% to 80% after 10 minutes.

5.3.4.2 BLOOD EXTRACTION

After recording sample weight, HCl (10 %; 2 ml) as well as the internal standard naproxen (10 μ g in 50 μ l methanol) was added to each vial and the mixtures vortexed for 50 seconds. Following centrifugation (15 minutes at 5444 x g; 4 °C), the supernatants were decanted and the pH adjusted to approximately 9, using ammonia solution (5 %; 2 ml). The relevant compound was extracted by adding chloroform (3 ml) and vortexing the mixture for 60 seconds. Solvent phases were separated by centrifugation (5 minutes at 5444 x g; 15 °C), the aqueous layer discarded and the organic fraction brought to dryness under a mild stream of nitrogen gas at room temperature (25 °C). The organic residue was re-dissolved in 250 μ l methanol and HPLC as well as LC-MS/MS analyses carried out. The mobile phases used for both analytical procedures were exactly the same as those for the brain tissue extraction.

5.3.5 INSTRUMENTATION AND ANALYSIS

HPLC analyses were performed using an Agilent® 1100 series HPLC equipped with a quaternary gradient pump, autosampler, diode array UV detector and Chemstation® Rev. A. 08.03 data acquisition and analysis software. The LC-MS/MS analyses on the other hand, were carried out using an Agilent® 1100 series HPLC with a binary gradient pump, autosampler and vacuum degasser coupled to an Applied Biosystems® API 2000 triple quadrupole mass spectrometer and Analyst® 1.4 data acquisition and analysis software.

Standard solution samples of each of the eight test compounds were prepared in analytical grade methanol and analysed using the above-described mobile phases. LC-MS/MS analyses were optimised by means of direct infusion of a standard solution using a Harvard syringe pump and selection of the two most prominent parent/daughter ion pairs for each component. The most prominent Multiple Reaction Monitoring (MRM) pair was used as quantifier and the other as qualifier. The same procedure was followed for the internal standard. Annexure B lists a full summary of instrumental settings and applied conditions for each compound tested.

5.4 LIPID PEROXIDATION STUDY (*IN VITRO*)

5.4.1 INTRODUCTION

Oxidation and aging are closely related (Kaizu *et al.*, 1993) and it is well established that oxidative stress can damage biological molecules. Lipid peroxidation is one of the most commonly discussed oxidation reactions and it may be defined as the ability of free radicals to alter the integrity of cell membranes. These alterations may then ultimately lead to increased permeability to ions such as Ca^{2+} resulting in cell destruction (Halliwell & Gutteridge, 1989). Polyunsaturated fatty acids are essential for normal neural cell membrane functioning because many membrane properties, such as fluidity and permeability, are closely related to the presence of unsaturated and polyunsaturated side chains. Lipid peroxidation causes a loss of membrane polyunsaturated fatty acids and oxidised phospholipids, as polar species contribute to increased membrane rigidity. Alterations in properties of plasma membranes may be responsible for the degeneration of neurons seen in neurodegenerative diseases (Farooqui & Horrocks, 1998).

The events in lipid peroxidation are illustrated schematically as follows:

- Cell damage $\rightarrow \cdot\text{OH} + \text{CH}_2 \rightarrow \text{C}\cdot\text{H} \rightarrow \text{conjugated diene} + \text{O}_2 \rightarrow \text{CHO}_2\cdot + \text{CH}_2 \rightarrow \text{C}\cdot\text{H} + \text{lipid peroxide}$.
- $\text{Fe}^{2+}\text{-complex} + \text{lipid peroxide} \rightarrow \text{CHO}_2\cdot$
- $\text{Fe}^{3+}\text{-complex} + \text{lipid peroxide} \rightarrow \text{CHO}_2\cdot + \text{H}^+ + \text{Fe}^{2+}\text{-complex}$.

An attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-\text{CH}_2-$), can initiate lipid peroxidation. An $\cdot\text{OH}$ can do this, as well as various iron-complexes. After abstraction from a methylene group, a carbon radical is yielded which then stabilises by molecular rearrangement to form a conjugated diene. This then combines with oxygen (which is hydrophobic and thus concentrates into the interior of membranes) to form a peroxy or “peroxy” radical ($\text{CHO}_2\cdot$). This radical can then abstract hydrogen from another lipid molecule or it may attack membrane proteins. Once the peroxy radical has formed it can abstract another hydrogen atom from a methylene group and combine with this hydrogen to form a carbon radical and lipid peroxide. This carbon radical may then react with oxygen to form a peroxy radical, continuing the lipid peroxidation cascade (Halliwell & Gutteridge, 1990).

The increased incidence of neurodegenerative diseases may be attributed to a pro-oxidative environment caused by smoking, alcohol abuse, UVA and UVB radiations, air pollution as well as inappropriate nutrition. The dependence of disease severity on an imbalance between oxidants and natural defences suggests that oxidative stress plays a pivotal role in the progression of neurodegenerative diseases and could serve as a useful target for treatment (Singh *et al.*, 2004). As described in chapter 4, section 4.1 certain NSAIDs possess 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.

5.4.2 ASSAY PROCEDURE

The thiobarbituric acid assay (TBA test) is the most prominent and currently used assay as an index for lipid peroxidation products (Garcia *et al.*, 2005). It 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 (fig. 5.7). This red adduct (MDA-TBA complex) has an absorption maximum at 532 nm (Janero, 1990).

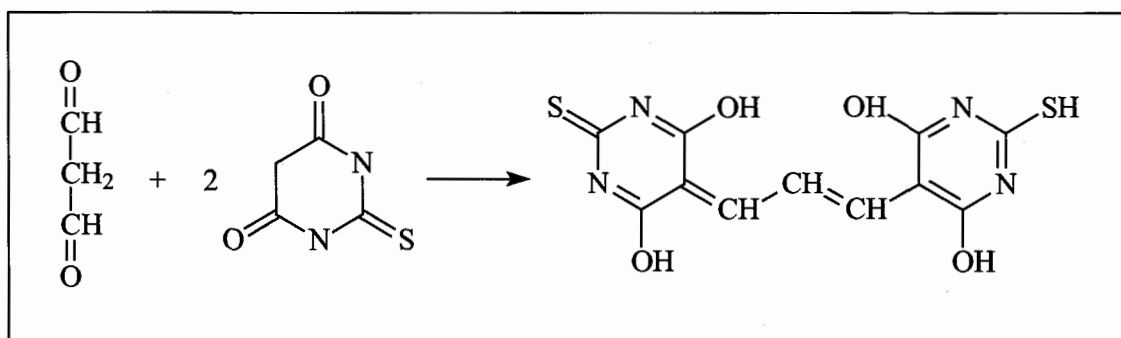
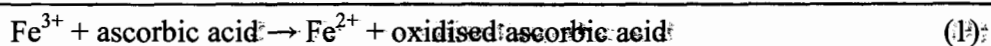


Figure 5.7: Reaction between MDA and TBA to form a red adduct (Janero, 1990).

The TBA test has however been widely criticised because of the low efficiency of fatty-acid hydroperoxide breakdown to MDA (Janero & Burghardt, 1988). Keeping this in mind, the TBA test was performed with a few modifications to improve its efficacy: Male Sprague-Dawley rats, weighing approximately 180-220 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 (eq. 2). 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 (eq. 1). 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 prooxidant in the presence of free iron, as is the case here (Aruoma, 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 artifactual 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. 0.5 ml BHT, 1 ml TCA and 0.5 ml TBA were then added to every test tube respectively 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. 2 ml buthanol was then added to every test tube and vortexed for 60 seconds. Centrifugation for 10 minutes at 2000 x g was subsequently followed by measuring the absorbancies at 532 nm with buthanol as blank.

Table 5.2: Sample preparation data.

<u>Test Tubes</u>	<u>10% Homogenate</u>	<u>PBS</u>	<u>H₂O₂ (5mM)</u>	<u>FeCl₃ (4.88 mM)</u>	<u>Ascorbic acid (1.4 mM)</u>	<u>Trolox (10 mM)</u>	<u>10% Ethanol</u>	<u>Test Compound (10 mM)</u>
<i>Control</i>	0.8 ml	0.2 ml	-	-	-	-	-	-
<i>A (H₂O₂+FeCl₃+asc. acid)</i>	0.8 ml	0.1 ml	0.05 ml	0.025 ml	0.025 ml	-	-	-
<i>B (Trolox+toxin)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	0.1 ml	-	-
<i>C (Ethanol+toxin)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	0.1 ml	-
<i>D (Acetylsalicylic acid)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>E (Ibuprofen)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>F (Compound 21)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>G (Compound 22)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>H (Compound 23)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>I (Amantadine.HCl)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>J (Compound 25)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml
<i>K (Compound 26)</i>	0.8 ml	-	0.05 ml	0.025 ml	0.025 ml	-	-	0.1 ml

All test compounds were dissolved in DMSO to give an end concentration of 10 mM for each one. Earlier lipid peroxidation studies done in our laboratory indicated that DMSO has no significant anti-oxidant activity, being in the range of ethanol; which has no known antioxidant activity. In this study Trolox, a model antioxidant, was applied as a positive control whereas ethanol was used as a negative control. Statistical analysis of the obtained data was employed using the Student-Newman-Keuls multiple range test.

5.5 CONCLUSION

Ester and amide prodrugs of acetylsalicylic acid and ibuprofen were synthesised, resulting in four novel polycyclic compounds. Characteristic signals observed for each specific compound, gave confirmation of their structure. A good example of this is where pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione was applied in prodrug syntheses. Here the characteristic ¹H NMR signals for both of the 'bridge' hydrogens were usually observed at approximately δ_H 1.51 and δ_H 1.86 ppm. In order to meet the objective of this study, the synthesised prodrugs were subjected to both blood-brain barrier permeability (*in vivo*) and lipid peroxidation (*in vitro*) assays. The results of these studies are discussed in chapter 6.

CHAPTER 6

RESULTS AND DISCUSSION

6.1 SYNTHESIS

Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione, was reacted with 3-aminopropanol to obtain the rearranged oxazinane cage structure. Acetylsalicylic acid and ibuprofen were subsequently conjugated to this cage structure by esterification, yielding the respective ester prodrugs. Amide prodrug syntheses were also employed by conjugating the NSAIDs to amantadine. Conjugation through both esterification and amide formation was achieved using activation chemistry in the form of different carbodiimides as well as acid chlorides where needed. The compound structures were confirmed by ¹H and ¹³C NMR, MS and IR.

6.2 BLOOD-BRAIN BARRIER PERMEABILITY OF SYNTHESISED COMPOUNDS

6.2.1 INTRODUCTION

As described in the previous chapter, both HPLC and LC-MS/MS 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 each compound. HPLC results were primarily used to determine the amount of salicylic acid (the main acetylsalicylic acid metabolite), acetylsalicylic acid and ibuprofen observed within blood and brain samples of mice injected with the test compounds. LC-MS/MS results were more extensively applied to quantitatively determine the percentage recovery and blood-brain barrier permeability of the test compounds.

6.2.2 RESULTS

Stock solutions of all of the test compounds were prepared in analytical grade methanol and were used to draw calibration curves for each compound. This was achieved by

preparing four different concentrations of each compound by diluting the relevant stock solution, resulting in concentrations of 100, 75, 50 and 25 ng/ml for each compound. Calibration curves and datasets for the test compounds are listed in annexure C. In figure 6.1 the calibration curve and dataset for the HPLC analysis of ibuprofen, is illustrated.

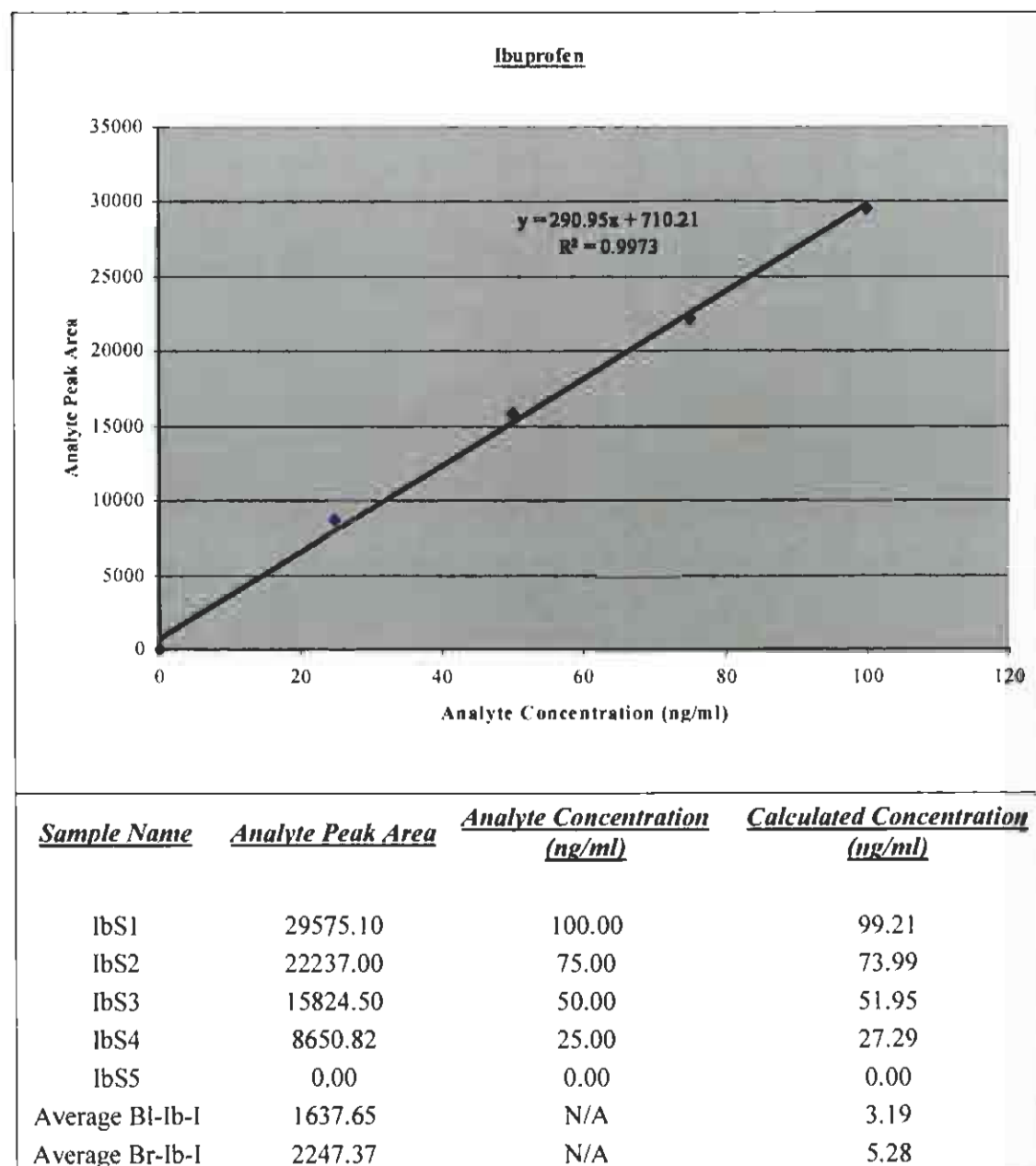


Figure 6.1: Calibration curve and dataset for ibuprofen (Bl = Blood, Br = Brain, Ib = Ibuprofen, I = Injected).

The average blood and brain concentration as well as percentage recovery from the blood and brain were determined using the LC-MS/MS results. These results were also applied to determine the brain to blood concentration ratios (table 6.1).

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. It was confirmed that acetylsalicylic acid, does not penetrate the blood-brain barrier to a great extent, presenting with a brain to blood concentration ratio of only 0.49. Most of the drug is observed as salicylic acid, the primary acetylsalicylic acid metabolite, presenting with a brain to blood concentration ratio of 1.06. The reason for the relatively high brain concentration is probably that once salicylic acid is produced in the brain as a result of hydrolysis, it is too hydrophilic to permeate back to the systemic circulation from the CNS.

The total salicylate concentration was calculated by multiplying the molecular mass ratio of salicylic acid to acetylsalicylic acid, with the average blood and brain concentrations of acetylsalicylic acid. From this it is clear that the distribution of the drug between the blood and brain does not favour brain delivery of the free drug.

After administration of compound **22**, the acetylsalicylate ester prodrug, the free drug was observed at about twice the concentration in the brain when compared to free drug administration. This indicated very favourable blood-brain barrier permeation for compound **22**. HPLC results indicated that salicylic acid was also observed in brain tissue after administration of compound **22**, but to a lesser extent than that observed for acetylsalicylic acid. This may be attributed to drug latention, where the acetyl group on acetylsalicylic acid is hydrolysed at a slower rate than normally because of the polycyclic molecule conjugated to it.

Table 6.1: LC-MS/MS results.

