The Identification of Bio-available and Active Components in Oxihumate

by

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Submitted in partial fulfilment of the
requirements for the degree
Philosophiae Doctor
(PhD)

in the Department of Pharmacology
Faculty of Health Sciences
University of Pretoria
Pretoria

April 2004

Acknowledgments

I am indebted to the many people who have made contributions, however small, to make this study possible. In particular, I wish to acknowledge the assistance, support and encouragement of the following people:

Professor Connie Medlen, my supervisor and mentor, who gave advice, guidance, encouragement and leadership. Her patience is greatly appreciated. She created the opportunity for me to carry out the study and this is appreciated very much.

Elmarie Oberprieler of the Pretoria Biomedical Research Centre for her patience, expert assistance with the animal work and baboon data collation. I would not have managed the baboon work without her helpful and committed attitude.

Mario Smuts, Johannes Teme and *Patrick Selahle* of the Pretoria Biomedical Research Centre for their arrangements and assistance with the availability and handling of the animals.

Dr Peter Buss, Dr Gerrit Steenkamp and especially *Dr Woodie Meltzer* who gave up their precious time as the allocated veterinarians from Onderstepoort for their professional assistance and advice.

Neil Rossouw of Radionuclide Production Department of the National Accelerator Centre (now iThemba Labs) for capable assistance with the isotope labelling procedures of the oxihumate.

Susan Malfeld and *Voula Stivaktas* of Immunology Department for assistance and training to run the flow cytometer.

Stephanie Harris of FRD who helped source specialist equipment and *Professor Mike Satekge* of the Nuclear Medicine Department at MEDUNSA for access to a gamma camera when we were left stranded with equipment failure at a critical point in our research.

Professor Jan Coetzee and *Andre Botha* of the Microscopy and Microanalysis Department; *Eric Palmer, Linda Prinsloo, Mila Maksa* of the Chemistry Department; *Professor Lizette van Rensburg* of the Human Genetics Department University of Pretoria; *Norman Arrangies,* of the Pharmacology Department of the University of Witwatersrand for making facilities available or running specialist analytical equipment for my research.

Lizelle Fletcher of the Statistics Department of UNISA for assistance and statistical analysis of data sets.

Elza, my wife, who lived through the stress of an aging fulltime student plus a PhD and still gave all her support and encouragement. Her help with digitising the figures and thorough technical proof reading was invaluable.

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Abstract

Key Words: Humic acid; oxihumate; chemical analysis; fractionation; HPLC; TLC; fluorescence spectra; UV spectra; IR spectra; infrared spectra; absorption; baboon model; rat model; distribution; pharmacokinetics; isolated gut segments; contact hypersensitivity; DNFB; chemiluminescence; CR3; human neutrophil adhesion molecules; anti-oxidant activity; anti-inflammatory; immuno-modulating

Humic acids are ubiquitous organic compounds found in soils and waters of the world. These organic molecules, derived from plant materials, have been isolated from many natural sources like seawater, marshes, soils, peat and coal. Despite extensive studies, the chemical structure of humic acids has not yet been elucidated and several theories have been proposed as to its structure. They have been found to have growth-stimulating effects on plants and medicinal properties in animals and humans.

Oxihumate is a semi-synthetic humic acid derived from a bituminous coal by a mild oxidation process. This synthetic humic acid has similar physical and chemical characteristics to humic acids isolated from different natural resources. Extensive toxicological studies have shown that there are no toxic effects to orally administered oxihumate at concentrations as high as 300mg/kg body weight.

In this study it was found that oxihumate could be sub-fractionated into at least seven sub-fractions using differential solubility in increasing concentrations of organic solvent, mixtures in which humic acid was reputed not to dissolve. Several analytical techniques were applied to each isolated sub-fraction in an attempt to determine the complexity and main chemical structures and to identify which sub-fractions possess the greatest anti-inflammatory properties. Thin layer and high pressure liquid chromatography, infrared, UV and fluorescent spectrophotometry, ash content and EDS microanalyses were performed on each isolated sub-fraction, all of which proved to still be complex mixtures of compounds.

The biological activity of the sub-fractions measured using immuno-fluorescent and chemiluminescent bioassays for anti-inflammatory properties indicated that the activity appeared to be associated with the complete mixture of compounds in the humic acid complex and not in the isolated or extracted sub-fractions.

Absorption studies using isolated rat gut segments and radioactively labelled oxihumate indicated that several compounds are absorbed from the lumen of the gastro intestinal tract

(GIT) and that the absorption rates depended on the GIT segment and reached a plateau within two hours.

As the GIT absorption studies showed uptake, the effect that orally administered oxihumate had on a dinitro-fluoro-benzene (DNFB) induced contact hypersensitivity rat model was tested and found to have a limited effect on the typical reaction at 61mg/kg. Brown coal humic acid and prednisolone, a steroidal anti-inflammatory, both showed significant and greater inhibition effects on the hypersensitivity response.

A further part of the study used 13 baboons that were dosed orally or rectally with radioactive iodide-123 labelled oxihumate. All the experimental baboons presented a distribution that compared almost exactly with that of free iodide controls. Initially however, the rectally dosed baboon showed a different pattern of distribution indicating uptake in the liver and gall bladder and only later showed the same distribution as the other animals. Excreted radioactivity was almost exclusively via the urine and this radioactivity was found to be mostly free iodide. The conclusions drawn from the baboon experiments was that the oxihumate appeared to be absorbed from the gut at a slower rate than the rate of metabolism of these absorbed compounds resulting in the release of free iodide.

The results of this study indicate that oxihumate can be sub-fractionated but that the isolated sub-fractions are still complex mixtures of compounds. The isolated sub-fractions appear to have less anti-inflammatory activity than the complete oxihumate indicating that the activity could be due to a combination of effects by different compounds. Oxihumate appears to have a unique combination of immuno-modulating properties that makes it a promising candidate as an anti-inflammatory agent.

OPSOMMING

Sleutelwoorde: Humiensuur; oksihumaat; chemiese analise; HDVC; dunlaag chromatografie; fluoressensie spektra; UV spektra; IR spektra; infrarooi spektra; absorpsie; bobbejaan model; rot model; verspreiding; farmakokinetika; geïsoleerde derm segmente; kontak hipersensitiwiteit; DNFB; chemiluminessensie; CR3; menslike neutrofiel adhesie molekules; anti-oksidant aktiwiteit; anti-inflammatoriese middel; immuno-moduleer middel.

Humiensure kom wyd verspreid voor en is in meeste grond en water aanwesig. Dié organiese molekules wat meestal van plant materiaal afkomstig is, is al uit verskeie bronne soos seewater, moerasse, grond, feen en steenkool geïsoleer. Ten spyte van uitgebreide studies en verskeie teorieë, is die chemiese struktuur van humiensure nog nie vasgestel nie. Dit is bekend dat hulle groei-stimulerende effekte op plante en terapeutiese eienskappe in diere en die mens het.

Oksihumaat is 'n semi-sintetiese humiensuur wat deur 'n matige oksidasie proses vanuit bitumineuse steenkool gemaak word. Hierdie semi-sintetiese humiensuur het soorgelyke fisiese en chemiese eienskappe aan humiensure wat vanaf verskillende natuurlike bronne geïsoleer is. Uitgebreide toksikologiese studies het gewys dat orale toediening van oksihumaat met konsentrasies van 300mg/kg liggaamsmassa, geen toksiese of newe effekte het nie.

In hierdie studie is bewys dat oksihumaat in minstens sewe sub-fraksies verdeel kon word, gebasseer op oplosbaarheids verskille in water met toenemende konsentrasies organise oplosmiddels, middels waarin humate na bewering onoplosbaar beskou te wees. Om die kompleksiteit, chemiese struktuure en anti-inflamatoriese eienskappe van elk van die geïsoleerde sub-fraksies te karakteriseer, is daar van verskillende analitiese tegnieke gebruik gemaak. Dun-laag en hoëdruk chromatografie, infrarooi-, UV-, en fluoressensie spektrofotometrie, as-inhoud en EDS mikroanaliese was op elke geïsoleerde sub-fraksie uitgevoer, wat bewys het dat al die sub-fraksies nog steeds komplekse mengsels van komponente is.

Die biologiese aktiwiteit van die sub-fraksies is gemeet deur immuno-fluoressensie en chemiluminessensie toetse wat aandui dat die hoogste aktiwiteit en anti-inflamatoriese eienskappe geassosieer word met die volledige humiensuur mengsel en nie by die geïsoleerde sub-fraksies nie.

Opname studies, waar geïsoleerde rot dermsegmente en radioaktief gemerkte oksihumaat gebruik is, het aangedui dat verskeie komponente wel opgeneem word uit die dermkanaal en dat die opname tempo afhankilik is van die spesifieke dermsegment. Afplatting van opname het binne twee ure plaasgevind.

Aangesien opname bewys is, is 'n vergelykende studie gedoen om die effek van 'n mondelinge toediening van 61mg/kg oksihumaat op dinitro-fluoro-benseen (DNFB) geinduseerde kontak hipersenstiewiteit in rot modele te toets. Daar is gevind dat slegs bruinsteenkool-humiensuur en prednisoloon, 'n bekende steroïedale anti-inflamatoriese middel, die hipersenstiewiteits reaksie betekenisvol geinhibeer het.

In 'n verdere studie is 13 bobbejane oraal of rektaal met radioaktief gemerkte oksihumaat gedoseer. Al die eksperimentele bobejane het 'n verspreiding soortgelyk aan die kontrole diere, waar net vrye jodied toegedien is, getoon. Die rektaal gedoseerde bobbejaan het wel in die begin 'n ander verspreiding getoon veral in die lewer en galblaas, maar dit het binne 24 uur dieselfde gelyk as al die ander. Radioaktiwiteit was slegs in die vorm van vrye jodied in die uriene uitgeskei. 'n Moontlike afleiding wat gemaak kan word is dat opname vanuit die spysverteeringskanaal stadiger is as wat metabolisme in die liggaam plaasvind en dus word net die vrygestelde jodied se verspeiding waargeneem.

Die resultate van hierdie studie toon dat oksihumaat wel gefraksioneer kan word, maar dat die geïsoleerde sub-fraksies nog steeds komplekse mengsels is. Die geïsoleerde sub-fraksies besit minder anti-inflamatoriese aktiewiteit as die volledige oksihumaat, moontlik omdat die anti-inflammatoriese effek 'n gekombineerde effek van verskillende komponente is. Dit blyk dat oksihumaat 'n unieke kombinasie van immuno-moduleerende eienskappe het, wat maak dat oksihumaat 'n belowende anti-inflamatoriese middel is wat verdere navorsing verg.

ABBREVIATIONS

AA	Arachidonic acid
APC	Antigen presenting cells
APCI	Atmospheric pressure chemical ionisation
AUC	Area under curve
CD	Clusters of differentiation (The number following the CD indicates which surface antigen is referred to).
CD4+	T-lymphocyte presenting the CD4 surface antigen. A T-helper cell
CD8+	T-lymphocyte presenting the CD8 surface antigen. A cytotoxic T-cell
CE	Capillary electrophoresis
CNS	Central nervous system
Con A	Concanavalin A
COX-1	Cyclooxygenase 1, prostaglandin H synthase type 1 (constitutive)
COX-2	Cyclooxygenase 2, prostaglandin H synthase type 2 (inducible)
CR3	Complement 3 receptor adhesion molecules, a leukocyte surface marker
DMSO	Dimethyl sulphoxide
DNFB	2,4-Dinitro-fluorobenzene
DTH	Delayed type hypersensitivity
E_4/E_6	The ratio of absorbance measured at 465nm divided by the absorbance measured at 665 nm for a compound in solution
eV	Electron volt
FAB	Fast ion bombardment
GC	Gas chromatography
GM-CSF	Granulocyte macrophage colony stimulating factor
HPLC	High performance liquid chromatography
HUVEC	Human umbilical cord arterial endothelial cells
ICAM-1	Intercellular adhesion molecule type 1
ICAM-2	Intercellular adhesion molecule type 2
Ig	Immunoglobulin
IL-	Interleukin- (the number refers to the particular interleukin)
INF-γ	Interferon –γ
IV	Intra-venous
kD	Kilo-Dalton, molecular mass/1000
LAK	Lymphokine activated killer cells

LPS	Lipopolysaccharide, a gram negative bacterial cell wall antigen
LTB_4	Leukotriene B_4
MALDI TOF	Matrix assisted laser desorption ionisation time-of -flight
MHC-II molecules	Major histocompatibility complex type 2 molecules
MO	Monocytes
MØ	Macrophages
mRNA	Messenger ribose nucleic acid
MS	Mass spectroscopy
NF-κB	Nuclear transforming factor - κB
NK	Natural killer cells, large granular lymphocytes
nm	nanometre
NMR	Nuclear magnetic resonance spectroscopy
PAF	Platelet activating factor, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphate
PAGE	poly-acrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor - 1
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostaglandin I_2
pH	Negative logarithm of the concentration of hydronium ions, a measure of the acidity of an aqueous solution
PHA	phytohemagluttinin
PMNL	Polymorphonuclear leukocytes
PPAR-α	Peroxisome proliferator-activated receptor-α
ROS	Reactive oxidant species
SEC	Size exclusion chromatography
sIgD	Soluble immunoglobulin D
sIgM	Soluble immunoglobulin M
Th 1	T-lymphocyte helper cells type 1
Th 2	T-lymphocyte helper cells type 2
TNF-α	Tumour necrosis factor – α, a pro-inflammatory cytokine
TNF-β	Tumour necrotic factor-β
t-PA	Tissue plasminogen activator
TxA_2	Thromboxane A_2
UV	Ultraviolet light
VCAM-1	Vascular adhesion molecule type 1

Chapter 1

Literature Review

1 Introduction

Natural humic acids are polymeric brown-black organic acids that are ubiquitous in nature, being found in almost all soils and surface water (Aiken, 1985; Berzelius, 1893; Davies, 1996; Hoppe-Seyler, 1889; Kononova, 1966; Stevenson, 1982). Humic acids are of the allomelanin class of compounds and are derived mostly from plant, but also animal, material. The chemical characteristics and physical properties of humic acids vary depending on the source from which they have been extracted. Humic acids present in soils and most fresh waters, are acknowledged to be important for soil fertility (Vaughan & Malcolm, 1985; Visser, 1986), are fairly refractive to chemical and biological decomposition (Hedges & Oades, 1997) leading to their dominance as the organic matter found in soils. Humic acids are also precursors to, or inclusions in many of the abundant natural resources like peat, bitumen, coal and petroleum. They are claimed to possess some therapeutic medicinal activities (Janecek & Chalupa, 1969; Priegnitz, 1986; Reichert, 1966; Visser, 1973; Ziechmann, 1996), a characteristic known from ancient times. Despite the abundance, the known effects and uses and the many years of research, a probable but not universally accepted chemical structure was only proposed relatively recently (Schnitzer, 1985; Schulten *et al*, 1991). The reason for the long delay in determining the structure is straightforward: humic acids are complex heterogeneous mixtures of compounds (Wilson *et al*, 1987), often containing unrelated inclusions, and which are undergoing continuous chemical metamorphosis.

Humic substance research has seen slow progress as humic acid structure and chemistry is difficult to unravel, even with the technology available today. Many new researchers that recently joined the search for the elusive structure of humic acid have added to the impetus of this research.

The term humic acid is generally used to describe the brown-black, polymeric, alkali-soluble organic acid fraction of humus that is found in geological sediments, soils, wetlands, surface and underground water, and that more recently has even been identified in certain living fungi and plant materials (Kühnert *et al*, 1982).

Because humic acids are ubiquitous in nature and can be extracted in varying proportions by the many subtly different methods used by researchers in the past, many compounds with slight chemical differences and various inorganic inclusions and organic aggregates have been identified as humic acid. The variations in the origin and extraction methods utilized resulted in humic acids with different solubilities, colours, and textures which were the primary properties used to characterize compounds until structural chemical analysis became readily available in the latter 1900's.

2 Historical background

Humus was originally named as such in 1761 by the German scientist Wallerius (1761) using the Latin name for soil: Humus (the term humus is generally applied to all organic compounds of plant origin found in the soil). Twenty-five years later the first extraction of a soluble sub-fraction termed "humic acid" was reported by Achard (1786) who used peat, a rich source of humic acids, for his experiments. At that time it was recognized that humus and the alkali extractable portion named humic acids contributed to soil fertility but the mechanism of action was still unknown and some wild assumptions were made in this respect.

One of the first comprehensive studies on humic acids that covered the chemistry, physical properties and its uses was published by Sprengel (1826) and highlights how little progress has been made since then with regard to chemical structure.

Synthetic substances that looked like humic substances and referred to as artificial ulmin were made as early as 1819 by Braconnot (1819) who added acids to starch or sucrose each of which then formed a dark precipitate that looked like the humic acids extracted from soils. Glucose was found to give the same type of products, and Malguti (1835) published his views of the transformation of carbohydrates to synthetic humic acid. In 1839 Mulder (Mulder, 1839) published his work on the synthesis of humic substances from cellulose, resulting in a then widely accepted assumption that humic acids were derived from polysaccharides.

By the late 1800's humic substances had been formed from many other types of organic molecules, some very simple and the polysaccharide theory was essentially discarded in favour of the newly developing idea of a micro-organism mediated transformation of plant material into humic substances. In the mid 1800, microorganisms were recognized as widespread in soils and the possibility that humic substances were formed in the soil by their action was offered as the origin of humic substances. This hypothesis better explained the then recently described ethers, esters,

ketones, anhydrides, furans, aromatic compounds as well as the nitrogen and sulphur that had been reported to be present in humic substances.

Schreiner and Shorey (1908; 1910), found organic compounds such as simple hydrocarbons, fatty acids, glycerides, resin esters, chitin, cellulose, xylans, sugar alcohols, sterols, lecithins, pyridines, amides, amino acids, purine bases, vanillin, numerous aliphatic and aromatic acids and elemental carbon in humus. Some workers (Marcusson, 1920; Gortner, 1916) still favoured the polysaccharide theory as late as 1914 as they found a strong correlation for the furan structure in both coal and humic substances. Other coal researchers however disputed this theory again by demonstrating that microorganisms rapidly consume polysaccharides (Fischer & Schrader, 1921) and that there could not be sufficient polysaccharides remaining in the soil to give the quantities of humic acids found. These same authors proposed that lignin was the precursor to humic substances, which started a new school of thought around the origins of humic acids.

Marcusson (1925) and Hilpert and Littman (1934), opposed the new lignin theory but it still gained popularity from both a microorganism based synthesis (Waksman, 1938) as well as from a chemical synthesis point of view. The presence of aromatic compounds provided support for the idea that lignins must be involved in the formation of humic substances, as polysaccharides contained no aromatic functionality.

Shapiro (1957) published a study of humic acid where he used chromatography and liquid phase infrared spectrophotometry. He also introduced organic solvents into the fractionation of humic acids that had until then been described as insoluble in any organic solvents. An ethyl acetate soluble sub-fraction of the humic acid that he isolated apparently had no aromatic character or compounds.

Gas chromatography (GC) became a popular method of analysis in the late 1950's but humic acid samples needed derivatization before this type of analysis could be done due to the requirement that the sample be volatile for the technique to work. To overcome this volatility problem, the oxidation method of Bone *et al.* (1934) was used to split the humic acid into volatile sub-fractions. Most compounds identified by GC were aromatic, which gave support to the lignin derived humic acid theory (Wright & Schnitzer, 1958). Several reports do however indicate that oxidation can result in the formation of aromatic compounds from certain reactive aliphatic substances (Reuter *et al*, 1983). Despite this shortcoming, chemical oxidation became a common method to aid in the analysis of humic substances especially when the oxidation products were further analysed by gas chromatography.

Kononova (1961) published her book that supported the lignin derived humic acid theory as did Schnitzer (1985) in a report in Nature, and Stevenson (Stevenson, 1982; Stevenson, 1994) published two editions of a book several years apart, both supporting the lignin derived humic acid theory despite mounting evidence that there were many aliphatic compounds not found in lignins present in humic acids.

In 1982 the International Humic Substances Society (IHSS) was established in an attempt to coordinate humic acid research, and to collect and maintain a reference collection of humic acid samples to give researchers access to standardized samples.

The aliphatic nature of humic acid has always been a contentious issue as it contradicted the generally accepted origin of the humic acids. However a marine humic acid described in 1972 was shown to be essentially aliphatic (Nissenbaum & Kaplan, 1972), and Harvey *et al.*, (1984) later proposed that marine humic acids were derived from fatty acids. Farmer and Pisaniello (1985) found no aromatic compounds in the samples of humic acids that they analysed by NMR. A year after this report, Ikan *et al.*, (1986) reported that they had also found mostly aliphatic compounds in the humic acids that they were studying. Few studies have been published on chemically reduced humic acids but those that have, have also indicated an aliphatic nature for the humic acids analysed (Mendez & Stevenson, 1966; Martin *et al*, 1987). The reports of humic acids that have exhibited little or no aromaticity are relatively few compared to the bulk of the data that points to aromatic structure, but it should be accepted that humic acids from different sources can exhibit different structures due to different starting material and environmental conditions in which they have matured and accumulated.

3 Structural considerations

Due to the variation in solubility of the various humic substances from different sources, there are large differences in the reported composition of the humic substances isolated from different soils, sediments and other environments. Added to this is the fact that humic acids tend to bind to each other physically in a relatively tight but random fashion (Wershaw, 1993), often incorporating molecules that are not strictly speaking humic substances such as lipids, carbohydrates, nucleic acids and amino acids (Khairy *et al*, 1996a; Khairy *et al*, 1996b). These non-humic compounds enter the complex at a rather early stage of the humification process, whereas the aromatic compounds tend to become detectable at the later stages of the process (Ziechmann *et al*, 2000). Humic acids also bind tightly to a number of minerals (especially silica and alumina) (Stevenson, 1994) and easily complex heavy metals (especially iron, copper, chrome and the lanthanide series) (Griffith & Schnitzer,

1975), which in turn affect their solubility and which can catalyse chemical reactions within the complex of compounds making up the humic acid (Liu & Huang, 2000). Figure 1-1 illustrates some of the typical organic functionality that occurs within the humic acid structure. This includes aliphatic, aromatic, heterocyclic and polyphenolic functionality. The figure in no way attempts to assign a structure to any humic acids.

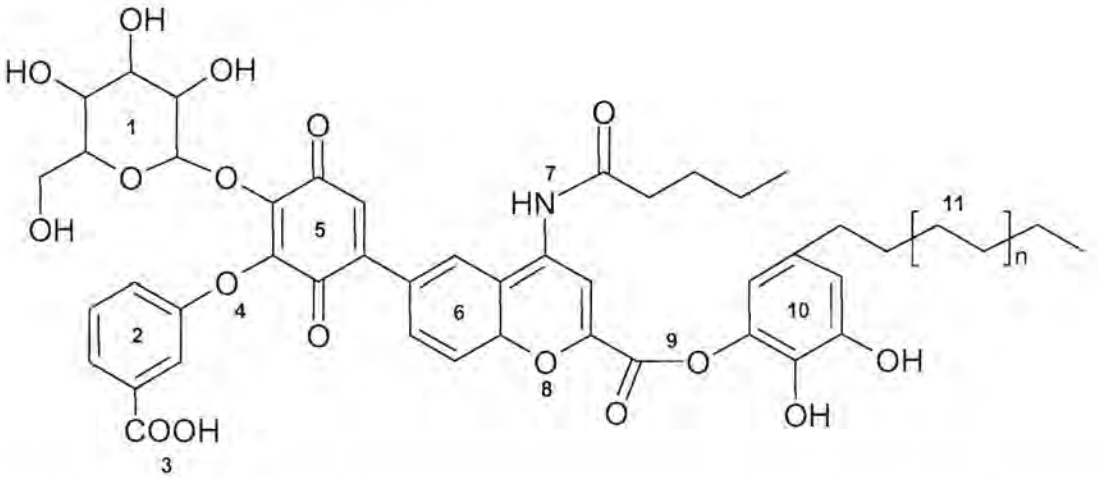

Figure 1-1: A diagram showing typical functional groups reported to occur in humic acids. This diagram in no way attempts to give a structure for a humic acid. 1, monosaccharides; 2, aromatic rings; 3, acid groups; 4, ether groups; 5, quinone groups; 6, fused aromatic rings; 7, amides; 8, heterocyclic rings; 9, esters; 10, polyphenols; 11, aliphatic carbon chains up to C34.

Coal is formed through a coalification process that involves a slow progression of reactions under distinct physical conditions that includes heat, pressure and submersion. The process takes place over many thousands to millions of years and is usually formed from humic acid rich deposits of rotting plant material (Ziechmann *et al*, 2000). This process of coalification can be reversed when coal undergoes weathering, usually when coal seams become exposed to oxygen and water. The medium to lower rank coals appear to spontaneously undergo this type of depolymerisation to form compounds that are humic substances, but which are probably not identical to the original compounds from which the coal formed (Rausa, 1994). Many of the compounds formed have typical chemical and physical properties of humic acids, although the more soluble fulvic acids and highly mineralized insoluble fractions are also present. The soluble products have high concentrations of carboxylic and aliphatic acids, phenolics and quinones (Wender *et al*, 1981).

4 Classification of humic substances

Humic substances were traditionally classified according to their solubility in aqueous medium at different pH values, but this was not a standardized method. Water-derived humic acids were precipitated at pH 2 whereas humic acids from soil were precipitated at pH 1. Generally, the weakest acids (highest pKa value) should be first to precipitate as the pH is lowered due to the suppression of ionisation. Unfortunately it is not this simple, as many other factors also influence the solubility of weak acids, such as the predominant counter ion, valence of the cations present, ionic strength, the presence of chaotropic agents, solvents or surfactants. These complications gave rise to different characteristics being reported for the isolated humic acids with respect to elemental analysis, ash content and solubility.

Humic substances are still generally divided into three classifications or sub-fractions based on the aqueous solubility of the humic substance (Odén, 1919). These are;

- fulvic Acids – the sub-fraction that is soluble in water at all pH values. They are generally reported as multivalent organic acids and have been found to have molecular mass varying from as low as 45 up to about 1000 Dalton.
- humic acids – the dark sub-fraction that is soluble in alkaline aqueous solutions but precipitates at pH of less than 2. They are not soluble in short chain alcohols. The reported molecular mass of humic acids have generally been found to be between 10 000 and 350 000 Dalton.
- humin – the water insoluble fraction. These compounds have very high apparent molecular mass and are more condensed molecules than the previous two fractions, being closer to peat and coal. They are often mineralized.

Ziechmann, (1993) defined a fourth fraction of humic substances as the haematomelanic acids, the fraction that is water and short chain alcohol soluble and with an average molecular mass between 5000 and 10 000 Dalton.

More recent methods of isolating humic substance sub-fractions with macroreticular resins has again given raise to a different grouping of compounds within the older general classification of humic substances (Leenheer, 1985; Malcolm & MacCarthy, 1992; Thurman & Malcolm, 1981). A column packed with XAD-8 resin is used first to extract the hydrophobic acids, hydrophobic bases and the hydrophobic neutrals from an acidified solution. The polar pass-through fraction is then separated into hydrophilic acids, hydrophilic bases and hydrophilic neutrals on an XAD-4 resin column. These columns can be used in tandem (Malcolm & MacCarthy, 1992) to isolate humic acids from aqueous

samples. This newer method appears to be the method of choice for the IHSS, although many laboratories still use the classical separation using pH adjustment only. This variation in isolation methodology remains a problem as it introduces yet another area of uncertainty into the structure of the humic acids.

5 Chemical analysis of humic acids

Humic acid can be isolated from many different sources and this has resulted in many compounds with fairly different chemical and physical properties being characterized. A major problem encountered is that classical chemical analysis has not been successful in the complete elucidation of the composition or the structure of humic acids. This has resulted in many hypotheses (Hayes, 1998; Steelink, 1999) as to the chemical and physical structure of the humic acids isolated from soils (Ghabbour *et al*, 1998), peat bogs (Francioso *et al*, 2001), lake sediments (Ishiwatari, 1985), coals (Novac *et al*, 2001), compost, animal excreta (Khairy, 1989), as well as from numerous aqueous environments (Nissenbaum & Kaplan, 1972). Making things even more complicated is that although it is generally accepted that humic substances are derived from well decayed plant material, there are reports of humic substances being isolated from living plants and senescent leaves (Wershaw *et al*, 1998b; Wershaw *et al*, 1998a; Ghabbour *et al*, 1994). Some bacteria and fungi have also been reported to synthesize humic substances intracellularly and release these substances during the decomposition of these organisms (Kühnert *et al*, 1982).

Although there are many easily hydrolysable bonds in humic acids, most bonds are difficult to cleave making humic acids fairly refractory to microbial as well as chemical decomposition (Ghabbour *et al*, 1998). The use of decomposition analysis where the humic acid is broken down into smaller molecules has generally required relatively harsh chemical treatment that resulted in complete breakdown of susceptible parts of the complex. This approach enhances the possibility of finding artefacts of the decomposition method rather than actual compounds making up the original material (Farmer & Pisaniello, 1985; Hayes, 1998; Lehtonen *et al*, 2001; Saiz-Jimenez, 1994).
Solubility becomes a problem at lower pH values (a characteristic that has been used in the broad sense to define humic acids) and humic acids isolated from environments that have been in constant contact with water have tended to have fewer lower molecular weight and polar soluble fractions.

A fairly generally accepted structure for humic acids is one of a dynamic heterogeneous complex of many different molecules at various stages of degradation (Baldock *et al*, 1992; Kononova, 1961; Novac *et al*, 2001; Stevenson, 1994) that easily complexes further with diverse organic molecules such as sugars, amines, fatty acids, waxes and aromatic compounds (Khairy *et al*, 1996a; Khairy *et*

al, 1996b), to metal ions (Nifant'eva *et al*, 1999; Ruiz-Has *et al*, 1998; Stevenson & Chen, 1991) or minerals (Wershaw, 2000). Each of these types of complexed compounds alters the solubility of the humic acid differently, probably because they bind to different regions of the acid, form cross-links between different acid molecules, or alter the exposed surface of the complex. Other proposals of the structure include "Maillard" products (a condensation reaction between reducing sugars and amines), "aromatic core and periphery" models, aliphatically linked aromatic rings and polymeric chains with substituting molecules. Humic acids are also reported to easily form micelles, even though the critical micellar concentration (CMC) is high – between 0.4% and 0.9% (Engebretson & vonWandruszka, 1994; Guetzloff & Rice, 1994; Wershaw, 1993; Sato *et al*, 1987b; Wershaw, 1994), a characteristic that makes separation by chromatographic means unpredictable and irreproducible unless all conditions with respect to sample concentration, pH, counter ions, injection volume, column regeneration etc are rigorously adhered to (Preuse *et al*, 2000).

The classic methods of chemical analysis used for humic acid are elemental analysis, ash content, the formation of coloured condensation products (a technique hampered by the intense colour of the starting humic acids) and titration of the acid groups and reactive hydrogen atoms.

5.1 Solvent solubility

Strictly speaking humic acids are only soluble in alkaline aqueous medium, as this characteristic is used to define these compounds. However humic acids from different sources have been shown to have sub-fractions that are soluble in organic and even acidic medium (Hayes & Graham, 2000). Using XAD 8 resin it was possible to remove amino acids and neutral sugars from a solution of humic acid in DMSO containing 1% HCl. This result implied that non-humic compounds are co-precipitated or adsorbed to the humic acids during the isolation procedure and that if conditions are chosen that promote adsorption of the humic acid to a non-polar resin, then the polar non-humic compounds can be removed (Hausler & Hayes, 1996). An extraction procedure where the humic acid solution is passed through XAD 8 and XAD 4 resins used in tandem was originally proposed by Thurman and Malcolm (1981). Six sub-fractions were isolated with this procedure with the neutral hydrophobic and hydrophilic compounds (adsorbed onto the XAD 8 and XAD 4 columns respectively) requiring Soxhlet extraction using ethanol or acetonitrile to remove them from the resins.

Another anomaly is that all the reported mild oxidation and reduction reactions have released products of humic acids that are soluble in organic solvents and that have been found to be small

molecules (up to 35% of the original mass of humic acids) (Burges, 1963; Burges *et al*, 1964; Lehtonen *et al*, 2001; Lobbes *et al*, 1999).

Strictly speaking the organic solvent soluble fractions should not be classified as humic acid but it appears as though these fractions occur in most humic acid samples, even though hydrolysis or oxidative or reductive reactions are sometimes required to release them.

In this investigation, where active compounds are being sought, use is made of the classic acid precipitation method followed by organic solvent fractionation to release the low concentrations of compounds from within the humic acid complex and to facilitate bioassays for anti-inflammatory and cellular adhesion molecule expression.

5.2 Chromatography

5.2.1 Thin Layer Chromatography (TLC)

Not many reports of TLC separations of humic acids have been published, as these acids tend to remain on or very close to the origin and streak extensively in almost all mobile phase combinations. They also tend to separate into only three or four distinct bands of dark colour. There are no visualising agents reported for humic acids as such, therefore the natural dark colour is used to identify the position of the compounds that have moved. However the position of several bands separated from humic acid can be visualized with iodine vapour or their fluorescence under long wavelength ultra-violet light.

Khairy (1980) used various thin layer media and found silica gel 60 best as stationary phase combined with a very polar mobile phase consisting of 70% of a concentrated (25%) ammonium hydroxide solution and 30% n-propanol to achieved preparative TLC separations of humic acids obtained from faeces. Generally 3 bands were obtained. In the above method, the humic acid was first extensively and sequentially pre-extracted with diethyl ether, acetone, ethanol and dioxane in a Soxhlet apparatus to remove "non humic" substances. Water and dimethyl formamide were used further to extract polar non-humic compounds from the "defatted" samples and the small amounts of humic acid that dissolved in these two solvents were recovered by preparative TLC or acid precipitation. This extensive extraction procedure removed any lipophilic and polar substances that were loosely bound to the humic acids.

5.2.2 High Performance Liquid Chromatography (HPLC)

Several HPLC techniques or modes have been applied to the separation and quantification of humic acids. Due to the complexity of the humic acids that affects the spectroscopic characteristics and the low solubility of humic acids in organic solvents there has been limited success in the fractionation of humic acids with most of the common techniques used. As humic acids are only soluble in alkaline aqueous media or highly polar organic solvents, the use of unbonded normal phase stationary phases is precluded. The successful modes of separation used for humic acid separations have included size exclusion chromatography (SEC), reverse phase chromatography and ion exchange chromatography.

SEC on HPLC has been used in an attempt to separate humic acids into different molecular mass ranges so that further analysis and characterisation can be done on individual compounds (Chin *et al*, 1994; Piccolo *et al*, 2000; Rausa *et al*, 1991; Saito & Hayano, 1979). Even using HPLC SEC, the resolution of the compounds has not been as successful as would be expected from a sample consisting of a simple mixture of compounds, leading to the conclusion that humic acids must be non covalently-bound polymer-like associations of molecules (Piccolo & Conte, 2000). It is interesting to note that the same pattern of separation has been reported when using low-pressure gel filtration or size exclusion chromatography techniques using various types of media e.g. Sephadex, Sephacryl, Biogel P150. The reported molecular mass for humic acids has varied from as low as 1.7×10^3 D to as high as 1.36×10^6 D. The larger mass fractions can be reversibly separated into smaller fractions by various treatments, especially treatment with organic acids (Piccolo & Conte, 2000; Conte & Piccolo, 1999). Several publications have pointed out that humic acids are surface-active compounds and that they form micelles above a critical concentration that could easily be reached on a column during separation (Engebretson & vonWandruszka, 1994; Guetzloff & Rice, 1994; Sato *et al*, 1987b; Wershaw, 1993; Wershaw, 1994). This characteristic alone would make molecular size dependent chromatographic separations very difficult and unpredictable.

Another characteristic that would affect chromatographic separation is that the solubility of humic acids appears to change in the presence of certain ions, especially the divalent and trivalent cations, and that the solubility is particularly sensitive to pH variations.

Another HPLC technique used is reverse phase chromatography. This mode of separation depends on an adsorption equilibration of lipophilic compounds onto the aliphatic 18-carbon chains bonded to the surface of the silica particles (Engelhardt, 1986). The silica particles have a fairly open and porous structure and the larger the effective pore size, the larger the molecules that can interact with

the inner aliphatic carbon groups of these particles. Several studies with fulvic and humic acids of different origin resulted in reported sample recoveries from reverse phase columns as low as 64% to as high as 300% (Woelki *et al*, 1997; Gremm *et al*, 1991; Frimmel *et al*, 1992).

A study done to determine the effect of the stationary phase pore size on the chromatograms was done and it was found that a pore size of 1000Å was ideal (Woelki *et al*, 1997). Pores of this dimension were found to exclude only molecules with a mass in excess of 2×10^6 D. Recovery of the injected sample was initially lower than with a smaller size pore stationary phase but by using a gradient and increased flow rate recoveries of ca. 90% could be achieved. An interesting observation in this study was that well resolved fractions that were collected, re-concentrated and re-chromatographed still exhibited most of the peaks found in the original sample separated, although the ratios of the peaks did vary in the new chromatograms.

It was also found that the concentration and volume of injection would affect the chromatogram (peak distribution), total recovery and the peak areas of eluted peaks (Preuse *et al*, 2000). The peaks were generally broad and unresolved, an indication that there are several very closely related compounds in the humic acid. Significant changes were seen in the chromatograms as the total amount of humic acid injected was increased. If the injected amount of humic acid was kept constant by varying the concentration and the volume (to accommodate the dilution) of the injected sample there were still major changes in the chromatogram.

Susic and Boto (1989) presented a method for quantitative analysis of humic acids using reverse phase chromatography and the fluorescent characteristic of the humic acids. In the development of their method, various bonded normal and reverse phase stationary phases were investigated as media for the separation. Amine and anion exchange media retained the humic acids completely. However bonded diol, cyano (normal phases) and phenyl (reverse phase) phases all resulted in only one early eluting peak with the peak width and shape dependant on the mobile phase used to elute the humic acid. In their method development, use of an octadecyl silyl (ODS) reverse phase stationary phase resulted in poor humic acid recovery from injected samples, even if the stationary phase was endcapped to prevent irreversible binding of the humic acids to exposed polar groups within the silica. Use of 0.002% or higher concentrations of ammonium hydroxide as well as the use of glass-lined columns was claimed to prevent this recovery problem. Sample cleanup or pre-concentration on ODS solid phase extraction cartridges resulted in more anomalies being observed. It appeared that there was a slow dissociation-equilibrium taking place within the ODS-SPE- retained humic acid, and that additional retained humic acid could be eluted from the SPE column after it had been

left long enough for the equilibration to be re-established, despite the fact that the column had already been exhaustively washed with the same eluting solvent.

A reverse phase HPLC separation of ether-extractable copper oxide oxidation-products of Swannee river and marine humic acids revealed the presence of 11 of the main lignic phenols at concentrations of 50 – 100 nmoles (Lobbes *et al*, 1999). Interestingly a comparison of the total lignic phenol concentrations as determined by GC/MS and HPLC showed a variation of less than 15% but the individual peak concentrations varied by much larger margins.