	<u>Average</u> <u>[Blood]</u> (ng/ml)	<u>Average</u> <u>[Brain]</u> (ng/ml)	<u>[Brain]/[Blood]</u>		<u>Average</u> <u>[Blood]</u> (ng/ml)	<u>Average</u> <u>[Brain]</u> (ng/ml)	<u>[Brain]/[Blood]</u>
Acetylsalicylic Acid	45.50	22.47	0.49	Acetylsalicylic Acid from Compound 25	13.84	6.81	0.49
Salicylic Acid from Acetylsalicylic Acid	6.95	7.37	1.06	Salicylic Acid from Compound 25	LOQ	LOQ	NC
Total Salicylates* from Acetylsalicylic Acid	41.45	24.60	0.59	Ibuprofen	61.37	52.51	0.90
Compound 22	LOQ	LOQ	NC	Compound 23	LOQ	LOQ	NC
Acetylsalicylic Acid from Compound 22	44.11	59.03	1.34	Ibuprofen from Compound 23	40.28	55.80	1.39
Salicylic Acid from Compound 22	LOQ	LOQ	NC	Compound 26	LOQ	LOQ	NC
Compound 25	17385.97	8585.11	0.49	Ibuprofen from Compound 26	30.45	11.83	0.39

LOQ = Obtained values were below the limit of quantification.

NC = Brain to blood concentration ratios were not calculable.

* = To calculate the total salicylate values the molecular mass ratio of salicylic acid to acetylsalicylic acid was used.

With administration of compound **25** (acetylsalicylamide prodrug), free acetylsalicylic acid was observed in both the brain and blood at roughly one third of the concentration of that observed after the free drug was administered. This observation may be due to the fact that the amide bond of the prodrug is less prone to hydrolysis than the ester bond. Less acetylsalicylic acid is thus observed at 1 hour after drug administration when compared to that for compound **22**, because at this specific time point less amide prodrug is hydrolysed to release acetylsalicylic acid. Compound **25**, on its own, exhibited a brain to blood concentration ratio of 0.49, which indicated that at 1 hour after administration there was approximately twice the amount of the compound in the blood than in the brain. This is contradictory to what was expected and might be explained by the percentage recovery from the blood being nearly twice that of the value from the brain, for this specific compound.

Free ibuprofen presented with a brain to blood concentration ratio of 0.90, which indicated more favourable blood-brain barrier permeation than observed for acetylsalicylic acid. Once conjugated to the pentacycloundecane structure to produce compound **23**, the brain to blood concentration ratio for ibuprofen increased to 1.39, indicating a higher ibuprofen concentration in the brain than that observed in the blood. Once again the amide prodrug, compound **26**, exhibited lower blood and especially brain concentrations than observed for the free drug and a ratio of 0.39 was obtained. This ratio is even lower than that observed for free ibuprofen, and may indicate a much lower rate of hydrolysis for the amide compound, **26**, in the brain.

6.2.3 DISCUSSION

From the obtained data it is concluded that the conjugation of acetylsalicylic acid and ibuprofen to the pentacycloundecane structure (compounds **22** and **23**) through esterification, substantially improved their blood-brain barrier permeability and thus attainable brain concentrations. Nielsen and Bundgaard (1989) stated that esterification renders the acetyl ester of acetylsalicylic acid extremely susceptible to enzymatic hydrolysis, with the half-life for deacetylation of acetylsalicylic acid esters being 1-3 minutes compared to a half-life of 2 hours for the free drug in human plasma. In this study acetylsalicylic acid was detected in the CNS at far higher concentrations than

salicylic acid after administration of the acetylsalicylate ester prodrug (**22**), indicating that this novel synthesised ester prodrug was a true prodrug in that it inhibited the hydrolysis of the acetyl ester on the free drug. The levels of the two ester prodrugs were below the level of quantification after the 1 hour time period, indicating that ester hydrolysis took place at a very fast rate after administration to the test animals. The lower free drug concentrations observed with the amide prodrugs (compounds **25** and **26**) could be attributed to incomplete hydrolysis of the amide bond, especially in the brain (Mark *et al.*, 1951). This could probably also explain the much lower brain concentration of the free drug observed for both prodrugs.

6.3 ANTIOXIDANT ACTIVITY OF SYNTHESISED COMPOUNDS

6.3.1 STATISTICAL ANALYSIS

All data are presented as means $\pm$ 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 at $p < 0.05$.

6.3.2 RESULTS

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 **22** and **26** both exhibit substantial antioxidant capacity in the presence of the toxin ($\text{H}_2\text{O}_2 + \text{FeCl}_3 + \text{ascorbic acid}$) (fig. 6.2).

Using the results obtained it is concluded that the novel synthesised prodrugs as well as the free drugs, exhibit a significant attenuation of lipid peroxidation when compared to the toxin (all presenting with $p < 0.001$). Acetylsalicylic acid and ibuprofen's abilities to attenuate lipid peroxidation are significantly increased by both their amide and ester prodrug forms (all having p values of < 0.001). Compound **22** (acetylsalicylate ester prodrug) has a significantly higher ability to inhibit lipid peroxidation than compound **21**, the cage structure used in its synthesis ($p < 0.001$). This is also true for compound **26** (ibuprofen amide prodrug), which attenuates lipid peroxidation more effectively than amantadine.HCl ($p < 0.001$). Compound **25** (acetylsalicylamide prodrug) did not attenuate lipid peroxidation to a significantly greater extent than observed for amantadine.HCl ($p <$

0.05). Compounds **23** (ibuprofen ester prodrug) and **21** exhibit no significant difference in their ability to attenuate lipid peroxidation, when compared to each other ($p>0.05$). This is also true when acetylsalicylic acid and ibuprofen are compared to each other ($p>0.05$).

From the results of the study the notion that both acetylsalicylic acid and ibuprofen possess certain antioxidant abilities, was confirmed. It was also observed that amantadine.HCl exhibited a certain amount of antioxidant activity in the presence of the toxin used. A further conclusion drawn from the study is that the synthesis of acetylsalicylic acid and ibuprofen prodrugs through esterification and amide formation with the pentacycloundecane cage structure and amantadine respectively, significantly increased the free drug's abilities to attenuate lipid peroxidation.

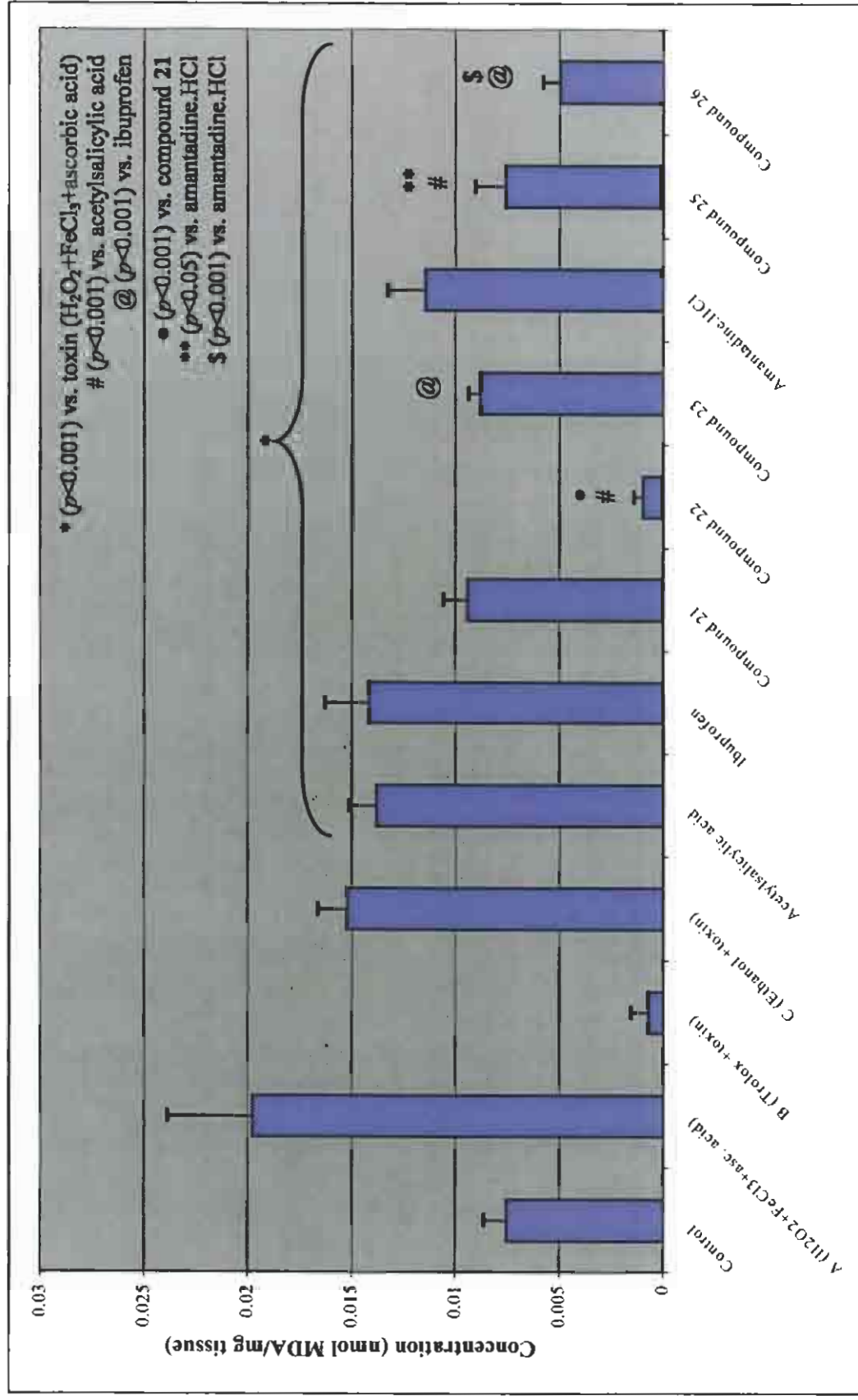


Figure 6.2: Antioxidant activity of the different compounds *in vitro*. Each bar represents the mean $\pm$ SEM.; $n=5$.

6.3.3 DISCUSSION

The modified TBA assay applied 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 H_2O_2 , FeCl_3 , ascorbic acid-toxin system. The study results also indicate that the novel synthesised prodrugs positively influenced the antioxidant activity of the free, unconjugated drugs. Decades of research have indicated the profound role oxidative stress plays in the pathogenesis of neurodegenerative disorders (Dykens, 1994; Gilgun-Sherki *et al.*, 2001; Floyd & Hensley, 2002) and these novel prodrugs may therefore be neuroprotective because of their increased antioxidant activity.

6.4 FINAL REMARKS AND CONCLUSION

In keeping with the specific purpose of this study, which was to apply polycyclic cage compounds as carrier molecules for neuroprotective NSAIDs, both *in vivo* blood-brain barrier permeability as well as *in vitro* lipid peroxidation assays were performed. Obtained results indicated that conjugation of acetylsalicylic acid and ibuprofen to the pentacycloundecane structure and amantadine was not only possible, but that this generally led to a significant increase in their blood-brain barrier permeability. Further conclusions drawn from the study was that both types of conjugates, i.e. ester and amide prodrugs, significantly increased the ability of the free drugs to attenuate lipid peroxidation. This was especially true for compounds **22** and **26**, with **22** being in the range of the model antioxidant, Trolox. The novel synthesised prodrugs therefore present with a multiple drug targeting action as the blood-brain barrier permeability and the antioxidant activity of the free NSAIDs were increased. In other words more of the drug was delivered to where it is needed and its ability to be neuroprotective was also increased at the same time. Furthermore, the amide type prodrugs (compounds **25** and **26**) may be dual acting drugs in that they deliver free acetylsalicylic acid and ibuprofen into the CNS, with amantadine, which has widely described neuroprotective activity.

Throughout the blood-brain barrier permeability assay it was indicated that the percentage recovery for the test compounds was extremely low. This may have