5.3 Ultraviolet/visible absorbance spectroscopy

Spectrophotometric and colorimetric analysis has often been carried out on humic acids as they are non-destructive and non-intrusive technique and can easily be repeated on the same sample under numerous slightly different conditions where pH or metal ion or the effect of added organic marker compound concentrations are varied. The use of dilute humic acid solutions for analysis by UV/visible spectrometry gave a general tendency, irrespective of the source of the humic acids: the absorption is intense at low UV wavelengths and decreases logarithmically with increasing wavelength. This effect, where a extended decreasing wavelength-dependant "absorption tail" exists can be described as an Urbach phenomenon (Urbach, 1953), caused by a structurally or thermally disordered system comprising numerous different wavelength absorption sites. Some humic acids do show broad low intensity absorption bands superimposed on the general absorption trend described above. These bands have been ascribed to contaminants and "immature humic acids" (Sato & Kumada, 1967).

Several absorption ratios have been used to give an indication of the degree of aromaticity of the humic acid under investigation although it must be pointed out that there has been no definite proof of this assumption. One advantage of using absorbance ratios is that it gives a characteristic that is independent of the concentration of the humic acid under investigation. The E4/E6 ratio (the ratio of absorbance at 465nm divided by the absorbance measured at 665 nm) (Welte, 1957) has become the most commonly used ratio in this regard and values of between 2.8 and 4.7 are fairly common for soil humic acids. Unfortunately no structural information can be obtained from these ratios.

5.4 Infrared spectroscopy

Fourier transform infrared spectroscopy is a rapid and convenient method of gaining information about the chemical structure of compounds. The chemical bonds connecting any two atoms in a

molecule are flexible and subject to various movements; stretch, twist, distortion and deformation when irradiated with energy that matches the energy required to allow the electrons involved in the bond to be excited to a high energy state (Gunzler & Gremlich, 2002). Each type of chemical bond has a characteristic combination of infrared absorption frequencies. By interpreting the absorption (or more commonly the transmission) spectrum it can be concluded that certain chemical bonds exist in a molecule being analysed e.g. aliphatic chain, aromatic rings, carbonyl groups and hydroxyl groups.

Due to the chemical complexity of humic acids and the high carbon content the spectra obtained are not as sharp and discrete as would be expected from clean chemicals. However infrared spectra of humic acids extracted from various sources have all shown most of the following chemical functional groups (with the typical IR absorption bands indicating their presence in brackets): free hydroxyl groups (3420 cm^{-1}), conjugated double bonds or substituted aromatic rings (3060 - 3050 cm^{-1}), aliphatic groups (CH stretch bands for CH_3: 2962 & 2872 cm^{-1}, and for CH_2: 2926 & 2853 cm^{-1}), carbonyl groups (1720 – 1710 cm^{-1}), aromatic rings (1630 cm^{-1}), carboxylate salts (1400 cm^{-1}) aliphatic asymmetric deformation (CH_2: 1465 - 1450, CH_3: 1380 - 1370 cm^{-1}) νC-O of acids, ethers or alcohols (1100 – 1000 cm^{-1}) and the presence of minerals or metal ions (1100 – 1000 & 525, 465, 415 cm^{-1}). This data was collected from Theng *et al.* (Theng *et al*, 1966; Theng & Posner, 1967), Khairy (1980), Tacacz *et al.* (Tacacz & Alberts, 1999) and (Pouchert, 1981).

5.5 Fluorescence spectrophotometry

Fluorescence is a phenomenon generally exhibited by humic acids and was originally believed to be due to the humic acid structure itself. Using this fluorescence characteristic, quantitation of humic acids has been determined using HPLC to separate the humic acid from other contaminants (Susic & Boto, 1989). This technique for quantitation of humic acids is however dubious as it has subsequently been proved that the fluorescence is caused by a small isolatable fraction of the humic acids and that this fraction can easily be separated from the bulk of the humic acids by size exclusion chromatography (Aoyama, 1999). Several workers have used fluorescence spectrometry to characterize humic acids (Alberts *et al*, 2000; Aoyama *et al*, 2000; Mobed *et al*, 1996; Spark & Swift, 1994; Tacacz & Alberts, 1999; Senesi *et al*, 1991) including the generation of total luminescence (3-D fluorescence) spectra. Determination of binding-coefficients of various fluorescent quenching compounds (Coolidge & Ryan, 2000; Engebretson & von Wandruszka, 1994) has also been used to determine the binding capacity of humic acids for various compounds. Humic acids have often been reported to have two discrete fluorescent bands and these vary depending on the source of the humic acid. A soil humic acid exhibited λ_{ex} 380 and 460nm which resulted in λ_{em} of

460 – 470nm and 510 – 530nm (Aoyama, 1999) while peat humic acids had a λ_{ex} 335nm resulting in λ_{em} 466 – 480nm with a small band at 512nm (Tacacz & Alberts, 1999). Humic substances from a Norwegian lake also revealed two fluorescent peaks; one with λ_{ex} 330 – 335nm and λ_{em} 437 – 480nm and the other with λ_{ex} 225nm and λ_{em} 426 – 428nm, a result claimed to be in common with humic acids from oceans, estuaries and rivers (Alberts *et al*, 2000). Suwannee River humic substances (a marsh drainage river) had λ_{ex} at 366nm and λ_{em} 450 – 465nm. These wavelength combinations however, appear to be dependant on the apparent molecular size of the humic acids, with the smallest molecules contributing most to the fluorescence.

5.6 Molecular mass determination

Indirect molecular mass measurement using size exclusion chromatography (von Wandruszka *et al*, 1999; Obenaus *et al*, 1966; Ouatmane *et al*, 2000; Piccolo & Conte, 2000; Rausa, 1994), ultra-filtration through membranes with select nominal molecular mass cut-off ranges (Trubetskoj *et al*, 1997), various electrophoretic methods (Duxbury, 1989) including poly-acrylamide gel electrophoresis (PAGE) (Trubetskoj *et al*, 1997; Trubetskoj *et al*, 1994; Trubetskoj *et al*, 1998) and capillary electrophoresis (DeNobili *et al*, 1998; Pokorna *et al*, 1999), and analytical ultra-centrifugation (Cameron *et al*, 1972; Flaig & Beutelspacher, 1968; Stevenson *et al*, 1953) has resulted in large variations of molecular mass being reported, with each mass range apparently having slightly different properties. When isolated, the properties of humic acids from the different mass ranges have generally been observed to revert back to the properties of the original humic acid with respect to molecular mass distribution if given time to re-equilibrate at high pH (Piccolo & Conte, 2000). This would indicate that there is an apparent dynamic complexation process or "supramolecular association" at work which could explain why it has been difficult to analyse humic acids by the classical chemical analysis techniques such as elemental analysis, colour analysis, solubility, total acidity, melting points, formation of specific derivatives and chemical degradation studies.

Mass spectrometry (MS) is a powerful technique used to determine the structure of compounds by identifying the mass of the whole molecule (the mother ion) and several sub-units ("daughter ions") of the compound, each dependant on the chemical functionality within the molecule. Pyrolysis-MS, where a sample to be analysed is heated until decomposition begins and the volatile products of the decomposition are directed into the MS for mass determination, was the first technique used to analyse humic acids by MS. The problem with this type of analysis is that although structural information is obtained for the compounds entering the MS, the pre-analytical thermal

decomposition often gave rise to new compounds that did not exist in the original sample and only a fingerprint of the original compound could be obtained.

Although structural detail of a molecule can be identified for single compounds when using electron impact ionisation, use of "soft" ionisation techniques has allowed determination of the masses of several compounds in mixtures without the requirement for separating the individual compounds from each other, but these mixtures have generally been fairly well defined or the compound of interest was known to be in the mixture. Many researchers have attempted direct molecular mass determinations of the compounds making up the humic acids using mass spectroscopy. Different "soft" ionisation techniques used include fast atom bombardment (FAB) on various matrixes (Brown *et al*, 1998), electrospray ionisation (ESI) (Brown & Rice, 2000) and matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF), (Pokorna *et al*, 1999; Haberhauer *et al*, 1999; Brown *et al*, 1998). Most of these studies revealed that the molecular mass of the majority of components in humic acid appear to have molecular masses of less than 1000 Dalton. Some researchers have found high mass compounds in humic acids from various sources but these results are not always reproducible, even with the same sample and same sample preparation (Haberhauer *et al*, 1999) and may indicate unpredictable ionisation or secondary effects in the separation chamber. The high occurrence of low molecular mass molecules could be due to impurities that reduce the ion yields or intramolecular dispersion involving transfer of the ionic charge in larger molecules causing them to loose their apparent charge. It may also be that humic acids are in fact loose complexes made up from only small molecules.

A major complication with regard to the molecular mass data collected so far is the different sources and pre-treatments of the humic acid samples used. This makes comparing the data from different laboratories very difficult.

5.7 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy (both proton 1H and carbon 13C) (Wershaw *et al*, 1998b; Wershaw *et al*, 1998a; Wershaw, 2000; Preston, 1996) has been fairly successful in determining structural detail, bearing in mind that the humic acids are complex combinations of different molecules in different ratios.

Solid state (i.e. no solvent added) ^{1}H- and ^{13}C-NMR of humic acids using the cross polarization magic angle spin (CP-MAS) technique has been used extensively and is claimed to give an indication of the ratio of aliphatic, anomeric and aromatic carbon atoms in the structure (Wershaw *et*

al, 1998b; Wershaw *et al*, 1998a; Preston, 1996; Mao *et al*, 1998). Liquid phase NMR where the sample is dissolved in a deuterated solvent reveals much more detail due to narrow radio frequency absorption bands but the baseline noise increases and the effect of the "inactive" carbons with long relaxation times is more obvious. It should be remembered that humic acid may be a complex mixture of many compounds with similar chemical structures and that there is a high ratio of inactive nuclei, so the overlap of peaks and distorted peak area ratios are to be expected which results in broader than expected peaks. Typical NMR spectra of humic acids look like distant mountain peaks rather than the sharp peaks of pure synthesized compounds. One problem of liquid phase NMR of humic acids is that humic acid is soluble in very few solvents other than deuterated water and that there are many active hydrogen sites within the humic acid structure that exchange for deuterium in the solvent thereby creating a large water or hydroxyl peak in the spectrum with simultaneous loss of these active hydrogens from the humic acid molecules.

5.8 Other spectroscopic techniques used to analyse humic acids

Raman spectroscopy, which is complementary to infrared spectroscopy, has often revealed graphite-like or amorphous carbon as major constituents of humic acids. Unfortunately not much useful information has been gleaned from Raman spectra. The intrinsic fluorescence of humic acids has also been found to interfere with Raman spectroscopy when using lasers for excitation.

Electron spin resonance (ESR) spectroscopy gives information with respect to the occurrence of free radicals in the humic acid molecules or in humic acid metal complexes and was apparently used in the original confirmation of the presence of quinone-hydroquinone radicals in humic acids.

X-ray absorption spectroscopy (Frenkel & Korshin, 1999; 2000) has been used to determine the type of complexation that takes place between humic acids and various metal ions or salts. Information with respect to electron structure, bonding geometry and neighbouring atoms can be gained from extremely small samples making this a powerful technique where very small areas of the humic acid are to be analysed.

6 Role of humic acid in soil

Even though the humic acid content of mineral soils is generally less than 5% it is an essential component of fertile soil and required for crop growth and agricultural success. For more than 150 years humic acid has been recognized as a plant growth-promoting component in soil and this was

the reason for much of the early research into humic acids, especially their chemical composition and physical properties (Sprengel, 1826).

Humic acids show benefits in the agricultural food production (Faust, 1996) with several presentations and papers documenting the growth stimulating and crop enhancement of up to 30% by plants grown in humic acid enriched soils. Many articles published on the subject of plant growth stimulation by humic acid was reviewed by Vaughan and Malcolm (1985), which points out the positive effect of humic substances on plant growth in general, with an increase in the growth at almost every stage of plant development evident. The growth parameter that appears to be most affected is an increase of the wet and dry mass of the plants (Vaughan & Linehan, 1976; Sladky & Tichy, 1959) attributed to an increase in the length of both the roots and the stems (Vaughan & Linehan, 1976; Tan & Nogamornbodi, 1979; Sladky & Tichy, 1959; Rauthan & Schnitzer, 1981; Malik & Azam, 1985; Furter *et al*, 1996; Furter *et al*, 1997) as well as an increase in number of leaves and flowers (Rauthan & Schnitzer, 1981), all of which manifest as a positive effect on crop yields (Varshney & Gaur, 1974). The mechanism of action has been attributed to many different characteristics and wild assumptions have sometimes been made.

Humic acids are reported to alter soil properties by making them more friable, by buffering soil pH, by allowing soils to swell with moisture retention, by complexing trace metals and making them more readily available to plants, and by releasing bound nutrients such as phosphates from clays (Visser, 1986; Day *et al*, 2000; Clapp *et al*, 1998; Chen *et al*, 1999).

Humic acids contribute to the formation and stabilization of soil aggregates, as well as adding to the nutritional value of the soil. They increase the water retention capacity, decrease soil density, control retention and release of micro and macro-nutrients and are involved in the carbon, nitrogen, phosphorus and sulphur cycles (Kononova, 1966). They act as buffers that regulate pH and bind many metal ions (Guminski *et al*, 1983; Vaughan & McDonald, 1971) in such a way that they are easily available for uptake by the roots of plants.

Within the plant tissues, metabolic cycles appear to be modified by humic acid and were demonstrated to occur both *in vitro* and *in vivo* (Vaughan & Malcolm, 1985; Vaughan *et al*, 1985; Visser, 1987). Samson and Vaughan (Samson & Vaughan, 1989) indicated that these metabolic changes may have been due to changes in the membrane porosity although an earlier study by the same group had implied that enhancement of the iron transport mechanism could have been the reason for the metabolic changes seen (Vaughan & McDonald, 1971).

Some researchers have found hormone like activity, although this is small (±100 times less) when compared to that of known plant hormones such as indoleacetic acid (Cacco & DellÁgnola, 1984; Chen *et al*, 1999).

At present there is a tendency for the agricultural sector to use synthetic fertilizers that are rich in nitrogen and phosphorus but public pressure is growing to use organic fertilizers such as humic acid (Faust, 1996).

7 Therapeutic and medicinal use of humic substances

Therapeutic and medicinal use of humic substances was already known in the Babylonian times, and during the Roman Empire mud baths were used to treat a number of ailments (Priegnitz, 1986). Humic acids were also used as folk remedies for a wide variety of ailments (Lotosh, 1991). "Shilajit", "asphaltum", "vegetable asphalt" or "mumie" are names used for humic substance found in the Himalayan and Caucasus mountains and has been used for centuries to treat numerous ailments and improve the immune system. A recent review article describes the many applications for which these compounds have been used (Schepetkin *et al*, 2002). Among the many indications for which these humic acid type compounds are used include: burns, injuries, bone fractures, dislocations, disease of the skin, neuralgia, arthritis, poisoning and as an anti-inflammatory, antibacterial, anticancer, a diuretic and an immune stimulating agent. Other diverse conditions claimed to respond to these compounds are diabetes, cholesterolemia, eczema, amnesia, epilepsy, asthma, dysmenorrhoea and digestive disorders - including ulcers.

Extranit®, Kalumat®, Kalumin®, Salhumin® and Sulumin® are examples of humic acid products that have been used for either humic acid baths or as topically applied gels. TPP® (Torf peat preparation), Torfot®, Humisol®, FiBS®, Shilagen®, Cystone®, Rumalaya® and Geriforte® are humic or fulvic acid containing medications manufactured for oral administration. Many other medications that are combinations of humic and herbal extracts are sold worldwide to treat many of the ailments mentioned above. Germany, Hungary, Poland, Russia and India appear to be the countries where most of these humic acid-containing medications are formulated.

In Europe, in the 19th century, many of the health spas extensively used peat mud baths especially for rheumatic and gynaecological conditions (Baatz, 1988; Kleinschmidt, 1988; Kovarik, 1988; Lent, 1988). Extracts, tinctures and infusions of peat were also given as a tonic for liver and gastric ailments (Kallus, 1964). This implies that humic acids were deemed safe for external as well as

internal applications as long as 150 years ago, which correlates with traditional uses of mumie in the East.

Peat from the Belgian town of Spa was used during the First World War (before penicillin was discovered) to treat wounds and amputations in field hospitals (van Beneden, 1971) The peat was applied directly to the wounds to prevent infections, relieve pain and facilitate healing (Haanel, 1924). In an experiment with rabbits, Biber and Bogolyubova (1952) reported faster wound healing in the rabbits injected with humic acid. Tazhimamentov *et al.* (1987) reported decreased suppurative wound healing times when humic acids were administered. Salz (1974) offered some thoughts on why humic acid could accelerate wound-healing rates. This author highlighted the bacteriostatic action, anti-inflammatory nature and steroid concentration altering characteristics of humic acids as well as an increased blood flow to the skin. Ghosal *et al.* (1988) and Rajic *et al.* (2001) eluded to the strong anti-inflammatory properties of 4'-methoxy-6-carbomethoxybiphenyl and the tirucallane type triterpenoids respectively, both of which are found in Shilajit, the Himalayan humic acid used traditionally as an anti-inflammatory remedy.

Experimental bone fractures demonstrated accelerated osteoid formation and mineralization if the humic acids were administered during the first week after the fracture, however if treatment was delayed until the second week, osteoid mineralization was reduced significantly (Tkachenco *et al*, 1979). Mumie extracts have also been reported to show positive effects on bone regeneration of fractures in children (Kelginbaev *et al*, 1973).

Adjuvant-induced arthritis in rats was suppressed by doses of crude mumie extract at concentrations of 50mg/kg (Goel *et al*, 1990). Carrageenan-induced foot oedema was also inhibited at the same concentration (Goel *et al*, 1990). Arthritis patients that were treated with humic acid baths showed decreased serum albumin and increased amino acid, globulin and properdin concentrations. The urinary corticosteroid concentrations were also found to be elevated (Reichert, 1966; Miehlke & Thürigen, 1961; Hiller, 1952; Hiller, 1953b) after humic acid bath treatments. It was noted that these elevated urinary corticosteroid concentrations returned to normal after the treatment regimen ended, implying that the changes were directly due to the treatment (Hartman, 1967). Eichelsdörfer (1976) pointed out that the mechanism of humic acid baths is still unknown and that several workers doubt that the benefits are due to the humic acid. However several commercially available humic acid based medications have been successfully and widely used for the treatment of arthritis and rheumatism.

Other balneotherapy combinations that have shown positive effects on rheumatoid arthritis patients consisted of mixtures of salicylic acid and humic acid in various ratios (2.5% humic acid and 47% salicylic acid) (Brandt, 1964; Miehlke & Thürigen, 1961). Some researchers therefore claimed that the positive effect has been due to the salicylic acid rather than the humic acid. The humic acid bath treatment is usually in the order of a 20-minute exposure to a bath at 38°C three times a week for up to 6 weeks.

Gastrohumit®, a bismuth salt and Huminit®, a calcium salt of humic acid are commercially available and used for the treatment of heartburn, gastric ulcers and acute gastroenteritis (Brandt, 1964; Kinzlmeier, 1954; Reichert, 1966; Schlepper, 1960; Weithaler, 1954). The calcium in the Huminit® had an immediate alkylating effect in the stomach but systemic alkylosis was avoided due to the buffering capacity of the humic acid. The humic acid was reputedly bound to the mucus in the stomach, leaching out slowly to give long lasting protection as opposed to the newer generation ion exchange type medication that gave only a few hours protection.

Gastrointestinal applications of humic acids in the veterinary discipline were very successful and included: treatment of enteritis in calves (Kühnert *et al*, 1980) and in piglets (Golbs & Kühnert, 1986; Gramsch, 1961), while successful treatment of diarrhoea, gastroenteritis, colitis and parvovirus was reported in dogs and cats (Bartels, 1986). None of these authors indicated any severe side effects, even after prolonged use, and found the humic acids to be as effective as commonly prescribed drugs. These effects have been ascribed to the toxin absorbing ability, anti-inflammatory, bacteriostatic and immune stimulating effects of humic acid. Kühnert (1989) also pointed out that there is no detectable residual drug after treatment and as there is negligible allergy incidence and no observed symptoms of overdosing, humic acids can easily be incorporated into the animal feed. This would be especially beneficial in meat producing animals where residual drugs are a problem.

Salz (1974), the developer of the topically applied Salhumin®-gel, successfully treated the following conditions with his product: osteochondritis, torn ligaments, lumbago, hip pain, chronic rheumatoid arthritis, arthrose, vertebrae pain, bruises, muscle spasms, oedema and phlebitis. These conditions are all related to inflammation, therefore it appears as though humic acid has anti-inflammatory properties and that the active components can be absorbed through the skin.
Tolpa Peat Preparation (TPP) a peat-derived product is sold in Eastern Europe as an immune enhancing agent. Studies have shown that TPP inhibits IgE induced anaphylaxis in mice (Wyczolkowska *et al*, 1993), enhances the humoral response to sheep red blood cells (SRBC) in mice in a dose dependant manner (Obminska-Domoradzka *et al*, 1993) and that TPP is effective in

the treatment of recurrent respiratory infections in human volunteers, reportedly due to enhanced granulocyte phagocytosis (Jankowski *et al*, 1993).

Taugner (1963) observed an impressive reduction in oedema in rats after intravenous injection of sodium humate. Klöcking *et al*, (1968) induced oedema experimentally in rats by two different methods and found that a peritoneal injection of humic acid reduced the oedema better than some proven medications to which the humate was being compared.

Salz (1974), Motohisha *et al*, (1974) and Iubitskaia and Ivanov (1999) reported analgesic and anti-inflammatory effects as well as serum lipid modulation and promotion of metabolic processes when using sodium humate in balneotherapy on osteoarthritic patients.

Anti-inflammatory properties for humic acids were reported (Kühnert *et al*, 1982; Ye *et al*, 1985) and gynaecological inflammatory conditions have been successfully treated by a group in Poland (Woyton *et al*, 1993).

Kühnert *et al*, (1982) described the anti-inflammatory action of humic acids as being as effective as that of dimethyl sulfoxide (DMSO), a well-known anti-inflammatory agent and drug carrier. Klöcking *et al*. (1968) ascribed the anti-inflammatory action of humic acid to the polyphenolic structure (based on the lignin derived humic acid theory) while Salz (1974) attributed it to the fact that it stimulated higher blood flow.

Klöcking (1994b) provided a feasible biochemical explanation of the anti-inflammatory properties of humic acids, basing their findings on earlier work by the same group where synthetic humic acids inhibited the lipoxygenase enzyme from rabbit reticulocytes strongly, while prostaglandin H synthase from sheep vesicular glands was only weakly inhibited (Schewe *et al*, 1991). Salts of humic acid and some synthetic humic acids were shown to inhibit the lipoxygenase and prostaglandin H synthase pathways involved in the conversion of arachidonic acid, released after membrane damage, into leukotrienes or prostaglandin H respectively. The lipoxygenase pathway resulting in the production of leukotrienes, lipoxins and other hydroxylated but unsaturated fatty acids, was blocked by low concentrations of the synthetic humic acids but required relatively high concentrations of the natural products to achieve the same inhibition effect. Leukotrienes act as mediators of the inflammatory processes affecting bronchial dilation, vascular permeability, chemotactic leukocyte infiltration, oedema and hypersensitivity reactions (Penrose *et al*, 1999). The prostaglandins are antagonists of the leukotrienes that show almost exactly the opposite effects

despite the fact that the origin of both these classes of potent biologically active C_{20} lipids is arachidonic acid. Natural humic acid appears to have a smaller effect on the prostaglandin synthesis pathway than the synthetic humic acids as approximately 1100µg/ml of natural humic acid was required to elicit a 50% inhibition of prostaglandin synthesis by a kidney homogenate (Breng *et al*, 1981).

It has also been found that the expression of protein type inflammatory mediators is inhibited (Gau *et al*, 2000). Intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E-selectin expression by cultured HUVEC were inhibited after treatment of the cells with humic acid and then stimulated by addition of a lipopolysaccharide (LPS). An immuno-fluorescence technique using fluorescent-labelled monoclonal antibodies against each of the above adhesion molecules was used and quantitation performed by measuring the fluorescent intensity of the cell-bound monoclonal antibody by flow cytometery. The LPS induced expression of all three of these adhesion molecules was inhibited in a dose and contact time dependant manner by the added humic acid. As the nuclear transcription factor kappa B (NF-κB) plays a central regulatory role for all three of these adhesion molecules, the effect of humic acid on the NF-κB activity was determined and found to have a similar dose and contact time dependant response as that of the adhesion molecules.

Inhibition of hyaluronidase activity that is associated with the anti-inflammatory response to humic acid baths is possibly due to an increase in the oestrogen concentrations in the blood (Sprunt *et al*, 1938; Vasterling, 1958; Wattenberg & Glick, 1949). This could either be from "contaminating" oestrogens in the humic acid (Lotmar, 1960; Taugner, 1963) or from stimulation of endogenous oestradiol and oestrone production by the adrenal cortex. These increased hormone concentrations are identified by the increase of up to 100% in urinary oestrogen excretion within 24 hours of balneology in both male and female patients (Hiller, 1952; Hiller, 1953b; Hiller, 1953a).

Human neutrophil functions, especially the respiratory burst that produces reactive oxygen species like hydrogen peroxide, were stimulated by two natural and one synthetic humic acid (Riede *et al*, 1991). There was however no stimulation of chemotaxis or chemokinesis.

Lange *et al.* (1985) found increased leukocyte counts and globulin concentrations in rats after oral administration of a combination of pesticides and humic acids. The increase in globulins was attributed to increased antibody production, a direct effect on the immune system, in response to the humic acid-pesticide complex. In another study on white rats by the same author (Lange *et al*, 1987), different humic acids alone were given as single intra-gastric doses, which resulted in changes in the

blood leukocyte profile. The changes were interpreted as indicating an effect on the immune system. Plasma protein concentrations and phagocytic activity were also analysed.

Soloveyva and Lotosh (1984) treated anaemia and hypercholesterolemia with humic acids and concluded that the action of humic acids could be attributed to a positive effect on liver detoxification, several enzyme systems and the immune system.

The beneficial effects of humic substances have been ascribed to the direct effect of the humic acids due to their surface effects and ability to bind or chelate compounds or metals (Marx & Heumann, 1999; Nifant'eva *et al*, 1999), adsorption of xenobiotics (Nielsen *et al*, 1997; Prosen & Zupancic-Krajl, 2000; Schulze *et al*, 1999) and to its polyacidic and polyphenolic nature (Woelki *et al*, 1997). Interaction with biologically important molecules like proteins, including peptides and enzymes, polysaccharides and lipids, including the steroids and lipophilic hormones, has been proposed as mechanisms of the humic acid action. The effect of humic acid has also been ascribed to the "contaminating" compounds that are bound to, or complexed within, the structure of humic acids and not to the humic acids themselves (van Beneden, 1971).

Studies on the anti-viral properties of humic acid and several synthetic humic acids formed by oxidation of phenolic precursors have revealed some interesting results. Natural humic acid was shown *in vitro* to have antiviral effects against both herpes simplex types 1 and 2 viruses (Klöcking & Sprössig, 1975; Klöcking & Helbig, 1991; Thiel *et al*, 1977; Thiel *et al*, 1981) and against Coxsackie virus (Klöcking & Sprössig, 1972). Synthetic humic acids formed by oxidatitive condensation of chlorogenic or caffeic acids showed even better antiviral properties than the natural humic acids (Eichhorn *et al*, 1984; Hils *et al*, 1986; Klöcking *et al*, 1983; Thiel *et al*, 1984). Other viruses tested for sensitivity to natural and synthetic humic acids are influenza type A and B (with selective sensitivities), cytomegalovirus, vaccinia, adenovirus which all showed sensitivity but poliovirus type 1, parainfluenza virus type 3, retrovirus type 1 and sindbis virus showed no sensitivity to any of the tested humic acids (Neyts *et al*, 1992). Inhibition of viral replication was evident at humic acid concentrations of between 20 and 100ug/ml. A topical application of a 1% ammonium-humate solution was effective against herpes virus infections in 90% of cases.
Several workers have tested HIV-1 and HIV-2 for natural and synthetic humic acid sensitivity. Schols *et al.* (1991) and Schneider *et al.* (1996), both found inhibition that appears to be due to inhibition of viral entry into the target cells, a process that involves the V-3 loop of the virus protein. It is possible that direct binding of the poly-anionic humic acid to critical regions the protein sheath

of the virus prevents cell attachment. Other mechanisms may be that humic acid binds to the viral attachment site on the host cells.

Studies done both *in vitro* and *in vivo* where the ability of humic acid to remove or counteract toxic compounds were studied (Fuchs *et al*, 1982; Fuchs *et al*, 1986; Golbs *et al*, 1984; Lange *et al*, 1985; Rottinghaus, 2000; Solovyva & Lotosh, 1984). Heavy metal, alkaloid, chemical and pesticide toxicity could be counteracted in varying degrees by the oral administration of humic acids. It was found that serum immunoglobulin concentrations increased if humic acids were administered together with the toxic compound (Lange *et al*, 1985) and that the detoxification activity of the liver appeared to have been stimulated (Solovyva & Lotosh, 1984). These authors attributed the *in vivo* antitoxic and prophylactic characteristic of humic acid to the combined effects that they have on the immunoglobulin concentrations and the liver activity.

The mutagenic effect of some well known carcinogenic compounds (benzo[a]pyrene and 3-aminoanthracene) was counteracted by humic acid, but this was found to be a desmutagenic effect where the mutagen was adsorbed onto the humic acid and therefore prevented from reaching the target tissue where it could exert its mutagenic effect. Larger molecular mass fractions of humic acid were found to be more effective in prevention of the mutagenesis in Ames test systems (Sato *et al*, 1986; Sato *et al*, 1987a; Sato *et al*, 1987b; Bernacchi *et al*, 1996).

Mao *et al* (1998) proposed that a possible mechanism of action of the humic acid compounds may have been due to their chemically reducing properties, thereby preventing oxidative radicals from exerting their documented mutagenic and carcinogenic effects.

8 Negative aspects of humic acid exposure

As far back as 1950 humic acid was implicated as a causative agent of goitre of the thyroid gland (Woodward, 1963; Hettche, 1955; Hettche, 1956; Galcenko, 1950; Burkat, 1965). These authors also implicate urochrome (a natural metabolic product of haemoglobin) as being a cooperative compound. The similarity between these compounds is that both humic acid and urochrome can complex copper ions ($Cu^{2}+$) and iodide, which in turn affects thyroxin synthesis and hence the thyroid gland is affected. However, Schierbaum (1966) and Janeček & Chalupa (1969) could not detect goitrogenic activity in thyroid glands of rats kept on a diet high in humic acid over extended periods.

Humic acids have also been implicated in the development of Black-foot Disease, an endemic disease in rural parts of south-western Taiwan where wells are the main source of drinking water. Black-foot disease is a vascular abnormality affecting the peripheral circulatory system to such an extent that the reduced blood flow to the extremities of the sufferer's results in small localized gangrenous areas and ulceration. The condition presents as cold feet and dark discolouring of the skin, resulting in "black feet" (Tseng *et al*, 1961). The well water, from which the implicated humic acids were extracted, also had relatively high arsenic concentrations. The metal complexing/chelating ability of humic acid results in an arsenic-humic acid complex that appears to result in a synergistic deleterious effect by the arsenic and humic acid on the vascular epithelial cells of the capillary walls (Lu *et al*, 1988; Gau *et al*, 2000).

In another study on Black-foot disease, it was demonstrated that tissue plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1) production by human umbilical cord arterial epithelial cells (HUVEC) in culture, were both induced by humic acids of natural and synthetic origin. t-PA is a proteolytic enzyme involved in conversion of plasminogen into plasmin, which in turn activates fibrinolysis and has a central role in plasma fluidity and plasma leakage. PAI-1 is normally released from activated platelets and is thought to be important in controlling fibrinolytic activity within thrombi. HUVEC cell cultures also showed changes on the cell surface and inhibition of growth with a simultaneous increase in mRNA concentrations after brief exposure to humic acids (Yang *et al*, 1996). Later studies on the same HUVEC model showed increased intracellular chelatable iron concentrations after humic acid exposure and this could be directly associated with an increased reactive oxygen radical formation. Formation of the oxygen radicals could be inhibited by intracellular antioxidants and intracellular iron chelators, but not by enzyme inhibitors or calcium chelators (Gau *et al*, 2001).

Hseu (2000) investigated the effect of humic acid on non-immune system blood cells in relation to Blackfoot disease. His study found that humic acid induced oxidation of normal membrane-proteins of human erythrocytes and that these were then exchanged for high molecular mass proteins resulting in echinocyte formation. Simultaneously haemoglobin inside the erythrocytes was oxidized increasing the oxidative stress on the cells.

Klöcking (Klöcking, 1994a) investigated the *in vivo* fibrinolytic and coagulation effects of a number of synthetic and of two natural humic acids in rats at doses of between 5 and 10 mg/kg. It is interesting to note that he found a decrease in PAI-1 activity in contrast to the results of Yang *et al*

(1996). However *in vitro* results using the same compounds indicated that some of the synthetic humic acid compounds had strong anticoagulation properties while others showed significantly shortened coagulation times.

Humic acids in water react with active chlorine to form carcinogenic compounds and are therefore seen as a problem in water purification plants throughout the world. Various chlorinated humic acid derivatives have been demonstrated to have mutagenic properties in the Ames test (Bernacchi *et al*, 1996; van Duuren, 1986).

9 Pharmacokinetics of humic acid

For any pharmaceutical compound to have an effect on a target organ or cell within a living organism, it must be absorbed from the point of administration and should be distributed effectively so that the target region, organ or cells become exposed to a therapeutically effective concentration of the pharmaceutical compound (Harvey, 1990).

In humans, the most acceptable and therefore the most common method of introducing a drug is via the enteral route involving oral administration of a dose at defined intervals. An effective uptake or absorption of the compound from the gastrointestinal tract (GIT) must occur before the compound would elicit any effect.

This absorption is dependent on several factors (Sommers, 2000);

- solubility
- lipophilic nature
- molecular size
- degree of ionisation
- pKa of the compound if it carries an ionisable group
- the rate of transit through the gastrointestinal tract
- enzymatic or chemical changes that may occur in the GIT

Once absorbed from the GIT, the compounds need to be effectively distributed to the target organ or cells and this is usually via the blood circulatory system but may initially be transported via the lymphatic system.

Blood is a complex suspension of numerous cell types and biologically active soluble components, including hormones, enzymes and lipids in a protein rich aqueous electrolyte solution. The primary function of blood is that of transport for nutrients, metabolites and respiratory gasses to and from all the cells in the body. Secondary functions are homeostasis (whereby pH, temperature and electrolyte concentrations are kept within approximately constant limits) and defence against foreign compounds and agents (Weiss & Jelkmann, 1989). Any compound that is introduced into the blood (indirectly by absorption from the GIT, subcutaneous or intramuscular injection or directly by intravenous injection) can complex or bind to the plasma proteins, enzymes or cells in the blood or may be transported in the serum as a soluble compound without interaction with any of the blood components (Harvey, 1990).

Many compounds are absorbed from the lumen of the gut by passive permeability, making use of the paracellular transport route, where the absorbed compound enters the interstitial space via the so called tight junctions between the mucosal enterocytes. This passive absorption is facilitated by osmotic, hydrostatic, chemical and electrical gradients but has a "pore" size limit of about 0.8nm in the jejunum reducing continuously down the length of the gut to only 0.25nm in the colon. This passive permeability is selective to some extent with cationic compounds being favoured above anions due to a negative charge of the surface of epithelial cells (Ewe & Karbach, 1989). Both diffusion and convection (i.e. "going with the flow") mechanisms are involved in this type of passive transport.

Some compounds are absorbed by an active transcellular transport mechanism where the compound is initially absorbed from the lumen either by diffusion across the cell membrane or actively sequestered and transferred by carrier proteins or in vesicles.

Some general characteristics of active transport are (Ewe & Karbach, 1989; Harvey, 1990);

- that metabolic energy is utilized in the process
- that inhibitors of metabolism stop the process
- that the process can be saturated, meaning that it can only transfer a limited amount of compound per unit time.
- that it is specific for particular compounds
- that compounds having similar chemical structure to the preferred transport compound can competitively inhibit the process.

- that maximal efficiency is only achieved when a coupled transport mechanism is in operation, meaning that there is an exchange of compounds from either side of the membrane.

Facilitated diffusion is a process where diffusion takes place faster than would be expected from either the concentration or electrical gradient that predominates. It is a form of active transport where only some of the above mentioned active transport criteria are met, such as a carrier protein being used but not metabolic energy.

Mixed transport occurs when there is a combination of mechanisms operating such as when an active transport process occurs at low concentrations of compound but when the process is saturated due to a high concentrations, passive diffusion rate exceeds the active uptake rate.

Pinocytosis is a process of absorption where the compound, or even a particle, is enclosed by an invagination in the cell membrane that then envelops the compound to form a small internalized vesicle. The vesicle then is transported across the cell to the basolateral membrane where it is expelled in the reverse manner to the initial engulfment. Pinocytosis is the mechanism by which intact particles, macromolecules and some proteins are absorbed.

Lipophilic compounds can enter the cell from the lumen by a diffusion mechanism whereas polar and charged (especially anionic) molecules require carrier proteins to assist the transfer. The compounds enter the epithelial cell cytosol (Harvey, 1990) from where they must again be transported across the basolateral membrane and deposited into the interstitial space and finally into the subepithelial capillaries from where they are rapidly transported further. The capillary venules surrounding the G.I.T. all drain into the hepatic portal system consisting of the superior mesenteric vein, inferior mesenteric vein, the gastric veins, the splenic vein and the portal vein (Netter, 2003). Compounds absorbed from the G.I.T. therefore pass through the liver prior to reaching the heart from where they are circulated to the rest of the body.

Absorbed compounds can complex with blood components or may be enzymatically altered even before they reach the liver (Harvey, 1990). One of the common interactions that occur is the binding of ionized and lipophilic compounds to the serum albumin proteins. This effectively reduces the apparent concentration of the compound in the blood, as the compound is no longer free to leave the blood and interact with the target organ. Antibodies and blood cells may also interact with the

absorbed compounds, which is equally deleterious for the therapeutic effect of the compounds for the same reason (Harvey, 1990).

Many non-nutritious compounds are initially altered by the liver where they are enzymatically metabolized by one of the cytochrome P-450 enzymes, an inducible mono-oxygenase enzyme group found in high concentrations in the liver, to form compounds with more polar functionality (Waterman *et al*, 1986). These enzymatically-altered compounds are often altered further by conjugation to highly polar molecules that enhance aqueous solubility and the excretion of these compounds by the kidneys.

The anionic nature of humic acids, the reported large molecular mass (Obenaus *et al*, 1966; Ouatmane *et al*, 2000; Rausa *et al*, 1991) and the fact that humic acids precipitate at low pH (Aiken, 1985; Hayes, 1998; Odén, 1919) are all factors which indicate that the absorption of humic acids would be slow if it occurs at all. There is doubt whether these compounds are in fact absorbed after oral administration and that any absorbed compounds are probably breakdown products of the administered humic acids.

If an *in vivo* therapeutic effect is to be achieved there must be sufficient absorption within a reasonably short time and the elimination half life must be long enough for the compound to reach a therapeutically active concentration in the blood and be able to reach the target organs. It follows that if the cellular immune system is the target of the active compound it is likely that contact with the target cells takes place as soon as the humic acid enters the venous capillaries following absorption from the lumen of the GIT and that a fairly rapid elimination half-life would not completely eliminate the therapeutic effects. Binding of the humic acid to serum protein would however render the concentration of the free active compound(s) very low and a therapeutic concentration of these compounds may then never be achieved.

The fact that physiologically measurable changes in hormone concentrations (Hiller, 1953a; Reichert, 1966) and immune system functions (Lange *et al*, 1985) together with the fact that there is apparent relief of the symptoms of rheumatoid arthritis after sufferers have taken humic acid orally, implies that humic acids or at least some of the physiologically active compounds in the humic acids are in fact absorbed from the gastrointestinal tract. Visser (1986) argued that there is no reason why humic acids could not be absorbed even though they are reported to have such a high molecular mass. Humic acids have been shown to be absorbed by animals after subcutaneous injection, to

circulate in the blood after intravenous administration with some of the humic acids bound to serum proteins (Klöcking *et al*, 1978) and appear to be metabolized in the liver (Visser, 1973).

Obenaus et al., (1965) demonstrated that humic acids bind to serum albumin *in vitro* and that metals like lead and iron increase this binding affinity. Immuno-electrophoretically it was shown that there were no changes in the immune effect of the humic acid bound albumin. Klöcking et al., (1967) demonstrated that this binding takes place *in vivo* in rats with 69% of an intra-cardially injected dose of humic acid bound within 20 minutes of administration.

Although two studies have been done using ^{14}C labelled humic acids, [a synthetic phenolic oxidation product by Lange *et al.*(1996) and a humic acid extracted from a fungus grown using ^{14}C glucose as the only carbon source by Visser (1973)], in an attempt to determine skin penetration depths or organ distribution, there have been very few studies on the pharmacokinetics of humic acids. Very few publications referring to experiments where the absorption of orally administered humic acids was determined, could be found in an extensive literature search. One reference compared different routes of administration of multi layer liposome-encapsulated humic acids on chickens. In these experiments liposomes containing the sodium salt of humic acid were injected subcutaneously, intra-cardially or given orally (Hampl *et al*, 1994). The sodium humate was labelled with ^{125}I prior to encapsulation and the liposomes were deemed to be stable, as 35 days of *in vitro* incubation in chicken serum did not show any liposome degradation (Hampl *et al*, 1992). Bioavailability of sodium humate was calculated as being highest after subcutaneous injection of the free unencapsulated sodium humate. The oral route of dosing resulted in approximately 30% of the dose being detected in the blood after liposome encapsulated humic acid was used. Unencapsulated sodium humate absorption was lower implying that the encapsulation did increase the absorption (Hampl *et al*, 1994). No data were given with regard to areas of accumulation of activity in the birds during the study. Bioavailability of sodium humate was calculated as being highest after subcutaneous injection of the free unencapsulated sodium humate.

Another study determined the penetration of humic acid through human skin and was performed *ex vivo* using synthetic ^{14}C-humic acids (Wohlrab *et al*, 1984). In this study it was found that almost 60% of a 1% humic acid ointment penetrated the keratin layer of the dermis while about 3% penetrated as deep as the epidermis within 30 minutes. It was concluded that therapeutic concentrations could be achieved by topical applications.

In vitro studies of ill defined mixtures are often not realistic as models that can be extrapolated directly to an identical *in vivo* effect due to over- or underestimation of the absorption of the active compounds or because a bias for absorbing only certain compounds exists, or due to selective binding and inactivation of only part of a mixture of compounds. The formulation of an administered dose can also play an important role in the rate and ability to cross membranes, an event that is not usually included in *in vitro* studies. The ability of humic acid to be rapidly absorbed from the gut is very important if it is to be used as an anti-inflammatory or for immuno-modulating therapy.

A baboon study using ^{123}I labelled oxihumate (Dormehl, 1998) and oral dosing revealed that approximately 11% of the activity was absorbed and that distribution appeared to be via the circulatory system. The uptake appeared faster when the dose was delivered directly into the duodenum using gastroscopic delivery. The areas that were reported to show accumulation of radioactivity after administering Iodide-123labelled oxihumate included the thyroid gland (possibly indicating free iodide), salivary glands, and the septum between the nostrils, the liver and the kidneys. Excretion was essentially via the urine and was persistent for up to 48 hours after which time the residual isotope activity became too low to detect. The liver and kidneys would be involved with the metabolism and excretion of the humic acid respectively, and except for the nasal septum these are areas where it could be safely speculated that free iodide would normally accumulate. Apparently none of the lymph nodes or organs of the immune system showed any uptake of activity.

No data could be found in the literature with respect to the normal distribution of free iodide in the baboon but discussions with several pharmacologists and physiologists (personal communications) pointed out that it is generally accepted that any drug that has covalently bound iodide within its structure, would be metabolized so that the iodide atom would be released. The thyroid gland and any other areas where iodide is known to accumulate, such as the salivary glands, eyes, testes etc would then scavenge the released iodide. Support for this theory can be found in several publications where *in vivo* administration of iodide labelled compounds have resulted in thyroid accumulation of iodide (Ercan & Senekowitsch, 1991; Sinn *et al*, 1990; Klett *et al*, 2003; Press *et al*, 1996)

The target organs and rate of elimination can give some idea of which organs and therefore an indication of the mode of action.

All potential drugs must be tested on animal models before human clinical trials may be initiated. The pharmacological parameters that are most often determined using animal models are the

toxicity, pharmacokinetics and the pharmacodynamics. The most common animal model used in the initial phase of testing are the mouse or rat models. Medical and pharmacological research makes extensive use of inbred or genetically deficient strains of mouse models (Festing, 1979), but the results from these models are often not directly applicable to humans. Despite this shortcoming murine models are popular due to availability, known susceptibilities, fast breeding, similar responses to the experimental procedures, ease of transferring techniques between laboratories and the low cost of maintenance compared to most other animal models. Extrapolation of data from rodent models to humans is not without problems due to anatomical, physiological, immunological and genetic differences. As rodent models are often from closely inbred or specific hybrid models they show no or very little variation to induced responses. This "homogenous" response is not always representative of the effect that would be observed in a general human population where heterogeneous responses can be expected.

In contrast the primates are the animals that are closest to humans with respect to physiology (including similarity in the nervous, respiratory, circulatory, digestive, immune and endocrine systems) and gross anatomy. Anatomical similarity is evident although the normal quadruped movement of the primates as opposed to the bipedal motion in the human results in some unique variations in gross anatomy (Swindler & Wood, 1982).

Despite the anatomical differences the baboon model remains the animal model that is closest matched to the human and which is fairly accessible for pharmacokinetic studies.

Some differences in the anatomy of the baboon digestive system exist when comparing the human and baboon. The most obvious difference is that the baboon has a well-developed caecum, whereas the human has an insignificant appendix as the equivalent organ. As the caecum is situated distal to the small intestine where most nutrient and drug absorption is expected to take place, this anatomical difference should have little effect on the pharmacokinetics of most drugs.

9.1 Iodide-123

Iodide is the most widely used radioactive label for proteins and phenolic type compounds that are used as tracers in radioimmunoassays and ligand derivatives for receptor binding studies. The most common iodide labelling reaction is a two-step process that involves *in situ* oxidation of a radioisotopic iodide salt to form molecular iodine that in turn reacts with phenolic groups (e.g. tyrosine and histidine residues in proteins) or other conjugated double bonds. This oxidation can be done chemically using chloramine-T or enzymatically using lactoperoxidase and hydrogen peroxide.

The latter technique has been used to label cell surface markers and due to the relatively mild reaction conditions, biological activity of the labelled surface markers was apparently not affected (Morrison, 1980). The labelled products are generally chemically stable. The oxidation of thiols and thio-ethers that can take place during the oxidative conversion of the starting iodide to iodine can be avoided by the use of a very mild biologically friendly acylation reaction that avoids this problem (Bolton & Hunter, 1973).

Iodine-123 is a cyclotron-generated gamma-emitting radioisotope of iodine with a half-life of 13.2 hours. This isotope has a single gamma radiation emission with an energy of 159 keV resulting from an electron capture event. These characteristics make ^{123}I a nearly ideal medical and pharmacokinetic radiotracer isotope. ^{123}I is commonly used for thyroid function and imaging studies in the free iodide form (Martindale, 1993), for renal imaging and perfusion studies as iodohippurate (Jorgensen & Ladefoged, 1987) as well as for cerebral imaging as iofetamine (Cohen *et al*, 1988).

Oxihumate (see below) has many available phenolic and activated aromatic rings, permitting successful electrophilic substitution by molecular iodine under mild conditions. The iodide labelled oxihumate prepared from iodide-123 and chloramine-T (Hunter & Greenwood, 1962) results in a relatively efficient labelling of numerous compounds in the oxihumate, but these labelled compounds cannot be rapidly isolated as individual labelled compounds by HPLC because of its complex chemical composition, its micelle forming characteristics and its low solubility in organic solvents. The alkaline salts are reasonably soluble in water although they precipitate quickly when lowering the pH of the solution to below pH 2.0 by addition of acids.

An earlier baboon study using labelled oxihumate did not identify any organs that could be associated with the immune system and only showed up the organs that would have been expected to accumulate free iodide. In spite of the typical iodide distribution being seen in the previous study (Dormehl, 1998), no attempt was made to analyse the labelled humic acid for free iodide but assumed that any residual free iodide would have been removed from the reaction mixture by the extensive washing procedure used.

One of the objectives of the present study was to repeat an earlier study where radiolabelled oxihumate was administered to baboons per os. The prime objective was to ascertain whether absorption of humic acid compounds did in fact take place from the gastro-intestinal tract. Additional data that was to be collected was kinetic data on the uptake and elimination of the

labelled compounds, to find possible immune system related target organs and to analyse the urine to find excreted labelled compounds.

10 The immune response

10.1 Overview

Immunity is the ability to ward off or protect the host against invading microorganisms or pathologic agents. The immune system is a complex combination of numerous cell types and bio-molecules, distributed throughout the host, especially in areas where contact with pathogens is high. Each component of the immune system, cellular or molecular, has a specialized role in this host defence. The immune system can be divided into two broad classes: an innate immunity involving immediate response and inflammatory processes and an acquired adaptive response that is antigen specific and involves immunological memory against a particular target antigen.

The innate response is the first line defence against exogenous invasions, especially against microbial assault, but it is also forms an integral part in the initiation of the adaptive response that follows a particular assault. The innate response is generally a localized response to an assault by microorganisms or foreign particles and involves the complement system of the blood, target independent phagocytic cells like the neutrophils also known as the polymorphonuclear leukocytes (PMNL), the macrophages (MØ) and their precursors the monocytes (MO) as well as non-phagocytising natural killer cells (NK) and the mast cells. All these cells can release inflammatory mediators and cytokines during the phagocytosis or when otherwise activated.

The adaptive response takes longer to be initiated and makes use of the T- and B- lymphocytes, cells which need to mature and proliferate in response to specialized antigen presenting cells (APC). The APC presents a processed antigen, from the invading agent, attached to the major histocompatibility complex class II molecules (MHC II) together with other stimulatory signalling molecules to immunocompetent Th 0 lymphocytes, which in turn proceeds through a series of differentiation steps depending on the cytokines released by the APC and accessory cells. This T-lymphocyte maturation results in either a cell mediated immunity (occasionally accompanied by a delayed type hypersensitivity) as a result of a Th 1 mediated response or a humoral immunity resulting from Th 2 mediated stimulation of B-lymphocytes to secrete immunoglobulins (Ig) which then bind to the exogenous microorganism causing disruption of cellular activity with suppression of microorganism growth and increases the recognition of the Ig-microorganism complex as being a foreign threat by the innate immune system.

10.2 Phagocytic cells of the innate immune system

10.2.1 Polymorphonuclear leukocytes (PMNL)

These are the most populous of the circulating leukocytes but also the shortest-lived cells in circulation. They are produced and mature in the marrow and are released into circulation where they spend as long as a week if they do not become stimulated. Stimulation results in the PMNL migrating by diapedesis (a sequence of attachment to the luminal vascular endothelium followed by a rapid spreading and flattening of the cells onto the endothelium followed by squeezing through the endothelium into the interstitial space) into the tissue where the stimulus originated. They are produced in the bone marrow in response to acute stress irrespective of where the stimulus originates, whether from infection, trauma, noxious stimuli, emotional stress, infarction or otherwise.

PMNL form the first line defence against any acute microorganism assault and is the first leukocyte to migrate (by means of amoeboid movement) into a lesion or region of infection. This migration is a well-coordinated chain of events involving chemotactic molecules (IL-8, PAF, LTB_4), adhesion molecules (β_2-integrins, ICAM-1, ICAM-2, the L-, P-, & E-selectins), the vascular endothelium, and several cytokines (IL-1, IL-8, TNF-α, GM-CSF). Many different receptors and molecules are involved, some with multiple functions e.g. L-selectin and CR3 on the PMNL and P- and E-selectins on the vascular endothelium (Witko-Sarsat *et al*, 2000). PMNL binding of endothelial P-selectin increases β_2-integrin mediated adhesion (via increased CR3 molecule expression) and also stimulates production of reactive oxygen species (ROS) (Ruchaud-Sparagano *et al*, 2000). Almost all the chemotactic molecules, phagocytic stimuli, activated complement molecules (C5a) and several cytokines (TNF-α and GM-CSF) stimulate expression of high levels of CR3 adhesion molecules by PMNL (Stewart & Hogg, 1996).

The functions of PMNL include phagocytosis of microorganisms, cell debris and denatured proteins, the release of proteolytic enzymes and highly reactive oxidant species (ROS) as well as the synthesis of cytokines, chemokines, and inflammation mediating lipid metabolites. Phagocytosis by PMNL always results in self-destruction, but this is a protective mechanism for the host. As the proteolytic enzymes and highly reactive oxygen species used by the PMNL to destroy the foreign particles cannot distinguish between target and host tissue, the PMNL engulfs the target particle and performs the destructive reaction in an intracellular phagosome thereby protecting the surrounding host tissue at its own expense.

Several cytokines are produced by PMNL, which appear to play an autocrine regulatory role on PMNLs. The main cytokines expressed by neutrophils are IL-8, a powerful PMNL chemotactic agent and inducer of degranulation, and TNF-α, an adhesion promoting and ROS inducing agent associated with inflammation (Cassatella, 1999). Exogenous inflammation suppressing cytokines (IL-4, IL-10 and IL-13) appear to inhibit the cytokine expression by neutrophils. This cytokine expression and control might open new avenues for drug targeting in chronic inflammatory conditions (Witko-Sarsat *et al*, 2000).

The arachidonic acid metabolites formed by PMNL are predominantly the leukotrienes, formed by the action of 5-lipooxygenase, with leukotriene B_4 (LTB_4) being the main metabolite (Alonso *et al*, 1998; Serhan, 1994). Recently it has been shown that prostaglandin E_2 (PGE_2) and thromboxane A_2 (TxA_2) are also synthesized by PMNLs via an inducible cyclooxygenase 2 (COX-2) in response to numerous known PMNL stimuli (Pouliot *et al*, 1998). The rate of upregulation of COX-2 in PMNL is dependant on the stimulant and differs greatly from that of the monocytes and macrophages that may have implications in the control of cell damage by PMNL in diseases like rheumatoid arthritis, sepsis and acute respiratory syndrome (Rocca & Fitzgerald, 2002). Like all inflammatory cells PMNL release platelet activating factor (PAF), which is a very powerful and omnipotent bioactive lipid. It potentiates the inflammatory response of PMNL and eosinophils, possibly through activation of specific G protein type receptors (Prescott, 1999).

10.2.2 Monocytes and macrophages

Monocytes and macrophages share several functions with other myeloid and lymphoid cells, illustrating the built in redundancy within the immune system, but are also the most adaptable cells. They are different stages of development of the same cell type, which are derived from the pluripotent stem cells in the bone marrow. These cells carry CD14 and CD68 cell surface markers. Initially newly formed monocytes circulate freely in blood and lymph (for 5 to 8 days) from where they migrate by diapedesis into almost all organs and body cavities where they differentiate to form resident macrophages that have varied function, properties and morphology depending on the tissue and conditions in their immediate environment (Hashimoto *et al*, 1999). Kupffer cells, Langerhans cells, microglia, dendritic cells and alveolar macrophages, although very different in morphology and function are all derived from monocytes. They are central to the host defence against pathogens, and like PMNLs are capable of phagocytosis, but without the self-destructive after-effects demonstrated by PMNL phagocytosis. They can also phagocytose many more microorganisms than PMNL.

Macrophages respond to circulating or tissue stimuli by secretion of cytokines, chemokines and lipid mediators of acute inflammation that results in the initiation of inflammatory processes, recruitment of leukocytes (especially PMNL). These cells can leave the circulatory system and return via the afferent lymph where they then interact with secondary lymphoid tissue (Gordon, 1999). They act as APCs to immunocompetent lymphocytes in the secondary lymphoid tissue but are not as effective as the dendritic cells (Bjercke & Gaudernack, 1985). They are found in elevated numbers in most areas of chronic inflammation or localized infection.

Bacterial lipopolysaccharides (LPS) and IL-1β induce COX-2 upregulation in macrophages but the Th 2 associated cytokines (IL-4, IL-10 and IL-13) suppress this induction (Berg *et al*, 2001).

Stimulated macrophages release pro-inflammatory cytokines and lipid inflammation mediators as well as express MHC class II molecules, all of which can condition dendritic cells or enable presentation of antigen directly.

10.3 Immune Mediators

Mediators play a central role in the immune response by linking the innate and acquired immune responses in a coordinated and controlled manner. They act as stimulators or amplifiers of specific responses and are central to immune cell trafficking as well as stimulation of cell proliferation and maturation in the myeloid and lymphoid tissue. Most mediators have small radii of action meaning that they only act on the cells in the immediate vicinity of the expressing cell, while a few diffuse throughout the system causing effect at distant organs or cells. Most mediators are associated with specific immunological stimuli and interact with specific high-affinity cell surface receptors. The immune mediators generally show pleiotropic activity (i.e. act on different types of cells and elicit several different responses) and exhibit redundancy (meaning that there are other mediators with the same or similar functions). They may have different effects on different cells or their effect may change depending on their concentration or the presence of other mediators. Mediators can be divided into the protein and lipid derived mediators.

10.3.1 Protein mediators

The protein mediators include amongst others the cytokines, chemokines, growth factors, interferons, adhesion molecules and tumour necrosis factors. The cytokines are expressed by myeloid and lymphoid cells and are ligands for receptors on other cells that are involved in the active immune response. This often results in a cytokine cascade where one cytokine initiates the

expression of another that again stimulates a third cytokine to be expressed. An example is the LPS induced TNF-α that causes the sequential cascade of IL-1 and IL-6 (Beutler & Cerami, 1987), which are all pro-inflammatory cytokines. The cytokines are generally stimulants of effects that take effect at gene expression level such as cell proliferation, differentiation, maturation or activation, but some cytokines inhibit these same effects. Cytokine production is regulated by inducing stimuli at gene transcription level with transient production and short radii of action. Their molecular mass is generally less than or equal to 30 kD (Vilcek & Le, 1994).

The interleukins are a subset of 18 cytokines that are produced mainly by the leukocytes and that act primarily on other leukocytes. Their functions are diverse, but mostly stimulatory for inflammatory processes (IL-2, IL-12, TNFα) or cell maturation (GM-CSF) although some are anti-inflammatory (IL-1β, IL-4, IL-6, IL-10, IL-13). They usually work in concert with other interleukins or cytokines.

The chemokines are a family of more than 40 polypeptides of less than 18kD that share many chemical and structural characteristics. They act as chemotactic attractants (at nanomolar concentrations) for the phagocytic cells of the innate immune response, enhance adhesion, stimulate lipid mediator synthesis, degranulation and the respiratory burst (Baggiolini *et al*, 1997). Chemokines fall into one of two classes; CC or CXC depending on the absence or presence of an amino acid between the two amino-terminal cysteine residues. The cell surface receptors for chemokines are involved in cell recognition; the CXCR4 chemokine receptor is used as an attachment site by the human immuno-deficiency virus (HIV-1) for entry into CD4+ T-lymphocyte cells (Feng *et al*, 1996).

The cell growth factors are stimulants of cell proliferation (especially of the myeloid cells in the bone marrow) and differentiation. GM-CSF is an example of a growth factor.

10.3.2 Lipid mediators

Lipid mediators, including prostaglandins, leukotrienes, thromboxanes, and platelet activating factor (PAF), are generally pro-inflammatory but can have a profound effect on cell maturation and expression of cytokines by cells in general. The first three types are all derivatives of arachidonic acid, which is released from membrane phospholipids by the action of the phospholipase A_2 enzymes. These mediators are active over short distances and have short half-lives. They exert their activity through surface membrane receptors.

Prostaglandins are formed from free arachidonic acid by the action of the cyclooxygenase enzyme 1 or 2. COX-1 is a constitutive enzyme while COX-2 is induced by numerous cytokines, endotoxins, phagocytic stimuli and growth factors (Smith & Langenbach, 2001). The main inflammatory related responses to prostaglandins are: fever, pain, oedema and regulation of leukocyte function. Pain and oedema are secondary effects due to hyperalgesia and increased blood flow respectively but the fever (pyrexia) and leukocyte regulation is a direct result of prostaglandins (Griffiths, 1999). There is an inhibitory effect on Th 1 helper cell formation by PGE_2 with the expressed cytokine profile being more inclined to the allergic response (Sergeeva *et al*, 1997). The main inflammation related prostaglandins are PGE_2 and PGI_2 and although PGD_2 and $PGF_{2\alpha}$ are also important they are more tissue specific (PGD is CNS and mast cell related while PGF is vascular smooth muscle related) (Griffiths, 1999).

Leukotrienes are formed by the action of 5-lipoxygenase on arachidonic acid (AA). This enzyme is found on the perinuclear membrane of most leukocytes and mast cells (Ford-Hutchinson *et al*, 1994). Two main classes of leukotrienes exist: the LTB_4 class of hydroxylated derivatives and the cysteinyl leukotrienes that include LTC_4, LTD_4 and LTE_4. LTB_4 enhances PMNL lysosome release and degranulation, mediates adhesion, migration and recruitment while B-lymphocytes are activated and enhanced production of immunoglobulins. It acts as an intracellular messenger acting on the peroxisome proliferator-activated receptor-α (PPAR-α) involved in fatty acid oxidation and adipogenesis. The cysteinyl leukotrienes have vasoconstrictive and smooth muscle constriction properties but augment vascular permeability (Penrose *et al*, 1999).

Two lipoxins (and two isomers that result from acetyl salicylic acid treatment) are also derived from AA by the action of 5-lipoxygenase and are lipid mediators, but in contrast have anti-inflammatory effects. They appear to be formed transcellularly i.e. synthesis occurs in two stages, each in a different cell. They are under IL-4 and IL-13 (i.e. inflammation suppressing) cytokine control and inhibit PMNL and eosinophil adhesion and migration but stimulates the same actions in monocytes and mediate vasodilation. They counteract the effect of prostaglandins and mediate nitric oxide generation in vascular smooth muscle (Bratt & Gyllenhammar, 1995; Serhan, 1999).

11 Oxihumate

11.1 Synthesis from coal

As mentioned above coal is formed slowly from humic substance rich material through a series of reactions. However, if conditions are right, coal can be converted back to a combination of fulvic

acids, humic acids, humic substances and highly mineralized compounds during the natural oxidative weathering of coal (Rausa, 1994). This natural process of oxidation of coal to form, amongst others, humic acids can be accelerated by chemical oxidation of the coal. By controlling the conditions used during the oxidation process, the yield of humic acids can be varied relative to the other products. Based on this fact a method for the production of a humic acid from a bituminous coal was developed (Bergh *et al*, 1997), patented (Cronjé, 1988; Cronjé *et al*, 1991) and used by a South African company – Enerkom (Pty) Ltd. - to reproducibly produce a formulated potassium salt of humic acid as a food supplement that was marketed as an immune stimulant (Dekker & Medlen, 1999b) under the name "Oximate". Another useful product formed during the process was fulvic acid, which could be applied in the agricultural and veterinary fields as well as formulated into a topical ointment for human use.

The patented process for the oxidation of bituminous coal was basically as follows;

- bituminous coal from a defined source was pulverized to a particle size of < 200μm.
- the powder was made into a slurry using water and pumped into a temperature- controlled high-pressure reaction vessel where the mixture was heated to 180°C under an atmosphere of oxygen maintained at a constant pressure of 4 MPa.
- the resulting exothermic reaction was then controlled to maintain the reaction temperature at 180°C while feeding oxygen at a high flow rate into the reactor for at least one hour. The reaction was stopped by shutting off the oxygen supply and cooling to ambient temperature.
- the reaction mixture was then filtered though an efficient high-pressure filter system to remove the insoluble humic acid and incompletely reacted coal fractions.
- the filtrate containing the fulvic acids was concentrated to a 24% solution in a reverse osmosis system.
- the retentate on the filter cakes contained incompletely reacted coal (termed oxicoal in the process) and humic acids that were resuspended in water and treated with an aqueous potassium hydroxide solution.
- this suspension was homogenized then dried in a spray-drying column to give the potassium humate powder.

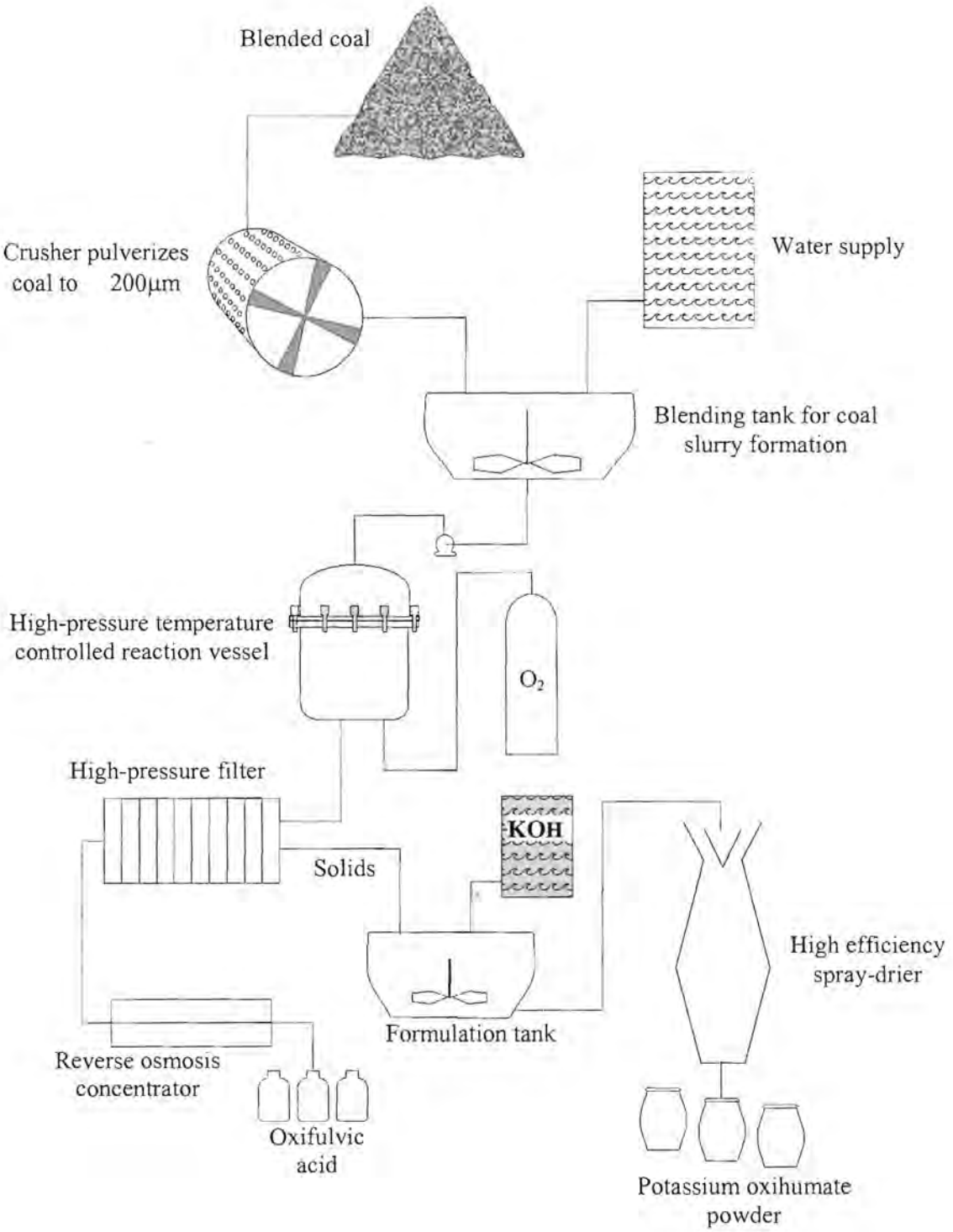

Figure 1-2: A diagrammatic representation of the patented process for manufacturing synthetic oxihumate from a bituminous coal (Cronjé, 1988; Cronjé *et al*, 1991)

To distinguish these synthetically produced products from the naturally formed equivalent compounds the fulvic acids and the potassium salt of humic acid were named oxifulvic acid and potassium oxihumate respectively.

Advantages that the above method has over other methods were that it was a rapid method that used no toxic compounds during the synthesis and that no toxic waste was generated. Due to the simplicity of the method it could easily be automated and the process could be adapted to batch or continuous flow processes. The wastage was also minimized as both the humic acid and fulvic acid fractions were recovered separately and could be used for different applications.

11.2 Therapeutic properties of oxihumate and oxifulvic acid

In vitro as well as *in vivo* toxicity tests have been performed on both oxifulvic acid and oxihumate using rat and dog models (Confidential Study Reports: Biocon (Pty) Ltd, Pretoria, South Africa, 1999 & 2001) where it was found that the sub-chronic long-term (90 day treatment) and the acute toxicity of oxihumate were low and that no toxic effects were exhibited up to oxihumate concentrations of 1000mg/kg/day during the test period. No drug related deaths occurred during any of the studies. Small changes in blood parameters (increased red blood cell size and haemoglobin content, increased serum globulin concentration, decreased circulating leukocytes, decreased serum inorganic phosphate) and a small but acceptable decrease of end body mass compared to control animals was found at concentrations greater than 100mg/kg/day. Under the conditions of the study the no-adverse-observed–effect-level (NAOEL) was concluded to be higher than 100mg/kg/day. The acute toxicity study using rats indicated that the oral LD_{50} was greater than 3456 mg/kg and the dermal LD_{50} greater than 4147mg/kg.

Oxifulvic acid studies indicated that it had antimicrobial activity against several known pathogens (van Rensburg *et al*, 2000) at concentrations of less than 15 g/l, was found to be effective as a topical cream (containing 5.3% oxifulvic acid) for the treatment of pyotraumatic dermatitis in dogs and cats as well as an inhibitor of contact dermatitis in animal studies using a mouse model (Dekker & Medlen, 1999a; van Rensburg *et al*, 2002). Another study evaluating the safety and efficacy of topically administered oxifulvic acid was done on atopic but allergic volunteers and was found not to cause sensitisation when applied topically nor did it alter any of a battery of safety parameters (Snyman *et al*, 2002). It was interesting to note that the formulation appeared to play a major role in the efficacy with the formulation containing the higher concentration of oxifulvic acid having less activity against the allergic response elicited by a challenge. This was ascribed to the lower pH of the cream with the higher oxifulvic acid concentration.

An *in vitro* study on the anti-viral activity of oxifulvic acid on Herpes Simplex Virus type-1 was conducted using BGM monkey kidney cells. These studies revealed that oxifulvic acid was toxic to the cells in culture at concentrations in excess of 1.25mg/ml. There was no virostatic effect on the HSV-1 virus in suspension but host cell entry was 75% inhibited and viral replication within the cells was completely inhibited at concentrations of 40µg/ml and 320µg/ml respectively (Williams *et al*, 2001).

The immunomodulating properties of oxihumate have been extensively investigated. The effect of oxihumate on the proliferation of lymphocytes stimulated with mitogens revealed that there was an increase in proliferation at oxihumate concentrations of more than 20µg/ml. This effect was seen *in vitro* and *ex vivo* in HIV positive individuals treated with oxihumate. It was determined that this increase in proliferation was probably due to increased IL-2 production and IL-2 receptor expression while IL-10 concentrations were suppressed (Jooné *et al*, 2003).

A two-week phase I clinical trial was performed using various orally administered oxihumate doses from 2 to 8 g per day. An increase in the weight of all the treated patients was found when compared to the control group receiving a placebo. There was however no significant improvement in either CD4+ counts or viral load (Botes *et al*, 2002) but this could have been due to the short duration of the study. No toxic effects were apparent during the treatment period or for one week after treatment had stopped. This study highlighted the non-toxic characteristic of oxihumate in humans. A later *in vitro* study on the anti-HIV properties of oxihumate (van Rensburg *et al*, 2001) demonstrated that replication of HIV was blocked through an inactivation of the virus particle that could occur after viral binding to the cell had taken place. This was identified as involving the binding of oxihumate to the V3 loop of the virus gp120 protein. No viral resistance developed against the oxihumate after as many as 18 passages.

In another study done using *in vitro* proliferating lymphocytes (Jooné, 2002), the concentrations of the following cytokines were determined in the culture medium: TNF-α, INF-γ, IL-2, IL-4, IL-6, IL-10 after exposing the cultures to various concentrations of oxihumate for 72 hours. By comparing the oxihumate induced cytokine profiles to equivalent controls, it was concluded that oxihumate induces mostly a Th 1 type of immune response. The expression of IL-2R – the cell surface IL-2 receptor (also referred to as CD25) was found to increase after oxihumate treatment.

Other immune related mediators, surface marker proteins and cell activities were also investigated for changes induced by oxihumate exposure. The effect of oxihumate on the concentrations of the

lipid immune mediators, prostaglandin E_2 and leukotriene B_4 were determined and showed a slight decrease in the former and a significant increase in the latter (Dekker & Medlen, 1999b). Prostaglandin E_2 is a pro-inflammatory mediator (stimulating pyresis, oedema and hyperalgesia) (Griffiths, 1999) that also has a suppressing effect on the Th 1 cytokine profile while LTB_4, which is also pro-inflammatory, especially with respect to neutrophils, stimulates the expression of IL-2, a Th 1 type cytokine (Penrose *et al*, 1999).

Due to phagocytic cell adhesion being one of the first stages in the inflammatory response, an *in vitro* investigation of the effect that oxihumate had on the expression of the CR3 adhesion molecules by PMNLs was included in the above study (Jooné, 2002). It was found that resting PMNLs were unaffected but that the expression of CR3 by activated PMNL (using either PMA or FMLP/cytochalasin B) was inhibited in a dose dependant manner. Additionally, the effect that oxihumate had on the binding of resting and stimulated PMNL to human ICAM-1 and human E-selectin molecules expressed on the surface of transfected hamster kidney cell lines were determined (Jooné *et al*, 2001). Again it was found that there was a dose dependant inhibitory response to oxihumate treatment.

To eliminate the possibility that the mechanism of action of oxihumate on the lymphocytes was due to disruption of the cell membrane integrity, the effect of oxihumate on sheep red blood cells, where the membrane is relatively fragile, was investigated and found to be minimal, which would exclude this membrane disrupting mechanism (Jooné, 2002).

12 Study design

Motivation

Various reports have been published on the lack of toxicity and possible anti-inflammatory properties of humic acid, but there is no conclusive evidence demonstrating that humic acid is absorbed when administered orally and that it is effective in the treatment of inflammation.

Unlike most other studies on humic acid, this study was done on oxihumate, the potassium salt of a semi-synthetic humic acid derived from a homogenous mixture of bituminous coal by a controlled process that ensures that the product is consistent from batch to batch. This product is easily available in large quantities, is relatively cheap and has already been registered as a food supplement.

Hypothesis

The immuno-modulating properties of oxihumate are attributable to only one or several discrete compounds that can be isolated *in vitro* and possibly also *in vivo* following oral administration of oxihumate.

Aims

The aims of this study are to determine whether;

- oxihumate can be sub-fractionated and that these sub-fractions can be chemically characterized
- biological activity of oxihumate can be assigned to one or more sub-fractions
- oxihumate can be absorbed from the gastro-intestinal tract
- oxihumate possesses anti-inflammatory activity *in vivo*

Objectives

The main objectives of the study are;

- to attempt to fractionate oxihumate using differential solubility of the semi-synthetic humic acid in aqueous organic solvent mixtures

- to chemically analyze the isolated sub-fractions using techniques such as thin layer chromatography, high-pressure liquid chromatography, fluorescence spectroscopy, infrared spectroscopy, UV/visible spectroscopy and the ash content.

- to assay oxihumate and any isolated sub-fractions for anti-inflammatory activity *in vitro* using two biological assays namely

    - expression of CR3, a pro-inflammatory adhesion molecule, on resting and stimulated human neutrophils
    - scavenging of pro-inflammatory oxidants formed by stimulated human neutrophils

- to perform an *in vitro* pharmacokinetic study using an isolated rat gut system to determine whether oxihumate can be absorbed from the gastro-intestinal tract

- to determine the anti-inflammatory activity effect of oxihumate and its sub-fractions in an *in vivo* contact hypersensitivity assay using a rodent model

- to perform an *in vivo* pharmacokinetic study in a baboon model to attempt to determine the target organs of absorbed humic acid components

Each phase of the research project depends on the outcome of the preceding phases and the *in vivo* studies will rely on the identification and isolation of an active fraction that can be confirmed *in vitro*.

Chapter 2

Chemistry of Oxihumate

1 Introduction

Many attempts have been made to analyse humic acid structure over the years but few have met with success. Oxihumate is a formulation containing oxidatively released humic acid salts derived from a homogenous mix of coal that is reputed to be non-varying and produced by a closely controlled reaction system, which implies that the product is exactly the same in each batch. However to avoid any complications that could possibly arise from variations, all the assays performed in this study were on a single retention batch of oxihumate.

The sub-fractionation of oxihumate relied the solubility differences in different solvent mixtures and were then analysed chemically. Seven major sub-fractions were isolated. The chemical analyses included TLC, HPLC, ash content, UV/visible spectra, infrared spectra, fluorescence spectra, and two fractions that were subjected to Raman spectroscopy. The isolated sub-fractions were further subjected to bioassays to determine anti-inflammatory activity.