influenced the obtained results and is an aspect that will have to be optimised in future studies. Great difficulty was also experienced with the extraction of the test compounds and more specifically the internal standard, naproxen. It was therefore decided to leave internal standard data out of consideration when the calibration curves were drawn. This aspect will have to be looked at when future studies are conducted. A possible solution might be to omit the neutralisation step during extraction from the biological material as the drugs used were mostly acidic and will therefore be extracted more effectively from an acidic environment. Another, more effective biological test model may also be considered for future blood-brain barrier permeability studies in which less variables are present that may have an influence on the test results. However, in successfully meeting the objectives of this study, 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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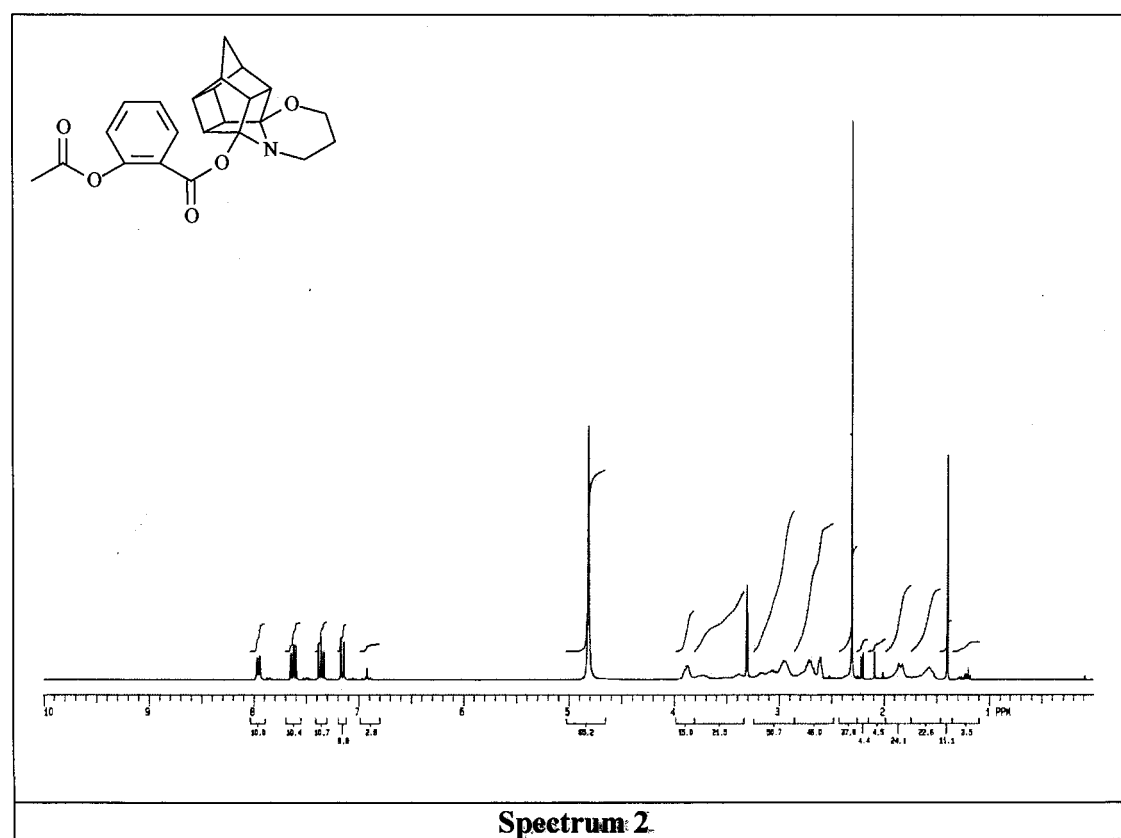
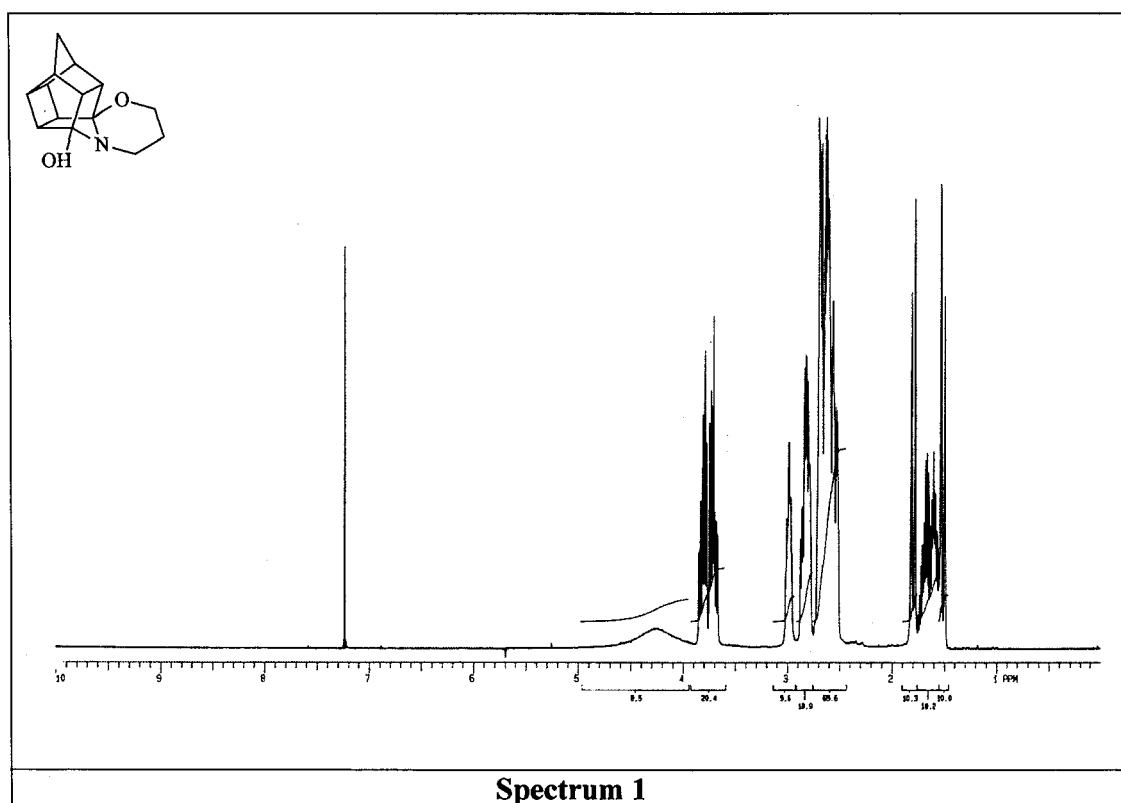
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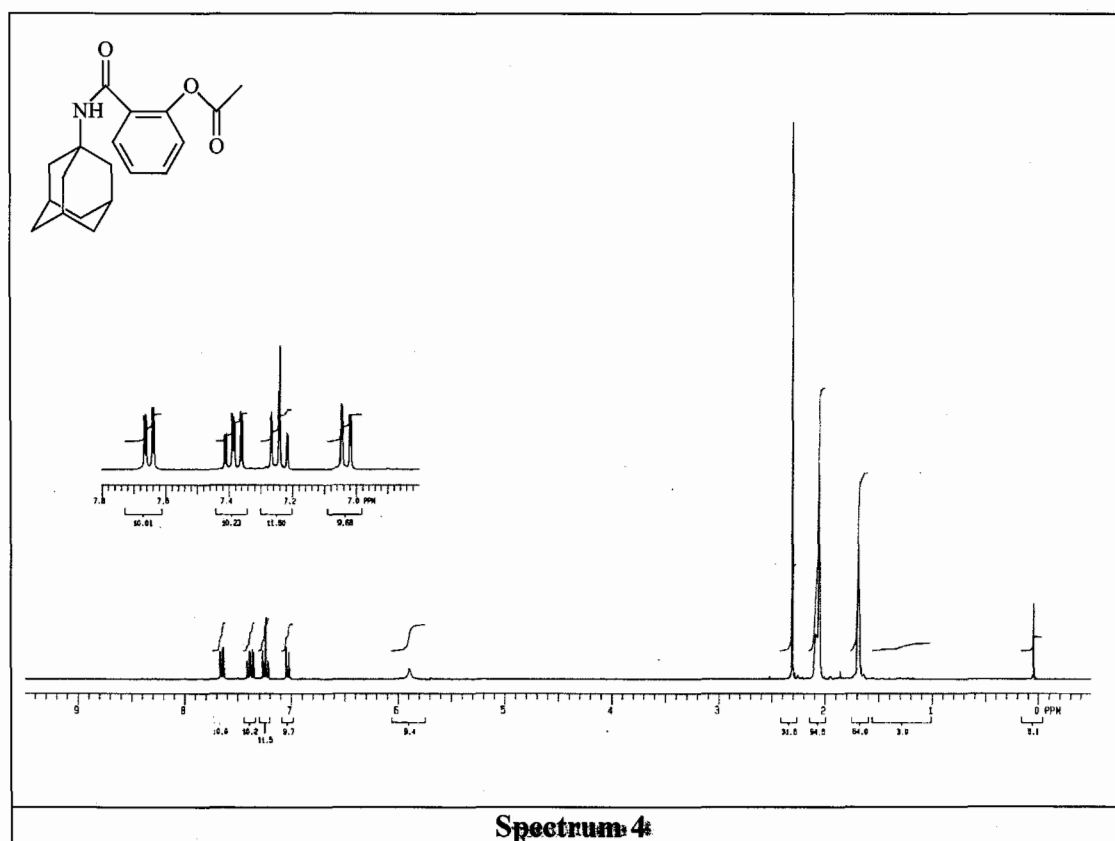
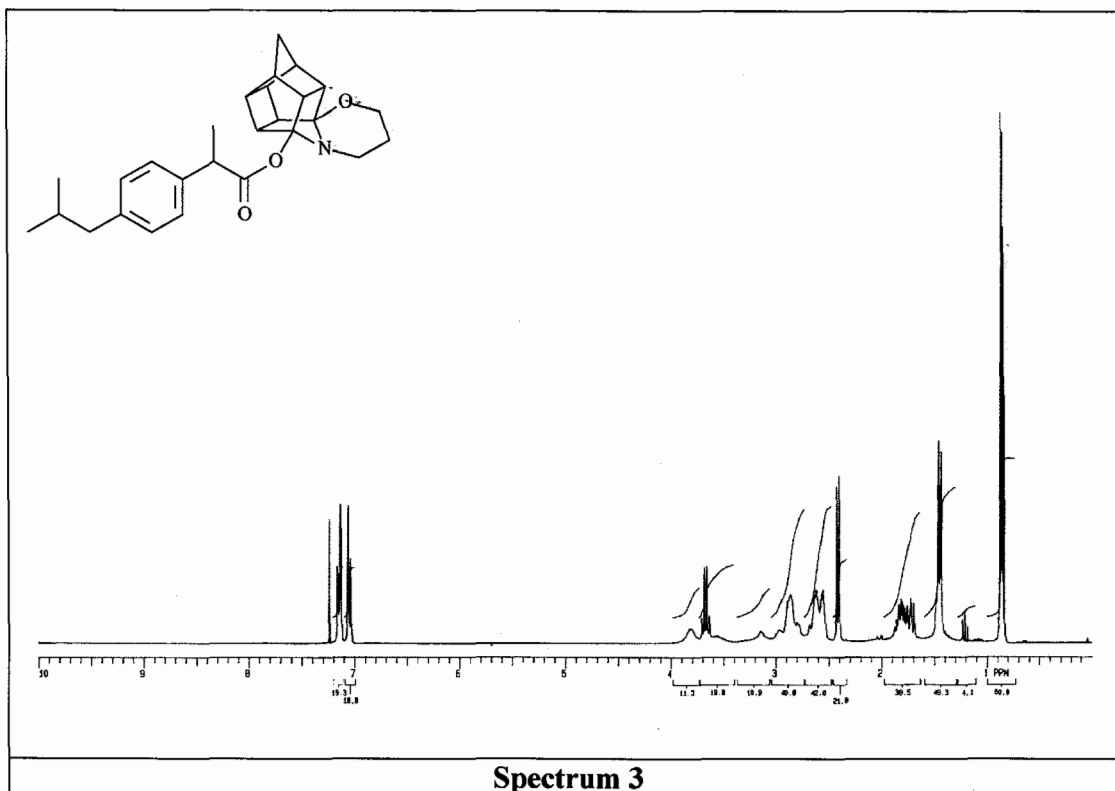
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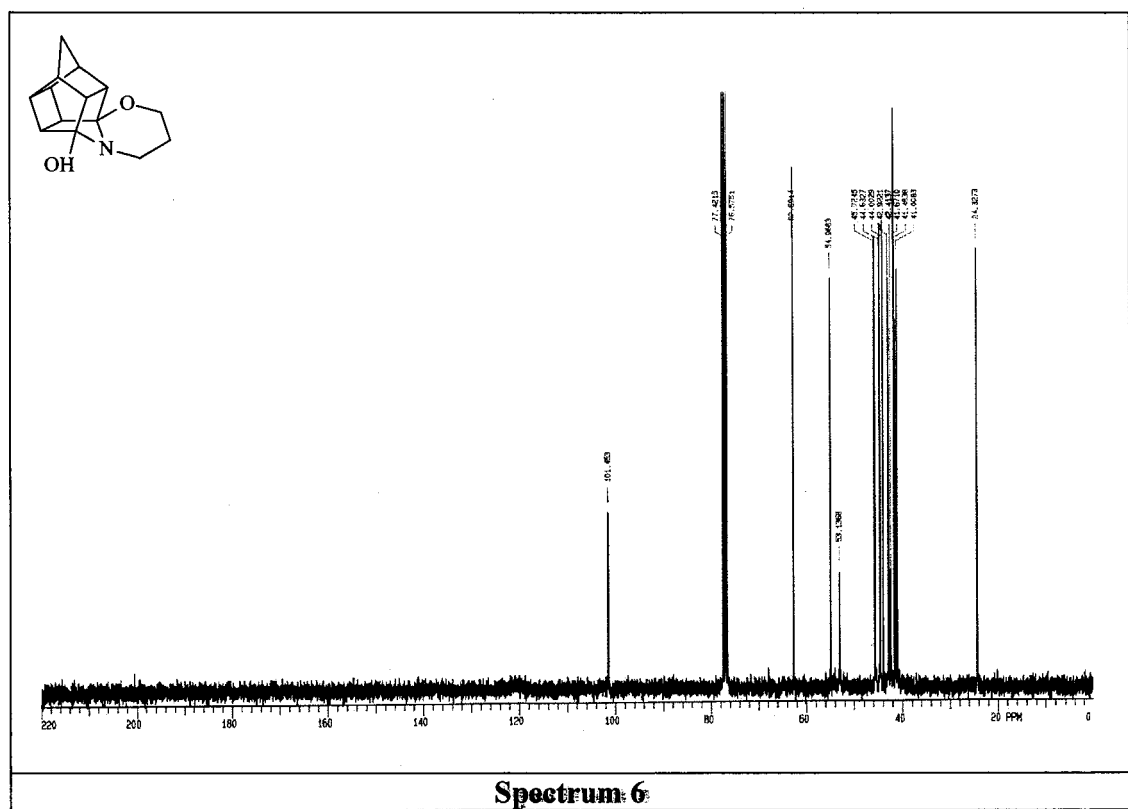
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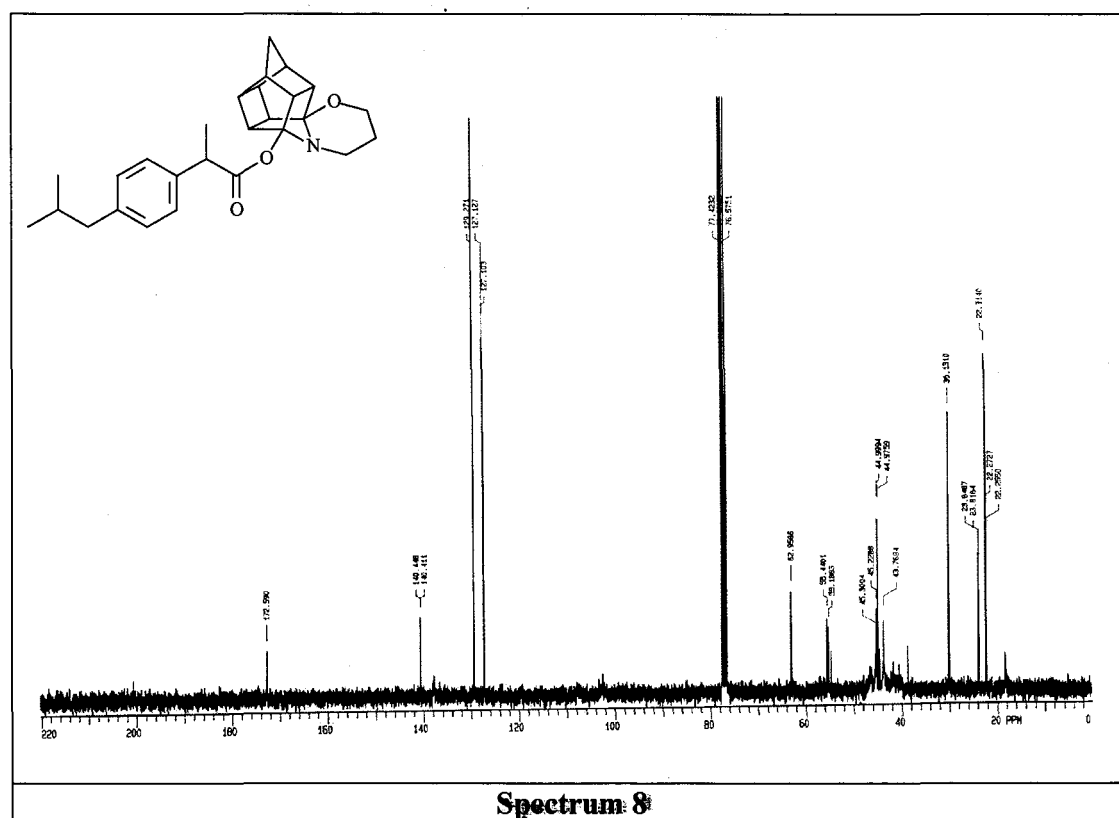
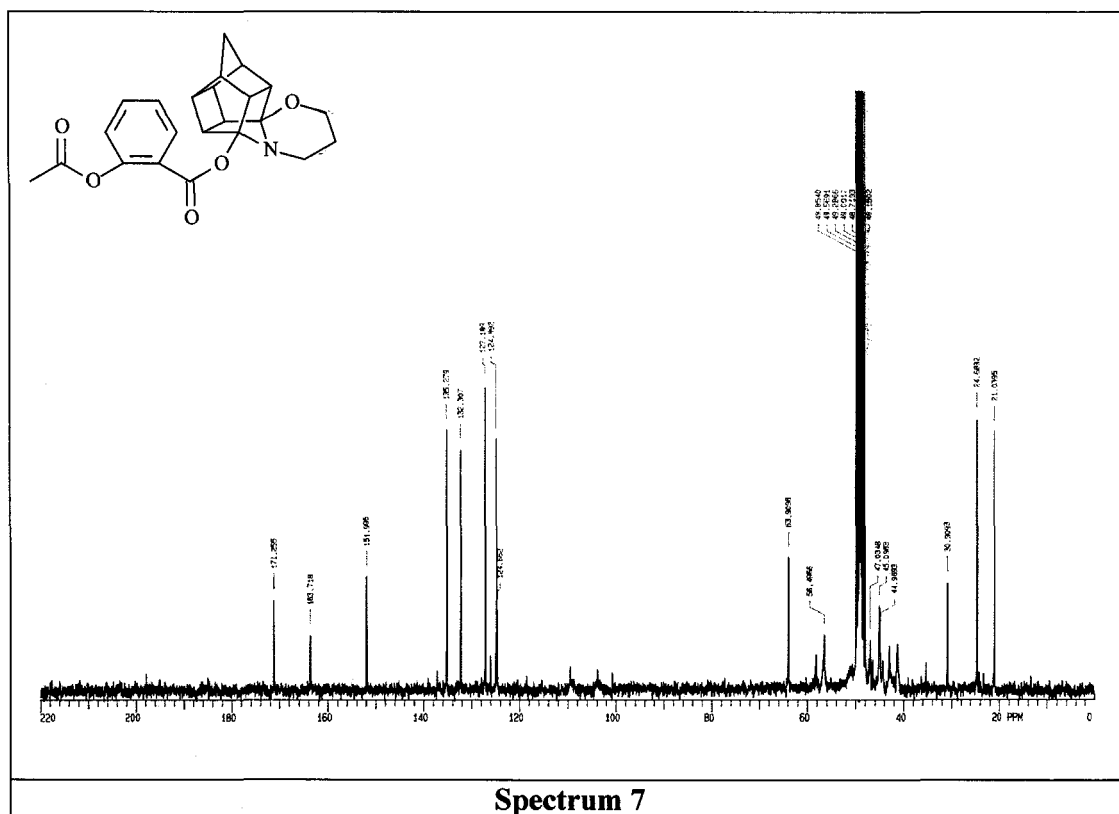
ZAMORA, J.M., PEARCE, H.L. & BECK, W.T. 1988. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Molecular pharmacology*, 33:454-462.