2 Materials and Methods

2.1 Materials

Oxihumate, a semi-synthetic humic acid formulation manufactured from a bituminous coal by a mild wet oxidation process (Cronjé, 1988) was supplied by Enerkom (Pty) Ltd. as a dry black powder of 200 to 400-mesh size. This is a formulated potassium salt of the humic acid fraction of the oxidation product.

All reagents used were of the best available grade or analytical reagent grade. Ammonium hydroxide solution (25%) and 32% HCl was from Merck, (Darmstadt, Germany). All solvents used were of analytical reagent or HPLC grade and supplied by Merck (Darmstadt, Germany) or Sigma Aldrich, (St Louis, MO, USA).

Silica gel 60 pre-coated glass or plastic TLC plates were purchased from Merck Chemicals, (Darmstadt, Germany). Initially both fluorescent (F-254) and non-fluorescent TLC plates were used but the fluorescent plates appeared to reveal more compounds.

Water was 18MΩ water produced from the municipal water supply after processing by an Elga Option 4 system fitted with a carbon polishing- filter.

2.2 Methods

2.2.1 Isolation of the humic acid fraction from Oxihumate

The procedure described here follows the standard acid precipitation method first introduced by Achard (1786) and was found to be reproducible and applicable to humic acids from various sources.

The oxihumate was dried in a drying oven at 110°C for 24 hours and allowed to cool to ambient temperature in a desiccator. Samples of this dried material were used to determine the ash content.

A 200 g aliquot of the dried oxihumate was suspended in 2000 ml distilled water and stirred at ambient temperature with a paddle stirrer for 4 hours at 500 rpm. After stirring was complete the suspension was allowed to stand overnight before transferring the upper layer into 500ml plastic centrifuge bottles and centrifuging at 3500*g* for 30 minutes. The supernatant was decanted and the sediment layer from the original suspension transferred to the centrifuge bottles and the centrifugation repeated. The supernatants of both centrifugation steps were combined and the sediments were washed by resuspending in distilled water and again centrifuging to remove the insoluble fraction that is referred to as oxicoal.

The combined supernatants were slowly acidified with 6M HCl to pH 1.0 while stirring continuously to avoid the formation of a thick slurry, allowed to stand for 2 hours and centrifuged at 3500*g* for 30 minutes to isolate the humic acid fraction. The precipitate was referred to as the humic acid fraction while the supernatant represents the fulvic acid fraction (present in the original oxihumate), which is by definition the acid soluble fraction.

2.2.2 Gravimetric determination of the concentration of humic acid

Determination of the concentration of the oxihumate and humic acids were done gravimetrically as follows;

- three aliquots of exactly 300.0ml were pipetted into large flat stainless steel pans that had been dried to constant weight at 110°C and cooled in desiccators.
- the solutions were then dried by heating in a forced air oven at 110°C for 24 hours followed by cooling in a desiccator. The pans with the residues were weighed and the original pan weight subtracted to give the dry mass of the 300.0ml aliquots. These weights in grams were divided by three to give a mass percentage solid.

The concentration of the various sub-fractions of the humic acids were done in the same way except that preweighed 50mm diameter Petri dishes were used and only 2 – 5ml of each sub-fraction used depending on the available volumes.

2.2.3 Ash content of humic acid sub-fractions

One of two methods was used to determine the ash content of the various isolated sub-fractions of oxihumate. In the case of the sub-fractions where there was more than 10g of material available, percentage ash was determined using 50mm flat open fused silica crucibles in a muffle furnace. The second method used was thermogravimetric analysis for the sub-fractions where there was not sufficient sample to perform the analysis in a muffle furnace.

2.2.3.1 Muffle furnace method

Marked silica ashing crucibles were dried at 110°C and cooled in a desiccator to constant weight. The material of which the ash content was to be determined was dried in a forced air-drying oven at 110°C for 24 hours and cooled in a desiccator. This gave a water free sample. Triplicate aliquots of 3,0g of the dried material (oxihumate or isolated sub-fractions) were accurately weighed into individual crucibles. These were loaded into a muffle furnace and the temperature increased from ambient to 500°C over 1.5 hours. The samples were maintained at 500°C for a further 1.5 hours before increasing the temperature to 850°C over 1.5 hours and maintained at this temperature for 3 hours.

The furnace switched off automatically and was allowed to cool without opening the door. When the temperature had dropped to approximately 200°C the samples were removed and placed in a

desiccator overnight. The samples were removed from the desiccator individually and weighed as quickly as possible to avoid any absorption of humidity from the atmosphere.

The percentage ash was calculated by using the formula

$$Percent\_Ash = \frac{\textbf{(Weight of crucible with ash)} - \textbf{(weight of empty crucible)} \times 100}{\textbf{Original weight of dried sample used}}$$

2.2.3.2 Thermogravimetric analysis

Due to the limited sample sizes available for the tetrahydrofuran precipitated and tetrahydrofuran soluble fractions these isolated sub-fractions were analysed for ash content using a Mettler TGA 851e thermogravimetric analyser with a gas flow controller. Approximately 10mg of each sample was accurately weighed into 70µl alumina sample pans with lids and heated from 30°C to 1000°C at 40°C/minute under a flow of 50ml/minute air. These samples were held at 1000°C for 15 minutes and the mass of the remaining residue used to calculate the percentage ash. These analyses were done in duplicate.

2.2.4 Sub-fractionation of the humic acid fraction of Oxihumate

The acid precipitated humic acid fraction was air dried at ambient temperature in flat pans in a stream of air. An aliquot of the dried humic acid fraction was then vacuum dried over potassium hydroxide and phosphorus pentoxide pellets before being further analysed. A 10g aliquot of the vacuum dried humic acid was suspended in a minimum amount of 1M NH_4OH and stirred until totally solubilized. This resulted in a fairly viscous dark brown solution that on standing formed a small dark precipitate that was removed by centrifugation at 3500*g*.

The first sub-fraction of humic acid was the 60% methanol (MeOH) precipitate. The volume of supernatant was measured and the required volume of methanol to adjust the concentration to 60% MeOH added slowly in 10 – 20ml portions while stirring continuously. If the methanol addition was not done slowly the mixture would thicken and the total fraction would precipitate, leaving a slightly coloured methanolic solution. After all the methanol had been added, the mixture was stirred for a further 2 hours and then left overnight before isolating the precipitated fraction by centrifugation at 3500*g* for 30 minutes. The 60% MeOH soluble fraction was decanted from the sediment. The sediment was washed once with 10 volumes of 60% MeOH and centrifuged again at 3500*g* for 30 minutes.

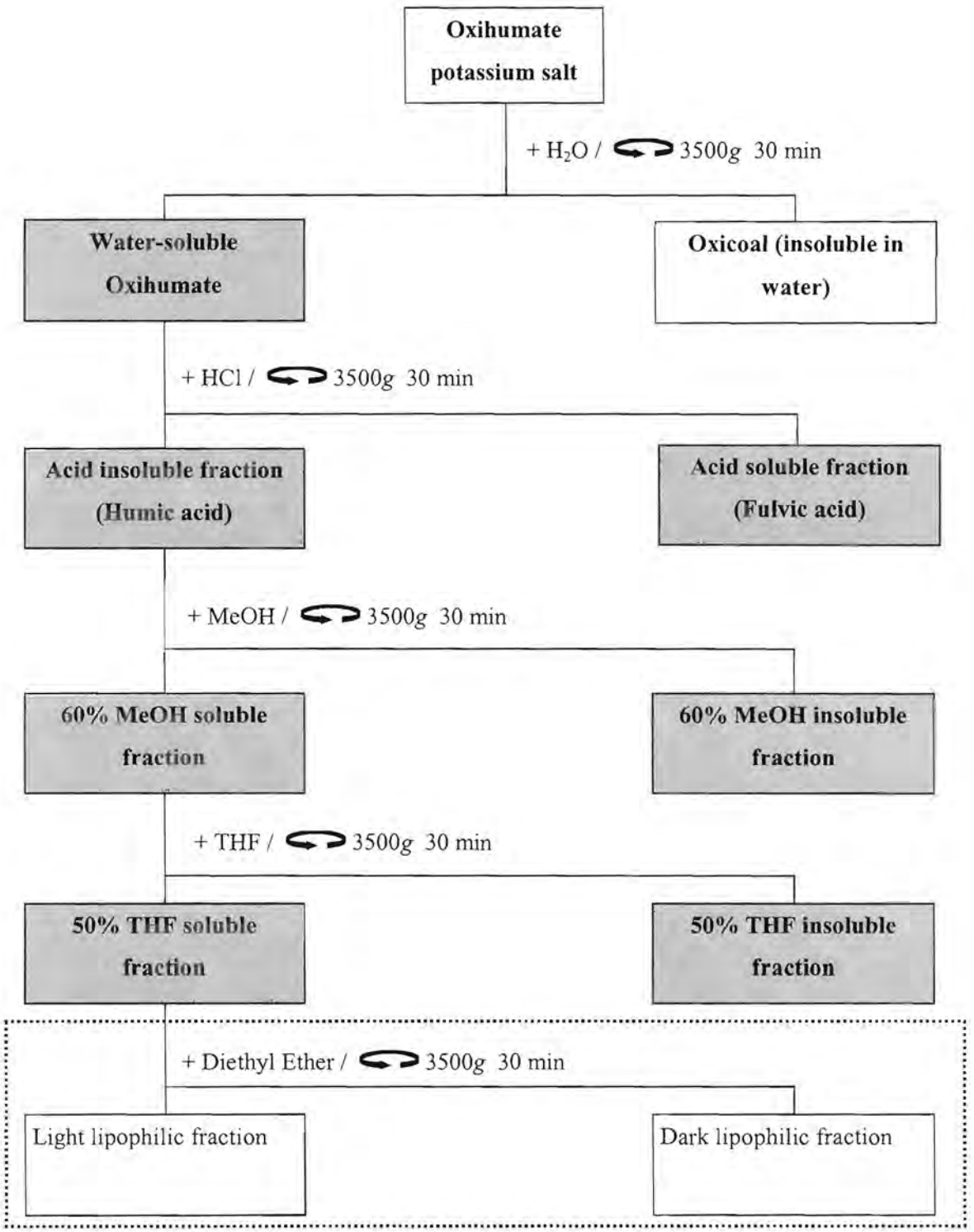

Figure 2-1: Scheme of the sub-fractionation of oxihumate to give 7 fractions. The seven main sub-fractions are shown in grey boxes. The oxicoal and diethyl ether treated lipophilic fractions were not further analysed during this study.

The precipitate was dried at 110°C overnight and weighed to determine the percent dry weight of the fraction. The 60% soluble fraction was adjusted to 50% tetrahydrofuran (THF) by slow addition of THF to the 60% MeOH soluble fraction while stirring continuously. The mixture was allowed to stand overnight before centrifuging at 3500*g* for 30 minutes to remove the precipitated material that was a brown to black amorphous sediment. The supernatant was a reddish-brown solution with a slight green fluorescence. This was air dried at ambient temperature to give a thick dark oil that is referred to as the tetrahydrofuran soluble fraction. The THF soluble fraction was washed with ether to give an orange brown oil that was referred to as the lipophilic fraction. The sub-fractionation sequence is summarized in Figure 2-1. The grey blocks are the sub-fractions that were further analysed.

2.2.5 Thin Layer Chromatography

Thin layer chromatography was performed on each of the isolated sub-fractions of oxihumate using pre-coated silica gel 60 F-254 plates of 20 x 20cm or 5 x 20cm. Initially fluorophore free plates were used but it was found that more compounds could be detected using the fluorescent plates. The glass plates were generally found to give better separations and could be loaded with up to 100µg per spot. Plastic plates could only be loaded with a maximum of 50µg per spot. Generally 2 – 5µl per spot was applied at least 1 cm apart at 1 cm from the lower edge of the plates which were then run in the ascending direction.

Initially several different solvent systems were attempted including those reported in the literature but it was found that the best system depended on the polarity of the fraction being analysed. The plates were run in standard glass TLC chromatography tanks lined with filter paper to ensure atmosphere saturation, in the ascending direction and were generally between 2 and 3 hours per run depending on the polarity of the mobile phase.

The following solvent systems were found to be good for separating the polar sub-fractions of oxihumate from each other:

Ammonium hydroxide solution (25%)-n propanol in the ratio of **7 : 3.**
The tank was pre-saturated for at least 30 minutes prior to developing the TLC.

Water-methanol-acetone-triethyl amine in the ratio of **6 : 6 : 6 : 0.1.**

Acetonitrile-water-ammonium hydroxide (25%) in the ratio **6 : 3 : 1.**

The following solvent systems were found to be good for separating the less polar sub-fractions of oxihumate;

Methanol:chloroform:n-butanol:water:aceticacid in the ratio of **9 : 9 : 3 : 3 : 0.5.**
Filter paper was used to line the tank that was pre-saturated for at least 30 minutes prior to developing the plates (about 2½ hours).

Acetone-n butanol-water-acetic acid in the ratio of **13 : 4 : 2 : 1.**

2.2.6 High Pressure Liquid Chromatography

High-pressure liquid chromatography was carried out on the complete oxihumate as well as the sub-fractions of the humic acid isolated from oxihumate. Although there are some methods published in the literature (Frimmel *et al* 1992; Preuse *et al*, 2000; Woelki *et al*. 1997) none of these were found to give acceptable resolution and reproducibility.

The separations were performed on a Hewlett Packard 1050 HPLC system consisting of a solvent degasser, automated fixed loop injector, column thermostat, quaternary pump, multi-wavelength UV/visible detector and fluorescence detector all of which were controlled from a data station running Chemstation® chromatography software.

All solvents were of HPLC grade and purchased from Sigma Aldrich, (St Louis, MO, USA) or Merck, (Darmstadt, Germany). Different manufacturers columns were used but it was found to have little effect on the chromatograms after about the first 20 injections through the columns. Generally a Phenominex SecurityGuard™ guard column fitted with a C_{18} cartridge was used together with a 150mm x 4.6mm column packed with 5µm C_{18} particles. The columns used were Phenominex "Luna" C_{18} (2), Cosmosil $5C_{18}$ MS-II (Nacalai Tesque Inc, Japan) and Supelco C_{18} (Sigma Aldrich). Column temperature was maintained at 45°C for all methods.

Detection by the variable multi-wavelength UV detector was simultaneously performed at two different wavelengths each with their own bandwidth and were 230nm with a bandwidth of 20nm for channel A and 270 nm with a bandwidth of 36nm for channel B. The fluorescence detection used 360nm for excitation wavelength and 440nm for the emission wavelength.

Several binary gradient programs were used to elute the injected samples (20µl or 50µl) at a flow rate of 1.0 ml $minute^{-1}$. The programs were as follows;

Program 1(only the percentage of eluent A is given, the balance was eluent B):

Eluent A: 0.1% trifluoroacetic acid in water

Eluent B: 0.1% trifluoroacetic acid in acetonitrile.

0 – 3.5 minutes	isocratic at 98% A
3.5 – 18.5 minutes	linear gradient to 75% A
18.5 – 24.5 minutes	linear gradient to 75% A
24.5 – 28.5 minutes	linear gradient to 3% A
28.5 – 30 minutes	isocratic at 3% A
30 - 32 minutes	linear gradient to 98% A
30 – 35 minutes	isocratic at 98% A

Program 2 (only percentage eluent A given, the balance is eluent B):

Eluent A: 33 mM Ammonium phosphate in water pH 8.0

Eluent B: acetonitrile.

0 – 3.5 minutes	isocratic at 99% A
3.5 – 20.5 minutes	linear gradient to 44% A
20.5 – 22.5 minutes	linear gradient to 4% A
22.5 – 24.5 minutes	isocratic at 4% A
24.5 – 28.5 minutes	linear gradient to 99% A
28.5 – 35 minutes	isocratic at 99% A

In some cases an automated fraction collector was used to collect the column eluent at 30-second intervals starting at injection time. The fractionated eluent from several consecutive separations of oxihumic acid were combined, concentrated rechromatographed on HPLC and by TLC as described in the section above.

2.2.7 Solid Phase Extraction Cartridges

As it appeared that the recovery of the compounds from the C_{18} HPLC column was low, a small study was done to determine the recovery from SPE cartridges packed with a similar stationary phase. The results were compared to controls where the samples were added directly to the eluent and dried in the same way as the SPE eluents.

For the controls 5.0 ml of a 1.0 mg/ml solution of the water-soluble oxihumate sub-fraction was added to 30 ml of a 1:1 mixture of eluent A: eluent B of the acidic solvent system used for the HPLC separations as described above. The mixture was mixed well and quantitatively transferred to

preweighed Petri-dishes (that had been dried to constant weight) and dried under a draught of air before transferring to a drying oven at 110°C for 48 hours.

Five clear plastic C_{18} SPE cartridges containing 500mg packing material were activated by washing with 2 x 2ml MeOH and then washed with 2ml distilled water followed by 5ml of eluent A from the acidic mobile phase system described above. One-millilitre samples of the same 1.0mg/ml solution of the water-soluble oxihumate sub-fraction used for the controls were applied to each cartridge and eluted slowly using 2ml of eluent A then 2ml of a 1:1 mixture of eluent A: eluent B and finally 2ml of eluent B from the same acidic solvent system. The eluents from all five SPE cartridges were combined and quantitatively transferred into a preweighed glass petri-dish, dried under a draft of air to evaporate the organic solvent, then for 48 hours at 110°C, cooled in a desiccator and reweighed. The experiment was repeated 3 times with new SPE cartridges each time.

As the cartridges appeared to retain much of the dark coloured compounds, they were then further washed sequentially with 2ml each of MeOH, acetonitrile, THF, ethyl acetate, chloroform and ether in an attempt to elute these coloured compounds. These eluents were combined and treated the same as the acidic eluents. Finally a slow wash with 4ml of a 1:1 mixture of acetonitrile:ammonium hydroxide was done. The total recovered mass from the cartridges was compared to the weight of the controls. These cartridges were washed again about 24 hours later with the last eluent and this displaced a significant amount of the colour from the cartridges.

2.2.8 Ultraviolet and Visible Spectroscopy

UV/visible spectroscopy was performed on a Perkin Elmer "Lambda 2" dual beam UV/visible spectrophotometer using UV WinLab version 2.0 (Perkin Elmer) software to control the spectrophotometer, collect data and graph the data. Distilled water was used as both the reference and blank solutions. Low-UV transparent quartz cuvettes of 10mm path length were used throughout for the analyses.

Generally the sub-fractions or solutions to be analysed were made up as solutions of 1mg/ml and centrifuged at 3500g for 30 minutes to remove any insoluble material. Further dilution to ensure that the absorbance at 200nm was below 2 absorbance units was done to ensure that the spectra were collected in the linear range of the spectrophotometer. The dilution factor and absorbance was then used to calculate the difference in absolute absorbance at 254nm and to normalize the data for each sub-fraction. Absorbance ratios between 465nm and 665nm for each sub-fraction were performed directly after the scans of the samples by the UV Win Lab software.

2.2.9 Fluorescence Spectroscopy

The seven isolated sub-fractions were made up to concentrations of 1mg/ml each in a 10 mM phosphate buffer at pH 7.2. The absorbance at 254 nm was determined for each sample and those with a high absorbance diluted to that of the sample with the lowest A_{254} This allowed normalization of the luminescence spectra. The samples were scanned in 1cm quartz cuvettes in a Perkin Elmer LS50B Scanning Fluorescence Spectrophotometer between 320 and 700nm with excitation wavelengths repeated with 5nm intervals from 220nm to 400nm. The data was collected to a data station and analysed with FL WinLab version 3.0 software (Perkin Elmer).

2.2.10 Infrared Spectroscopy

Infrared spectra were obtained from KBr windows made by grinding 0.5 – 2 mg of the powdered samples together with 100mg anhydrous KBr until homogenous and pressing 12 mm disks at 320 $kg.cm^{-2}$ in a hydraulic press. The KBr disks were scanned a minimum of 32 scans between 4000 and 400 wavenumbers with a 2 cm^{-1} resolution on a Bruker "Vector 22" spectrophotometer and the data collected and analysed with OPUS version 2.2 software (Bruker).

2.2.11 Raman Spectroscopy

Raman spectra of two of the humic acid fractions were collected on a Diol XY Raman Spectrometer using the 514.5 nm laser line of a 100mW Coherent Innovate 90 Argon ion laser. A backscattering configuration through a microscope fitted with a 50x objective was used at a resolution of $2cm^{-1}$. A liquid nitrogen cooled CCD detector recorded the signal that was then automatically baseline corrected using Dilor Labspec software.

2.2.12 Energy Dispersive Elemental Analysis (EDS)

The ash from each of the major sub-fractions was further analysed by EDS using a Jeol JSM 5800LV scanning electron microscope fitted with a 20KeV X-ray emission source and a Noran Vantage 3rd generation detector. A take-off angle of 35° and a data collection time of 100 seconds were used. At least three different areas of each sample were analysed due to the highly localized nature of the analysis (only $0.01mm^2$ per analysis). Representative samples of ash from major isolated sub-fractions were prepared by sputter-coating a thin layer of gold over a sample of vacuum dried ash mounted on an adhesive strip applied to a sample carrier. At least 3 different areas of ash surfaces were selected and analysed for all elements with atomic number greater than 20 using the emitted k, l and m X-ray lines. The data was collected and analysed with Noran Vantage System 4 Software and the quantitative weight percentage of each of the elements present calculated.

3 Results

3.1 Isolation of the humic acid fraction from Oxihumate

The humic acid fraction of oxihumate was isolated from the formulated potassium oxihumate salt by initial solubilisation in distilled water. Only about 38% of the material was found to dissolve in water although heating the water or increasing the pH of the solution to pH 8 with ammonium hydroxide or triethyl amine could increase this percentage to about 42%. Prolonged stirring did not increase the solubilized amount significantly.

The precipitation of the humic acid fraction by acidification of the aqueous solution resulted in varying percentages of soluble material with the highest concentration remaining in solution when the acidification was carried out slowly while stirring continuously. Between 88% and 92% of the water-soluble fraction precipitated as humic acid. This fraction was then further analysed and sub-fractionated by differential solubility in mixtures of organic solvent. The acid soluble fraction was by definition fulvic acids.

3.2 Gravimetric determination of the concentration of humic acid

Gravimetric determination of the concentration of humic acid appears to be the only reliable method for this determination as all the chromatographic methods can only give qualitative data about the mixture and the recoveries are not reproducible. Triplicate gravimetric analyses generally had a very small variance (less than 0.5%).

3.3 Ash content of humic acid sub-fractions

The ash content of the different isolated sub-fractions is summarized in Table 2-1. Generally it was found that the ash content of the major fractions did not vary much although the colour and texture of the ash from the different fractions were quite different. Glass formation was evident with the acid soluble fraction and it appears as though the major cation present in this fraction is excess potassium. The other fractions tended to form soft loose ash of variable colour. Thermogravimetric analysis of the THF precipitated and THF soluble sub-fractions both gave hard dark grey residues that appeared to be incompletely ashed despite the high temperature and the time at this high temperature. A typical thermogravimetric analysis of the THF soluble fraction is shown in Figure 2-2. The upper section (A) shows a curve of the actual residual percentage mass of the sample verses time in minutes using the left Y-axis for the scale, while the dotted curve represents the temperature of the sample and uses the scale of the right hand Y-axis. Note that the temperature reaches 1000°C at 25 minutes and was held constant for a further 15 minutes. The lower curve, Box (B), shows the rate of percentage mass change per second during the analysis. It can be seen that there are three

distinct temperature ranges during which the mass loss was rapid and that even after 10 minutes at 1000°C a slow mass loss was still observed. The residual mass at the end of the analysis was 30%, which is very high for a pure organic compound.

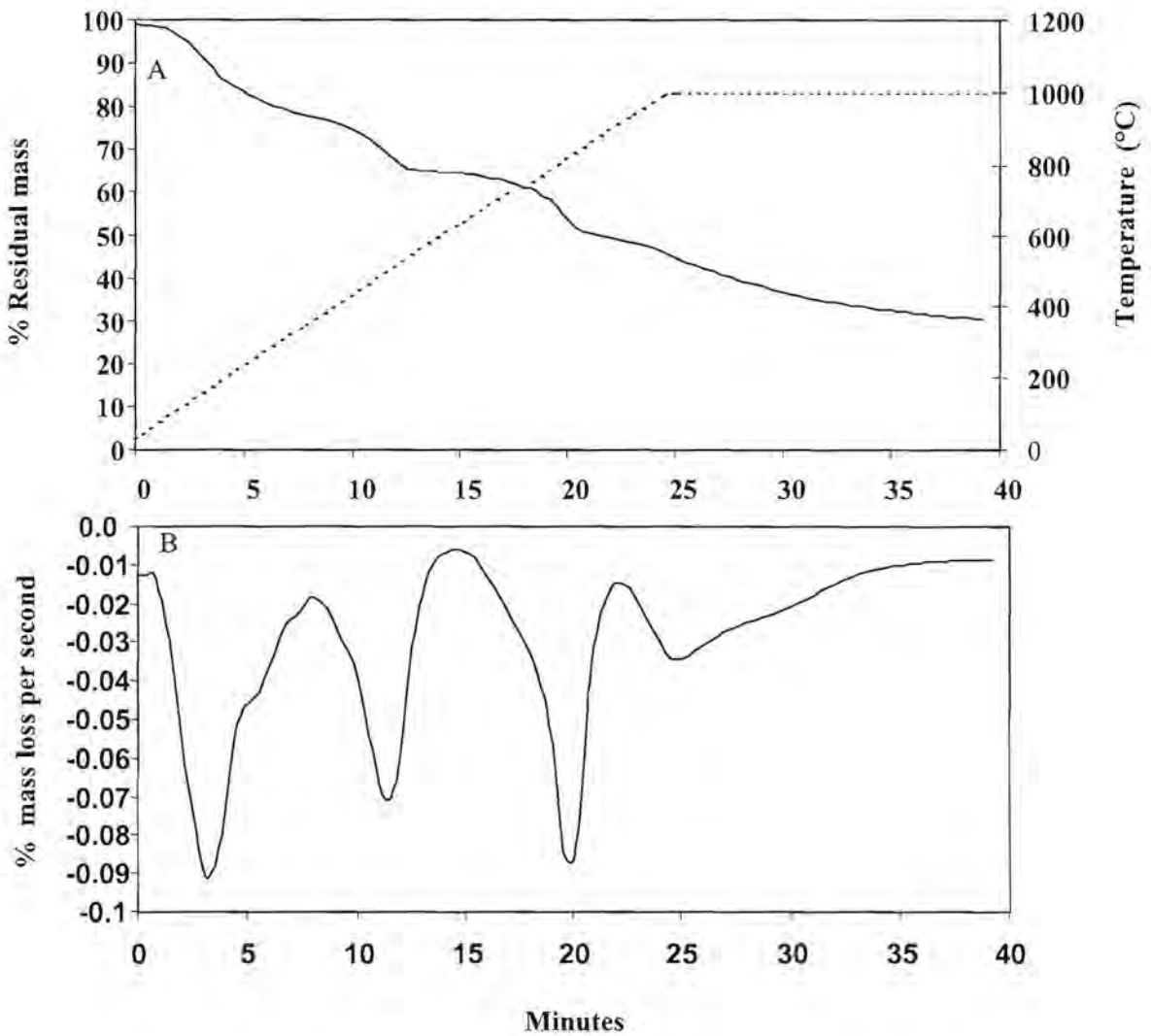

Figure 2-2: A graphic presentation of thermogravimetric analysis parameters for the THF soluble sub-fraction of oxihumate. The solid line in Box A shows the percentage residual mass, using the left ordinate for scale, of the sample while the dotted curve shows the actual temperature of the sample using the right side ordinates scale. The curve in Box B shows the rate of % mass lost per second during heating verses time. Note that there is still significant mass loss for at least 10 minutes after reaching 1000°C at 25 minutes.

Table 2-1: Table summarizing the ash content of different oxihumate sub-fractions. The data is from 5 determinations for each fraction except for the last two sub-fractions where analysis was by thermogravimetric analysis and only two analyses were done (indicated in brackets).

Sub-Fraction	**% Ash**
Potassium Oxihumate (starting material)	26 – 32%
Water-soluble fraction	25 – 34%
Acid precipitate	20 – 22%
Acid soluble fraction	67 – 90%
60% methanol precipitate	17 – 20%
60% methanol soluble fraction	16 – 20%
THF insoluble fraction	13.7% (2)
THF soluble fraction	30.1% (2)

3.4 Sub-fractionation of the humic acid fraction of Oxihumate

The sub-fractionation of oxihumate was found to be fairly reproducible with respect to yields when carried out slowly. Rapid pH changes or organic solvent additions tended to cause a rapid coagulation of the solutions and very little material was then left in solution. Initially triethyl amine salts were made but it was found that later removal of the triethyl amine, which was toxic to the cells used in the bioassay, was very difficult and tedious when compared to ammonia. The most variable fraction was the acid soluble or fulvic acid fraction but this could have been due to large percentages of inorganic material in the fraction. The percentage weight of the seven different fractions and some of the physical characteristics is presented in Table 2-2 below.

Table 2-2: The mass percentages of the different sub-fractions of oxihumate are presented as a percentage of oxihumate and of the fraction from which the sub-fraction was isolated. The physical colour and appearance of each fraction is also indicated

Fraction	Percent of Oxihumate	Percent of parent fraction	Physical appearance
Oxihumate	100	100	Dull black powder
Water soluble fraction	36 - 42	36 - 42	Matt black powder
Acid soluble fraction	6 - 12	25 - 34	Light brown sticky crystals
Acid insoluble fraction	26 - 31	63 – 72	Black powder with a sheen
60% Methanol soluble	3 - 6	15 – 20	Thick dark brown oil
60% Methanol insoluble	20 – 22.5	82 - 90	Lustrous black chunks
Tetrahydrofuran insoluble	1 - 4	20 - 30	Thick dark brown tar
Tetrahydrofuran soluble	2 – 5	72 - 80	Thick red brown grease
Diethyl ether soluble	0.5 – 0.8	12 - 16	Light -orange brown oil

3.5 Thin Layer Chromatography

Thin layer chromatography was the most successful method of quickly gaining information about various fractions or sub-fractions of oxihumate. Several of the separated compounds were visible due to their intense dark colour but many other compounds could not be identified without further visualising techniques. The separated compounds were generally resistant to charring by acids. However, the use of fluorescent TLC plates and UV light inspection permitted detection of several compounds that could not be visualized with general spray reagents. Many compounds in oxihumate showed native fluorescence under long UV light (360nm), varying in colour from bright blue through to a dirty pink. A green and a white fluorescent compound were detected in the THF soluble

fraction. The colour of the fluorescence appears to be dependant to some extent on the concentration of the compound in the separated spots with the more concentrated compounds tending to show dark orange to brown fluorescence.

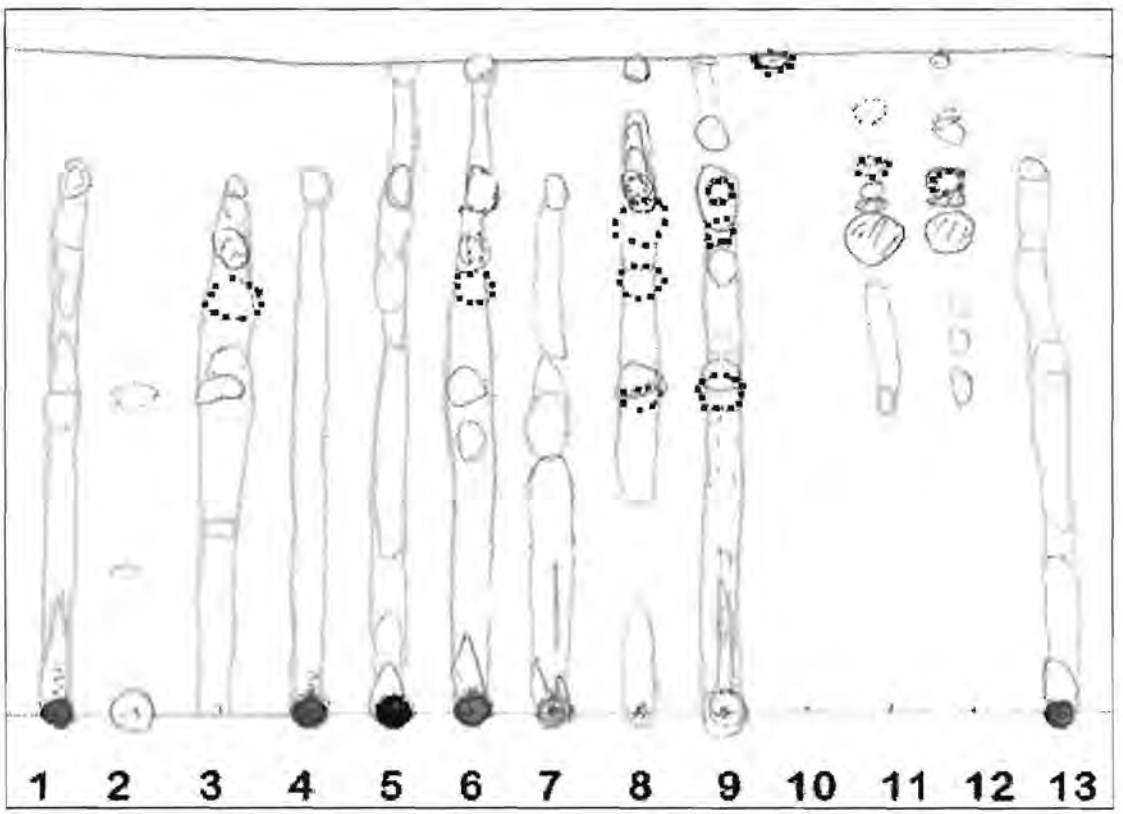

Figure 2-3: TLC separation of the sub-fractions of oxihumate on silica 60 using the methanol: chloroform:butanol:water:acetic acid system. The areas marked by a dotted line stain with I_2 vapour. The stripped areas are strongly blue fluorescent under both 254nm and 360nm light. Lanes are 50µg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

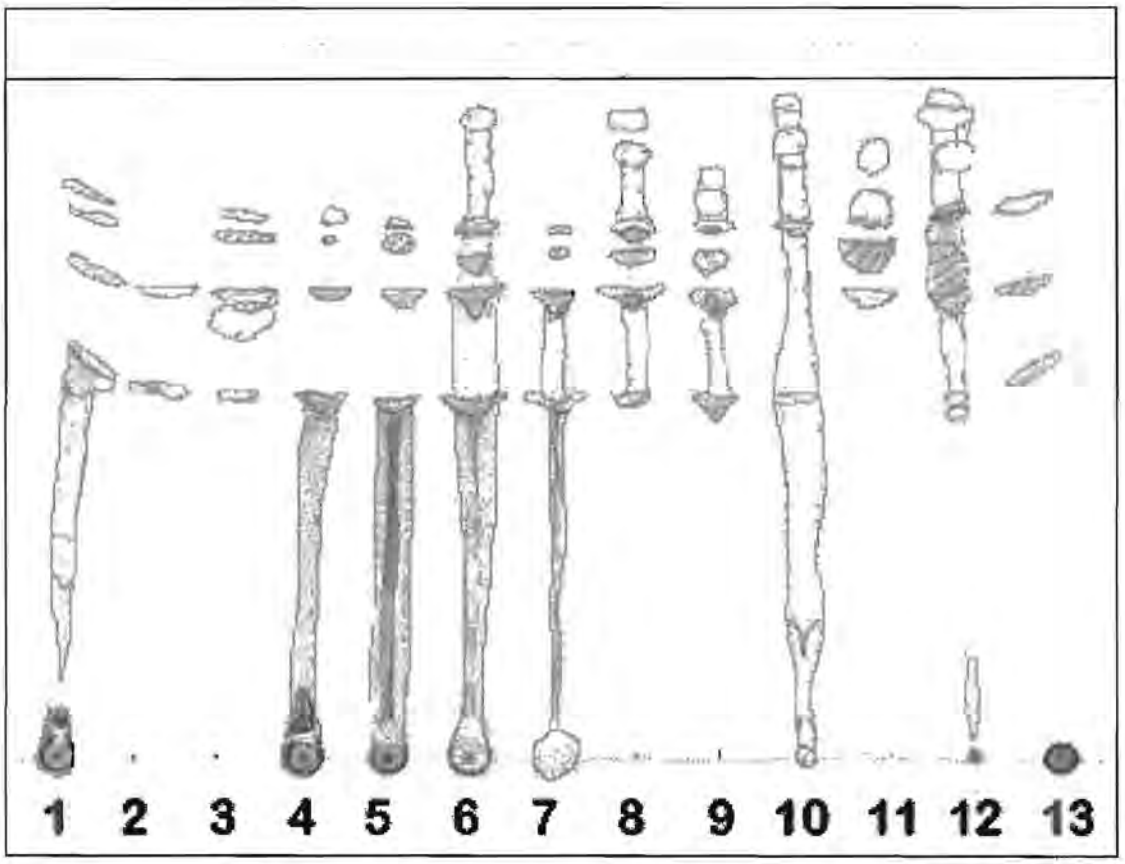

Figure 2-4: TLC separation of the various solvent sub-fractions of oxihumate on silica 60 using the acetonitrile:water:NH4OH system. The areas marked by dotted lines stain with I_2 vapour. Lanes are 50µg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

Typical TLC separations run under different conditions are shown in Figures 2-3 through 2-5, which highlights the differences between the different solvent systems that proved most useful in the separation of the various sub-fractions of oxihumate. The acid solvent system did not move any of the dark compounds from the origin and the non-polar compounds present in the sub-fractions were well resolved with *Rf* values between 0.4 and 0.75. The more polar compounds tended to streak in this development system and were seen as weakly dark-orange fluorescing streaks.