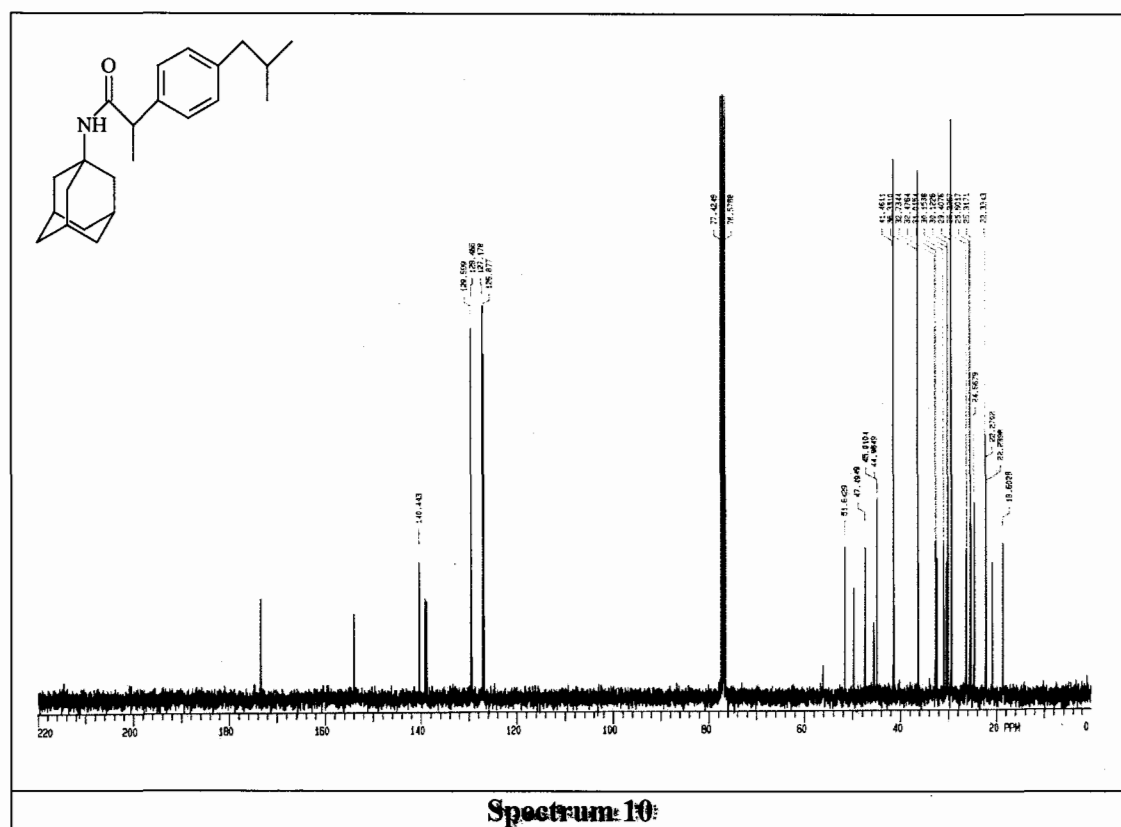
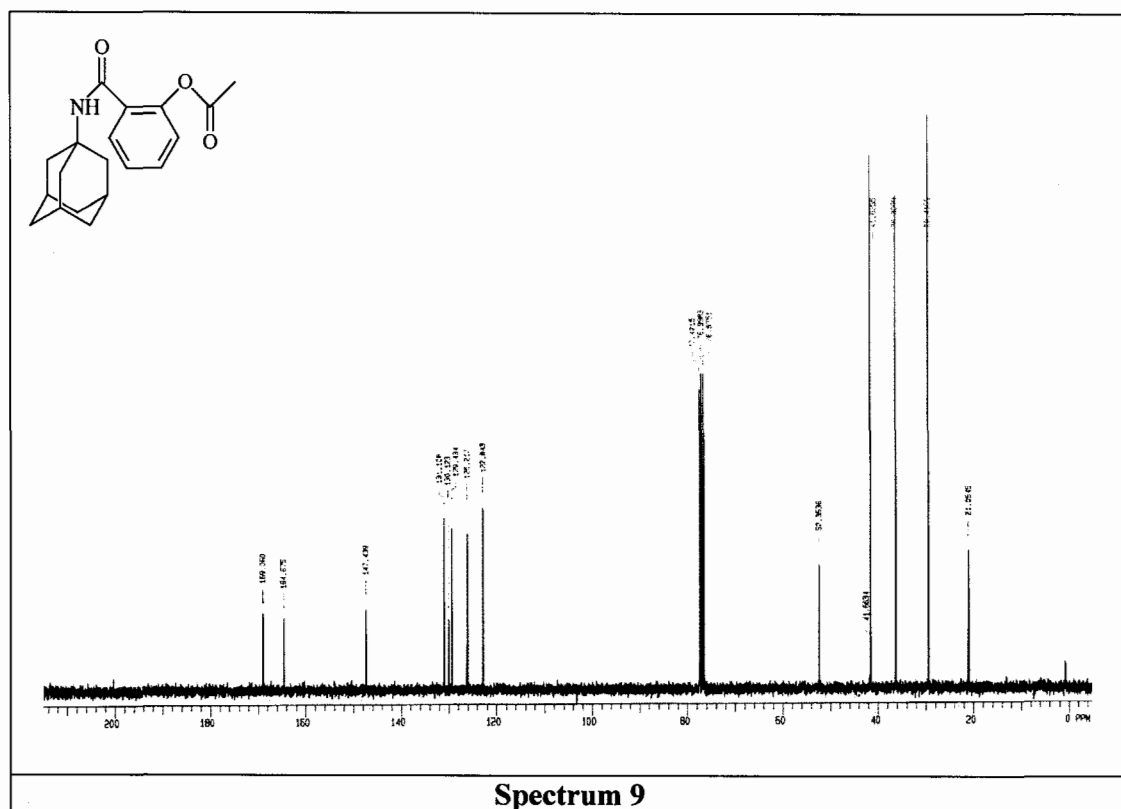
ANNEXURE A
SPECTRAL DATA:
 ^1H NMR, ^{13}C NMR, DEPT, MS, IR



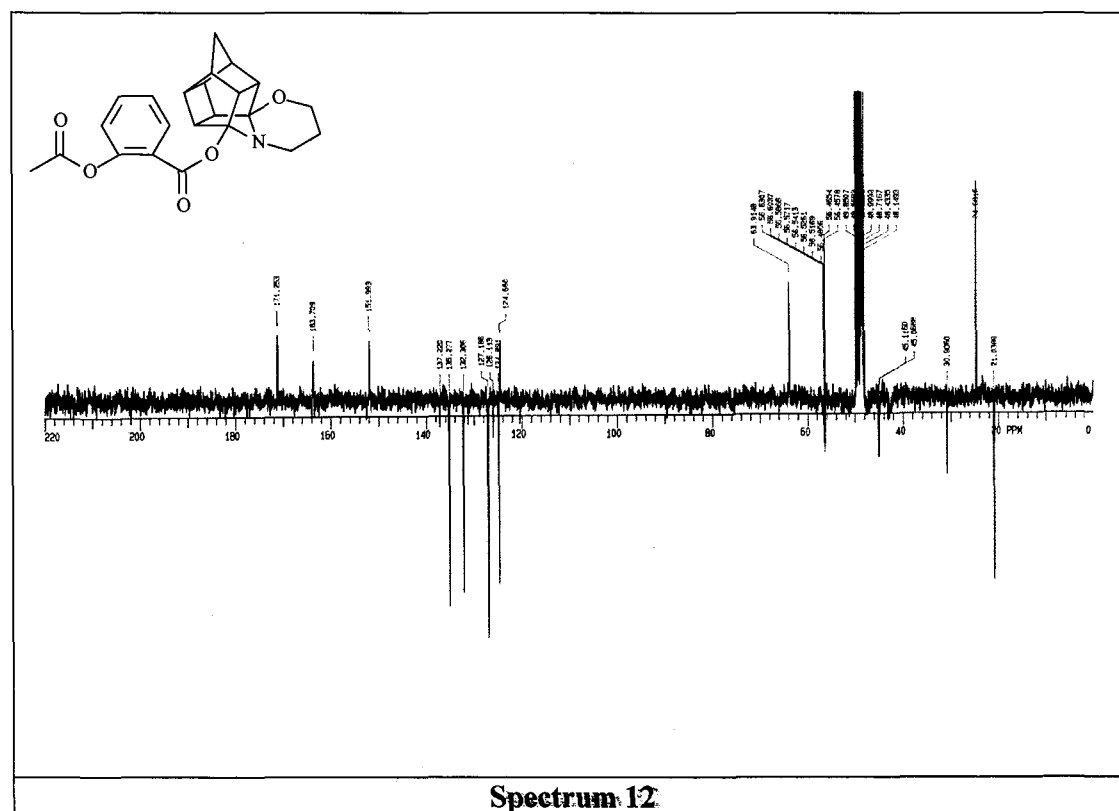
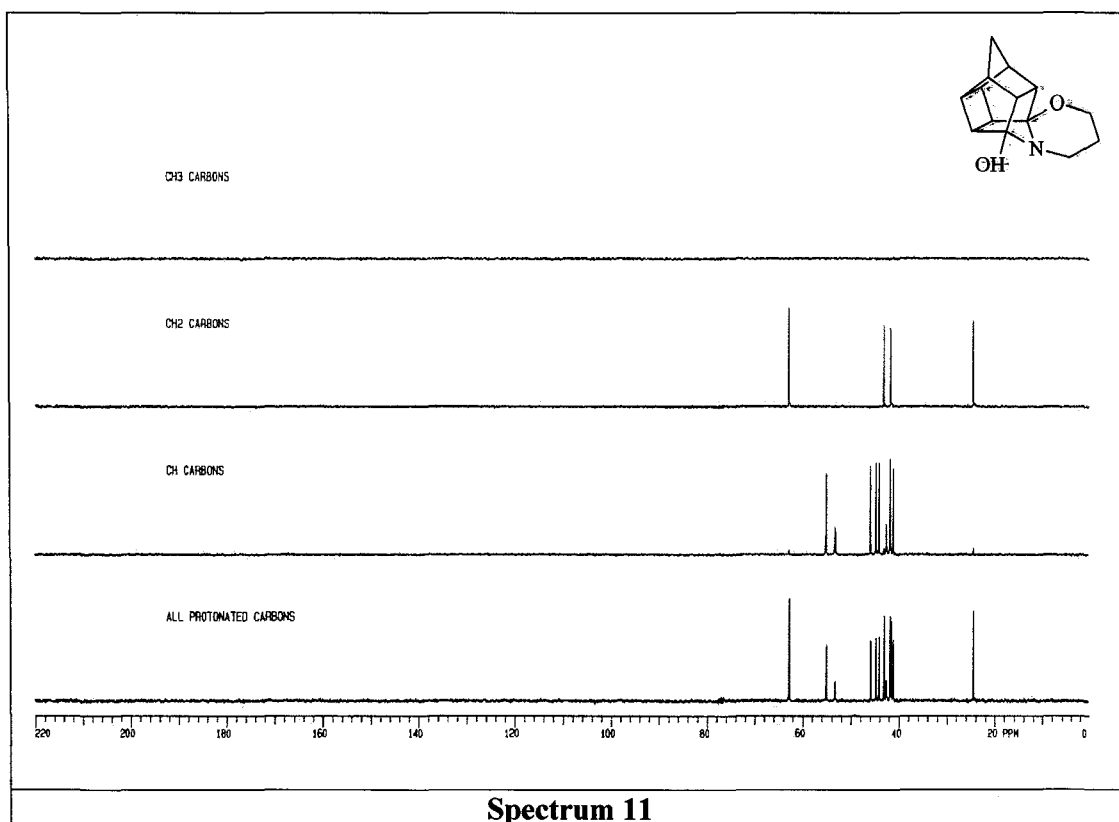


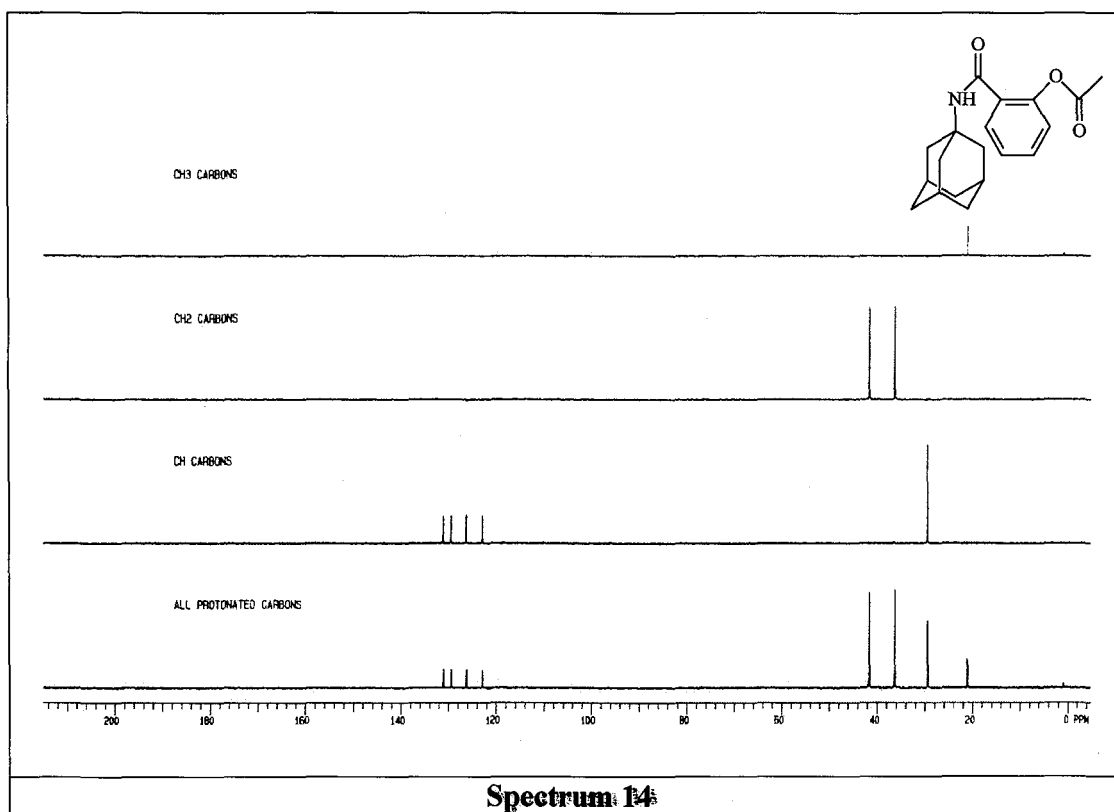
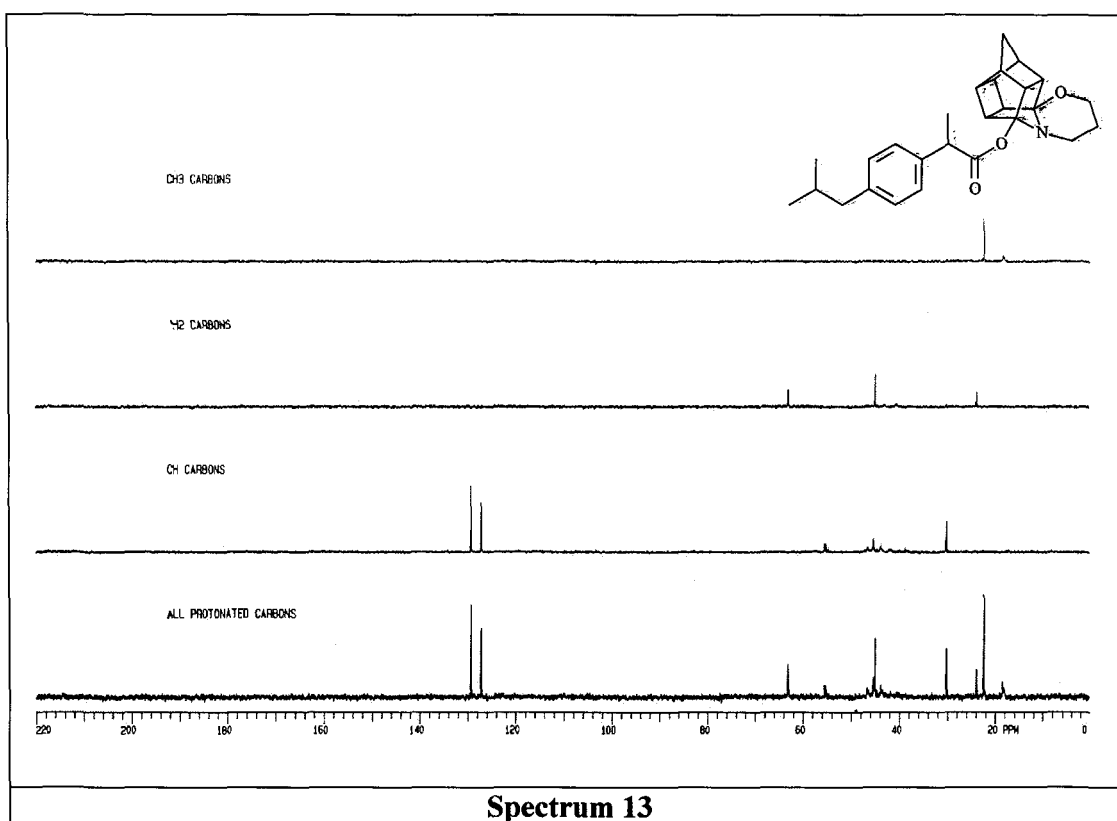




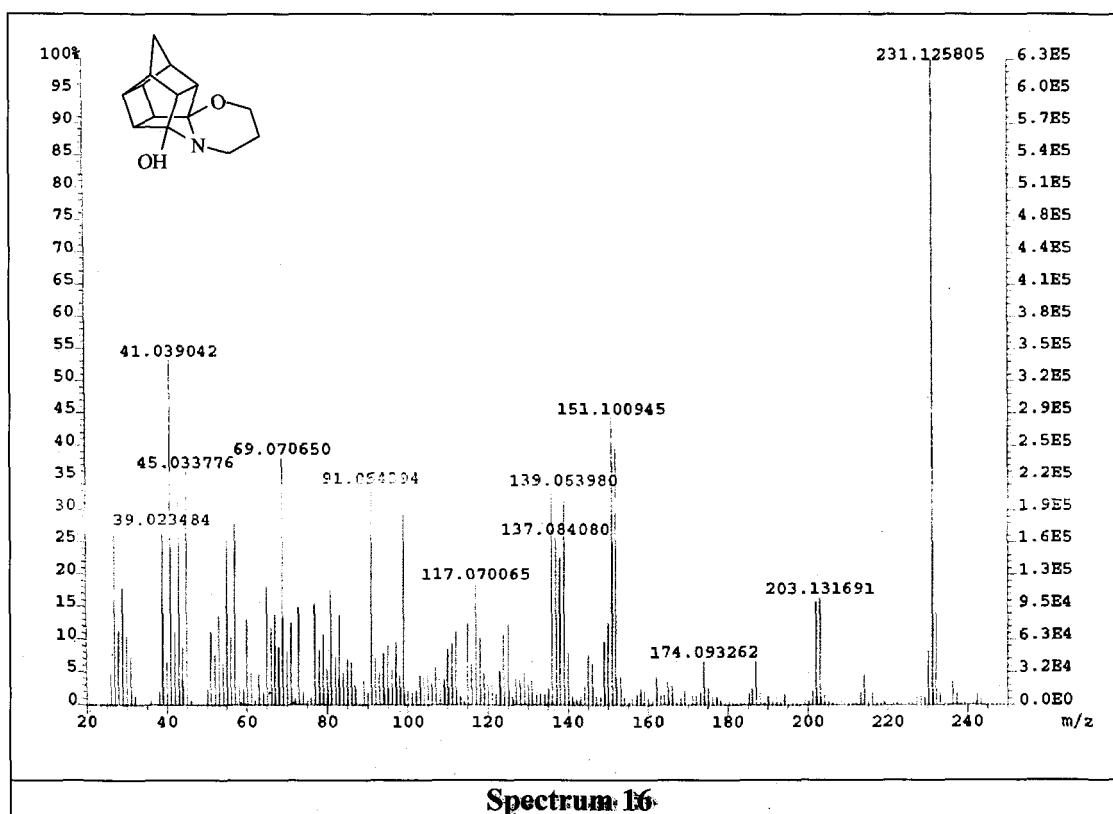
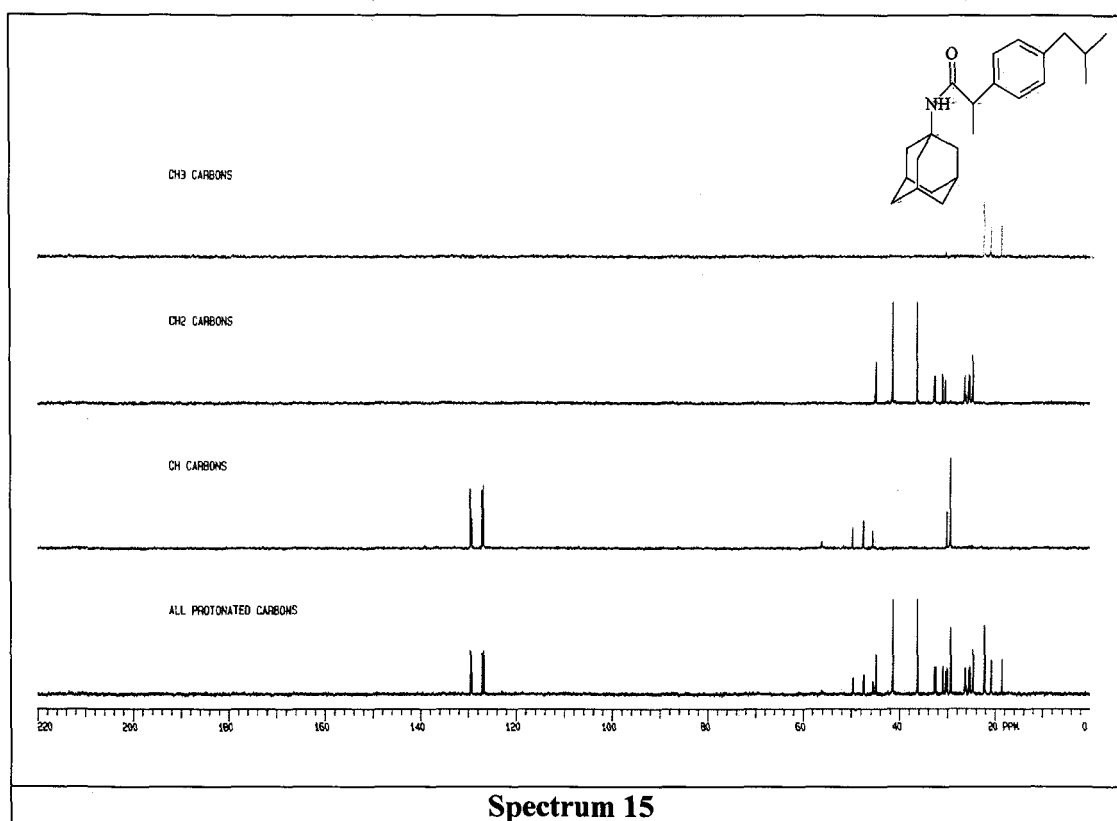


SPECTRAL DATA

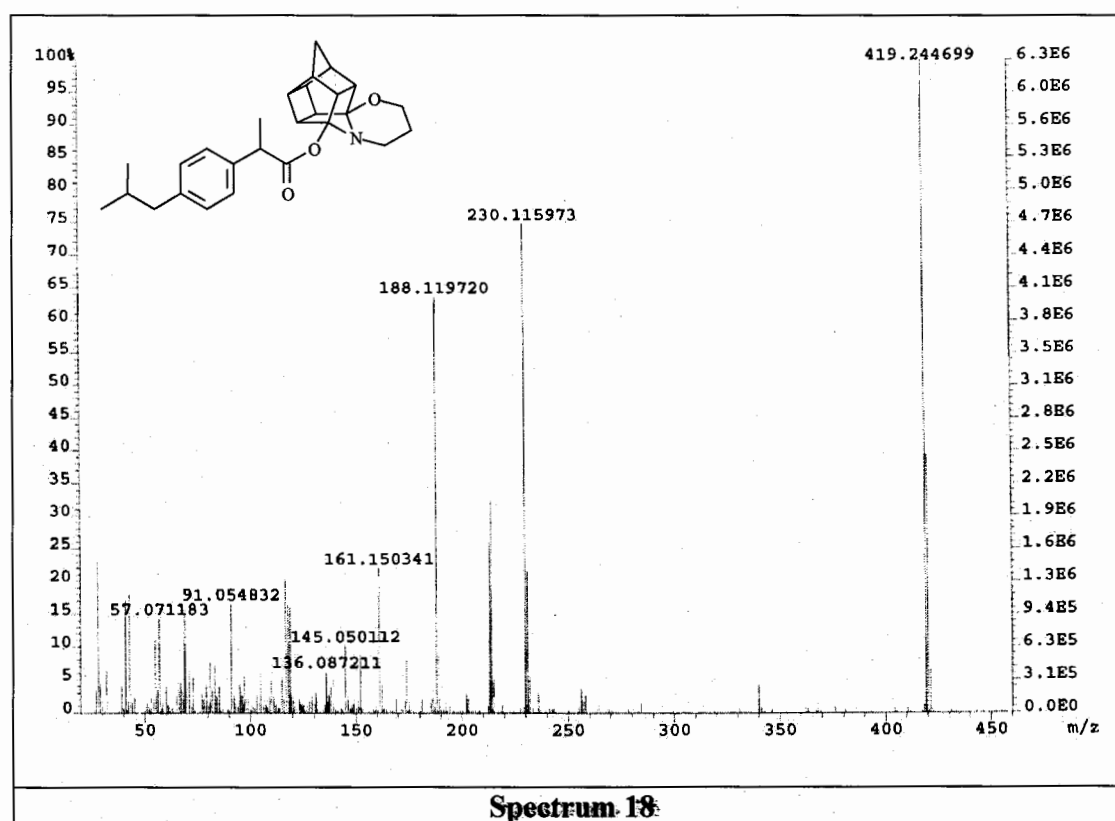
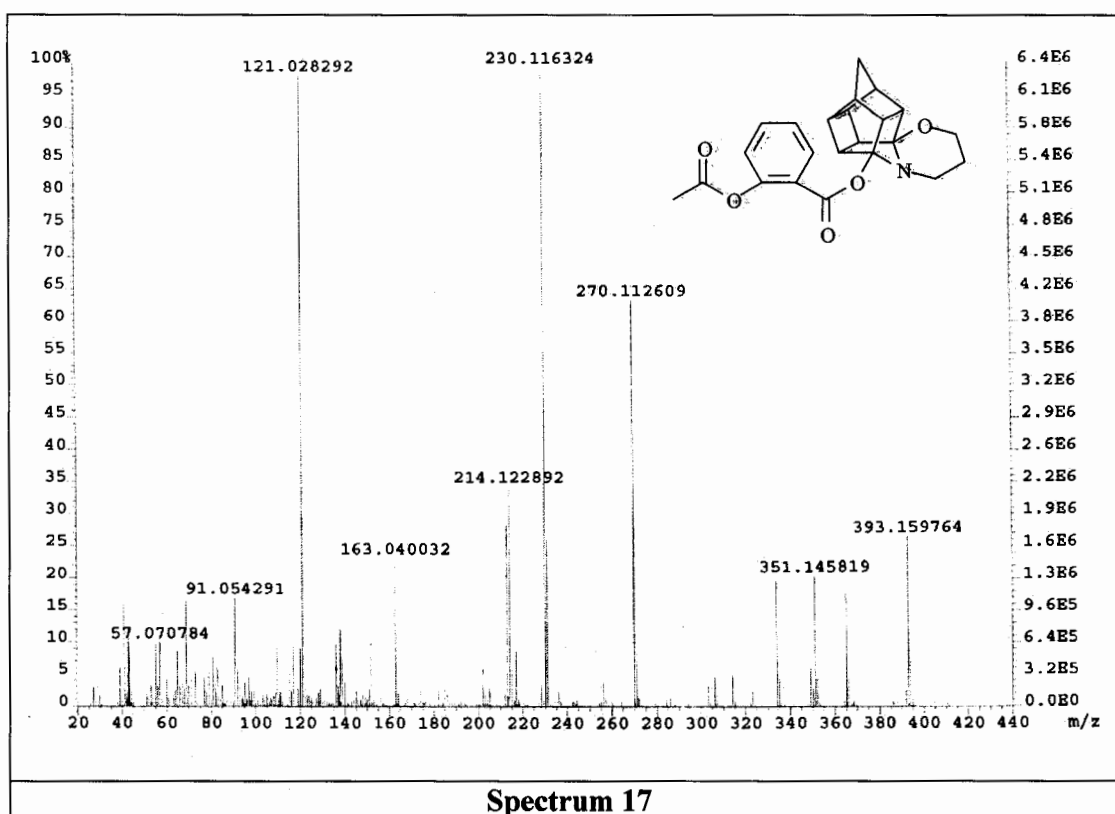