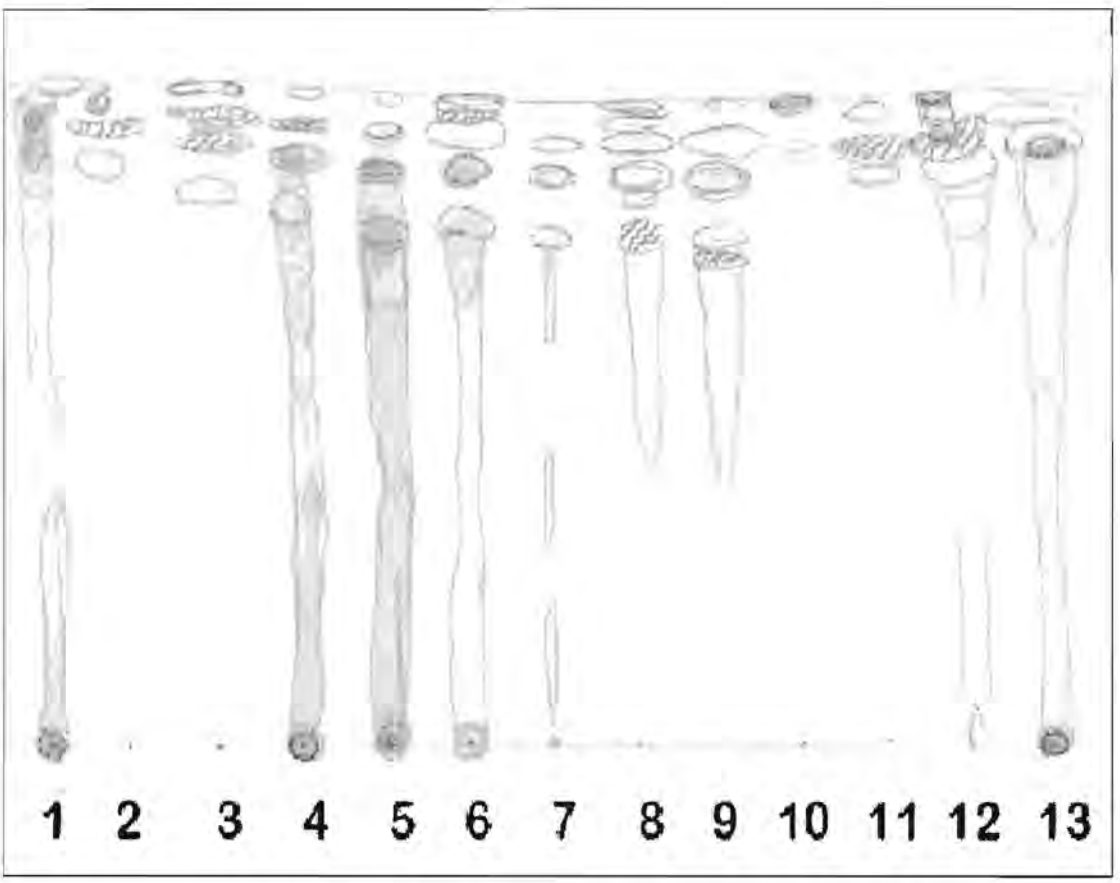

Figure 2-5: TLC separation of the various solvent sub-fractions of oxihumate on silica 60 using the NH4OH propanol system. Note that the majority of the separated compounds run at Rf values of 0.75 and above. Some of the dark coloured compounds have also moved from the origin although they streak severely. Lanes are 50µg of: 1, oxihumate; 2, H^+ soluble fraction; 3, methanol soluble from H^+ fraction; 4 H^+ precipitated fraction; 5 MeOH precipitated fraction; 6, MeOH soluble fraction; 7, THF precipitated fraction; 8, THF soluble fraction; 9, Ethyl acetate soluble fraction; 10, petroleum ether soluble fraction; 11, fraction of combined blue fluorescent compounds; 12, chloroform soluble compounds; 13, oxihumate. Lanes 1 & 13 are the same except that lane 1 was not dried prior to developing.

The alkaline solvent system using acetonitrile:water:ammonium hydroxide appeared to create three solvent fronts on the plates and there were a number of compounds that would move with these apparent fronts. The non-polar compounds tended to run in the zone above the solvent fronts with the fluorescent compounds moving on these pseudo- fronts. There was development of the brown coloured compounds from the origin of the dark coloured sub-fractions but not from the water-soluble oxihumate starting material, even when the applied spot was not permitted to dry on the origin. The dark compounds tended to streak severely and were found mostly at *Rf* values of less than 0.5.

The highly alkaline solvent system using ammonium hydroxide and propanol tended to move more of the dark coloured compounds but at the same time resolution of the less polar compounds was lost with most of these compounds having *Rf* values of 0.75 and above. Many of the dark compounds could be separated into individual spots but there was still a streak of dark colour from the origin to the slowest moving compounds at about *Rf* 0.75. The majority of the dark compounds in unfractionated oxihumate were again not moved from the origin, although there were two dark compounds running at *Rf* 0.89 and 0.94.

Few of the compounds in oxihumate stained with iodine vapour and only a few of the very lipophilic compounds could be visualized with vanillin/H_2SO_4 spray reagent (see Figure 2-6). Charring with sulphuric or chromic acid revealed few compounds, and essentially the same compounds that were visualized by vanillin/sulphuric acid.

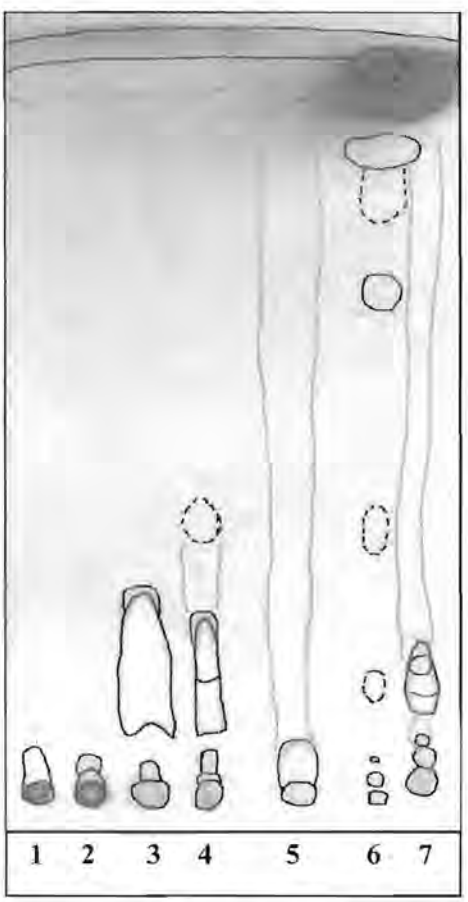

Figure 2-6: A TLC separation of various oxihumate sub-fractions developed using the acetonitrile :water:ammonium hydroxide system and visualized with vanillin/sulphuric acid. Note that only the petroleum ether soluble sub-fraction contains long-chain aliphatic compounds in detectable quantities. Lanes were 50µg of: 1, oxihumate; 2, acid precipitated fraction; 3, MeOH soluble fraction; 4, acetone soluble fraction; 5, chloroform soluble crystalline fraction; 6, petroleum ether soluble fraction; 7, chloroform soluble oil fraction.

3.6 High Pressure Liquid Chromatography

HPLC separations of humic acids on reverse phase columns varied according to the pH of the eluent. A significant fairly sharp pass-through peak (sometimes followed rapidly by a second sharp peak) dominated the first minutes of the chromatogram and was followed later by a large, broad, almost featureless peak eluting over an extended period of several minutes and which was the major feature of the chromatograms of most of the oxihumate sub-fractions when using the alkaline elution system. Several undetected UV transparent compounds were found to elute after this major peak when a fraction collector was used to collect the eluent and the fractions dried under a stream of air. These compounds were found to be waxy lipophilic compounds undetected by monitoring the eluent at a wavelength of as low as 220nm. The dominant peak was a very dark coloured compound or mixture of compounds.

When using the acidic elution system the pass-through peak was very small to insignificant. The main dominant peak that eluted from about 10 minutes and later was better resolved when using the acidic elution than the alkaline conditions, yet still appeared as a broad flat peak with small resolved peaks superimposed on it.

Figures 2-7 and 2-8 illustrate typical chromatograms of the water-soluble fraction of oxihumate separations on the same reverse phase column under the acidic and alkaline elution conditions as described in the materials and methods section above. The chromatograms shown are superimposed traces of a fluorescence detection channel and the two separate UV detection channels at 230±15nm and 270±20nm respectively.

In contrast to previous reports, rechromatography of single peaks isolated from the fractionated HPLC eluent resulted in essentially the collected peaks being eluted, although these peaks appear to be no better resolved than in the original separation of the oxihumate itself. Figure 2-9 clearly illustrates this phenomenon for four of the recombined HPLC fractions collected. TLC analysis of these isolated fractions on normal phase silica plates did however indicate that several components are still present in all these peaks, confirming that they are still not pure compounds.

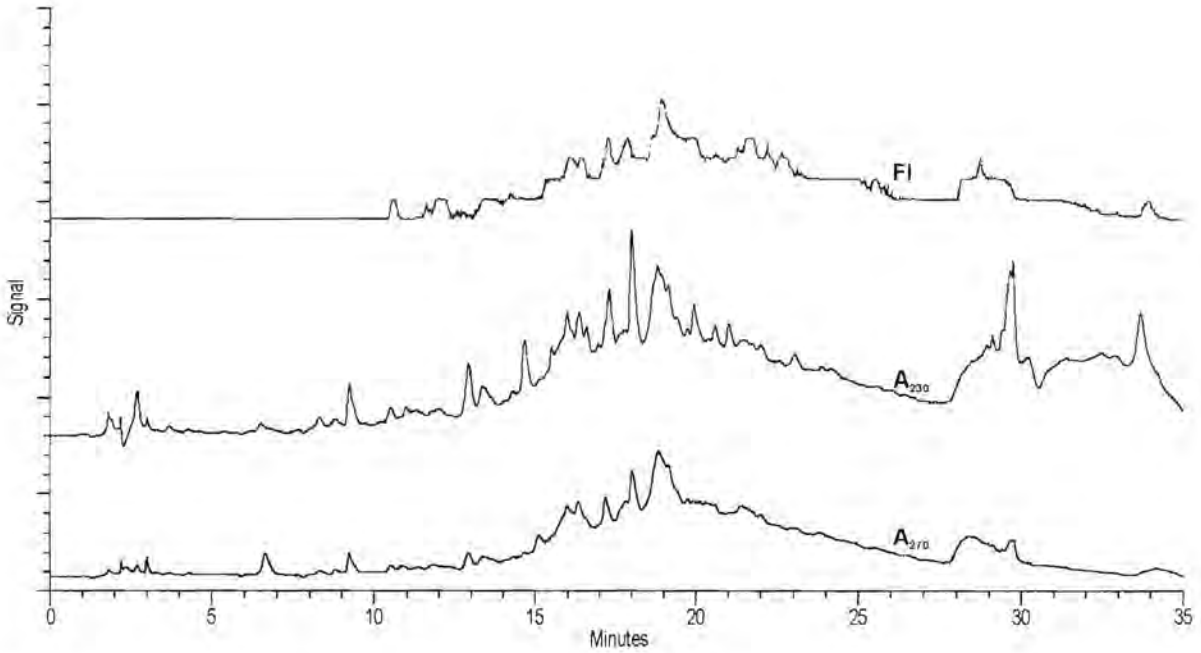

Figure 2-7: HPLC separation of the water-soluble fraction of oxihumate on a C_{18} column using the 0.1% TFA buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). A new C_{18} column was used for this separation.

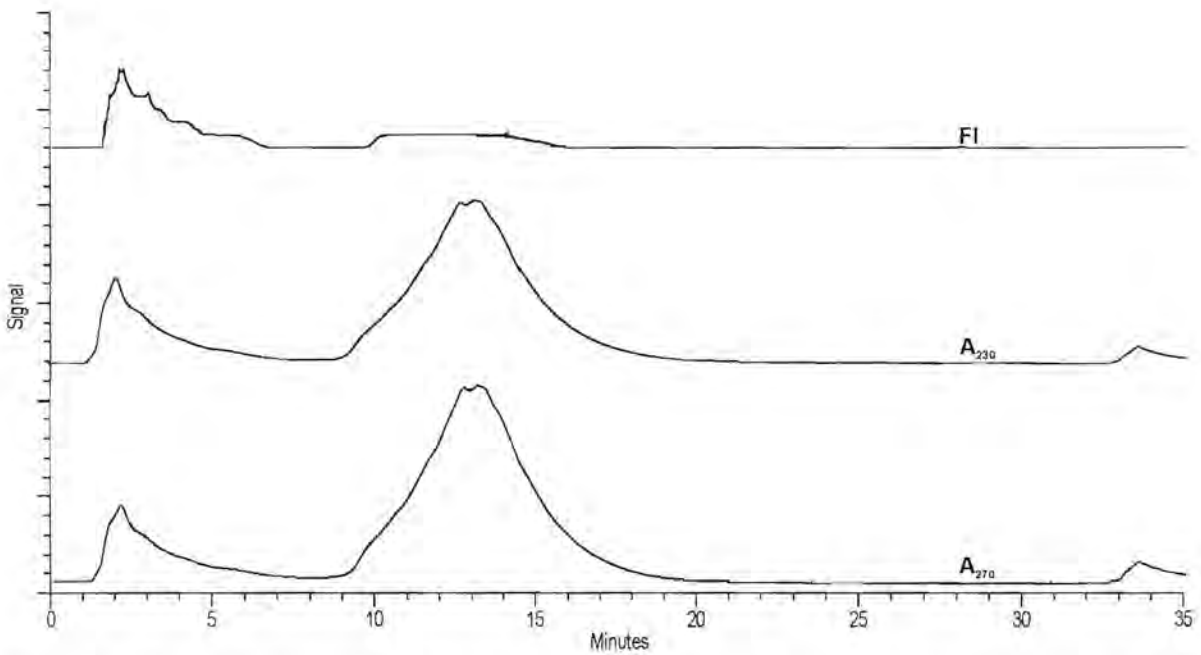

Figure 2-8: HPLC separation of the water-soluble fraction of oxihumate on a C_{18} column using the pH 8 buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The same column as used for the acidic system in Figure 2-7 was used for this separation.

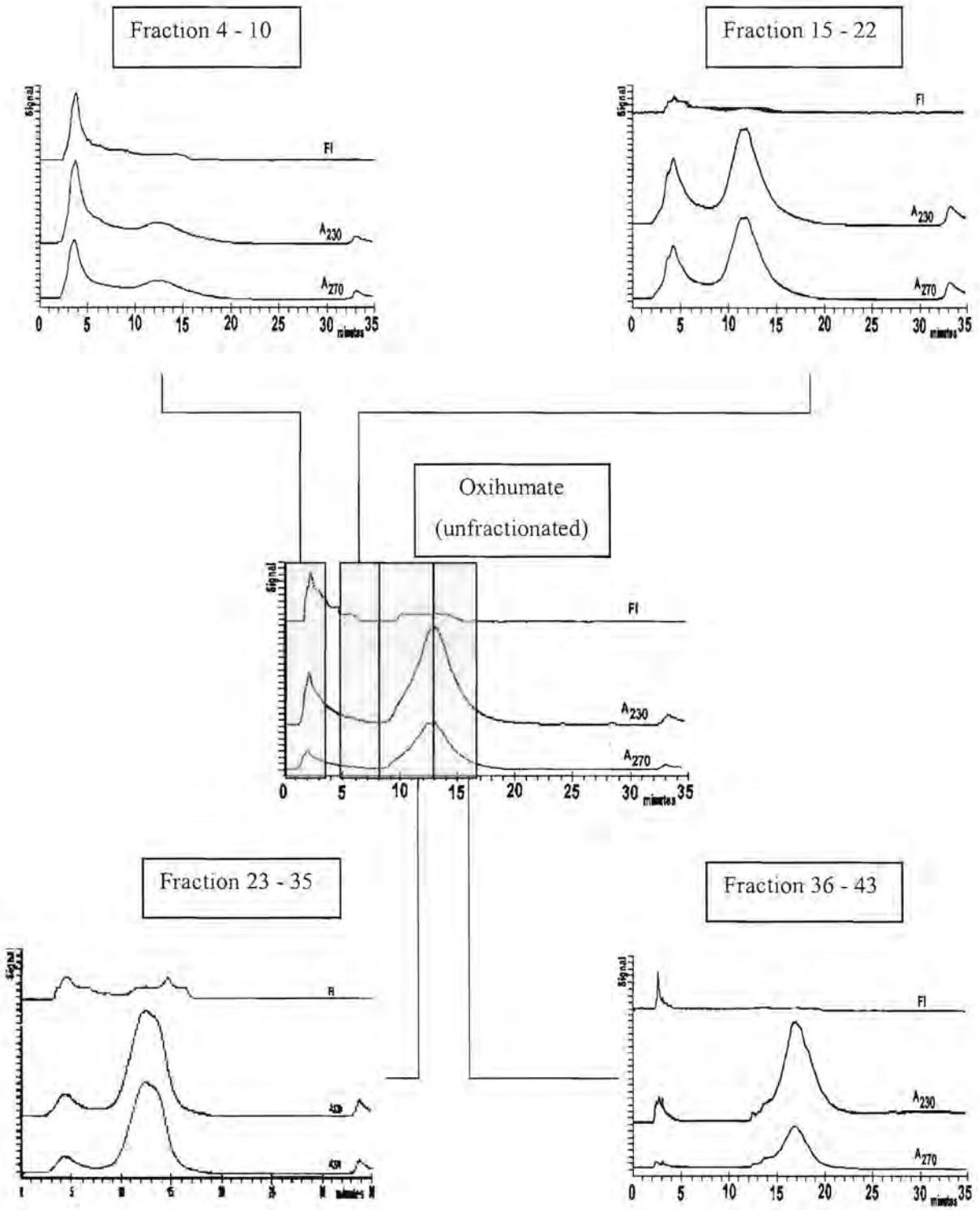

Figure 2-9: Chromatograms demonstrating the separations achieved when re-chromatographing fractions collected during the HPLC separation of oxihumate (centre chromatogram). The chromatograms are labelled with the fraction numbers that were combined before rechromatography.

Figure 2-10 illustrates a TLC separation of the isolated HLPC eluted peaks and it can be seen that there are still several compounds in each of the isolated HPLC peaks.

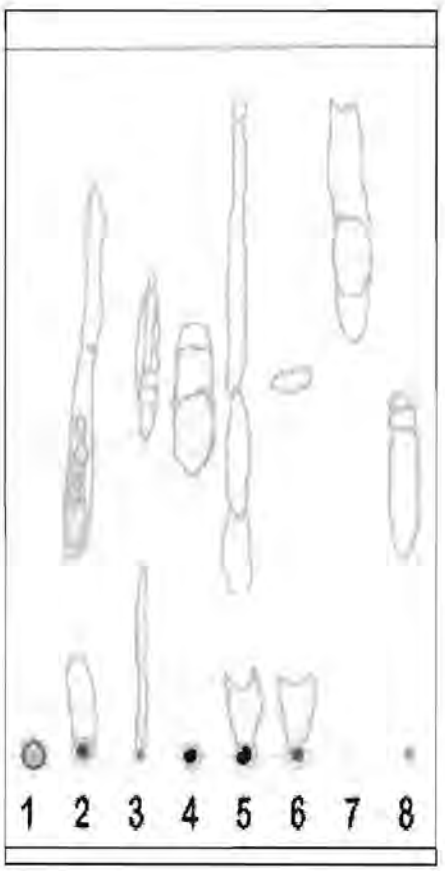

Figure 2-10: A TLC separation of humic acid fractions eluted from a C_{18} HPLC column. Each lane has 50µg of the following sub-fractions 1, oxihumate; 2, fractions 4 – 10; 3, fractions 11 – 15; 4, fractions 15 – 23; 5, fractions 24 – 34; 6, fractions 35 – 44; 7, fractions 45 – 66; 8, column wash.

Sub-fractions isolated from oxihumate by differential solubility were also separated by HPLC using ODS columns and were found to have most of the peaks seen in the chromatograms of the starting material although the ratios of the peak areas varied greatly. The more lipophilic fractions were free of the pass-through peaks but the more polar fractions always exhibited lipophilic peaks. Figures 2-11 through 2-17 illustrate the HPLC reverse phase separations of the seven oxihumate sub-fractions isolated by differential solubility. Differences in peaks that eluted at similar retention times but occurring in chromatograms of different sub-fractions could sometimes be demonstrated by the differences in the fluorescent properties of the peaks.

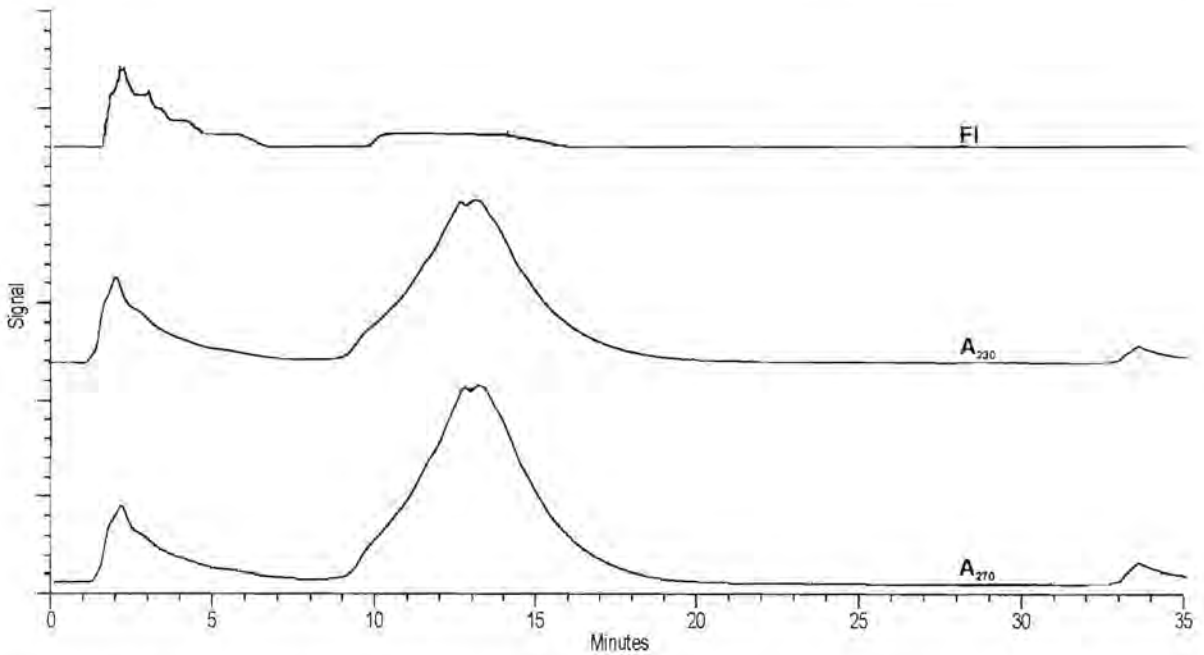

Figure 2-11: HPLC separation of the water-soluble fraction of oxihumate (1) on a C_{18} column using the pH 8 buffer system. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$).

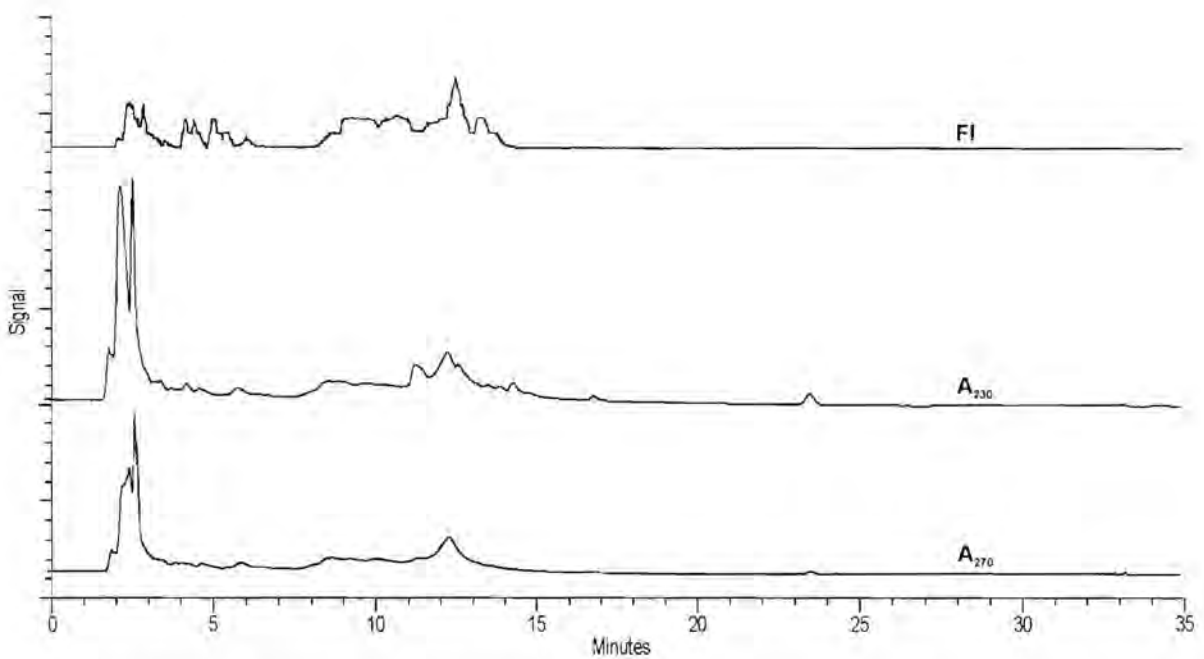

Figure 2-12: HPLC separation of the acid soluble fraction of oxihumate (2) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The dominant humic acid peak does not appear in this chromatogram.

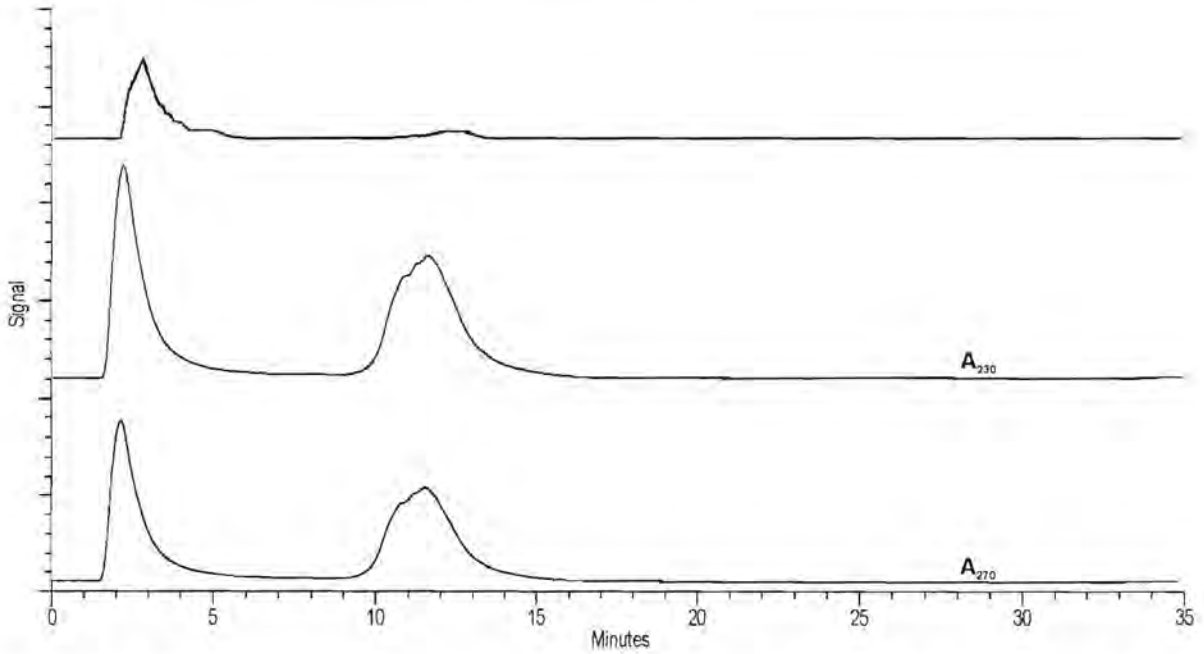

Figure 2-13: HPLC separation of the acid insoluble fraction of oxihumate on a (3) C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The UV traces show a narrower main peak than that of the water-soluble fraction and the fluorescent peak on the void appears as the major peak.

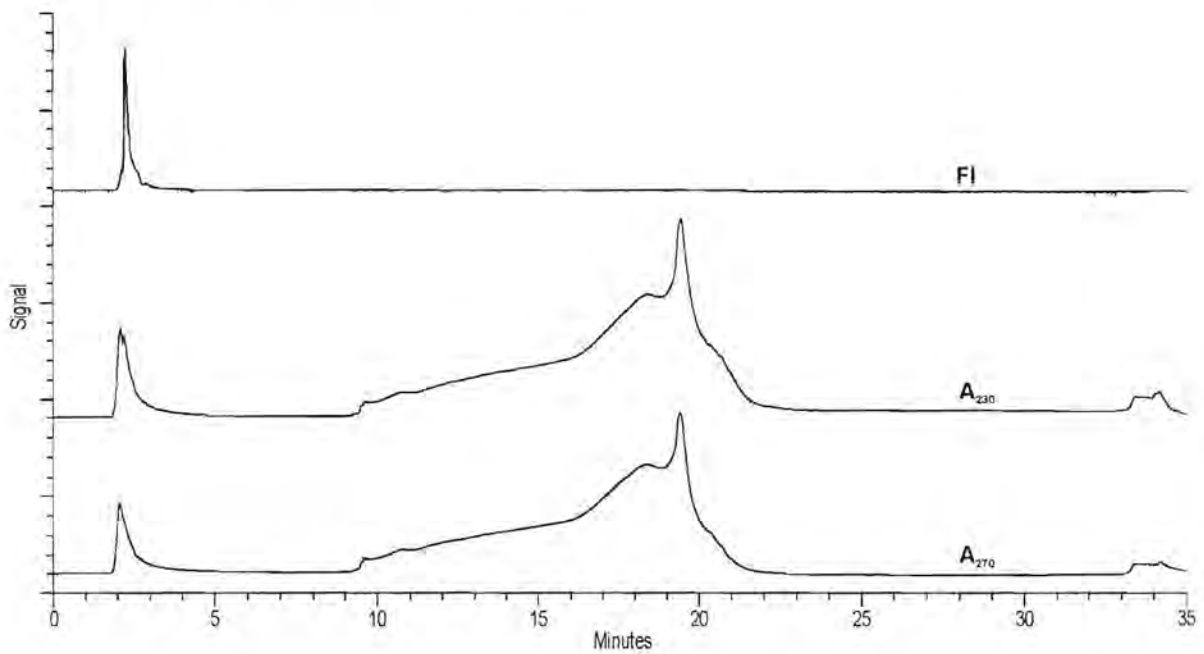

Figure 2-14: HPLC separation of the 60% methanol insoluble fraction of oxihumate (4) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The only fluorescent compounds elute on the solvent front and the dominant peak of the humic acids is present with a sharp peak superimposed on this peak.

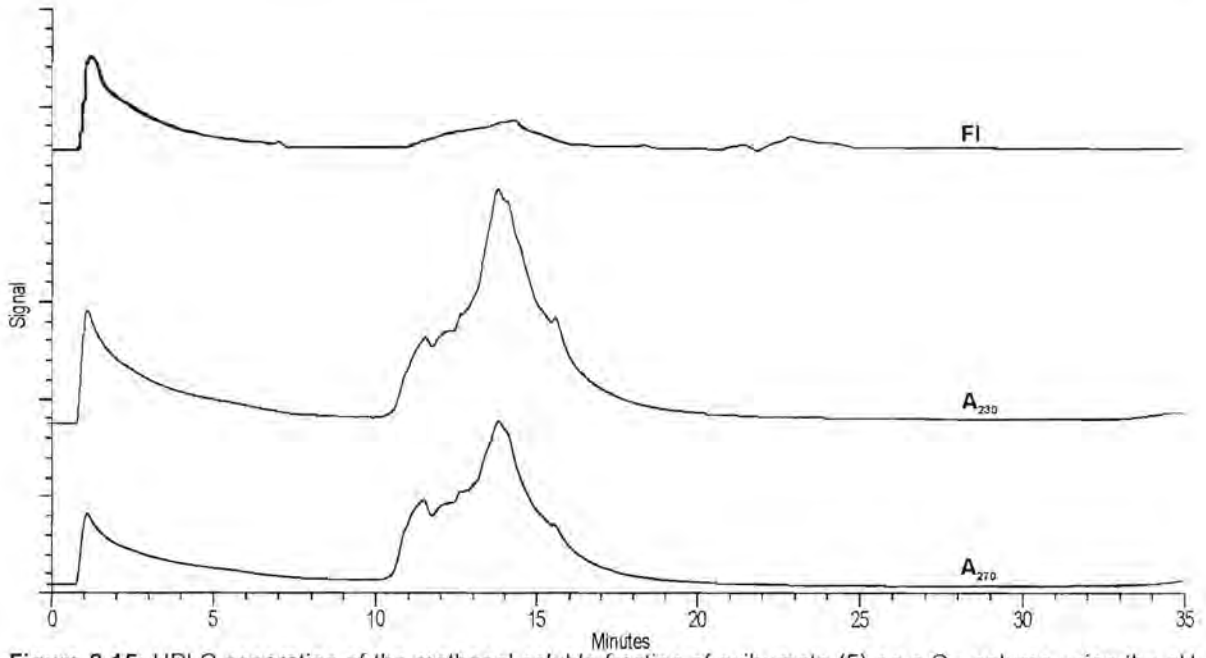

Figure 2-15: HPLC separation of the methanol soluble fraction of oxihumate (5) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The main fluorescent peak elutes on the solvent front with a broad unresolved non-fluorescent peak elutes between 10 and 15 minutes.

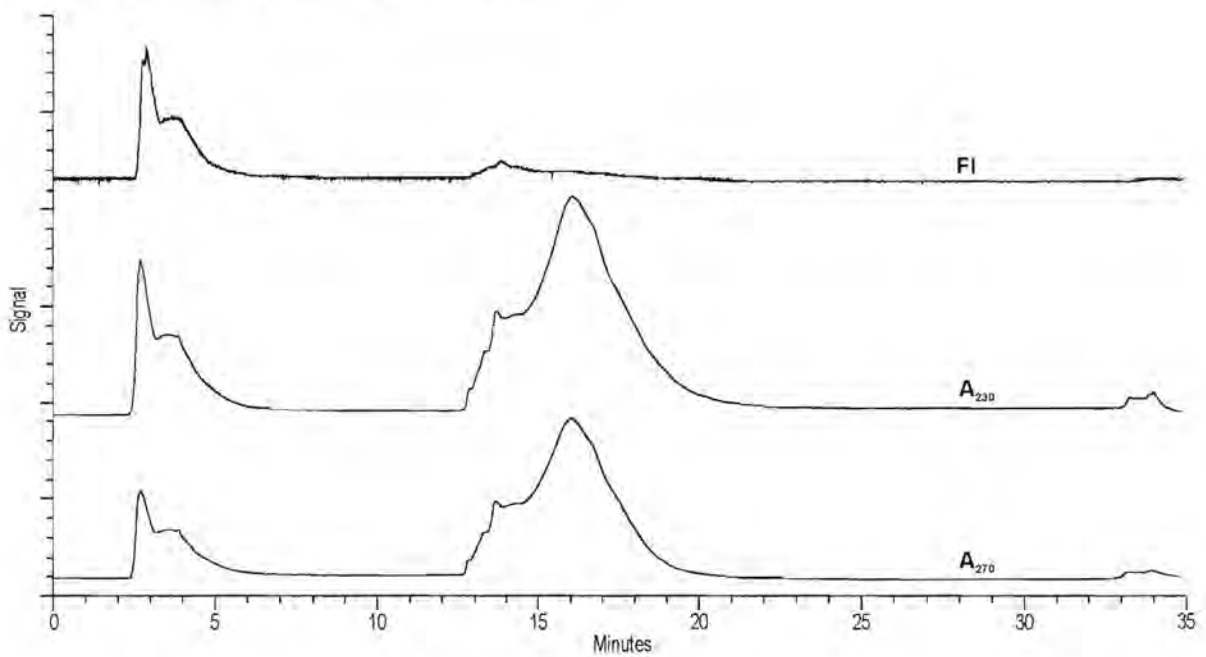

Figure 2-16: HPLC separation of the tetrahydrofuran insoluble fraction of oxihumate (6) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (Fl); UV at 230 ($\mathbf{A}_{230}$) and UV at 270nm ($\mathbf{A}_{270}$). The dominant peak is again the large peak eluting between 12 and 20 minutes – a shift in elution times of the other dominant peak of the fractions. A sharp fluorescent peak elutes on the solvent front implying the presence of polar compounds.

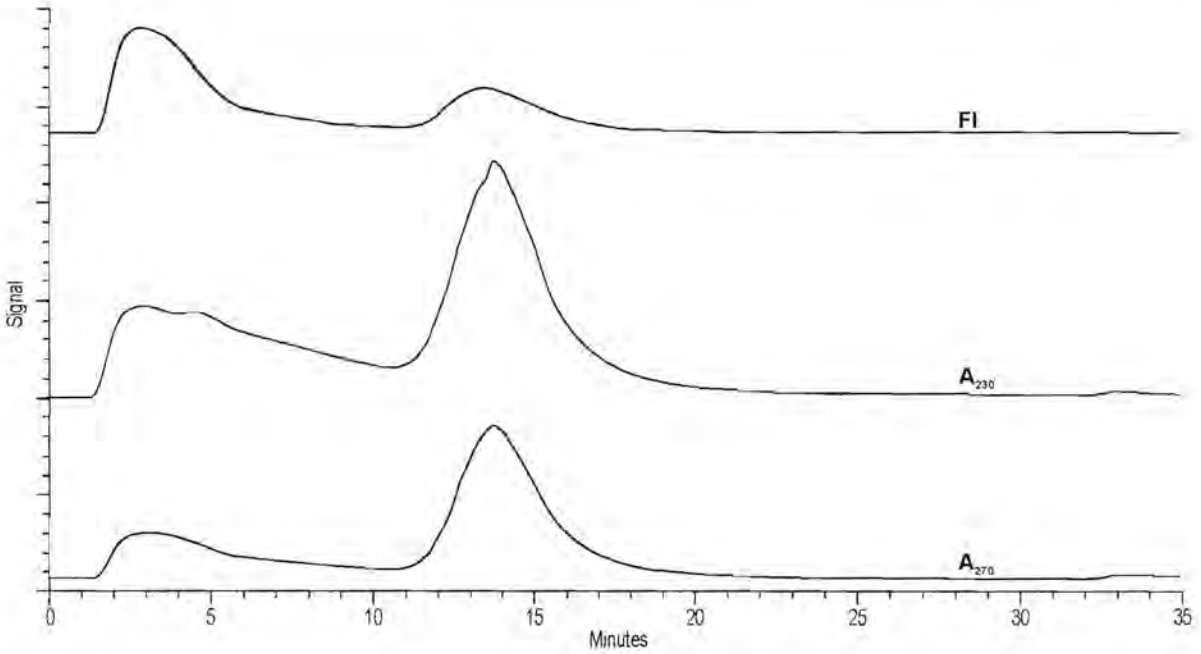

Figure 2-17: HPLC separation of the tetrahydrofuran soluble fraction of oxihumate (7) on a C_{18} column using the pH 8 buffer system for elution. The traces are from top to bottom: fluorescent detector (FI); UV at 230 (A_{230}) and UV at 270nm (A_{270}). The dominant peak appears sharper and elutes slightly earlier than the THF precipitated fraction. An early eluting strongly fluorescent peak is present in addition to the main peak.

3.7 Solid Phase Extraction

The results were a combination of the eluents from five SPE cartridges as initial results indicated that the method could not give accurate results when the recovered material was less than 1mg as the weighing error then became too large.

The recoveries of the controls where samples were added to the mobile phase and dried were all more than 100%. The acid eluent recovery from the SPE cartridges was about 60 – 70% without correcting for any increase in the recovery seen in the control samples. The results are summarized in Table 2-3 below.