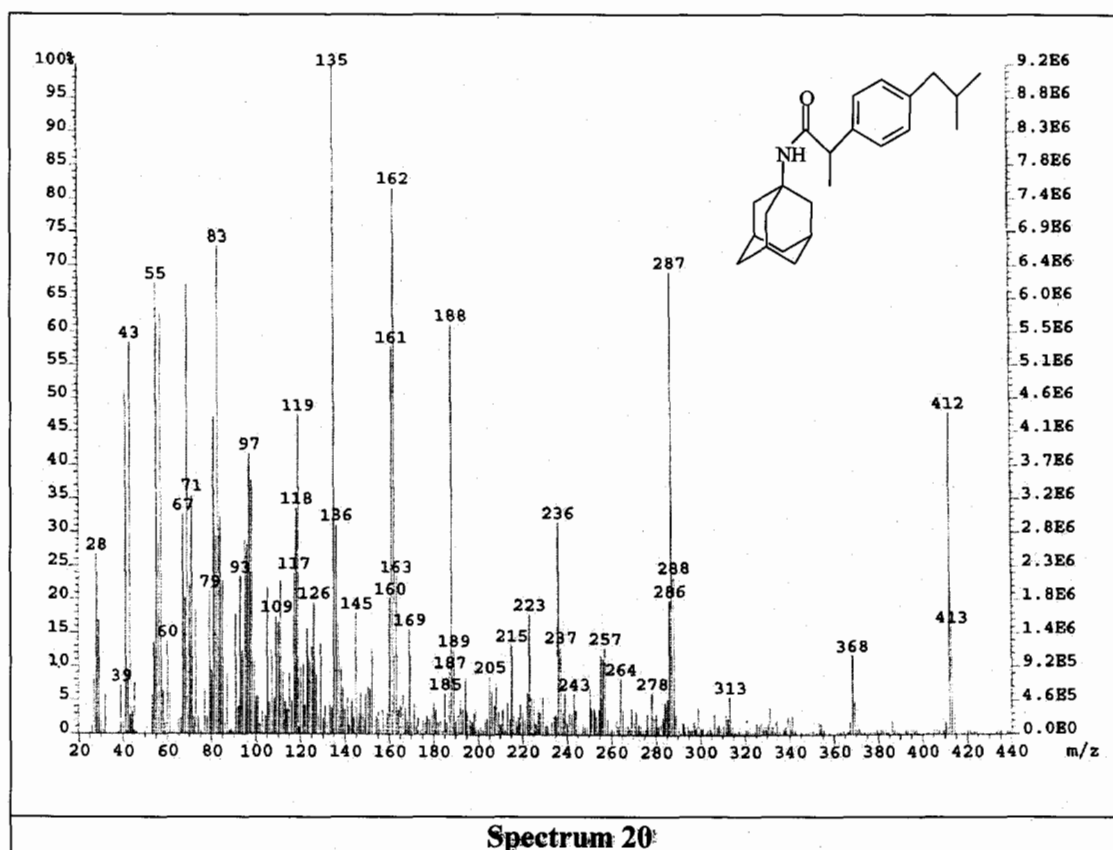
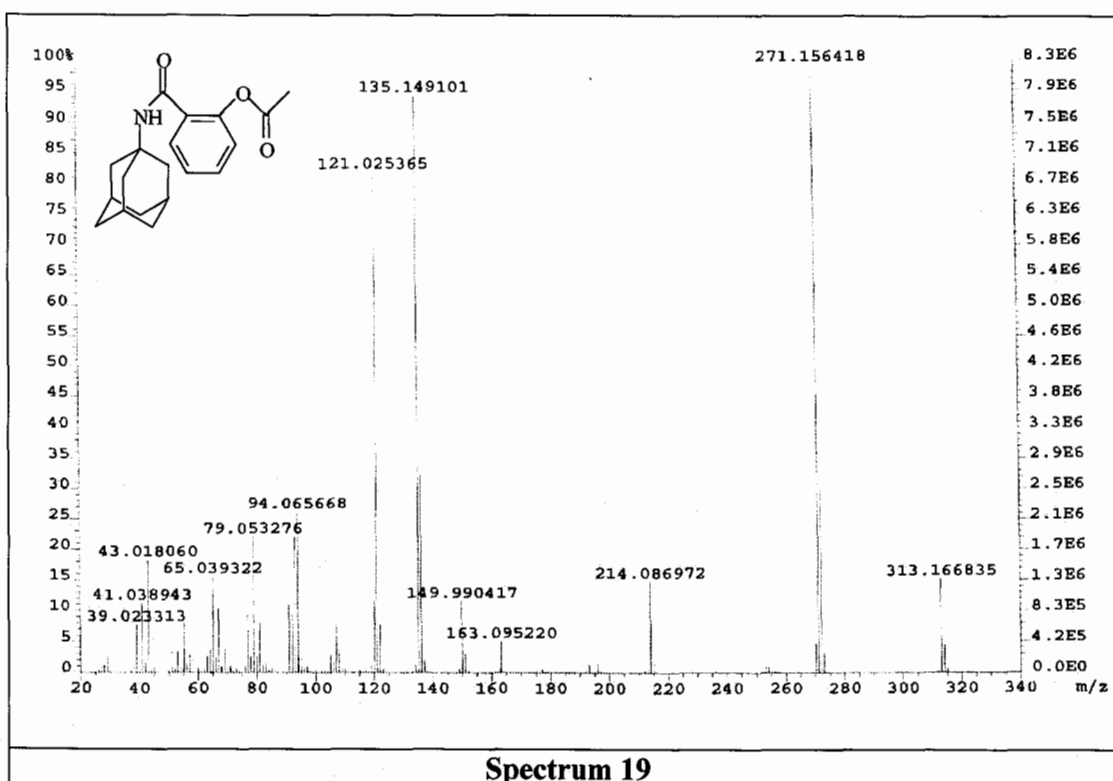
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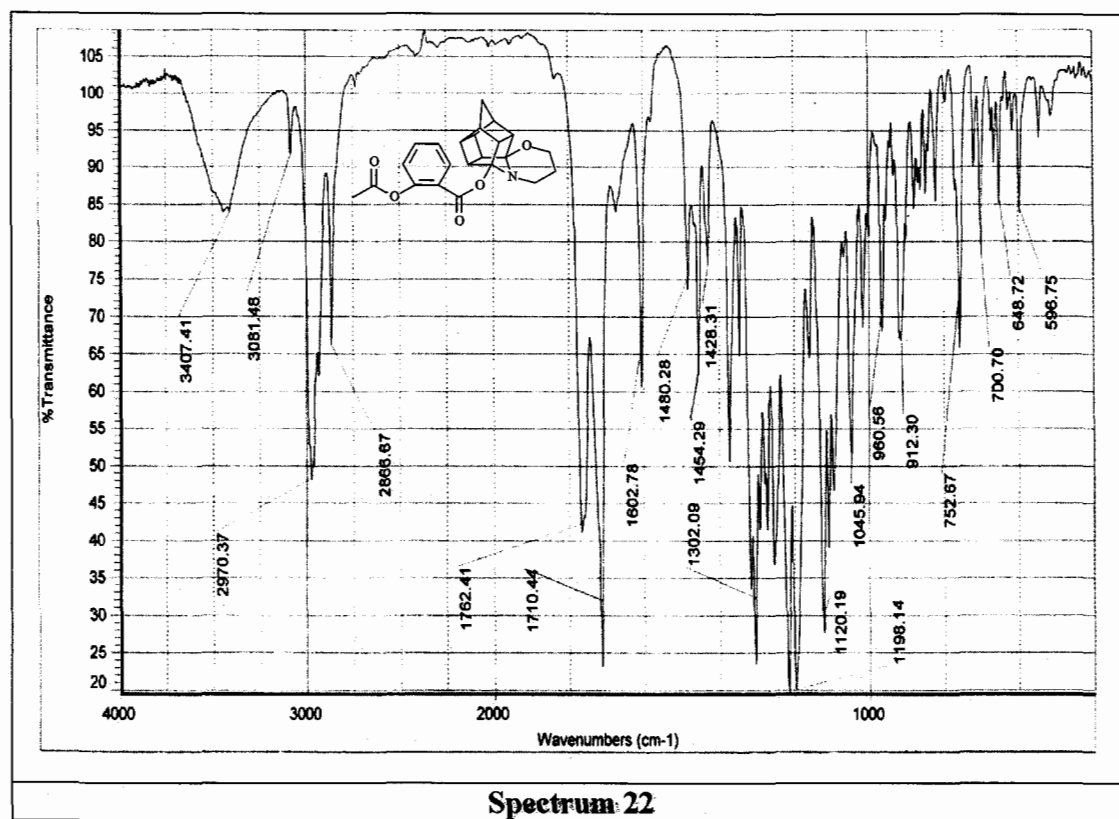
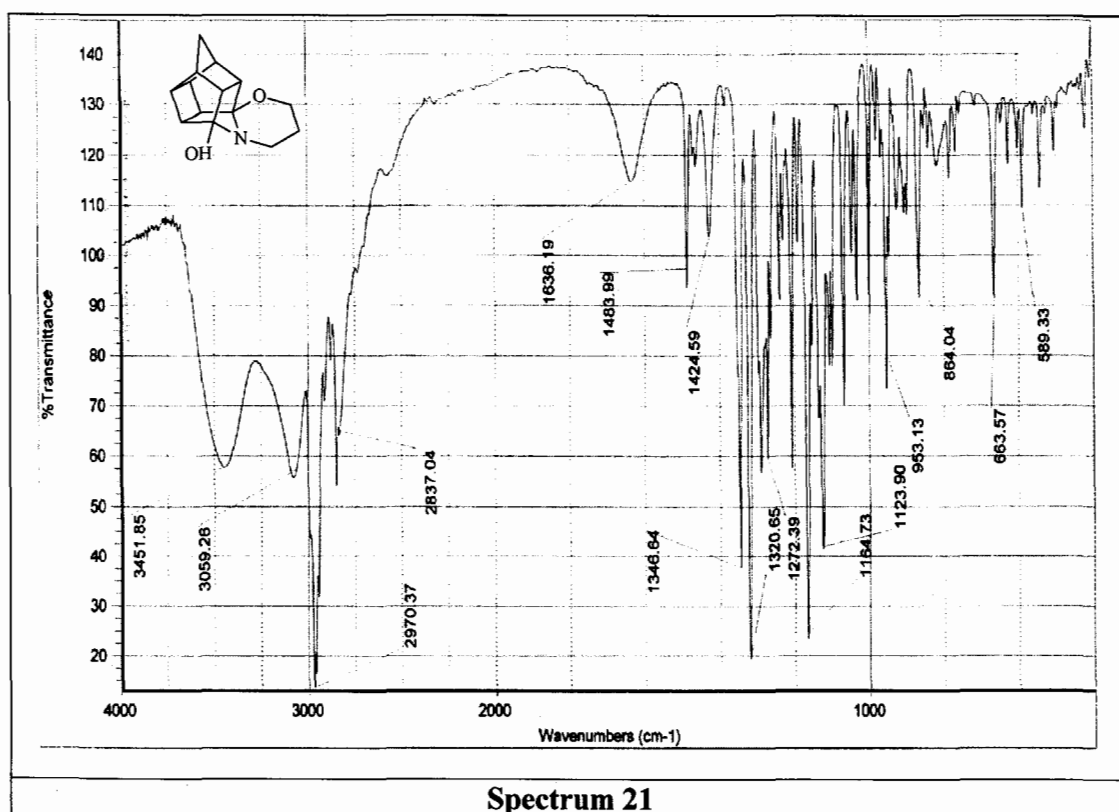


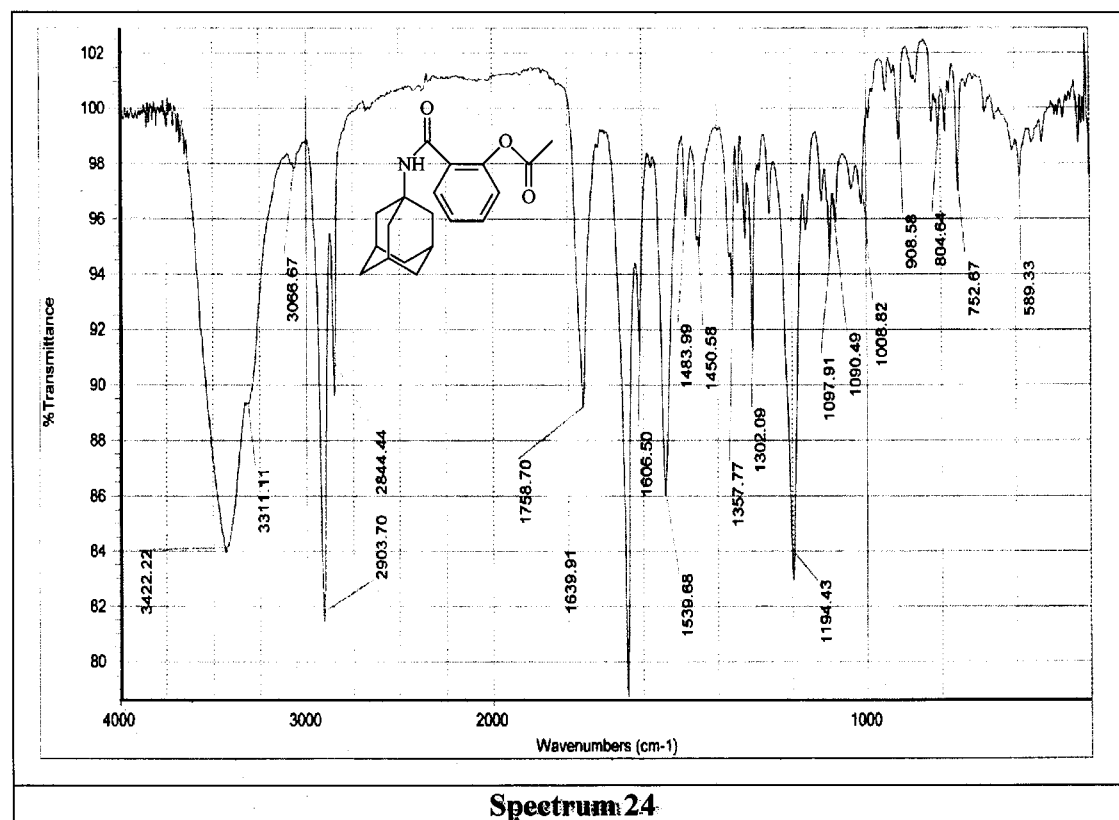
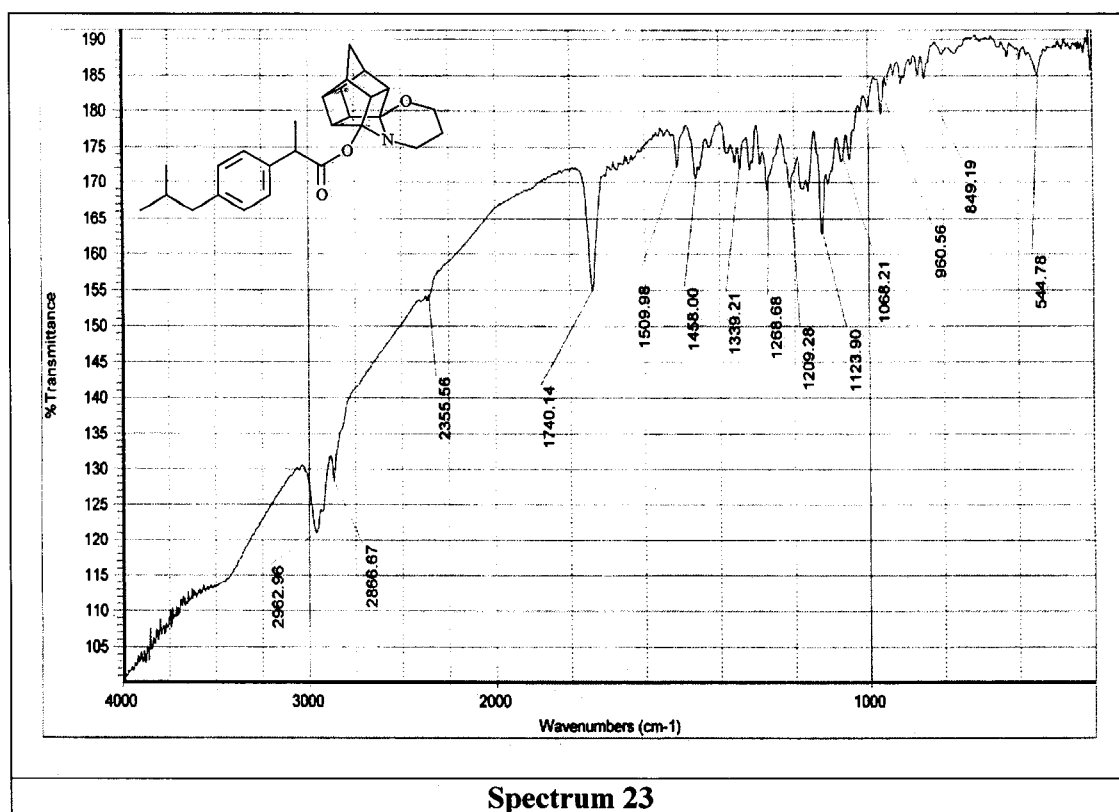
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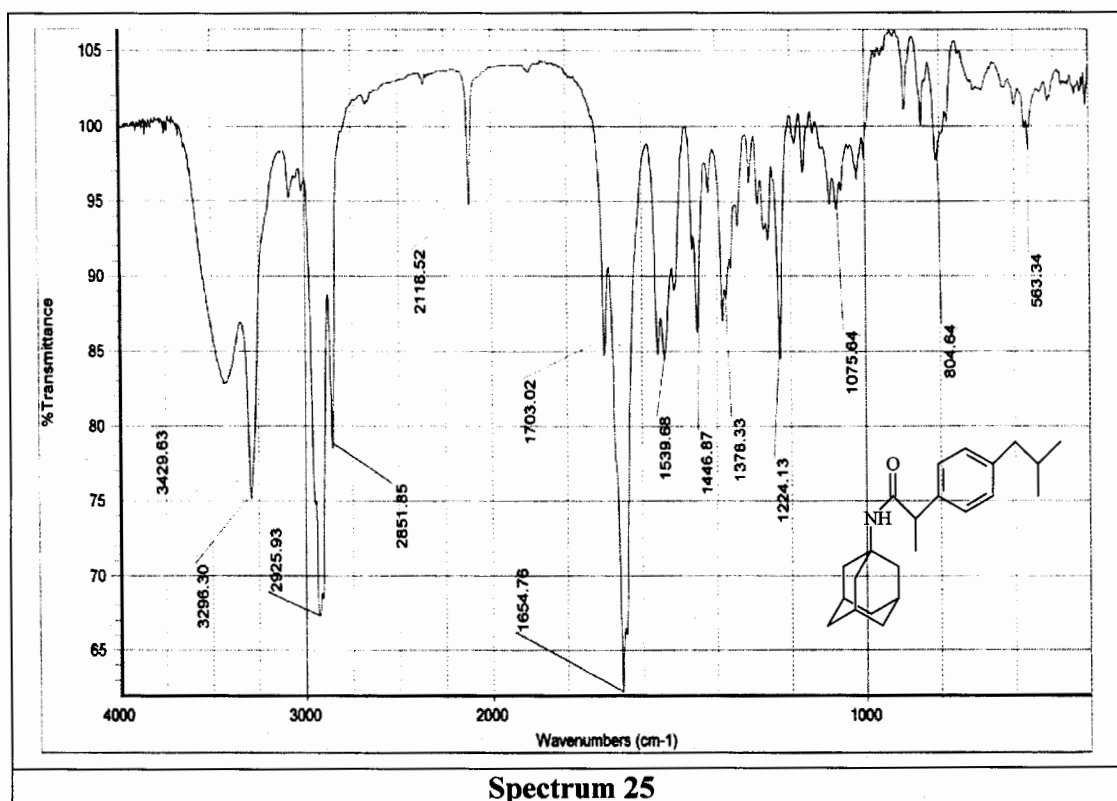


SPECTRAL DATA









ANNEXURE B

LIQUID CHROMATOGRAPHY TANDEM MASS

SPECTROMETRY:

ANALYTICAL CONDITIONS

Table B.1: Parameters used in the analysis of the novel test compounds.

		<u>Declustering Potential</u>	<u>Focusing Potential</u>	<u>Entrance Potential</u>	<u>Collision Cell Entrance Potential</u>	<u>Collision Energy</u>	<u>Collision Cell Exit Potential</u>
Compound 22	1 st Ion	-1.00	-310.00	-4.00	-14.00	-20.00	-18.00
	2 nd Ion	-1.00	-310.00	-4.00	-14.00	-16.00	-28.00
<i>Acetylsalicylic Acid</i>	1 st Ion	-26.00	-350.00	-10.50	-8.00	-26.00	-12.00
	2 nd Ion	-26.00	-350.00	-10.50	-8.00	-20.00	-22.00
<i>Salicylic Acid</i>	1 st Ion	-6.00	-350.00	-10.50	-10.00	-22.00	-30.00
	2 nd Ion	-6.00	-350.00	-10.50	-10.00	-36.00	-10.00
Compound 25	1 st Ion	-31.00	-350.00	-10.50	-12.00	-44.00	-22.00
	2 nd Ion	-31.00	-350.00	-10.50	-12.00	-52.00	-26.00
<i>Acetylsalicylic Acid</i>	1 st Ion	-26.00	-350.00	-10.50	-8.00	-26.00	-12.00
	2 nd Ion	-26.00	-350.00	-10.50	-8.00	-20.00	-22.00

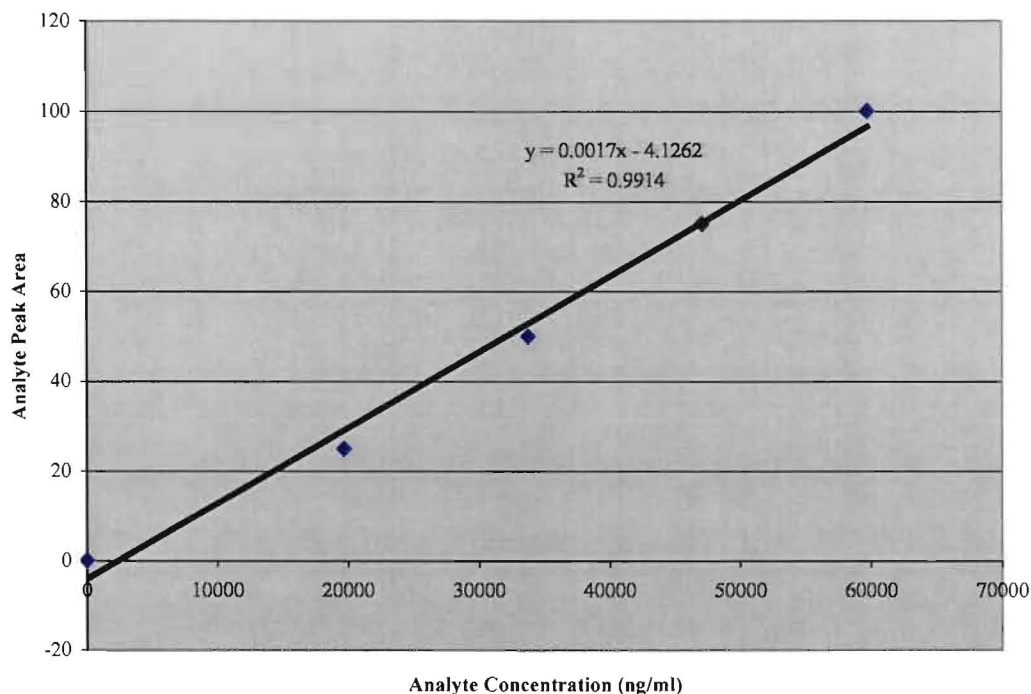
		<u>Declustering Potential</u>	<u>Focusing Potential</u>	<u>Entrance Potential</u>	<u>Collision Cell Entrance Potential</u>	<u>Collision Energy</u>	<u>Collision Cell Exit Potential</u>
Salicylic Acid	1 st Ion	-6.00	-350.00	-10.50	-10.00	-22.00	-30.00
	2 nd Ion	-6.00	-350.00	-10.50	-10.00	-36.00	-10.00
Compound 23 (+ Ion Mode)	1 st Ion	51.00	330.00	9.50	16.00	49.00	18.00
	2 nd Ion	51.00	330.00	9.50	16.00	97.00	2.00
Ibuprofen	1 st Ion	-56.00	-350.00	-10.50	-13.65	-6.00	-10.00
	2 nd Ion	-56.00	-350.00	-10.50	-13.65	-6.00	-10.00
Compound 26	1 st Ion	-21.00	-350.00	-10.00	-12.00	-20.00	-8.00
	2 nd Ion	-21.00	-350.00	-10.00	-12.00	-18.00	-26.00
Ibuprofen	1 st Ion	-56.00	-350.00	-10.50	-13.65	-6.00	-10.00
	2 nd Ion	-56.00	-350.00	-10.50	-13.65	-6.00	-10.00

		<u>Declustering Potential</u>	<u>Focusing Potential</u>	<u>Entrance Potential</u>	<u>Collision Cell Entrance Potential</u>	<u>Collision Energy</u>	<u>Collision Cell Exit Potential</u>
Compound 21 (+ Ion Mode)	1 st Ion	36.00	370.00	12.00	8.00	43.00	6.00
	2 nd Ion	36.00	370.00	12.00	8.00	71.00	2.00
Compound 21	1 st Ion	-16.00	-350.00	-10.50	-14.25	-20.00	-8.00
	2 nd Ion	-16.00	-350.00	-10.50	-14.25	-20.00	-8.00
Amantadine.HCl (+ Ion Mode)	1 st Ion	26.00	370.00	12.00	6.00	41.00	8.00
	2 nd Ion	26.00	370.00	12.00	6.00	55.00	8.00
Naproxen	1 st Ion	-16.00	-350.00	-10.50	-14.25	-20.00	-8.00
	2 nd Ion	-16.00	-350.00	-10.50	-14.25	-20.00	-8.00

All of the analyses were done in the negative ion mode, except where indicated otherwise.

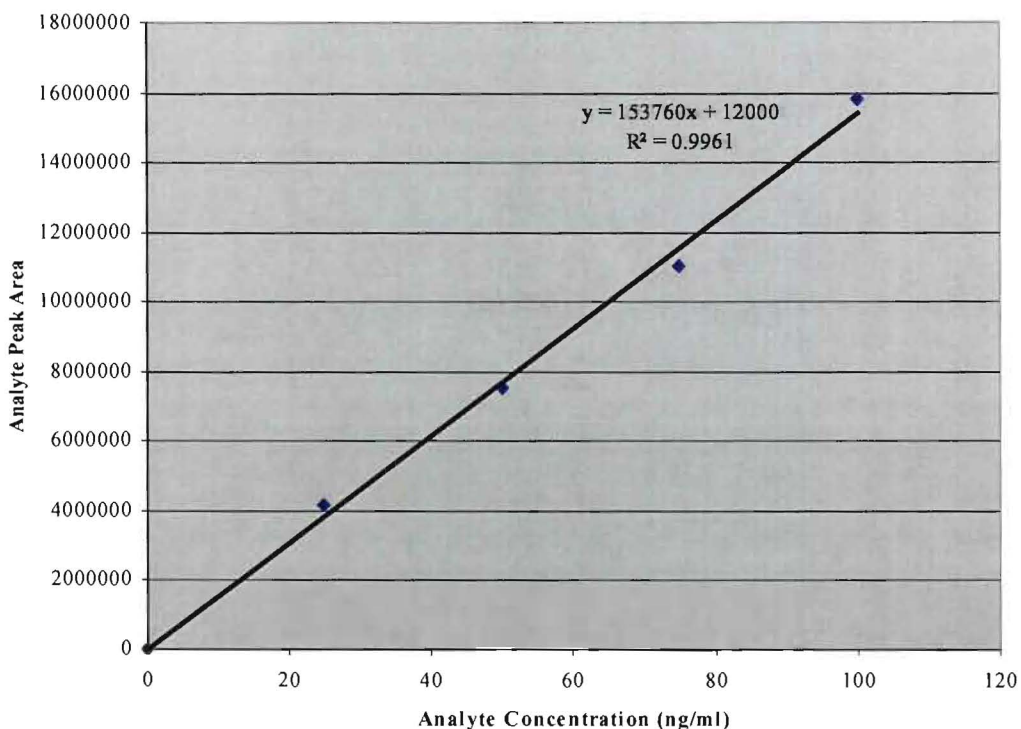
ANNEXURE C
CALIBRATION CURVES AND DATA SETS:
TEST COMPOUNDS

CALIBRATION CURVES AND DATA SETS



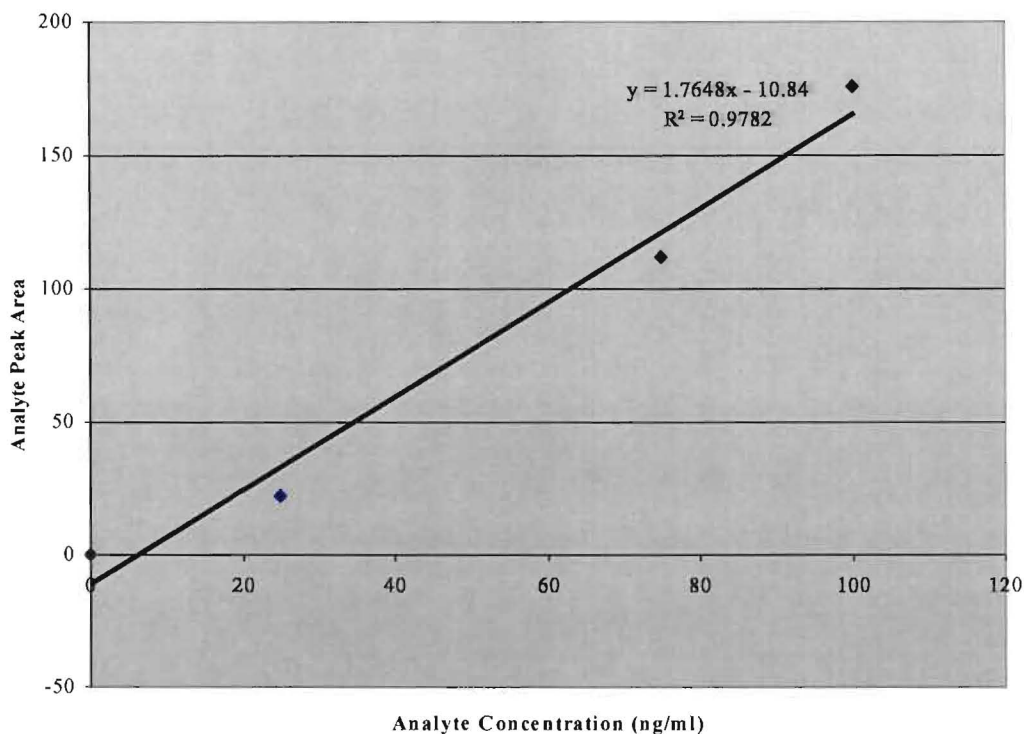
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AspS1	59700.00	1610000.00	0.04	100.00	35120074.24
AspS2	47100.00	1650000.00	0.03	75.00	27708309.53
AspS3	33800.00	1740000.00	0.02	50.00	19884780.12
AspS4	19700.00	1770000.00	0.01	25.00	11590662.47
AspS5	0.00	1770000.00	0.00	0.00	0.00
Bl-Asp-K1	491.00	9710.00	0.05	N/A	291250.71
Bl-Asp-K2	466.00	13700.00	0.03	N/A	276544.82
Bl-Asp-K3	596.00	19300.00	0.03	N/A	353015.41
Br-Asp-K1	1110.00	76100.00	0.02	N/A	655368.35
Br-Asp-K2	5070.00	42600.00	0.12	N/A	2984780.12
Br-Asp-K3	623.00	48300.00	0.01	N/A	368897.76
Bl-Asp-I1	999.00	24500.00	0.04	N/A	590074.24
Bl-Asp-I2	3110.00	22400.00	0.14	N/A	1831838.94
Bl-Asp-I3	5470.00	23200.00	0.24	N/A	3220074.24
Br-Asp-I1	1250.00	28400.00	0.04	N/A	737721.29
Br-Asp-I2	978.00	73400.00	0.01	N/A	577721.29
Br-Asp-I3	907.00	27900.00	0.03	N/A	535956.59
Acetylsalicylic Acid (1st Ion)					

CALIBRATION CURVES AND DATA SETS



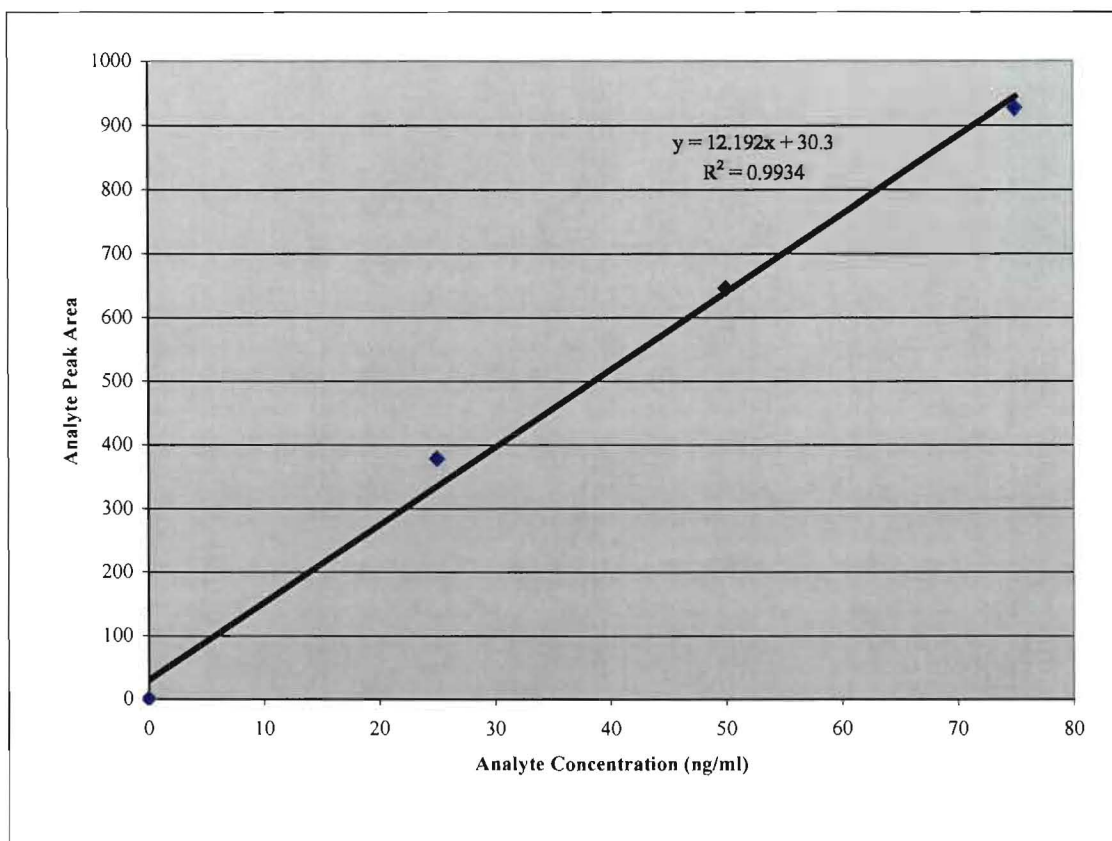
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AspS1	15800000.00	675000.00	23.41	100.00	102.68
AspS2	11000000.00	634000.00	17.35	75.00	71.46
AspS3	7540000.00	669000.00	11.27	50.00	48.96
AspS4	4160000.00	658000.00	6.32	25.00	26.98
AspS5	0.00	658000.00	0.00	0.00	0.00
Bl-Asp-K1	0.00	13100.00	0.00	N/A	-0.08
Bl-Asp-K2	0.00	16200.00	0.00	N/A	-0.08
Bl-Asp-K3	0.00	21100.00	0.00	N/A	-0.08
Br-Asp-K1	0.00	76700.00	0.00	N/A	-0.08
Br-Asp-K2	0.00	48000.00	0.00	N/A	-0.08
Br-Asp-K3	0.00	48300.00	0.00	N/A	-0.08
Bl-Asp-I1	0.00	25900.00	0.00	N/A	-0.08
Bl-Asp-I2	800000.00	24400.00	32.79	N/A	5.12
Bl-Asp-I3	1360000.00	23800.00	57.14	N/A	8.77
Br-Asp-I1	660000.00	28900.00	22.84	N/A	4.21
Br-Asp-I2	1130000.00	73400.00	15.40	N/A	7.27
Br-Asp-I3	1160000.00	30100.00	38.54	N/A	7.47
Salicylic Acid in Acetylsalicylic Acid (1st Ion)					