Negligible amounts of dark coloured compounds were recovered from the organic solvent washes of the cartridges although a transparent waxy residue accounting for about 20% of the mass was recovered in this eluent. Some dark compounds were eluted by the ammonium hydroxide:acetonitrile solution, which were clearly visible, but the recovered mass was too small to determine. Interestingly, elution of the cartridges with the same eluent after a further 24 hours displaced more of the dark colour but could not displace all the retained coloured material.

Table 2-3: Summary of the recoveries of the water-soluble oxihumate sub-fraction from C_{18} SPE cartridges. The controls were performed by adding 1.0ml sample to the acidic eluent and drying the samples as for the SPE eluents. The experiments are all a combination of the eluents from 5 separate SPE cartridges.

	Acid elution (%)	**Organic phase elution (%)**	**Ammonium hydroxide: acetonitrile (%)**
Control 1	122	-	-
Control 2	132	-	-
Control 3	116	-	-
Experiment 1	62 Dark brown	16 White wax	Too small to determine Dark brown/black
Experiment 2	72 Dark brown	20 White wax	Too small to determine Dark brown/black
Experiment 3	66 Dark brown	20 White wax	Too small to determine Dark brown/black

3.8 Ultraviolet and Visible Spectroscopy

Although limited information could be derived from the UV/visible spectra it was found that there were some distinct differences in the spectra of the different sub-fractions derived from the same humic acid starting material. The dilution factors required to ensure that the absorbance at 200nm was below two Absorbance Units was different for each fraction and is summarized in Table 2-3. The $E_{4/6}$ absorbance ratios were calculated for the diluted solutions after normalising the absorbance at 254nm. These ratios are summarized in Table 2-4. Although the general trends of the absorbance spectra were similar for all the oxihumate sub-fractions with the absorbance decreasing logarithmically with increasing wavelength there were distinct differences in the spectra. This is illustrated in Figure 2-18 where the UV spectra are overlaid in a staggered pattern to enable differentiation of the different sub-fractions spectra. It can be seen that the acid soluble and the THF soluble sub-fraction have a faster decrease in absorbance than all the other sub-fractions and that the THF sub-fraction has a distinct broad shoulder between 250 and 300nm. This shoulder can only just be discerned in most of the sub-fractions and does not appear to influence the $E_{4/6}$ ratio that is commonly used to characterize humic acids.

Table 2-4: Dilution factors required to normalize the absorbance at 254nm of 1mg/ml solutions and the absorbance at 465nm, 665nm and the E4/6 ratio.

Sub-fraction	Dilution factor	A_{465}	A_{665}	$E_{4/6}$
Oxihumate	24	0.135	0.054	2.513
Acid solubles	1	0.066	0.044	1.514
Acid precipitate	27	0.108	0.028	3.935
Methanol precipitate	30	0.212	0.107	1.981
Methanol solubles	12	0.117	0.055	2.108
THF precipitate	3	0.140	0.058	2.410
THF solubles	8	0.093	0.052	1.777

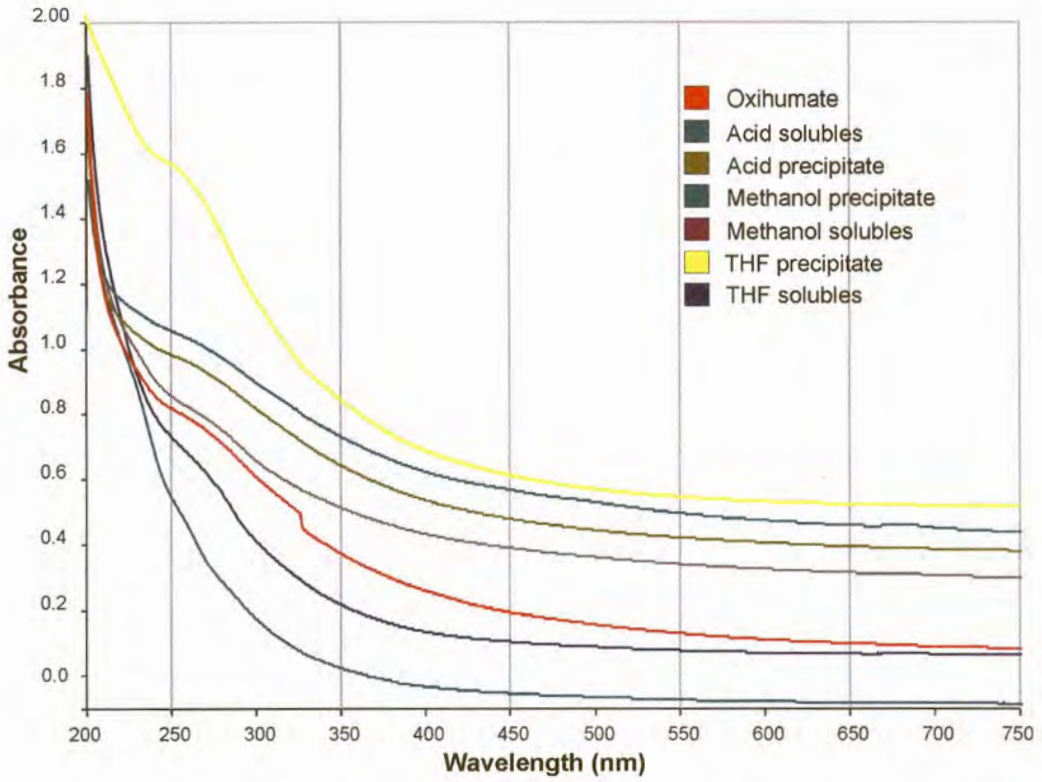

Figure 2-18: Staggered UV/visible spectra of the seven sub-fractions of oxihumate. The absorbencies were normalized at 254nm. Note the similarity and featureless absorbance above 350nm for most of the sub-fractions.

3.9 Fluorescence Spectroscopy

Fluorescence spectroscopy was performed on each sub-fraction at 36 different excitation wavelengths and a 3D emission profile generated from the individual scans using the FL WinLab software version 3.0, the same software that was used to control the instrument and collect the raw data.

It was found that the fluorescent intensity of the various sub-fractions of oxihumate varied greatly from each other although all 7 of the isolated sub-fractions exhibited a maximal emission as a broad almost symmetrical peak centred on 432nm. Figure 2-19 is an overlay of all seven isolated sub-fractions to illustrate the different fluorescent maxima when the samples were excited with light at 280nm.

The acid soluble sub-fraction, that would represent the fulvic acids, had a fairly intense fluorescence centred on 432nm with a broad shoulder appearing around 385nm when the excitation wavelength was at about 280nm.

The THF precipitated sub-fraction showed an intense fluorescence with the main emission centred slightly higher at 440nm This emission wavelength appeared to be independent of the excitation wavelength as the same emission peak was seen over a very wide range of excitation wavelengths.

The THF soluble sub-fraction was the most fluorescent sub-fraction and required a further dilution of at least 1:30 more than the other fractions to remain within the scale limits set for the remaining fractions. The main fluorescent emission wavelength for the THF soluble sub-fraction was centred on 375nm with a second emission peak centred on 432nm and a slight shoulder was seen at around 480nm.

The THF precipitated and THF soluble sub-fractions both revealed the 480nm shoulder when excited at wavelengths below 300nm. The methanol soluble sub-fraction, like the THF soluble sub-fraction, had a fluorescent emission peak centred at 375nm although it was broad and weak and was a secondary peak in the methanol soluble sub-fraction. The fluorescent intensity could be severely affected by the dark colour of the sub-fractions and the fact that this fluorescent emission falls within the region where intense UV absorption occurs. This could account for the low fluorescence displayed by the darker sub-fractions.

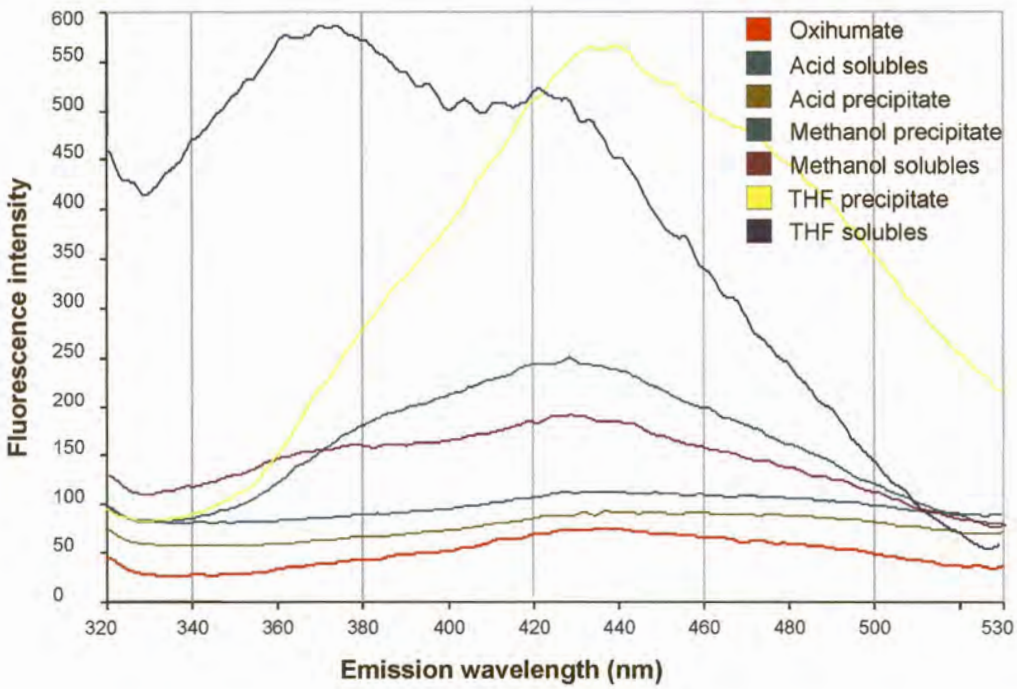

Figure 2-19: Fluorescent emission spectra of the seven sub-fractions of oxihumate, all recorded using an excitation wavelength of 280nm.

3.10 Infrared Spectroscopy

Infrared spectroscopy of the seven different sub-fractions provided the most chemical structural information. Except for the THF soluble sub-fraction, the similarity between the spectra of the different sub-fractions was obvious, with the main absorption bands appearing in all the sub-fractions.

The broad band from 3500 to 3200 cm^{-1} is due to hydrogen bonded υ(O-H) stretch of carboxylic, alcohol and phenol groups. The small bands at 2950 – 2840 cm^{-1} are the asymmetric and symmetric υ(C-H) stretch of aliphatic chains.

The intense bands at 1720 cm^{-1} υ(C=O) seen in the THF soluble sub-fraction can be seen as an insignificant inflection on the oxihumate spectrum but becomes more visible in the sub-fractions isolated from the organic solvent modified solutions. This band is assigned to the carbonyl groups of α, β unsaturated or aromatic esters or for ketones.

The very intense band with a maximum absorption between 1570 to 1685 cm^{-1} is the dominant peak in all the dark coloured sub-fractions and is probably due to υC=O of carboxylic acid salts that overlaps with the υ(C=C) of aromatic and conjugated double bonds. Alkyl chains are associated with bands that occur between 1400 - 1462 cm^{-1} for δ(CH_2) and CH_3 deformations at 1375 cm^{-1}.

Phenolics are indicated by the bands around 1250 - 1280 cm^{-1} due to υ(C-O) and the small sharp band resulting from free υ(O-H) at 3690 cm^{-1} that is at the high extreme for this stretching absorbance, occurring when there is alkyl substitution of the phenolic ring. The sharp but medium intensity bands between 1030 - 1095 cm^{-1} are due to υ(C-OH) and υ(C-O-C) of ethers and alcohols with the lower wavenumbers associated with aromatic ethers.

The combination of bands from 400 – 800 cm^{-1} would point towards terminal double bonds in alkanes or substituted phenolic compounds. As a complication, Si-C bonds and several covalent sulphur compounds demonstrate absorption in this region. All these bond types could well occur in oxihumate.

Figure 2-20 is a staggered overlay of infrared spectra of all 7 sub-fractions of oxihumate and illustrates the strong similarity of spectra, implying that the different sub-fractions must be composed of very similar structural entities. The only two spectra that have obvious differences are the acid soluble sub-fraction that would be the fulvic acid sub-fraction and the THF soluble sub-fraction. Both these sub-fractions lack the strong absorption band at 1587 cm^{-1} but do show a strong absorption band at 1720 cm^{-1} indicating the presence of carbonyl compounds that are not carboxylic salts.

The broad absorption bands, especially in the fingerprint region, would indicate that there is a mixture of compounds with similar but not identical structures. The fact that all the dark coloured sub-fractions showed an intense absorption band over a broad region from above 1720 cm^{-1} to below 1550 cm^{-1} and that the fulvic acid and THF soluble sub-fractions had less intense absorption at the upper limit of this region (above 1700 cm^{-1}) could indicate that the latter two sub-fractions account for the absorbance at the higher wavenumbers and can be extracted from the complex due to a weaker association with the bulk of the humic acid complex.

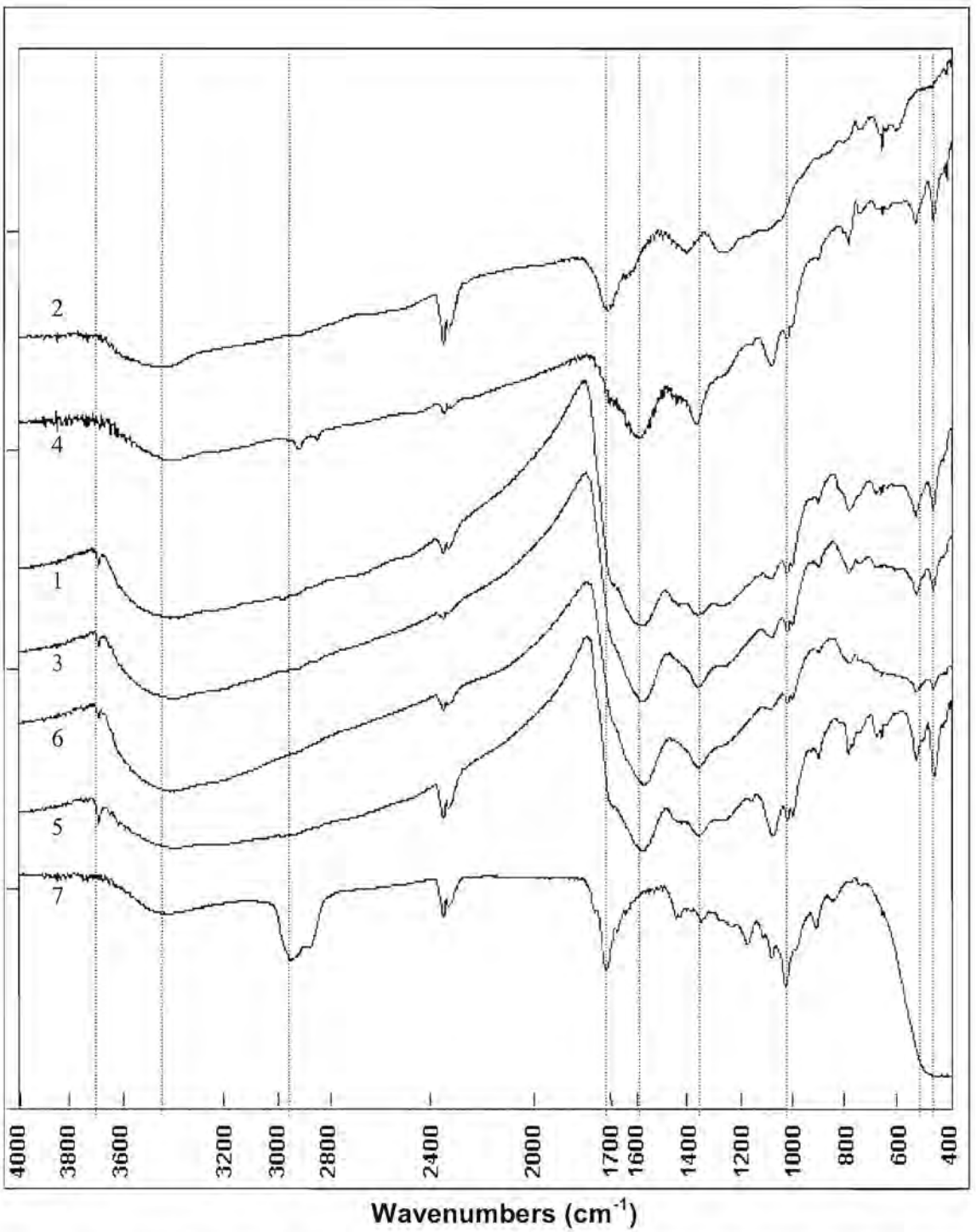

Figure 2-20: Staggered overlays of the infrared spectra of the seven sub-fractions of oxihumate. The spectra are for the following sub-fractions: 1, oxihumate; 2, acid soluble; 3, acid precipitate; 4, MeOH precipitate; 5, MeOH soluble; 6, THF precipitate; 7, THF soluble. The vertical dotted lines indicate peaks that are common to most spectra and correspond to 3695 cm^{-1}, 34 41 cm^{-1}, 2952 cm^{-1}, 1720 cm^{-1}, 1596 cm^{-1}, 1375 cm^{-1}, 1038 cm^{-1}, 537 cm^{-1}, 457 cm^{-1}. The peaks at 2358 and 2343 cm^{-1} are due to CO_2.

3.11 Raman Spectroscopy

Raman spectroscopy did not reveal any useful information with regard to chemical structures or functionality. A problem experienced with this technique was the intense fluorescence exhibited by the oxihumic acid under the argon ion laser. The greater the fluorescence the less defined the spectra became. Another observation was that the small area of the samples of the oxihumate sub-fractions exposed to the intense laser beam appeared to bleach to an off-white colour during the analysis.

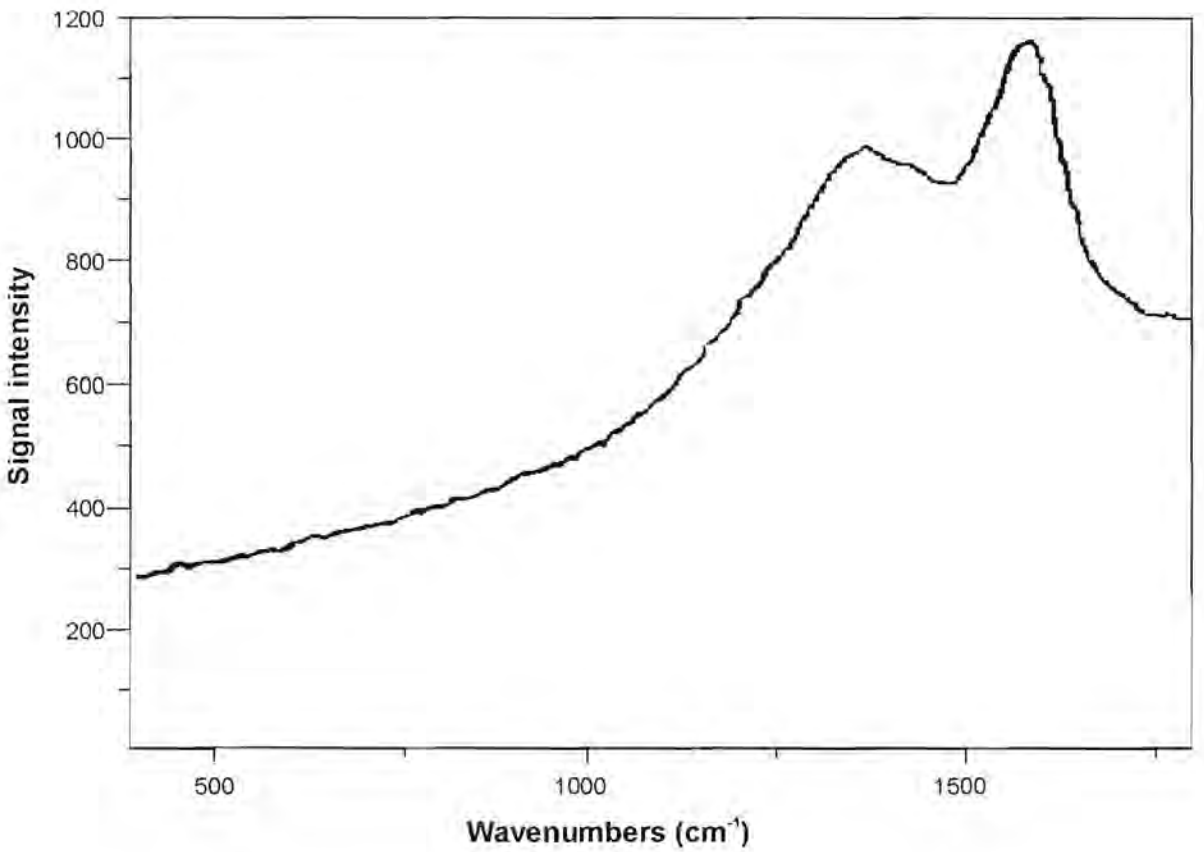

Figure 2-21: A typical Raman spectrum of the water-soluble fraction of oxihumate. Note that the peaks are relatively broad and that there is an apparent baseline drift.

The Raman spectra of all the fractions revealed only two fairly broad bands centred around 1370 and 1591 cm^{-1} and a typical trend of decreasing background absorbance with decreasing wavenumbers. These bands can be assigned to polycondensed aromatic functionality and amorphous carbon structures. The fact that the relative intensities of these absorption bands in the different isolated sub-fractions changed only marginally again implies that the basic chemical structure of all the sub-

fractions is very similar. The acid soluble fraction exhibited a slightly different Raman spectrum that could be explained by the very intense fluorescence and high ash content implying a high inorganic salt content. A typical Raman spectrum of water-soluble oxihumate is presented in Figure 2.21.

3.12 Energy Dispersive Spectroscopy (EDS)

EDS analysis of the ash obtained from the various isolated sub-fractions revealed a high content of potassium and silica in most of the fractions. The major salts that were detected in the samples were aluminium, silica, sulphur and potassium. Minor components were chloride, sodium, titanium and iron.

The elements found in the different fractions varied from sub-fraction to sub-fraction but it was also found that there was a fairly large variation within the same sample due to the small area of the ash particle that could be analysed at a time. A typical EDS spectrum of an ash sample is illustrated in Figure 2-22. The results of the elemental composition of the ash from the different isolated sub-fractions is summarized in Table 2-5.

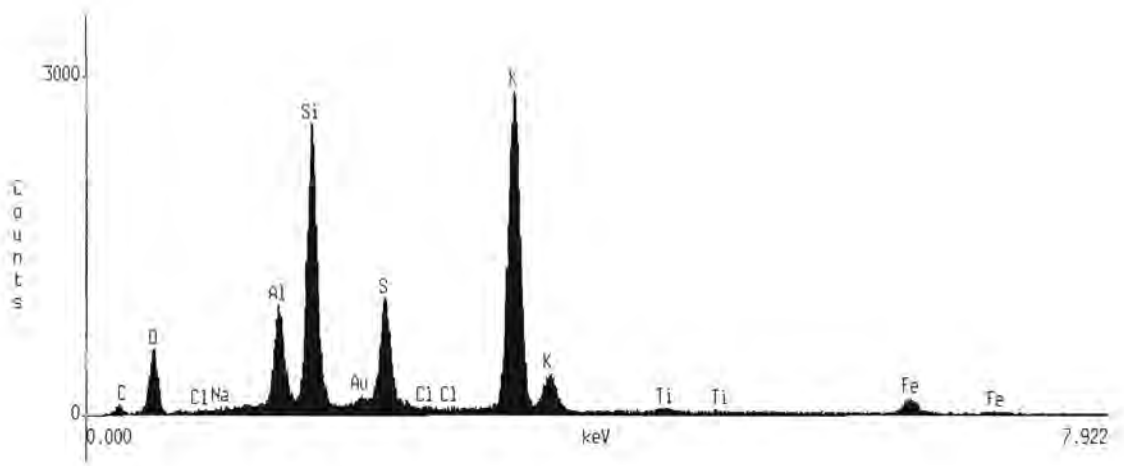

Figure 2-22: A typical EDS spectrum of an ash sample, in this case of the methanol precipitated sub-fraction of oxihumate. The area analysed per observation is about 100 x 100 µm. Note that there are two emission lines for most elements being the k and the l x-ray lines.

Table 2-5: A summary of the percentage inorganic element composition of five of the different isolated sub-fractions as determined from their ash. The ash was obtained by heating the samples in a muffle furnace at 500C and 850°C as described in the ashing method. Samples were analysed by EDS in several different areas to obtain an average element composition. Only the detected elements are reported in the table. ND: not detected.

Sample / Element	237 H2O sols	H+ sols	H+ ppt	MeOH ppt	MeOH sol
Na	0.08	ND	0.04	0.14	0.05
Mg	ND	ND	0.01	0.03	ND
Al	1.10	0.08	0.35	1.98	1.76
Si	6.93	0.03	4.68	6.29	4.21
P	ND	ND	ND	0.02	0.02
S	1.83	0.64	0.66	0.46	1.79
Cl	0.17	39.58	3.29	0.24	0.04
K	20.42	43.54	11.07	6.23	7.65
Ca	0.10	0.35	0.11	0.09	0.08
Cr	ND	ND	0.11	0.01	ND
Fe	1.03	0.01	1.26	2.23	1.19
Ti	0.14	ND	0.12	0.31	0.18
Percent ash	31.8	84.2	21.7	18.0	17.0

4 Discussion

Humic acid structure has been an elusive parameter to characterize despite the introduction of new technologies and instrumentation over the last 50 years. Arguments have been put forward as to the structure, origin and mechanism of formation but consensus has not yet been reached (Hayes, 1998; Novac *et al*, 2001; Wershaw, 1993; Wilson *et al*, 1987). One popular hypothesis at present is that humic acid is in fact a complex mixture of chemical compounds that are strongly associated or weakly bound to each other (Conte & Piccolo, 1999; Piccolo & Conte, 2000). The original material from which the humic acid was formed would determine to a large extent which compounds would most likely be found in the humic acid complex and conditions of formation would determine the physical characteristics (Ziechmann *et al*, 2000). The environment and conditions to which the humic acid mixture has been exposed would also play a role in the final chemical compounds

present, the minerals incorporated and other inclusions found in the humic acid complex and therefore play a role in their physical properties. The data presented in this study supports the hypothesis that humic acid is a complex of many different compounds held together in a tight association.

The relatively high ash content and the refractory behaviour of the humic acid to combustion at temperatures (as high as 1200°C) indicate that there is probably some highly stable condensed aromatic compounds or volatile inorganics in the humic acids, especially in oxihumate, which is derived from coal by a relatively mild oxidation process. Thermogravimetric analysis has revealed that there is a fairly large percentage of refractory organic matter that requires extended time at or above 1000°C before total combustion occurred. The bulk of the ash appears to be potassium salts, which would be expected as potassium hydroxide was added during the formulation of the oxihumate. Even the THF soluble fraction that had been through four solubility dependant fractionation steps of increasing concentrations of organic solvents still exhibited a high ash content of 30%. The high percentage of organic solvent in this fraction's mother liquor is unlikely to solubilize any pure inorganic salts, indicating that the inorganic ions involved must be complexed to large lipophilic organic acid compounds. The acid soluble fraction (the fulvic acids) ashed to give almost pure potassium chloride, implying that the organic material is all burned away and that the silica and alumina salts are almost quantitatively precipitated during the acid treatment used to isolate this sub-fraction. In the sub-fractions where a "glass melt" formed during the ashing process, it was found that the predominant element in the glassy residue was silica. Potassium, silicon, aluminium, sodium, sulphur, chloride and iron were the major inorganic ions found in oxihumate ash by EDS micro-assays as summarized in Table 2-5.

The percentage solubility of the various sub-fractions was affected by the rate of the change from the soluble to the insoluble state; rapid changes were characterized by precipitation of almost all the compounds in solution while slow changes left more of the soluble compounds in solution. The fact that up to 5% of the soluble portion of oxihumate was soluble in aqueous tetrahydrofuran but that this sub-fraction cannot be extracted directly from the oxihumate by the same solvent mixture indicates that the changes need to be done subtly and slowly to avoid co-precipitation or re-inclusion into the chemical complex/association.

The use of TLC to gain qualitative information of the sub-fractions of oxihumate was the fastest and most successful analytical technique. Many of the compounds extracted from the oxihumate proved to be highly fluorescent or UV absorbing at 254nm, which presented a convenient method to detect

these compounds on TLC plates if impregnated with a fluorescent agent. Not many compounds could be visualized with "universal" visualizing agents like chromic acid nor did many of the separated compounds stain with iodine vapour. The most successful visualizing agent for the more lipophilic sub-fractions (those that were soluble in methanol, THF or ether) was vanillin/sulphuric acid, a reagent that visualizes long chain aliphatic alcohols, steroids, essential oils and phenols. Several visualizing reagents used revealed no compounds whereas others could visualize only one or two compounds that had separated from the bulk of the very dark material. Antioxidant visualizing reagents like reduced Fast Blue or 0.05% $KMnO_4$ or 10% molibdophoshoric acid revealed a limited number of compounds in the various sub-fractions except the acid soluble sub-fraction (fulvic acids) which appeared to be comprised of several strong antioxidants.

Most of the dark material did not move off the origin of silica gel TLC plates. When dark material did move from the origin it streaked severely indicating poor solubility in the solvent systems used. The more alkaline and polar the solvent system, the more coloured material moved from the origin, but the dark compounds always exhibited severe streaking. This is illustrated in Figures 2-4 and 2-5, which show the acetonitrile:water:ammonium hydroxide and the ammonium hydroxide:propanol mobile phase separations respectively. The acetonitrile:water:ammonium hydroxide system runs with a series of four apparent solvent fronts, each pseudo-front carrying with it a mixed band of compounds. This would imply that solubility is limited and that only the compounds that are solubilized move on the pseudo-front. The fastest moving front is the least polar and would carry the non-polar lipophilic compounds whereas the zone behind the slowest moving front is the most polar and alkaline that carries the polar and coloured compounds from the origin. A distinct increase in the apparent amount of coloured material that moves from the origin was observed for the isolated sub-fractions of oxihumate with the least polar sub-fractions leaving almost no coloured compounds on the origin. The ratio of colourless compound to coloured compound increases in the isolated sub-fractions that are soluble in the more non-polar organic solvents. This may result from the coloured compounds being soluble in the mobile phase when there is a high enough concentration of non-polar compounds present. This increase in their solubility results in an increase in the movement of these polar coloured compounds.

When using the ammonium hydroxide:propanol system, more of the coloured compounds move from the origin due to the strong solvating effect and the highly polar nature of this solvent system as well as deactivation of the silica stationary phase. A disadvantage of this solvent system is that all the non-polar compounds move in a relatively tight band between *Rf* 0.75 and *Rf* 1.0 - the solvent front. Although the dark coloured compounds move from the origin more readily than with the less

polar acetonitrile:water:ammonium hydroxide solvent system, the same trend is seen that the less polar isolated sub-fractions still show more of the dark coloured compound moving off the origin. Again it could be speculated that the more lipophilic compounds are solvating the polar dark coloured compounds and this is why more of the latter compounds move off the origin. Another observation that supports the solvation effect is that if the oxihumate fraction is applied to the plate and not allowed to dry before developing, more of the coloured compounds move from the origin.

Contrary to the results with alkaline solvent systems, the acidic solvent systems all gave good separations of the non-polar compounds in the more lipophilic sub-fractions but did not move any coloured compounds from the origin. In the case of the water-soluble oxihumate sub-fraction only a trace of these non-coloured compounds could be detected moving from the origin – implying that the complete humic acid complex is very strongly associated and not easily disrupted. Although the lipophilic sub-fraction of oxihumate accounts for about 5% of the soluble mass of oxihumate, the amount of detectable material separating from the bulk of the material left on the origin appears to be far less than 5% of the mass. The coloured compounds in oxihumate do not move off the origin as these compounds are insoluble in acidic solvents and solubility is a prerequisite for chromatographic separation. The non-polar compounds in the isolated sub-fractions did however move from the origin and were well resolved over a wide region of *Rf* values from 0.4 to 1.0 (the solvent front). Some compounds in the isolated sub-fractions appeared to streak over the full length of the separation, indicating poor solubility. The most lipophilic compounds moved with the solvent front, stained with iodine vapour and coloured with the vanillin/sulphuric acid visualizing spray, both indications that they were probably aliphatic type compounds as reactive phenolics would have bleached the iodine vapour due to iodination reactions taking place.

The acidic solvent systems proved particularly useful to determine the free iodide content of radioactively labelled fractions of oxihumate as the bulk of the oxihumate remained on the origin while the free iodide had an *Rf* of approximately 0.73. From the autoradiograms made from TLC plates of ^{123}I labelled oxihumate, several previously minor unseen compounds were revealed that ran between the origin and the iodide as well as a compound that ran very close to the solvent front at an *Rf* (0.92) which is higher than that of iodide. This observation alone points to the fact that the humic acid in oxihumate contains several chemically different compounds, some that carry a functionality that can easily react with iodine and that these compounds can easily be separated from the bulk of the humic acid.

HPLC is a technique that in general has not proved to be very successful for the separation of compounds in humic acids to date. Several attempts in the past have all shown that there is an exclusion peak and a major peak that elutes over a fairly long time with limited resolution (Susic & Armstrong, 1990; Schulze *et al*, 1999; Preuse *et al*, 2000; Frimmel *et al*, 1992). This effect was seen in the HPLC separations performed on C_{18} columns in this study. It was found that the recovery of the injected sample was low and that the column backpressure would increase rapidly after about 50 injections with a simultaneous deterioration in the resolution of the eluted peaks. A washing sequence of the column at this stage could not improve the resolution and had only a minor effect on the backpressure. However if the column washing sequence was performed after 20 injections a waxy residue could be eluted and the increase in backpressure on the columns was delayed. The resolution however still deteriorated progressively. If the columns with high backpressure were disassembled it was found that the stationary phase had almost completely stained dark brown with only a short length at the outlet end showing only slightly stained particles. This dark colour could not be completely washed from the stationary phase even if using concentrated ammonium hydroxide, a treatment that could be used to clean C_{18} SPE cartridges fairly effectively.

The recovery of the material from the HPLC column was therefore not quantitative and the material that was remaining on the column was altering the chemistry of the column, which caused the rapid decrease in column efficiency.

The compounds eluting in the first peak appeared to be polar and contained the highest concentration of fluorescent compounds (except for the acid soluble sub-fraction). The compounds eluting later (between 10 and 16 minutes) and which made up the main unresolved peak became progressively darker. This large unresolved peak represented most of the dark compounds eluted from the column. Ultraviolet transparent compounds eluted at the end of the gradient and these compounds were waxy and very lipophilic. As these compounds were UV transparent they were not detected by either the UV or the fluorescent detector but were found when a fraction collector was used to collect the eluent. These undetected compounds eluted at acetonitrile concentrations of greater than 70% and were found to be waxy white solids with no distinct smell.

It was found that C_{18} HPLC eluted peaks collected by fraction collector were not as soluble as they were prior to chromatographic separation. This could have been due to breaking up a complex or association of compounds or the result of denaturing the compound similar to what can occur to some proteins when chromatographed on C_{18} columns.

Re-chromatography of the HPLC eluted fractions on silica TLC plates using an acidic mobile phase showed that the dark compounds remained at the origin, implying insolubility in acidic medium. When alkaline mobile phases were used severe streaking occurred, implying low solubility or ionic compounds that bind strongly to the silica.

The general pattern of HPLC separation of oxihumate is similar to that reported in the literature for humic acids (Frimmel *et al*, 1992; Gremm *et al*, 1991) using C_{18} columns and similar mobile phases. This elution pattern was found to be reproducible and appeared to have a slightly better resolution than those reported above, especially with respect to the second major "peak" eluting from 10 to 16 minutes. The number of injections through the column however had a major role in the resolution of the different compounds in the oxihumate and it would be difficult to compare the methods due to the different sources of the analysed humic acids because the number of injections through the column was not reported in the above literature.

The isolated sub-fractions (separated according to solubility differences) also gave reproducible elution patterns. Despite the reproducibility, quantitation was not possible as the sample recovery varied greatly from fraction to fraction.

Complications in attempting to quantitate the compounds in oxihumate (and other humic acids) are that the very lipophilic compounds and the C_{18} HPLC column washings were not detectable by the UV or fluorescence detectors. A further complicating factor is that the eluted compounds were not completely resolved. The absolute absorbance for individual compounds could therefore not be determined and calibration curves were unreliable.

The use of a C_{18} HPLC column and an acidic mobile phase (0.1% trifluoroacetic acid in both water and acetonitrile) resulted in good resolution of the compounds of the main peak, eluting between 10 and 16 minutes, but the recovery was very low with almost none of the dark coloured compounds being eluted. This method was later only used when sub-fractions had been previously separated by low-pressure column chromatography and the bulk of the dark compounds had already been removed from the sample. The UV transparent lipophilic compounds were strongly retained when using the acidic mobile phase and, like with the alkaline mobile phase, needed to be washed from the column after 20 injections. This result would imply that these lipophilic compounds are very non-polar and unaffected by the pH of the mobile phase indicating an absence of any ionic functionality in these compounds.

Due to the low recoveries on HPLC, especially using acidic mobile phases, a small study was done in an attempt to determine the percentage recovery using C_{18} SPE cartridges. Although the method used would not give precise results it was a far better method than comparing peak areas on the HPLC system with and without a column as the retention behaviour of any compound distorts the apparent concentration of individual compounds grossly. The UV transparent compounds in the oxihumate were only detected after collecting the eluent and drying these fractions. These compounds, although adding to the mass of the injected sample would not be detected by the HPLC system detectors with or without an analytical column and would therefore have distorted the apparent recoveries in a positive direction.

The fact that the control samples appeared to have gained mass is probably due to the potassium in the oxihumate fraction forming salts with the stronger trifluoroacetic acid in the eluent. Although the eluent is completely volatile, potassium trifluoroacetate would not be volatile and would then add to the mass of the sample. If this increase in mass of the control samples were factored into the percentage recovery of the SPE eluent, then the actual recoveries of the oxihumate from the SPE cartridges would be only 60% at most. The waxy residues that were eluted by the organic solvents accounted for a further 15 – 20% (corrected for the controls) of the mass, which means that there is still approximately 20% of the mass of the sample retained on the cartridges.

It was obvious that the coloured compounds were being strongly retained on the C_{18} cartridges and that they were not easily recovered. This same effect was seen on the HPLC columns, where the dark compounds eventually contaminated the entire length of the stationary phase resulting in poor resolution. The retained compounds however appeared to be in a slow equilibrium that would allow displacement of some of the dark compounds to occur under strongly alkaline conditions, conditions that result in deterioration of silica based stationary phases. This phenomenon was also reported by Susic and Boto (1989) who reported that SPE columns had unpredictable behaviour and appeared to establish a new equilibrium on standing.

It would appear from these results that there are several compounds with very different characteristics present in the oxihumate and that these compounds must have a mutual effect on the other compounds in the oxihumate. The solubility, chromatographic retention, UV absorbance, fluorescence and colour are all affected by the stripping out of some of the compounds from the humic acid complex.