CALIBRATION CURVES AND DATA SETS



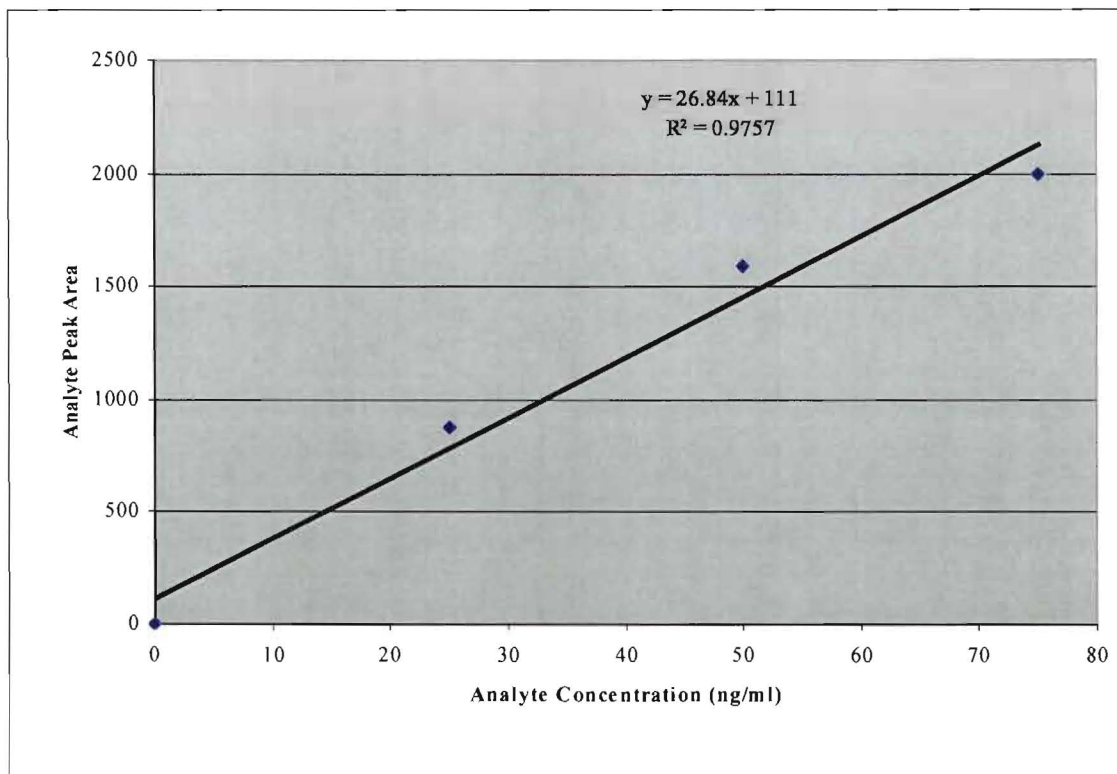
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C22S1	175.60	658000.00	0.0003	100.00	105.64
C22S2	112.00	666000.00	0.0002	75.00	69.61
C22S3	50.60	602000.00	0.0001	50.00	NC
C22S4	22.00	628000.00	0.0000	25.00	18.61
C22S5	0.00	628000.00	0.0000	0.00	0.00
Bl-C22-K1	48.30	16500.00	0.0029	N/A	33.51
Bl-C22-K2	19.90	26600.00	0.0007	N/A	17.42
Bl-C22-K3	70.10	24400.00	0.0029	N/A	45.86
Br-C22-K1	106.00	99800.00	0.0011	N/A	66.21
Br-C22-K2	48.50	67100.00	0.0007	N/A	33.62
Br-C22-K3	71.30	45900.00	0.0016	N/A	46.54
Bl-C22-I1	56.50	28700.00	0.0020	N/A	38.16
Bl-C22-I2	34.50	1050.00	0.0329	N/A	25.69
Bl-C22-I3	110.00	35400.00	0.0031	N/A	68.47
Br-C22-I1	103.00	49200.00	0.0021	N/A	64.51
Br-C22-I2	158.00	987.00	0.1601	N/A	95.67
Br-C22-I3	19.00	26000.00	0.0007	N/A	16.91
Acetylsalicylic Acid in Compound 22 (2nd Ion)					

CALIBRATION CURVES AND DATA SETS



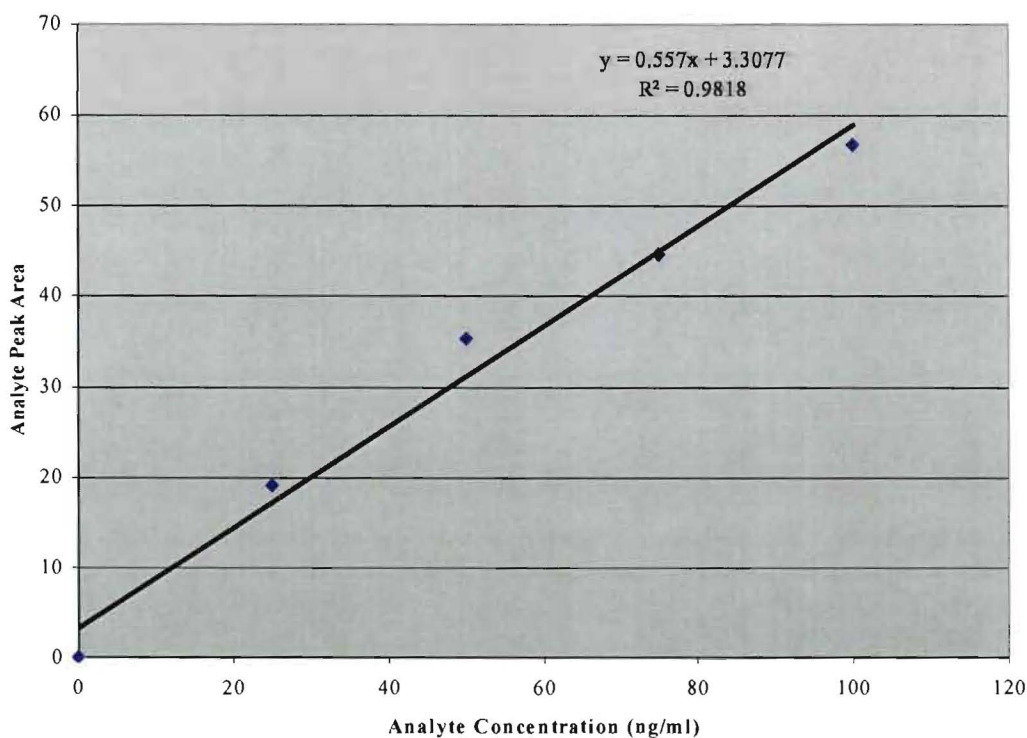
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C25S1	614.00	2200000.00	0.0003	100.00	NC
C25S2	927.00	2220000.00	0.0004	75.00	73.55
C25S3	645.00	2180000.00	0.0003	50.00	50.42
C25S4	378.00	2530000.00	0.0001	25.00	28.52
C25S5	0.00	2530000.00	0.0000	0.00	0.00
Bl-C25-K1	212000.00	12300.00	17.2358	N/A	17385.97
Bl-C25-K2	225000.00	13600.00	16.5441	N/A	18452.24
Bl-C25-K3	146000.00	16100.00	9.0683	N/A	11972.58
Br-C25-K1	56100.00	40200.00	1.3955	N/A	4598.89
Br-C25-K2	204000.00	59000.00	3.4576	N/A	16729.80
Br-C25-K3	92500.00	4060.00	22.7833	N/A	7584.46
Bl-C25-I1	197000.00	18500.00	10.6486	N/A	16155.65
Bl-C25-I2	291000.00	494.00	589.0688	N/A	23865.63
Bl-C25-I3	148000.00	12000.00	12.3333	N/A	12136.62
Br-C25-I1	49100.00	585.00	83.9316	N/A	4024.75
Br-C25-I2	147000.00	31300.00	4.6965	N/A	12054.60
Br-C25-I3	118000.00	229.00	515.2838	N/A	9675.99
Compound 25 (1st Ion)					

CALIBRATION CURVES AND DATA SETS



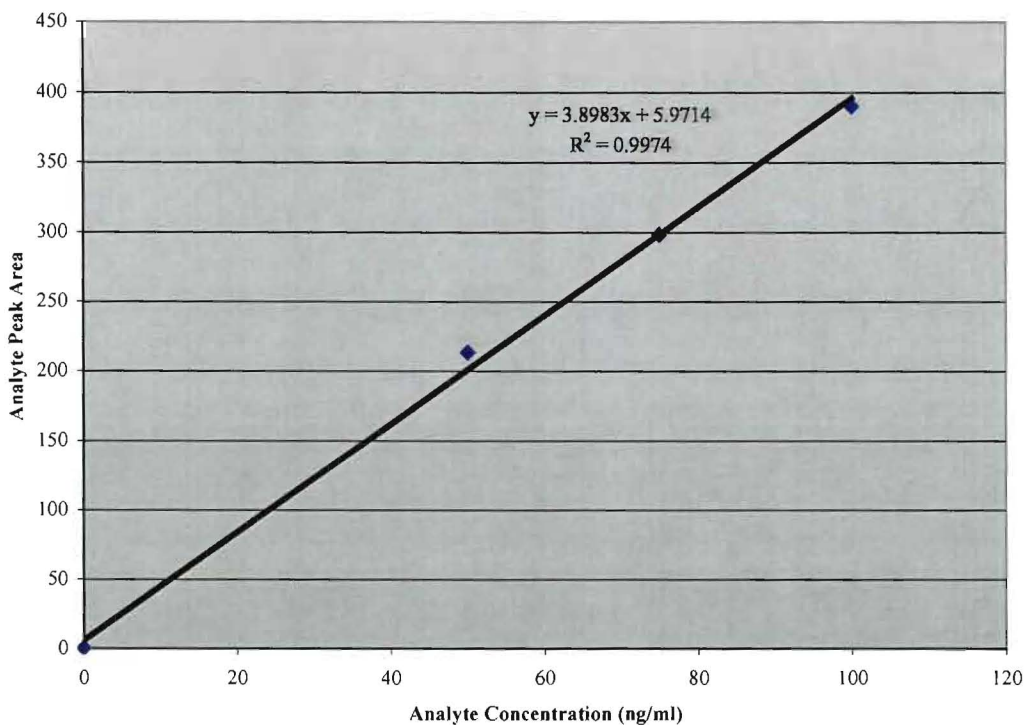
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C25S1	4760.00	2200000.00	0.0022	100.00	NC
C25S2	2000.00	2220000.00	0.0009	75.00	70.38
C25S3	1590.00	2180000.00	0.0007	50.00	55.10
C25S4	880.00	2530000.00	0.0003	25.00	28.65
C25S5	0.00	2530000.00	0.0000	0.00	0.00
Bl-C25-K1	170.00	9240.00	0.0184	N/A	2.20
Bl-C25-K2	88.70	10100.00	0.0088	N/A	-0.83
Bl-C25-K3	383.00	13900.00	0.0276	N/A	10.13
Br-C25-K1	264.00	40200.00	0.0066	N/A	5.70
Br-C25-K2	357.00	59000.00	0.0061	N/A	9.17
Br-C25-K3	346.00	4060.00	0.0852	N/A	8.76
Bl-C25-I1	587.00	17100.00	0.0343	N/A	17.73
Bl-C25-I2	187.00	494.00	0.3785	N/A	2.83
Bl-C25-I3	673.00	10800.00	0.0623	N/A	20.94
Br-C25-I1	329.00	585.00	0.5624	N/A	8.12
Br-C25-I2	418.00	30200.00	0.0138	N/A	11.44
Br-C25-I3	134.00	229.00	0.5852	N/A	0.86
Acetylsalicylic Acid in Compound 25 (2nd Ion)					

CALIBRATION CURVES AND DATA SETS



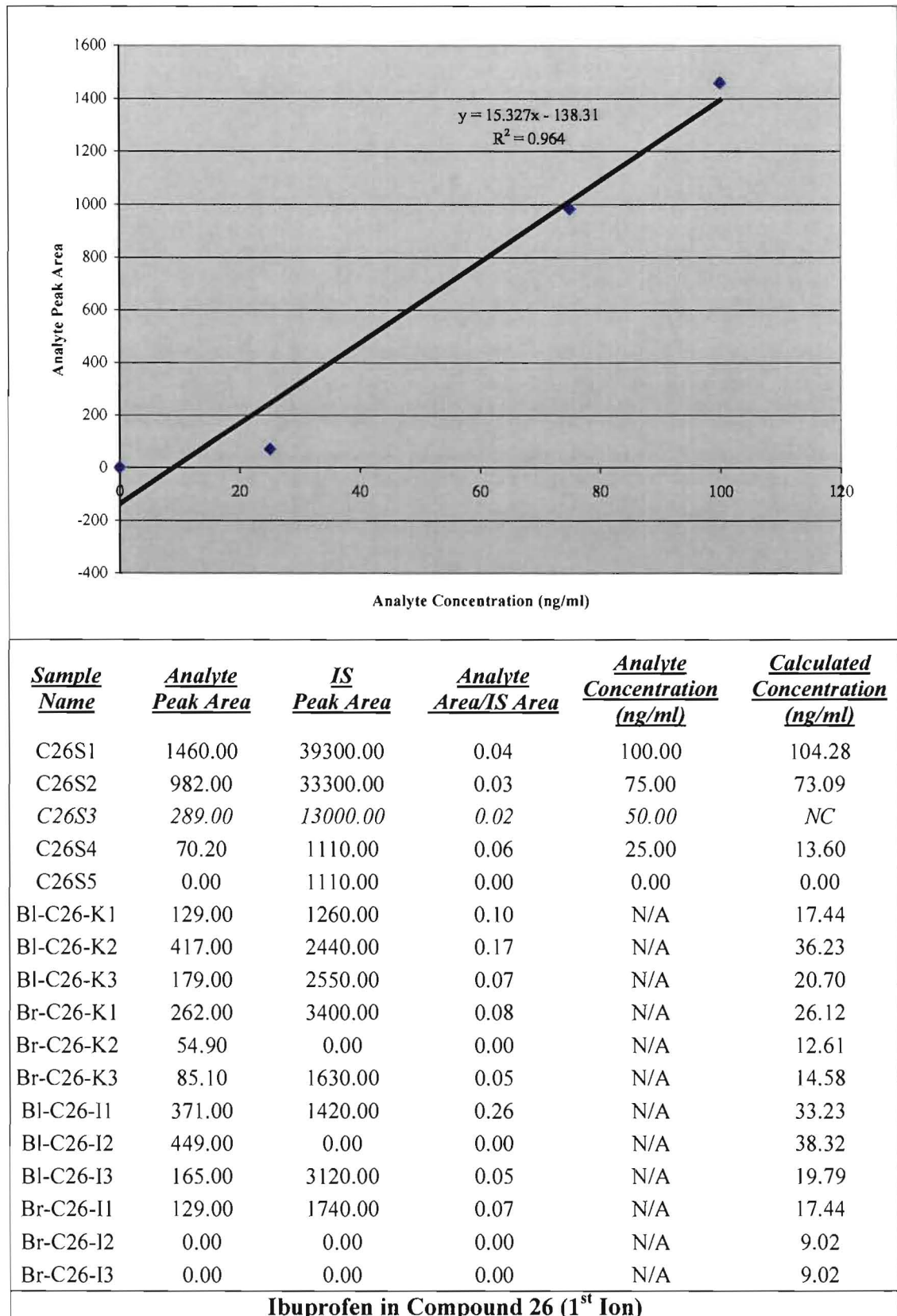
<u>Sample Name</u>	<u>Analyte Peak Area</u>	<u>IS Peak Area</u>	<u>Analyte Area/IS Area</u>	<u>Analyte Concentration (ng/ml)</u>	<u>Calculated Concentration (ng/ml)</u>
IbS1	1630000.00	28700.00	56.79	100.00	96.03
IbS2	1350000.00	30200.00	44.70	75.00	74.32
IbS3	1050000.00	29800.00	35.23	50.00	57.32
IbS4	621000.00	32600.00	19.05	25.00	28.26
IbS5	0.00	32600.00	0.00	0.00	0.00
BI-Ib-K1	9260.00	506.00	18.30	N/A	26.92
BI-Ib-K2	9840.00	730.00	13.48	N/A	18.26
BI-Ib-K3	7730.00	407.00	18.99	N/A	28.16
Br-Ib-K1	15100.00	2350.00	6.43	N/A	5.60
Br-Ib-K2	25800.00	2180.00	11.83	N/A	15.31
Br-Ib-K3	21700.00	3860.00	5.62	N/A	4.15
BI-Ib-I1	13300.00	282.00	47.16	N/A	78.74
BI-Ib-I2	26600.00	981.00	27.12	N/A	42.74
BI-Ib-I3	28300.00	741.00	38.19	N/A	62.63
Br-Ib-I1	75100.00	2350.00	31.96	N/A	51.44
Br-Ib-I2	113000.00	5260.00	21.48	N/A	32.63
Br-Ib-I3	138000.00	3120.00	44.23	N/A	73.47
Ibuprofen (1st Ion)					

CALIBRATION CURVES AND DATA SETS



<u>Sample Name</u>	<u>Analyte Peak Area</u>	<u>IS Peak Area</u>	<u>Analyte Area/IS Area</u>	<u>Analyte Concentration (ng/ml)</u>	<u>Calculated Concentration (ng/ml)</u>
C23S1	390.00	25500.00	0.02	100.00	98.51
C23S2	298.00	33100.00	0.01	75.00	74.91
C23S3	213.00	38300.00	0.01	50.00	53.11
C23S4	281.00	28700.00	0.01	25.00	NC
C23S5	0.00	28700.00	0.00	0.00	0.00
Bl-C23-K1	181.00	237.00	0.76	N/A	44.90
Bl-C23-K2	254.00	1060.00	0.24	N/A	63.62
Bl-C23-K3	39.80	939.00	0.04	N/A	8.68
Br-C23-K1	254.00	3490.00	0.07	N/A	63.62
Br-C23-K2	411.00	2710.00	0.15	N/A	103.90
Br-C23-K3	71.00	2350.00	0.03	N/A	16.68
Bl-C23-I1	191.00	85.20	2.24	N/A	47.46
Bl-C23-I2	251.00	355.00	0.71	N/A	62.86
Bl-C23-I3	135.00	67.70	1.99	N/A	33.10
Br-C23-I1	232.00	27.30	8.50	N/A	57.98
Br-C23-I2	0.00	204.00	0.00	N/A	-1.53
Br-C23-I3	215.00	2720.00	0.08	N/A	53.62
Ibuprofen in Compound 23 (1st Ion)					

CALIBRATION CURVES AND DATA SETS



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