Fluorescence spectroscopy revealed that all the isolated sub-fractions had some fluorescent compounds present but that the THF soluble sub-fraction accounted for most of the fluorescence of oxihumate. Two particularly strongly fluorescing compounds were present in this sub-fraction. The general appearance of the sub-fraction was a brown oil with a slight fluorescent green tinge when seen in sunlight and gave a very strong blue fluorescence under both 254nm and 360nm UV light. On TLC it could be shown that several compounds in the THF sub-fraction had native fluorescence that was only visible under a 360nm light. The emission colour of these compounds varied from almost white through blue and green to orange and "brown". The colour and intensity of the fluorescence appeared, at least to some extent, to be dependant on the concentration of the spots and how well the spots were resolved. When the isolated sub-fractions were scanned in a spectrofluorometer, the compounds were still a complex mixture of compounds and a cumulative fluorescent effect was observed. There is a strong possibility that interference and quenching effects play a major role in the observed fluorescent spectra due to the many fairly similar chemical structures that would be present in the sample (Senesi *et al*, 1991). The excitation and emission maxima reported by Alberts (Alberts *et al*, 2000; Tacacz & Alberts, 1999) for humic acids from aqueous environments matched those found for oxihumate in this study the closest, although there were small differences. Luminescence spectra (3D spectra) (Mobed *et al*, 1996) reveal that a broad emission band between 375 and 500nm existed even when the excitation wavelengths were varied by more than 100nm. The maximal emission wavelength is approximately 430nm for all the sub-fractions despite the fact that they are mixtures of several compounds. This could indicate that there are a limited number of strongly fluorescent compounds or that there is a reproducible quenching or interference mechanism present.

In concentrated samples it was seen that the scattered excitation and second order harmonic wavelength emission were not present in the emission spectra although when the same sample was further diluted, both these emission wavelengths were exhibited as strong peaks. This could indicate that a strong quenching effect is evident. Furthermore the fact that the more concentrated samples show darker and even a "brown" fluorescence on TLC also indicates some form of quenching, the mechanism of which is not known at this time.

Infrared proved to be a very useful technique in determining the presence of chemical functionality in oxihumate although the results are not as well defined as would be expected if a single compound were under scrutiny. The intense absorption in the "carbonyl region" appears to be fairly universal for all humic acids, irrespective of where they are sourced (Khairy, 1989; Theng & Posner, 1967). The spectra of the THF and the fulvic acid sub-fractions were very different from all the other sub-

fractions in the carbonyl region. The fairly sharp absorbance by these two sub-fractions at 1720 cm^{-1} would indicate the presence of esters or unsaturated ketones. The THF sub-fraction also has a strong absorbance band at 1038 cm^{-1} which is in the absorbance region of the C-O stretch of esters, alcohols and ethers. The value is low for typical simple esters but could be due to α-unsaturated esters or α-unsaturated free acids. Aromatic ethers show a typical combination of peaks at 1250 cm^{-1} and 1030 cm^{-1} a combination of bands that is also seen in the THF sub-fraction. An alternative structure also absorbing in this region is internal tertiary carbon groups of substituted alkanes.

The fulvic acid sub-fraction also shows the strong ester absorption band at 1720 cm^{-1} but lacks the C-O stretch band of the alcohols, ethers and esters. This sub-fraction shows a shoulder at 1630 cm^{-1} that falls into the alkene, substituted aromatic ring, carboxylic acid salt or amine absorption region. The 1410 cm^{-1} absorption however, reinforces the carboxylic salt structure. The high ash content and high potassium concentration in the ash would again point towards the carboxylic salt structure.

The presence of strong hydrogen bonded hydroxyl absorption could result from acidic or alcohol groups, both of which fit the generally accepted chemical functionality that would be found in humic acids. The old debate that has not been resolved is whether the structure is predominantly aromatic or aliphatic (Farmer & Pisaniello, 1985; Ikan *et al*, 1986; Schnitzer, 1985; Stevenson, 1994). This cannot be seen from the IR spectra of the oxihumate nor from most of its dark coloured sub-fractions. The THF soluble sub-fraction was the only sub-fraction to show a distinct aliphatic CH_3 and CH_2 peaks at 2950 cm^{-1} and 2850 cm^{-1}. The aromatic C-H stretching bands (3000-3100 cm^{-1}) did not stand out in any of the sub-fractions, which raises the question whether this functionality is really involved in the main chemical structure. However, if there were few aromatic C-H bonds present due to extensive substitution of the aromatic rings these classic aromatic absorption bands would be very small to insignificant.

The insignificant aliphatic C-H stretching in all the fractions except the THF sub-fraction (which is rich in aliphatics) cannot be explained by the same argument. Even though the THF soluble sub-fraction accounts for only 5 – 6% of the mass, the aliphatic band should be visible in all the sub-fractions from which this sub-fraction was isolated. The literature generally does not describe the effect that mixtures of compounds would have on the spectra of each other and whether some form of quenching could occur. From the results of the UV/visible, fluorescent and Raman spectra, where distinct interference or quenching effects appear to be evident, it would not be surprising if some form of interaction, which includes quenching or energy transfer, does occur in infrared spectra.

The Raman spectroscopy was relatively disappointing in that the bands were broad and there was a strong baseline shift. These effects were attributed to quenching or interference. A problem experienced with the Raman spectra of oxihumate sub-fractions was the strong fluorescence exhibited by the different isolated sub-fractions that appeared to interfere with the scattered light pattern of the spectra. The spectra obtained for the water-soluble sub-fraction pointed to polycondensed aromatic ring structures and/or amorphous carbon. If this is considered together with the IR spectra it would appear that there are nearly no hydrogens bound to aromatic rings, leaving highly oxygen and carbon substituted ring structures. In this case there would be many C=C bonds which also absorb in the 1500 - 1600 cm^{-1} region, the region of very strong absorbance in the IR spectra of oxihumate. This region of absorbance overlaps the carbonyl absorption of carboxylic acid salts, which makes the peak assignments difficult. The fact that the absorption bands are mostly broad, again implies that the humic acid is probably a complex mixture of slightly different compounds. The fact that the THF soluble sub-fraction can be separated into at least 30 different compounds on TLC, many which are UV absorbing or fluorescent, again supports the concept that humic acid is a tight association of many different compounds.

Although not shown, liquid phase NMR spectra that were performed on oxihumate during this study showed a very complex combination of resonance bands, including aromatic and aliphatic hydrogens and carbons, but the spectra were not of good quality, had very large solvent (H_2O) peaks and could not give quantitative data with respect to the relative proton or carbon numbers. What was obvious was that there were many different resonance bands of compounds present in different apparent concentrations and that there were many "inactive" carbon atoms present in the complex. These NMR results confirmed the presence of both the aliphatic and aromatic functionality in oxihumate.

If all the chemical and physical data collected for oxihumate and its isolated sub-fractions is considered it would appear to support the hypothesis that humic acids are in fact tightly associated or loosely bound chemical complexes. Some compounds can be separated from the structure under certain conditions but the bulk of the humic acids remain in the complex due to similar chemical structure or physical properties. It would appear that the remaining humic acid, after extraction of the least tightly bound compounds, would re-equilibrate and this would allow more compounds to be extracted from the complex. The presence of aromatic functionality could not easily be confirmed by the techniques used to determine chemical structures during this study although there are several characteristics such as the refractive nature of the oxihumate to combustion in air, the strong UV absorption and the fluorescence that would imply that such structures do exist in the complex.

This study has used several analytical techniques that have not been used in combination by any other research group reporting on their humic acid analysis so far. The individual and combination of techniques all point to a complex mixture of compounds and not to a single chemical structure that could be isolated and characterized as "humic acid". Taking into account the fact that these compounds are derived from plant material that would be a mixture of many different chemical compounds to start, and that these compounds have been through a degradation process and chemical alteration due to environmental and physical conditions over a period of several million years it would not be surprising to find that there are still many different compounds in the complex making up humic acid. Despite the fact that there are sub-fractions that can be extracted and that these can be further separated chromatographically into many compounds it is uncertain whether these compounds were chemically bound within the humic acid structure or just associated with a core compound that is not soluble.

Another complicating fact is that there is a relatively high ash content for a compound that is organic. This can be explained by the binding of inorganic salts and metal ions but there appears to be a large percentage of silica and aluminium oxides, both elements associated with soil or clay. This would indicate that the organic compounds are "contaminated" with soil or that degraded soil is complexed by the humic acids. By washing these isolated sub-fractions with hydrofluoric acid it should be possible to remove most of the silica and this would indicate the amount of clay in the humic acid.

Considering the data collected during this study, it would be reasonable to state that humic acid, and especially oxihumate derived from a bituminous coal by an oxidation process, are complex mixtures of many individual chemical compounds held together by either weak chemical bonds or merely in a tight association with each other and that the environment and treatment of this complex mixture would determine the physical and chemical characteristics of the humic acid as a whole.

Chapter 3

Bioassays of Oxihumate and Oxihumate Sub-fractions

1 Introduction

As humic acids show anti-inflammatory and immunomodulating properties, the different isolated sub-fractions of oxihumate were assayed for activity using *in vitro* bioassays to determine which of these sub-fractions had the greatest activity. Three bioassays were used to test oxihumate and the isolated sub-fractions. These bioassays were conducted on isolated granulocyte populations (that will be referred to as neutrophils in this chapter) or mixed leukocyte populations, where the lymphocyte and monocyte populations were not separated from the granulocyte populations.

It has been demonstrated that humic acid inhibits the expression of the complement receptor 3 (CR3), an inflammatory associated marker, by stimulated neutrophils in a dose dependant manner yet appears to have almost no effect on resting neutrophils (Jooné, 2002). As expression of CR3 is one of the initial responses to stimulation of neutrophils and that this expression is critical to continued activity of the neutrophils, a bioassay monitoring the effect that isolated sub-fractions of oxihumate had on CR3 expression by neutrophils was deemed to be a suitable test to determine which sub-fraction had the greatest anti-inflammatory activity.

The second bioassay used in this study was a chemiluminescent assay. The chemiluminescence assay of neutrophils is a technique used to measure the release of reactive oxygen species generated by these cells during the respiratory burst that takes place after stimulation by various foreign, chemical or particulate stimulants. The inhibitory or promotional effects that test compounds have on the release of oxidative species after exposure to known stimulants can be monitored using the same chemiluminescence assay (Allen, 1986).

If differences could be demonstrated in the response of cells exposed to test compounds relative to control cells not exposed to the same test compounds, an assumption could be made as to the possible pro- or anti- inflammatory effects of the test compounds which in this study was the different isolated sub-fractions of oxihumate.

To rule out that the effects the sub-fractions had on cellular functions was due to an effect on cell viability a rapid, known viability staining technique, that was compatible with a flow cytometer, was used (Tas & Westerneng, 1981; Darzynkiewicz & Li, 1996).

1.1 Complement receptor 3 (CR3) expression assay

On activation neutrophils and monocytes express complement receptor 3 (CR3), a β_2 integrin that is expressed exclusively by haematopoietic cells and which is an adhesion molecule important for cell sticking, diapedesis and phagocytosis. The expression of CR3 is one of the first measurable responses on the surface of the cells that occurs after stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA), formyl methionyl leucyl phenylalanine (FMLP) and tissue necrotic factor α (TNFα) and can be used as a marker of cell response in inflammatory reactions (Harlan, 1993). The CR3 receptors consist of two polypeptide chains: an α chain of 165kDa and a β chain of 97kDa. The α chain is the CD11b cell surface marker molecule whereas the β chain is the CD18 cell surface marker. These receptors can be expressed very rapidly, apparently due to expression of preclustered internal reserves (Petty *et al*, 1987). Normally CR3 binds to fibrinogen or ICAM-1 and E-Selectin, the latter two present on the surface of activated endothelial cells of the vascular system, as an initial stage of diapedesis of the neutrophils. The inflammatory process depends on the migration of large numbers of neutrophils from the vascular system to the affected area where they release oxidative reactants and cytokines that cause the inflammation (Kishimoto *et al*, 1999).

In this study the cellular expression of CR3 by neutrophils was quantitated by a flow cytometric method based on the method originally reported by Rabinovitch & June (1990) and adapted by Jooné (Jooné, 2002). Phycoerythrin (PE) conjugated monoclonal antibodies against the CD11b cell surface marker molecules were added to neutrophils in both the resting and stimulated state. By adding an excess of fluorescent antibody it was certain that all the CR3 receptors on the neutrophil membrane surfaces were fluorescently marked. Analysis of the cellular fluorescence intensity by a flow cytometer provided a quantitative measure of the total CR3 on the surface of the neutrophils. An Epics XL-MLC flow cytometer (Beckman Coulter, Fullerton, CA) equipped with an air-cooled argon laser operating at 488nm was used in this study. The region in the scattergram representing the neutrophils was gated and only the fluorescence within this gated region analysed. The fluorescent peak channel position in the histogram was used to quantitate the expressed CR3.

1.2 Chemiluminescence assay of neutrophil respiratory burst

Chemiluminescence is the non-thermal release of light from specific molecules that are easily elevated into a higher energy state by chemical reactions, of which the oxidation reactions are the most common (Allen, 1986). Analysis of many reactive compounds and biological reactions can be achieved by means of chemiluminescent assays, which is more sensitive (up to 1000x) and specific than spectrophotometric methodologies. Chemiluminescence can replace the use of radioisotopes in several common assays, making the use of this technique faster, safer and less demanding on facilities and equipment.

Most reactive oxygen species are only weakly or not at all luminescent making the direct measurement of these compounds difficult. Scintillation counters have been used to determine the concentration of the weakly luminescent oxidants but a more convenient and far easier method that also allows for kinetic analysis is to add a labile photo-emitter such as luminol or lucigenin to the reaction mixture (Thorpe & Kricka, 1986). These luminescent reactants or photosensitizers react easily and very rapidly with most reactive oxidant molecules releasing a defined amount (a quantum) of visible light per reaction, which is directly proportional to the concentration of reactive oxidant species present.

The luminescent reagents are relatively stable and non-toxic. In biological assays a reasonable specificity can be achieved (Allen, 1986). Turbid samples and samples of intense colour can still be analysed despite quenching of the response by colour absorption or scatter although this does complicate quantitation.

Luminol (5-amino-2,3 dihydro-1,4-phthalazinedione) is a synthetic photo-emitter used for detecting oxidants produced by cells containing peroxidase or nitrous oxide synthase enzymes. Luminol reacts with peroxide molecules or oxygen radicals with the simultaneous release of a brief flash of light at 425nm. Molecular oxygen cannot induce this reaction but there are a number of inorganic oxidative catalysts that can do so. Certain metal ions and metal complexes such as haematin accelerate the reaction with oxygen (Allen, 1986).

Lucigenin (bis-N-methylacridinium nitrate) reacts in a similar way but has a requirement for divalent cations to react. The reaction is accompanied by a simultaneous release of a brief flash of light directly proportional to the concentration of the oxidative species present in the mixture. Peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical ($HO\cdot$) produced by the myeloperoxidase enzyme reaction are all easily detected using this reagent (Allen, 1986).

The simplicity of the assay system however means that it cannot discriminate between whether an added test compound alters the cellular production of oxidants or whether the respiratory burst is normal, forming oxidative species as expected, but that these oxidative species are scavenged directly by the test compounds.

The discrimination between scavenging and inhibition is done by enzymatic generation of superoxide anion by the xanthine/xanthine oxidase reaction and monitoring the chemiluminescence. Xanthine oxidase is the enzyme that catalyses the conversion of xanthine to uric acid with the simultaneous release of two superoxide anion molecules. This enzyme system can be used to generate superoxide anions *in situ* so that the scavenging of these reactive oxygen species by compounds with unknown activity can be determined. By adding lucigenin to a mixture containing xanthine oxidase and xanthine, the enzymes substrate, the released superoxide anion can be quantitated accurately in a chemiluminometer. To determine whether a compound has superoxide scavenging characteristics it can be added to the assay mixture and any apparent decrease in the concentration of the measured superoxide would be evident from a reduction in the luminescence of the lucigenin as compared to a positive control where the test substance is replaced with buffer. The test compound was then added to the mixture and the change in chemiluminescence monitored. If the test compound acts as an antioxidant it would scavenge the superoxide anion and the concentrations of the enzymatically-generated oxidant would appear to be reduced after the addition of the test compound.

The respiratory burst of neutrophils, which is responsible for the formation of reactive oxidation species, can be elicited by several stimulants. Each stimulant has a unique mechanism of action, which also affects the time for the response to reach a maximum.

Two commonly used stimulants are;

- formyl methionyl leucyl phenylalanine (FMLP), a fast acting synthetic tripeptide that mimics the peptide precursors associated with bacterial infections and which is both chemotactic (at low concentrations) and a stimulant of the full respiratory burst of neutrophils (at higher concentrations) (Panaro & Mitolo, 1999).
- phorbol 12-myristate 13-acetate (PMA), a plant-derived tumour promoting lipophilic compound that activates the protein kinase C linked receptors and is a strong stimulant for nitric oxide release. Although a slower acting stimulant than FMLP it results in a full respiratory burst (Saitoh & Dobkins, 1986; Schuman, 1989).

Three different chemiluminescent assays were done.

- the first assay was done to determine whether there was any stimulation of the respiratory burst by the various sub-fractions of oxihumate on resting human neutrophils. In this assay the test compounds were added to the tubes just prior to initiation of monitoring. Measuring of chemiluminescence started immediately after the addition of the test compound and collected continuously for 80 minutes. In this assay no stimulant was used and was replaced by 100 µl HBSS.
- the second assay was done to determine the effect of the isolated oxihumate sub-fractions on the respiratory burst induced by the addition of the synthetic chemotactic tripeptide formyl methionyl leucyl phenylalanine (FMLP). In this assay the cells were pre-incubated with the sub-fractions for 20 minutes prior to the addition of the stimulant. A background luminescence was obtained and the stimulant added and monitored for a further 3 minutes. A similar experiment using PMA as the stimulant was done but the monitoring period was then 80 minutes due to the slower stimulation rate of PMA.
- the third assay was done to determine the antioxidant activity of the various sub-fractions by determining the scavenging effect that these sub-fractions had on superoxide anions generated by the xanthine/xanthine oxidase enzyme system.

2 Materials and Methods

2.1 Materials

Xanthine Oxidase (EC 1.1.3.22) was purchased from Sigma Diagnostics, (St Louis, MO, USA). Just before the assays were performed a diluted enzyme solution was prepared by adding 20µl of enzyme solution to 2,0ml (approximately 0.25 units/ml) Hanks Balanced Salt Solution containing calcium and magnesium and buffered with HEPES at pH 7.4 and kept on ice.

Xanthine was purchased from Sigma Diagnostics, (St Louis, MO, USA). For the superoxide scavenging assay a solution of 1.52mg/ml (10mM) was made up in distilled water. Just before the assay was to be done this was diluted to 2.2mM by adding 35µl of this solution to 125µl HBSS.

Heparin was purchased from Sigma Diagnostics, (St Louis, MO, USA). Three hundred milligrams was dissolved in 100ml-distilled water and the solution filter sterilized. The solution was stored at 4°C in sealed tubes. 100µl of the solution is equivalent to 5 units.

Histopaque 1077 was purchased from Sigma Diagnostics, (St Louis, MO, USA) and stored at 4°C. Aliquots for cell separations were removed under sterile conditions.

Ammonium chloride, sodium hydrogen carbonate and ethylene diamino tetra-acetic acid disodium salt EDTA were of analytical reagent grade purchased from Merck, (Darmstadt, Germany). 8.30 g ammonium chloride, 2.00 g sodium hydrogen carbonate ($NaHCO_3$) and 0.148 g EDTA were carefully weighed out and dissolved in 1000 ml deionized (18MΩ) pyrogen free water. This solution was usually used as is but when sterile conditions were required the solution was filter sterilized.

Hanks Balanced Salt Solution with calcium, magnesium and buffered with HEPES buffer but without phenol red indicator was purchased from Highveld Biological (Pty), LTD. (Johannesburg).

RPMI-1640 tissue culture medium was purchased from the National Institute for Virology in Johannesburg, South Africa, in sealed sterile 1 litre bottles. This medium was used as is or was fortified with heat inactivated foetal calf serum as described below.

Foetal calf serum purchased from Sterilab, Johannesburg, South Africa, was inactivated by heat treatment to give complement free serum (HI- FCS). Incubating the sealed bottles of foetal calf serum at 56°C for 45 minutes achieved heat inactivation. After allowing the still sealed bottles to cool to ambient temperature the serum was transferred under sterile conditions to sterile centrifuge tubes and centrifuged at 1500*g* for 25 minutes to remove any coagulated protein. Heat inactivated FCS was added to the HBSS or RPMI 1640 to a concentration of 10% by volume to give standard working solutions for neutrophil work.

Phosphate buffered saline was purchased as ready mixed dry powder (FTA-buffer from BBL Microbiology Systems, Becton Dickenson and Company, USA). Exactly 1000ml of deionized water was added to 9.23 g of the powder as per instructions.

Cell counting fluid was made by adding 100µl of a 0.1% solution of gentian violet (Gurr) to 2ml glacial acetic acid and diluting to 100ml with distilled water.

Lucigenin was purchased from Sigma Diagnostics, (St Louis, MO, USA). A solution of 1mg/ml in PBS or indicator free HBSS was made up and aliquoted into 5ml aliquots that were kept at –18°C until required.

Luminol was purchased from Sigma Diagnostics, (St Louis, MO, USA). Eighteen milligrams luminol were dissolved in 1.00ml DMSO and 100µl aliquots stored at –18°C. A 100µl aliquot was diluted to 10ml with HBSS just prior to use and kept on ice.

FMLP was purchased from Sigma Diagnostics, (St Louis, MO, USA) and dissolved in DMSO at a concentration of 2.5mg/ml, aliquoted and stored at –18°C. This stock solution was diluted to 10µM just prior to use by diluting 1:500 using HBSS.

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Diagnostics, (St Louis, MO, USA) and dissolved in absolute ethanol (Merck, Darmstadt, Germany) to a final concentration of 1 mg/ml. The solution was divided into 100µl aliquots in glass tubes, sealed and stored at –70°C until required. Just before use, this solution was further diluted in HBSS to a concentration of 1µg/ml and stored on ice. The diluted solution was stable for less than 30 minutes.

2.2 Methods

2.2.1 Isolation of Neutrophils from human blood

The method used for the isolation of neutrophils is the Ficoll-Hypaque method that was first described by Böyum (1968). Briefly the method is as follows: Venous blood was collected from healthy consenting adults into evacuated potassium EDTA tubes by venipuncture if small volumes of blood are used. For larger volumes (more than 50 ml) blood was collected into FENWAL Blood–Pack blood bags to which 5 units of preservative free heparin per ml blood had been added prior to collection.

The freshly collected blood was poured into 50ml graduated plastic centrifuge tubes to a volume of 35 ml. Fifteen millilitres of Histopaque-1077 (Sigma Diagnostics, St Louis, MO, USA) was under-layered at the bottom of the tube using a syringe with a long blunt needle. The tubes were centrifuged at 520 *g* for 30 minutes at 20°C after which the portion of the upper layer containing the plasma and platelets was removed and discarded. The lymphocyte/monocyte layer was carefully removed together with the bulk of the Ficoll-Hypaque layer. The neutrophil layer was directly above and in contact with the red blood cell concentrate at the Ficoll-Hypaque boundary. The layer was gently removed with minimal inclusion of red cells and diluted to at least six times the volume with cold 0.83% ammonium chloride solution, allowed to stand on ice for 10 minutes during which time the red blood cells haemolysed, before centrifuging at 480 *g* for 10 minutes at 10°C to sediment the granulocytes. If there was still evidence of red blood cells in the pellet the ammonium

chloride haemolysis step was repeated. The pellet was washed with a suitable medium (RPMI with 10% FCS for the CR3 expression experiments or Hanks Balanced Salt Solution containing calcium, magnesium, 10% FCS and HEPES buffer, pH 7.4 but without phenol red indicator for chemiluminescence assays), centrifuged at 480*g* for 10 minutes at 10°C and resuspended in cold medium to approximately $1/10^{th}$ of the original volume of blood.

When mixed leukocyte populations were used for the tests instead of a purified granulocyte population, the separation on Histopaque-1077 was omitted and the whole blood was diluted directly to at least 6x its volume with the 0.83% ammonium chloride solution. The rest of the isolation procedure remained the same as for the neutrophil isolation. A second ammonium chloride wash step was routine when using whole blood.

The cells were manually counted (50µl cell suspension added to 450µl counting solution and allowed to stand for at least two minutes before counting in a Neubauer cell counting chamber at 400 x magnification) and the cell suspension further diluted to a concentration of approximately 10 x 10^6 neutrophils per ml with medium.

2.2.2 Cell Viability Assay

For cell viability studies the isolated neutrophil population was separated and diluted to 10 x 10^6 cells per millilitre as described above. For each isolated sub-fraction a test tube was prepared containing 50µl of this cell suspension and 400µl RPMI 1640 medium containing 10% heat inactivated FCS. Two extra tubes were included as controls to which no test compound was added. These suspensions were pre-incubated at 37°C for 15 minutes. One of the control tubes was treated with 50µl FMLP as a stimulated control. Each experimental tube was dosed with 50µl of RPMI containing varying concentrations (shown in Table 3.1 below) of the relevant test compounds (to match their concentrations in the oxihumate) and incubated with gentle mixing every 5 minutes for a further 30 minutes at 37°C.

Table 3-1: Summary of the different sub-fractions of oxihumate used for the bioassays and the final concentrations in the test solutions of each used for the assays.

Sub-Fraction	Concentration (µg/ml)*
H2O solubles	100
H+ solubles	30
H+ precipitate	60
MeOH precipitate	50
MeOH solubles	12.5
THF precipitate	8
THF solubles	5
Blank	0

* The concentrations used for each sub-fraction are equivalent to the ratio at which these sub-fractions occur in the water soluble oxihumate, so it would be expected that the active compounds would be present in the same concentrations as when using 100µg/ml oxihumate. The blank has no humic acid compounds present.

The tubes were then centrifuged at 480*g* for 5 minutes and the supernatant gently decanted. One hundred microlitres of a 1mg/ml solution of propidium iodide in PBS was added to the pellet that was gently re-suspended by swirling the tubes and incubated at 37°C in the dark for 10 minutes. The suspension was diluted with 600µl Isoflow® (Beckman Coulter) and the cells analysed on an Epics XL-MLC flow cytometer (Beckman Coulter) equipped with an Argon ion laser. In one experiment RPMI 1640 medium containing no FCS was used to determine whether the exclusion of FCS did have an effect on the cell viability.

The neutrophil population in the scattergram was gated and this area analysed for cells exhibiting propidium iodide fluorescence that indicated binding. An aliquot from the unstimulated control tube was removed and treated with a propriety cell lysing detergent solution (Beckman Coulter) for 10 minutes as a positive control for propidium iodide binding. The percentage of the cells showing propidium iodide binding relative to the total number of cells counted in the gated area was taken as the percentage non-viable cells.

2.2.3 Quantitation of complement receptor 3 (CR3) expression

Isolated neutrophils or mixed leukocyte populations were made up to 10 x 10^6 cells per millilitre as described above. Two 5ml plastic test tubes for each test compound, one for resting cells and one for stimulated cells, as well as equivalent control tubes were prepared by dispensing 50µl of this

cell suspension into 400µl aliquots of RPMI 1640 medium containing 10% HI-FCS per tube and pre-incubated in a water bath at 37°C for 15 minutes. The test compounds were replaced by HI-FCS fortified RPMI in the two control tubes; otherwise all additions were identical to the test compounds. After the pre-incubation period, 50µl isolated sub-fraction as test compound was added to both the tubes of cell suspension per paired set, gently mixed and incubated for a further 20 minutes. After the second incubation time the stimulated tube from each pair was treated with 50µl of a 1.0µg/ml solution of freshly prepared PMA in RPMI. 50µl RPMI alone was added to the paired resting cell tube. After a further 20 minutes incubation time the tubes were quickly transferred to an ice bath and 100µl aliquots removed and added to corresponding clean counting tubes to which 5µl of PE conjugated anti-CD11b antibody had been added. Isotypic background controls were provided by the addition of 100µl aliquots from the resting or stimulated control tubes to 5µl PE conjugated anti-mouse IgG. After 10 minutes the suspensions were diluted to 600µl with Isoflow® and the suspensions analysed within an hour on a Epics XL-MLC flow cytometer (Beckman Coulter) equipped with an air cooled Argon ion laser. Time per analysis was set to 100 seconds. The neutrophil population in the scattergram was gated and this region analysed for fluorescent intensity. Quantitation of the CR3 was directly related to the fluorescent signal measured using the median peak position.

2.2.4 Chemiluminescence assays of neutrophil generated oxidants

Three millilitres of neutrophils at 10 x 10^6 cells/ml suspension was transferred to a clean test tube and diluted with an equal volume of 1 mg/ml lucigenin solution. This cell suspension was incubated on ice for half an hour.

The test was carried out in clear disposable plastic luminescence tubes. A blank, a negative control and a positive control were included in each experimental set. To each tube in the test set was added 600µl of the Hanks Balanced Salt Solution without phenol red but containing calcium, magnesium, 10% FCS and HEPES buffer - pH 7.4 and 200µl of the lucigenin equilibrated cell suspension. These cuvettes were equilibrated at ambient temperature (22°C) for 5 minutes before being loaded into a preheated (37°C) BioOrbit 1251 Chemiluminometer carousel where they were incubated for a further 20 minutes.

When the isolated sub-fractions were tested for their stimulatory effect on neutrophils, a further 100µl of HBSS was added to the cell suspensions before incubation at 37°C. At the end of the 20-minute incubation period, a 100µl aliquot of the appropriate concentration of isolated sub-fraction

was added and monitoring initiated. This resulted in a delay of approximately 60 seconds. Monitoring was continued for 80 minutes.

In two different experiments the cells were pre-treated with the isolated sub-fractions by adding aliquots of 100µl sub-fraction in appropriate concentrations to the cell suspensions before incubation at 37°C. After 20 minutes incubation, monitoring was initiated and 60 seconds later the cells were stimulated by the addition of 100µl of either 10µM FMLP or 1µg/ml PMA. In the case of FMLP stimulation the monitoring was stopped after 3 minutes while the PMA stimulated cells were monitored for the full 80 minutes.

When the neutrophils were pre-incubated with the test compounds, these compounds were added to the cuvettes (100µl of the appropriate concentration of test compound per cuvette) just prior to the pre-incubation time in the chemiluminometer at the appropriate time before the test began (and three minutes apart when stimulated with FMLP), vortexed briefly but gently to ensure proper mixing and replaced into the preheated carousel.

The blank contained all reagents but no cells (an aliquot of the lucigenin treated cells was centrifuged at 500 *g* for 10 minutes at 10°C and 200µl of the cell free supernatant used instead of the cell suspension), in the negative control the stimulant was replaced by 100µl HBSS and in the positive control the test compound was replaced by 100µl of HBSS.

After the preincubation the tubes were again briefly vortexed and the monitoring for 3 minutes initiated. Ten seconds later 100µl of the FMLP solution was introduced by automated dispenser and mixed. The release of reactive oxidants was seen as an increase in the chemiluminescence.

2.2.5 Superoxide anion scavenging assays using the xanthine oxidase generation system

All solutions were made up freshly as described under the materials section and kept on ice. The enzyme activity after dilution was tested in the same way as a positive control to ensure that the activity was high enough to be easily detected and to eliminate background noise but low enough to give a steady state reaction time of more than 3 minutes.

The following reagents were added to chemiluminescent tubes and incubated for 15 minutes at 37°C in a BioOrbit 1251 Chemiluminometer carousel;

- 160µl HBSS containing 2.2mM xanthine
- 20µl Lucigenin solution (1mg/ml).

After the pre-incubation period, monitoring was initiated for a total of 2 minutes. The tube mixer was programmed to mix throughout the monitoring period. Fifteen seconds after initiation of the monitoring 100µl xanthine oxidase enzyme solution was added via an automated dispenser.

Forty seconds after initiation of monitoring, a 40µl aliquot of test compound in distilled water (at a concentration of either 40µg/ml or 400µg/ml) was added by syringe directly into the tubes. In one experiment the concentration effect of the acid precipitated sub-fraction was evaluated by varying the concentration of this sub-fraction from 0µg/ml to 50µg/ml.

A negative and positive control and a blank were included for each series of tubes. The intensity of the luminescence 25 seconds after addition of the xanthine oxidase was used as the maximum response and the intensity 20 seconds after addition of the test compounds used to indicate the antioxidant activity.

3 Results

3.1 Cell isolation

The isolation of neutrophils using Histopaque-1077 was found to be a quick effective method for this isolation and about 15 x 10^6 neutrophils per 10 ml blood could be retrieved within an hour and a half of drawing the blood. The use of mixed leukocyte populations for the CR3 expression testing was acceptable because the flow cytometer can be gated specifically to monitor different cell populations which are then further analysed for fluorescence, an indication of the effect that the test compounds had on the cell population within the gated zone.

3.2 Cell Viability

Neutrophils were exposed to the different isolated sub-fractions of oxihumate at the relative concentrations at which they were present in oxihumate. This meant that the concentration varied from 100µg/ml for the water-soluble fraction to 5µg/ml for the THF soluble fraction. In one experiment it was found that the addition of 10% FCS to the medium in which the cells were suspended provided a protective effect on the cells.

Table 3-2: Comparison of the cell viability after 30 minutes incubation of isolated neutrophils in the presence of the various concentrations of the different sub-fractions of oxihumate. This assay used the propidium iodide exclusion method and a flow cytometric analysis.

Sub-Fraction	Concentration (µg/ml)	% Viability	% Viability + 10% FCS
H2O solubles	100	93.9	99.3
H+ solubles	30	94	99.3
H+ precipitate	60	90	99.5
MeOH precipitate	50	94	99.1
MeOH solubles	12.5	92	99.4
THF precipitate	8	93	99.4
THF solubles	5	95	99.5
Blank	0	87.8	99.4
Control	Lysing solution	0.5	0.3

None of the sub-fractions showed any appreciable reduction in the cell viability after 30 minutes incubation at 37°C. Table 3-2 summarizes the percentage viable cells and the concentrations of the sub-fractions of oxihumate used for the experiment.

3.3 Quantitation of CR3 expression

The expression of CR3 by human neutrophils could be monitored with relative ease when using the flow cytometric method and fluorescently labelled antibodies raised against the CD11b region of this receptor. The scattergram of the cell suspension could be used to gate the neutrophil population specifically and this region was then analysed further for the fluorescent intensity of the neutrophils due to bound antibody. A fluorescent PE-labelled anti-mouse antibody was used as an isotypic background control.

Neither the dark colour of the cell suspensions caused by the isolated sub-fraction nor the use of mixed leukocyte populations appeared to affect the results obtained from the flow cytometer.

Figure 3.1 below shows a typical scattergram of a cell suspension of isolated neutrophils that are untreated and unstimulated (left side of image) and a histogram for CR3 bound antibody within the gated area (right side of image) marked A in the scattergram. Increased binding of antibody resulted in a shift of the position of the histogram to the right. The position of the fluorescence histogram

along the x-axis for resting cells varied depending on the donor and the degree of stimulation due to the isolation procedure

The number of events counted in the stimulated cell samples appeared to be less than the corresponding resting cells indicating that either the stimulated cells had adhered to the tube surface or that the cells had undergone a change that altered the size and shape of the cells and hence fell outside of the gated region.

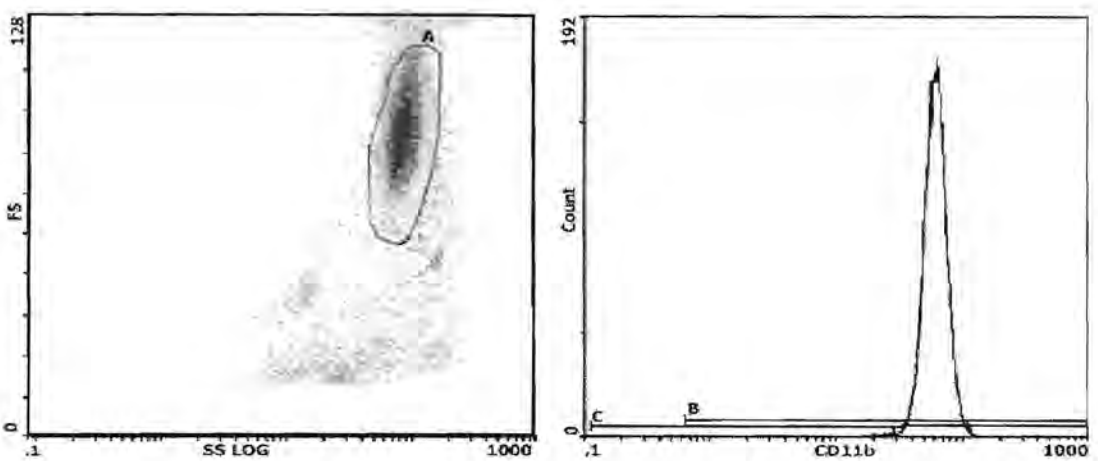

Figure 3-1: A typical scattergram and histogram of a control sample of isolated neutrophils. The scattergram on the left has been gated for the neutrophil population, which is indicated by the area enclosed by the line marked "A". The histogram on the right indicates the fluorescent intensity of the cells within the gated area of the scattergram. Note that the scales are semi-logarithmic.

The fluorescent intensity of the resting control cells was generally about one fifth to one third of that of the stimulated controls. If duplicate analyses were done on aliquots of the same isolated cells the repeatability of the fluorescent signal was very close, but the repeatability from the same donor over a period of time showed greater variation, especially with respect to signal of the resting control cells.

The bar graph in Figure 3-2 illustrates the effect that the different sub-fractions of oxihumate had on the expression of CR3 by both resting cells and cells stimulated by the addition of PMA after a 20 minute pre-incubating with the various concentrations of isolated sub-fraction as set out in Table 3-1 above. Firstly it can be shown that the stimulation of the cells with PMA results in a significant increase in the CR3 molecule concentration on the cell membrane. Secondly, there is a significant

inhibition of the expression of CR3 molecules by the stimulated cells when exposed to three of the seven isolated sub-fractions of oxihumate, i.e. the water-soluble fraction, the acid precipitated fraction and the methanol precipitated fraction. The water-soluble sub-fraction of oxihumate appears to have the greatest inhibitory activity. The acid soluble sub-fraction, the methanol soluble sub-fraction and the two sub-fractions derived from the latter all appear to have no effect on the expression of the CR3.

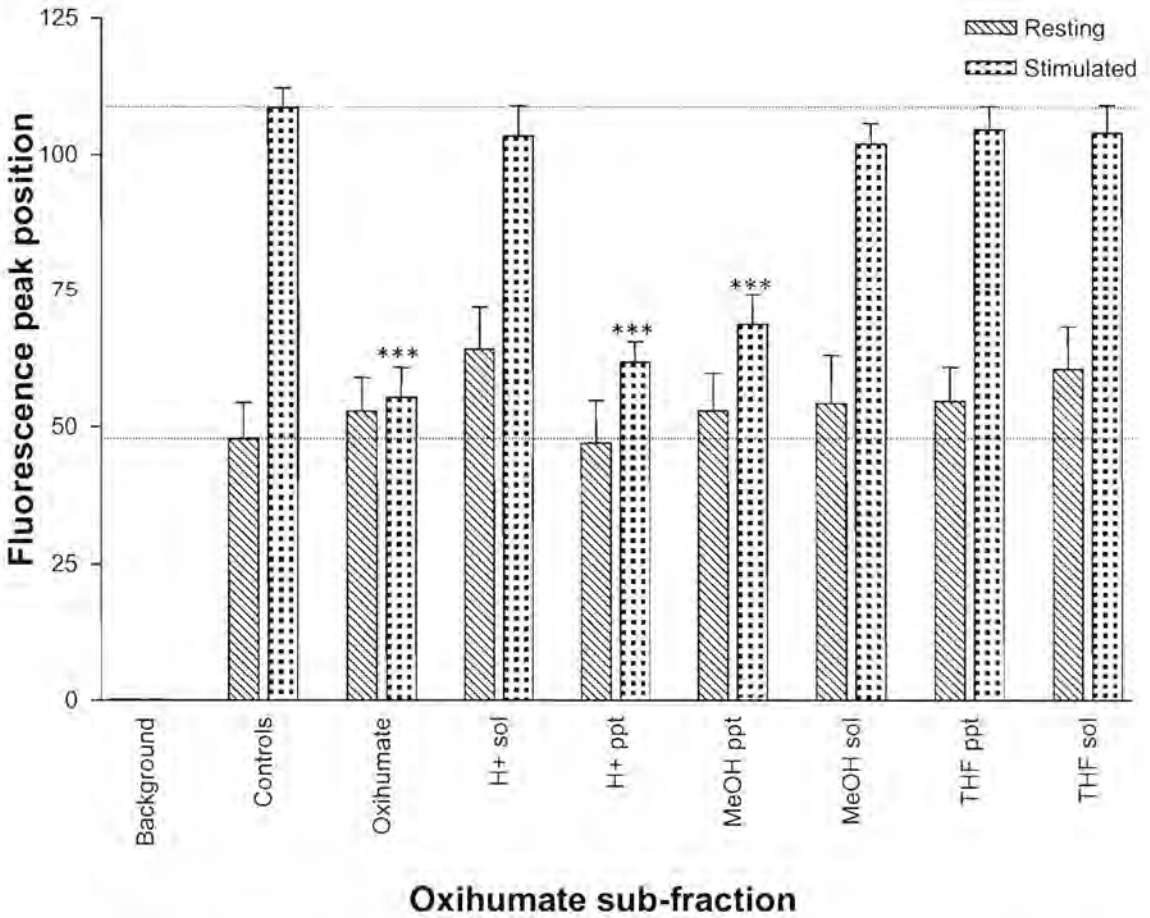

Figure 3-2: The effect of different isolated sub-fractions of oxihumate on the expression of CR3 on the surface of neutrophils. The sub-fractions were added to the neutrophils at the relative concentrations that match the mass of the sub-fractions in the oxihumate. The resting cells were pre-incubated with the test compounds only while the stimulated cells were further stimulated by the addition of PMA to the cell suspension after pre-incubating with the test compounds. The two horizontal dashed lines indicate the resting and stimulated averages for control neutrophils not exposed to any test compounds. Each bar is the average of 5 different experiments with SEM error bars. *** indicates $p < 0.001$ compared to the relevant control using the Students T test.

3.4 Chemiluminescence assays of neutrophil generated oxidants

The chemiluminescence exhibited by human neutrophils due to the release of reactive oxygen species after exposure to the different isolated sub-fractions of oxihumate in medium containing 10% foetal calf serum revealed interesting results. Figure 3-3 shows a series of overlaid luminescence curves after exposure of resting neutrophils to the various sub-fractions in the concentrations indicated.

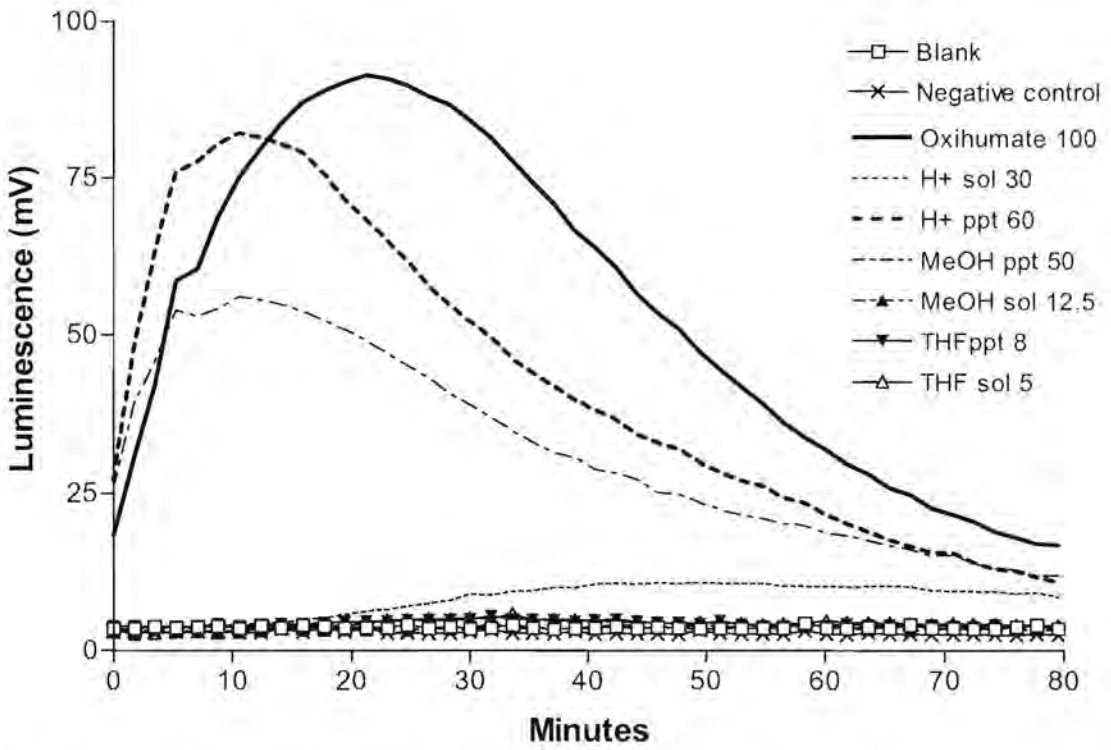

Figure 3-3: Overlaid plots of chemiluminescence against time for human neutrophils in HBSS containing 10% FCS and lucigenin. No stimulants were added to the cell suspensions so the release of reactive oxidation species was due to the isolated sub-fraction added to the cells when monitoring was initiated. The concentrations of the added sub-fractions are given in µg/ml in the legend. Only the water soluble, acid precipitated and methanol precipitated fractions showed any significant stimulatory activity.

The concentration of the water soluble, acid precipitated and methanol precipitated sub-fractions of oxihumate were high enough to result in fairly dark coloured solutions that were expected to show quenching, however these were the sub-fractions that showed the highest stimulation of the cellular release of oxidative reactants. Of the other sub-fractions, only the acid soluble sub-fraction showed any effect and this was small by comparison to the above sub-fractions that elicited a strong

response. The time to reach the maximal release of oxidative reactants was between 12 and 20 minutes.

When the neutrophils were pre-incubated at 37°C for 20 minutes with the different isolated sub-fractions, at concentrations to match the relative mass present in oxihumate, before stimulation with PMA, it was again found that differences in the response could be detected by chemiluminescence. All the sub-fractions showed an increase in the total release of reactive oxygen species combined with a faster initial response. This effect is illustrated in Figure 3-4, which shows the overlaid chemiluminescent responses of neutrophils that had been pre-incubated for 20 minutes with the different isolated sub-fractions prior to stimulation with PMA.

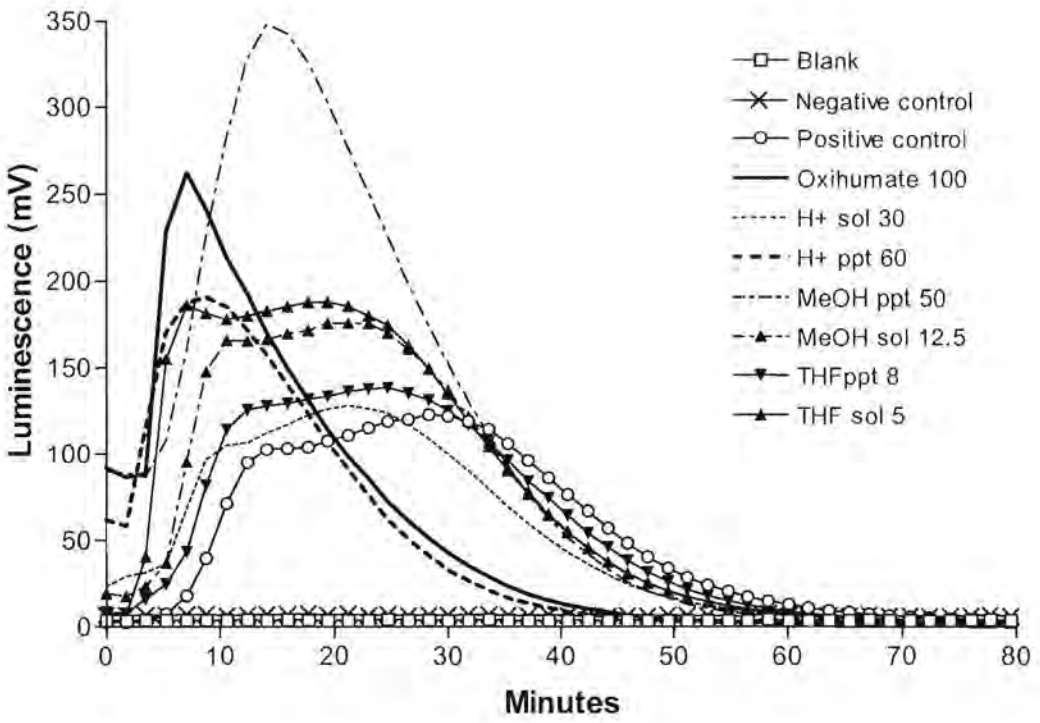

Figure 3-4: Overlaid luminescence response curves of neutrophils that were pre-incubated with the different fractions of oxihumate for 20 minutes prior to stimulation with PMA. The water soluble, acid precipitated and MeOH precipitated sub-fractions showed a single peak whereas the other sub-fractions showed a broad overlapping double peak. All sub-fractions had a greater response and a faster initiation than that of the positive control.

The time for the initial reaction to reach a maximum varied from 8 minutes for the water-soluble and THF soluble sub-fractions to 16 minutes for the positive control and THF precipitated sub-

fraction. The time to reach the maximum response for the second or follow-on response, when present, varied from 21 minutes for the THF soluble sub-fraction to 32 minutes for the positive control.

The water soluble, acid precipitated and methanol precipitated sub-fractions showed a different curve profile with a single rapid release of oxidants followed by a prolonged steady decrease in activity. The remaining four sub-fractions (more lipophilic) showed a similar curve profile to the positive control, which appeared as two broad overlapping peaks, except that initiation was faster and the responses slightly larger.

The chemiluminescence profiles for neutrophils that were exposed to the various isolated sub-fractions of oxihumate prior to being stimulated with FMLP demonstrated an interesting trend as illustrated in Figure 3-5. The blank (without cells) and the negative control (no FMLP) demonstrated no increase in the chemiluminescence above the background. The positive control, which included no test compound, gave a relatively smooth bell shaped curve reaching a maximum in approximately 65 seconds. The methanol soluble, tetrahydrofuran precipitated and tetrahydrofuran soluble sub-fractions all demonstrated curves very similar to the positive control but with a more rapid initial increase and slight increase in the maximum luminescence. The acid soluble sub-fraction showed a broader curve profile than the positive control but the maximum luminescence appeared to be unaffected.

In contrast to these results, the water soluble and acid precipitated sub-fractions had a background luminescence prior to addition of the FMLP which then caused a small decrease followed by a rapid short burst of luminescence lasting from 17 to 25 seconds followed again by a broad slow increase that was centred around 40 seconds. This second increase in luminescence was much lower than that of the positive control and decreased more rapidly than the control. The initial short burst and the second broad increase had approximately the same maximum luminescence intensity. This change in the profile and shift in the time of the maximum was only seen with the less refined and darker sub-fractions. Figure 3-5 below shows the overlaid signals of the blank, negative control, positive control and the seven different isolated sub-fractions at final concentrations equivalent to that at which they occur in the oxihumate and summarized in Table 3-1 above. Note that the monitoring time for the neutrophils stimulated with FMLP is only 3 minutes (and shown as seconds on the abscissa of the graph) whereas that for the PMA stimulated neutrophils is 80 minutes.

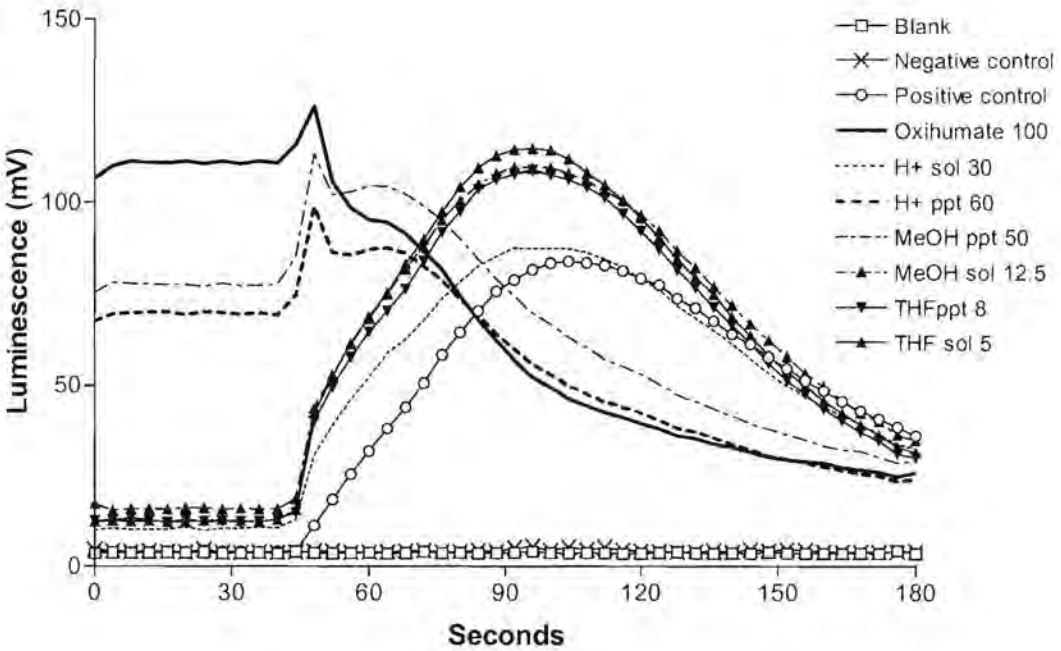

Figure 3-5: Overlaid chemiluminescence response curves of neutrophils that were pre-incubated with the different fractions of oxihumate for 20 minutes prior to stimulation with FMLP. The water soluble, acid precipitated and MeOH precipitated sub-fractions showed significant background activity and gave a small peak with a rapid onset followed by a much smaller and broader second peak before decreasing steadily. The other sub-fractions showed a single broad peak similar to the positive control except that the initiation was more rapid and the total response slightly greater. The concentrations of the sub-fractions used are given in the legend.

It is interesting to note that similar curve profiles but very different luminescent maxima were observed for neutrophils treated with the different sub-fractions for only 3 minutes prior to stimulation with FMLP. This is illustrated in Figure 3-6 below. In the case of the water soluble and acid precipitated sub-fractions, the initial background luminescence was much lower, the initial peak was smaller and the second peak more prominent. The methanol-precipitated sub-fraction showed a similar initial curve but the second peak was much larger, and overlapped the first peak and exhibited a luminescent maximum similar to that of the positive control although it occurred earlier. The acid soluble sub-fraction again showed a broader curve profile with a similar maximum to the positive control. The remaining three sub-fractions showed curve profiles similar to that of the positive control except that there appeared to be a slightly faster initiation of the curve and slight inhibition of the maximum relative to the control.

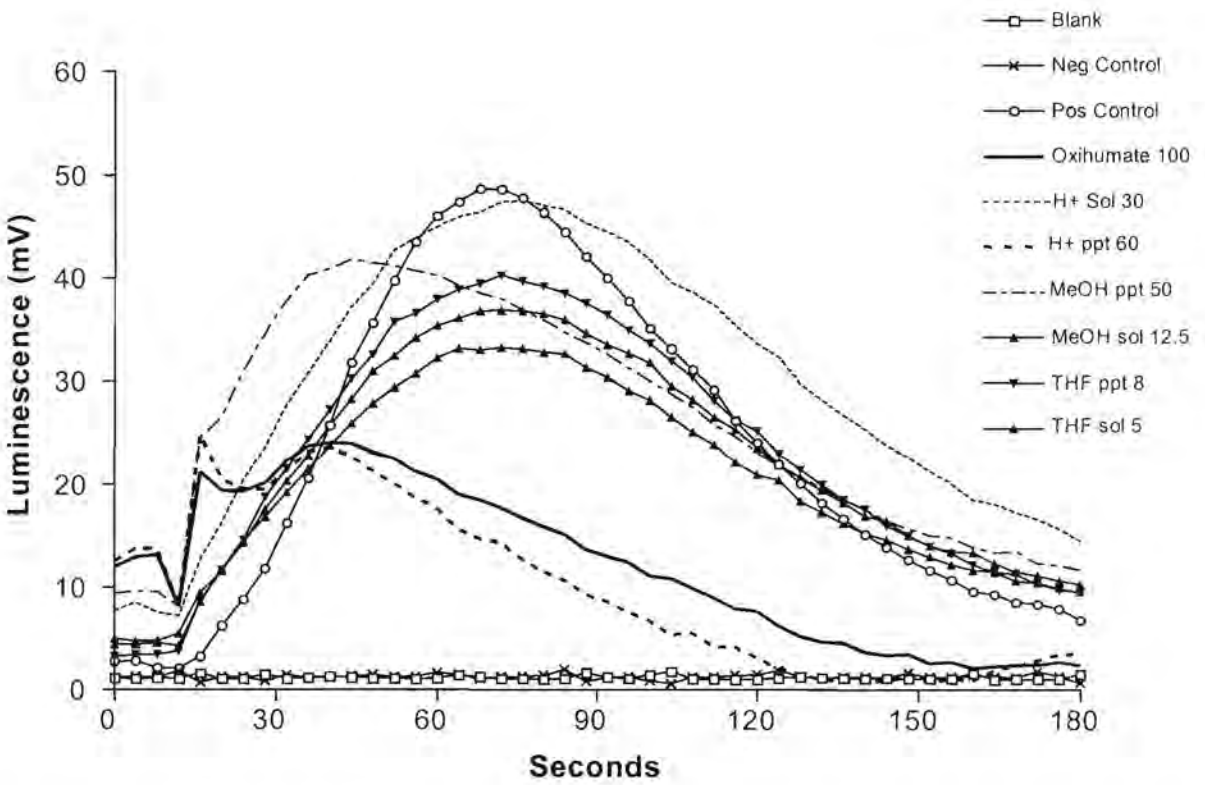

Figure 3-6: Overlaid chemiluminescence response curves of neutrophils that were pre-incubated with the different sub-fractions of oxihumate for only 3 minutes prior to stimulation with FMLP. The blank and negative control show no release of reactive oxidants while the positive control is the response of the neutrophils without exposure to any test compounds. The water soluble oxihumate, acid precipitated and methanol precipitated sub-fractions appear to cause a rapid release of an oxidant which is then followed rapidly by a second response that is less intense than that of the control and other sub-fractions. The remaining sub-fractions do not effect the time or duration of the response but only appear to affect the intensity of the response.

3.5 Superoxide anion scavenging assays using the xanthine oxidase generation system

A definite trend could be seen in the superoxide scavenging effect of the different isolated sub-fractions. The less refined sub-fractions (i.e. water soluble, acid precipitate and methanol precipitate) showed the greatest scavenging activity. This effect is illustrated in Figure 3-7 and Figure 3-8 below, where the concentration of the test compounds (5µg/ml and 50µg/ml respectively) were varied in an attempt to minimize the quenching effect of the dark sub-fractions. The bars represent the results of three different experiments with their SEM.

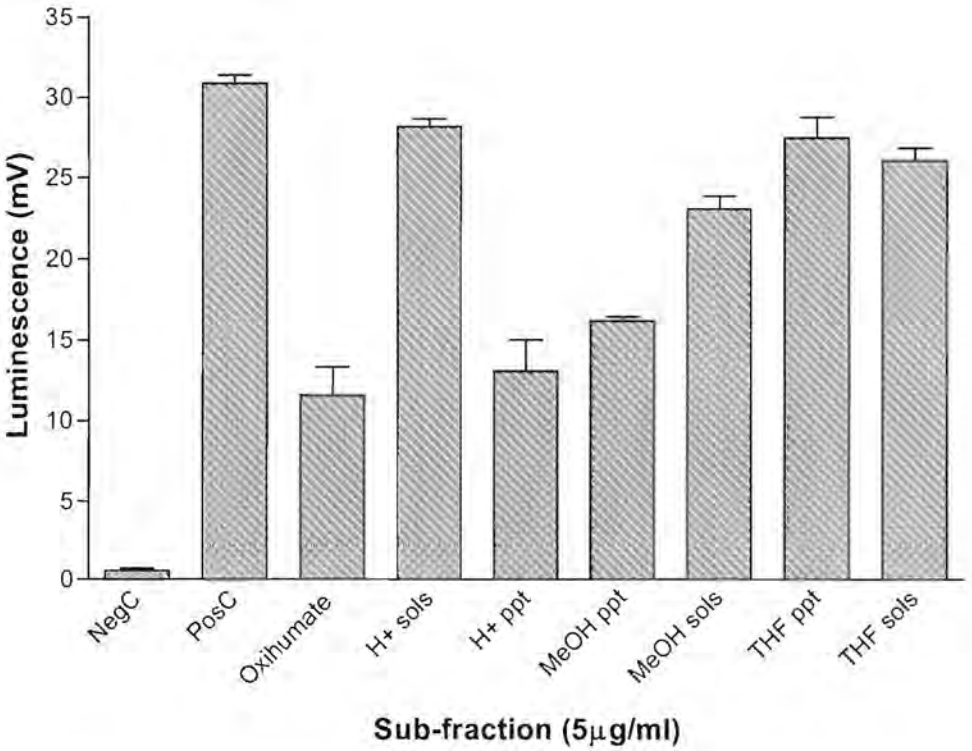

Figure 3-7: The superoxide scavenging effect of a final concentration of 5µg/ml of the various isolated sub-fractions of oxihumate compared to a negative and positive control. The most active sub-fractions appear to be the least refined sub-fractions. The negative control had no enzyme to generate the superoxide added and the positive control had no test compound added. The chemiluminescence is directly dependant on the concentration of superoxide anion present in the solution. Summary of three experiments with SEM bars.

It can be seen from these two figures that there is a concentration effect in addition to the variation in the effect of the different sub-fractions. The higher concentrations show greater quenching but the trend of the antioxidant activity for the different fractions remains constant.

As the water-soluble sub-fraction, the acid precipitated sub-fraction and the methanol precipitated fractions were the darkest coloured fractions it was thought that the apparent antioxidant effect of these fractions might have been due to quenching of the emitted luminescence.

The absorbance at 450nm of each test mixture was determined and is summarized in Table 3-3.

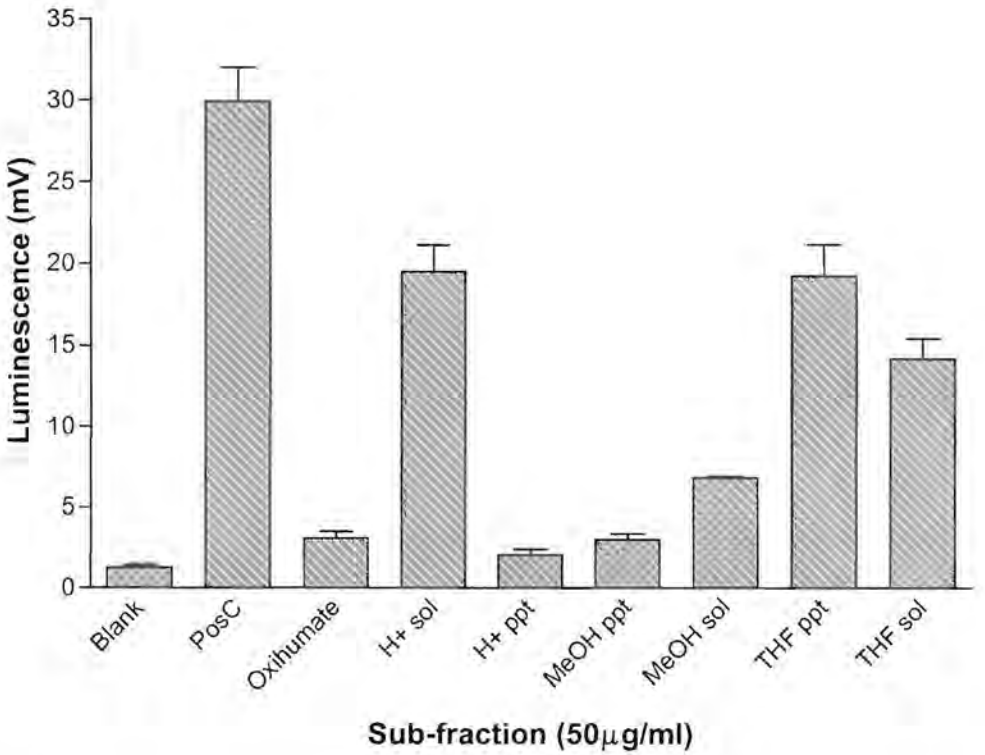

Figure 3-8: The superoxide scavenging effect at a final concentration of 50μg/ml of the various isolated sub-fractions of oxihumate compared to a negative and positive control. The most active sub-fractions still appeared to be the least refined sub-fractions. The negative control had no enzyme to generate the superoxide added and the positive control had no test compound added. The chemiluminescence is directly dependant on the concentration of superoxide anion present in the solution. Each bar is a summary of three experiments with SEM bars.

Table 3-3: Summary of the absorbance at 425nm of the different test solutions after addition of all the reagents. This could indicate that the apparent antioxidant activity was due to quenching rather than scavenging of the superoxide anions.

Fraction	A_{425} @ 5μg/ml	A_{425} @ 50μg/ml
Control (no test compound)	0.046	0.072
Water soluble oxihumate	0.150	0.505
Acid solubles	0.087	0.069
Acid precipitate	0.150	0.662
Methanol precipitate	0.154	0.772
Methanol solubles	0.125	0.333
THF precipitate	0.052	0.081
THF solubles	0.049	0.042

In addition to the measurement of the absorbance at 425nm one fraction (the acid precipitated sub-fraction) was tested at 5 different concentrations to determine whether there was a linear dose response. A definite dose response was observed but this response was not linear. Figure 3-9 illustrates the concentration effect relative to a control where no enzyme was present to generate superoxide anions. The dose response showed the largest apparent difference in activity between the untreated solution and the lowest concentration tested.

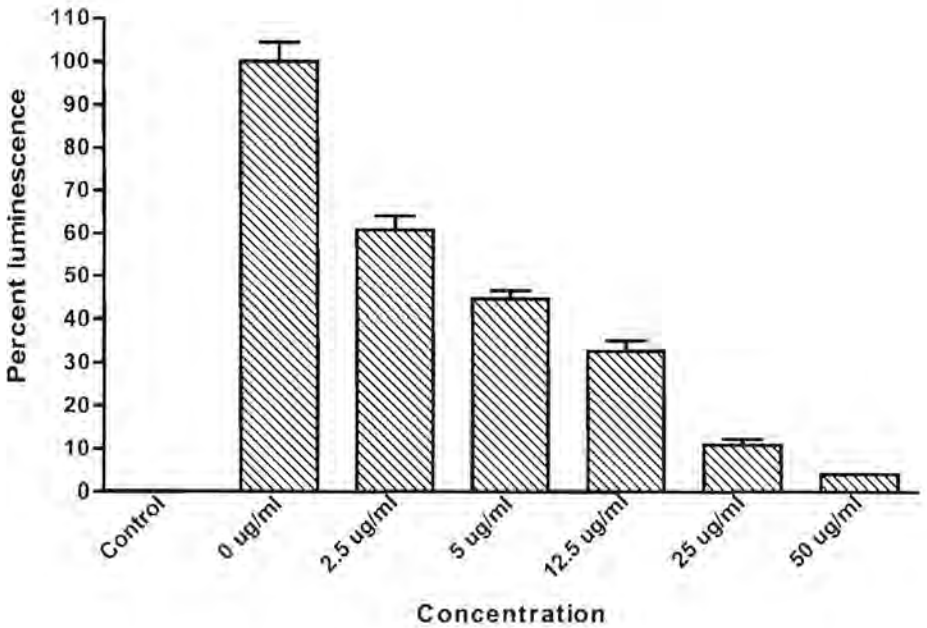

Figure 3-9: Bar graph showing the concentration effect exhibited by the acid precipitated sub-fraction on the superoxide anion scavenging. The control contained no superoxide generating enzyme system and is therefore also used as the background value. Each bar is the average of 5 experiments with SEM bars.

4 Discussion

Bioassays are important methods to determine the efficacy and the effects of unknown compounds that show potential as therapeutic drugs. The correct choice of an appropriate bioassay is critical for the determination of activity of the test compounds.

In this study the emphasis has been on the anti-inflammatory effects of oxihumate and the choice of bioassays was done to determine the effect on neutrophils, the most common inflammatory cell in all inflammatory processes (Witko-Sarsat *et al*, 2000; Jooné *et al* 2003). indicated that the anti-inflammatory effects of oxihumate could be ascribed to its inhibitory effects on the expression of

CR3 by activated neutrophils as well as the adhesion of these cells to ICAM-1. By using neutrophils isolated from healthy volunteers in this study, similar effects were observed for oxihumate as well as with some of the isolated sub-fractions. In the case of oxidant production by stimulated neutrophils, oxihumate and several of the isolated sub-fractions had either a priming or an anti-oxidant effect.

The different isolated sub-fractions were generally tested at the relative concentrations at which they occur in oxihumate. This was done to give an indication of the contribution of each sub-fraction to the total anti-inflammatory effect of oxihumate.

The first experiments were done to determine whether there were any deleterious effects on the neutrophil viability when exposed to the different sub-fractions. If there were indications that the oxihumate sub-fractions did affect the neutrophils viability then the results of the expression of CR3 receptors and the oxidative burst could not have been assigned to the test compounds alone. As no obvious toxic or membrane disrupting effects were detected and more than 90% of the cells appeared to be viable after 30-minute exposure to all the different sub-fractions, it could safely be assumed that the responses seen in the bioassays were due to the test compounds. If this fact is considered together with the finding that the oxihumate binds extensively to plasma proteins it could safely be assumed that the sub-fractions absorbed from the GIT would not exceed the concentrations of free oxihumate used in the study and cannot cause cell damage.

The expression of CR3 molecules was strongly inhibited by the less refined sub-fractions, namely the water-soluble sub-fraction, the acid precipitated sub-fraction and the methanol-precipitated sub-fraction. The remaining sub-fractions showed insignificant changes from the controls, which would imply that the activity is associated with the complete mixture of compounds and not with the minor compounds that could be extracted from the oxihumate. The three lipophilic sub-fractions would represent the most soluble and most extractable compounds in oxihumate and although they account for almost 40% of the water-soluble mass of oxihumate, they appear to have a very small and insignificant effect on the expression of CR3. It should be noted that the acid soluble sub-fraction, which represents the fulvic acids, also contains a high percentage of inorganic salts, which would not be expected to have any influence on the cellular expression of CR3 molecules.

The possibility that the dark colour of these active sub-fractions could have contributed to the apparent inhibition was tested in our laboratories using similar dark compounds and found not to have any effect on the measurements (unpublished results). A concentration effect has been

demonstrated for oxihumate (Jooné, 2002) and the same effect was also found for the more active sub-fractions tested in this study.

As expression of CR3 molecules is required to enable adhesion of the neutrophils to the vascular epithelial cell prior to diapedesis it would imply that diapedesis would also be inhibited. It has been shown *in vitro* that oxihumate does inhibit the binding of treated neutrophils to transfected baby hamster kidney (BHK-126-16 and BHK 331-7) cells that over-express human the E-selectin and ICAM-1 adhesion molecules respectively (Jooné *et al*, 2001). If adhesion is inhibited in the presence of the oxihumate, then extravascularisation of the neutrophils would be suppressed and the inflammatory response would be expected to be decreased.

The second luminescence bioassay performed was to determine the effect of the different sub-fractions on the respiratory burst of the neutrophils. It is well known that the oxidative burst plays an important role in the inflammatory process, and if suppressed, a very limited inflammatory response follows (Rosen, 1993).

The different isolated sub-fractions of oxihumate showed variable effects on resting neutrophils. As seen with the inhibition of the expression of CR3 molecules it was the darker less refined sub-fractions that showed the greatest effects. On the other hand, there appeared to be a stimulation of the formation of reactive oxygen molecules by the same sub-fractions, as measured by the luminescence assay using lucigenin which is reputed to be selective for superoxide anion radicals (Allen, 1986). The water soluble, acid precipitated and methanol precipitated sub-fractions all showed a large increase in luminescence of resting neutrophils with a broad skewed peak reaching a maximum between 10 and 25 minutes. The acid soluble sub-fraction showed a small flat and very delayed activity peak only reaching a maximum at approximately 50 minutes. This initial background activity of the neutrophils had a dramatic effect on the response of the cells to further stimulation by PMA or FMLP and caused a high background reading when the cells were pre-incubated with the most active sub-fractions for 20 minutes prior to the stimulation. The remaining three sub-fractions (methanol soluble, THF precipitate and THF soluble sub-fractions) showed no significant effect on the neutrophils although further stimulation with PMA resulted in responses greater than, and faster than, that of the positive control samples. This would imply a priming of the cells sensitising them for the stimulant. When the neutrophils were stimulated with FMLP after the 20 minute pre-incubation the effect was even more dramatic, with the water soluble, acid precipitated and methanol precipitated sub-fractions showing very high background luminescence followed by a small very rapid burst followed by a second smaller but broader peak which tapered

off to baseline levels. The remaining fractions showed a much greater response that was similar to that of the positive control sample except that the luminescence responses were greater than that of the positive control. This again pointed to priming or sensitising of the cells to the stimulant. In the case where the cells were pre-incubated with the isolated sub-fractions for only 3 minutes there was a similar curve profile for the active sub-fractions to that of the 20 minute pre-incubated cells but the remaining fractions still showed a curve very similar to the positive control and in this case, with a slight inhibition of activity relative to the control. This would imply that the sensitisation seen in the pre-incubated cells was time dependant and was therefore not merely a blocking action on a receptor.

The decrease in total luminescence of the cells exposed to the active sub-fractions could result from a number of factors. It may be due to exhaustion of the energy levels in the cells, depletion of the oxygen in solution, a blocking effect of particular receptors or the blunting of the effect of one receptor by the response of a different receptor. The later effect is apparently due to crosstalk between different receptors and their signaling pathways, which can result in desensitization of one stimulant by another. Normally, signals delivered by "end target-derived" chemoattractants are dominant and override "regulatory cell-derived" attractants, such as bioactive lipids (LTB_4) or chemokines (IL-8) (Kitayama *et al*, 1997; Foxman *et al*, 1999).

Inflammation is the result of the release of reactive oxygen species and pro-inflammatory mediators at the inflammatory focus. A "drug" that eliminates any of these neutrophil formed and released compounds would have some anti-inflammatory properties. Destruction of the reactive oxygen species would occur if the oxihumate sub-fractions could act as antioxidants, thereby reducing the amount of cell damage in surrounding tissue that results from excess reactive oxygen species in the inflammatory focus.

To determine if oxihumate could act as an antioxidant, the activities of the different isolated sub-fractions were tested to determine the relative antioxidant activity towards the superoxide anion, one of the reactive oxygen species formed by neutrophils during the respiratory burst (Klebanoff, 1999). In these antioxidant assays it was again found that the least refined sub-fractions showed the greatest activity as antioxidants when added to the reaction mixture at the relative concentrations equivalent to what is found in the oxihumate complex. The acid soluble and THF precipitated sub-fractions showed very limited activity while the remaining sub-fractions showed varying degrees of antioxidant activity as seen from the bar graphs in Figures 3-7 and 3-8. There was an obvious dose response for all the sub-fractions. In these assays a strongly coloured reaction mixture could result

in quenching of the emitted luminescence. As the luminescent flash is emitted at 425nm the absorbance of the different sub-fractions were determined at 425nm at the same concentrations used in the antioxidant assays. These absorbance values are summarized in Table 3-3 and it can be seen that the methanol-precipitated sub-fraction had the highest absorbance but the activity of the water-soluble and acid precipitated sub-fractions proved to have the greatest antioxidant activity. This would then imply that the quenching was either not so great as to have an effect on the result or that the antioxidant activity of the dark sub-fractions are very much higher than the other fractions and that this increased activity is partially quenched by the colour. The dose response of the acid precipitated sub-fraction is shown and although there is an obvious dose response it should be noted that the effect is not linear with respect to the concentration of the sub-fraction. The greatest effect is demonstrated for the lowest concentration used. It is possible that the colour of the sub-fractions played a role in the non-linear response observed. The luminescence curve profiles obtained during this study showed that enzyme activity was still present after addition of the sub-fraction, and that the slope of the curve was similar to that of the control sample. This observation eliminates the possibility that the reduction in signal was due to inhibition of enzyme activity.

These results would therefore support the findings that oxihumate complex does have anti-inflammatory properties and that these appear to operate at different points in the inflammatory process. Not all the compounds present in the oxihumate complex are active and it seems that some of the sub-fractions can cause some form of priming of the neutrophils although this would implicate oxihumate as a pro-inflammatory agent, a contradiction to the rest of the results. The antioxidant activity although present appears to be localized only in the complete complex, a result which seems to be reinforced by all the other bioassays used in this study.

As the mechanism of action of humic acids has not yet been elucidated it is difficult to speculate on which bioassay is the most important in determining the most active sub-fractions as there is a possibility that some the sub-fractions that show limited activity may be very active as enzyme inhibitors or interfere with the production and release of inflammation mediators. It was not the aim of this study to determine the mechanism of action of humic acid but to attempt to find active compounds in the complex mixture of compounds that make up the oxihumate complex.

Chapter 4

In vitro Absorption of Oxihumate by Rat Gastrointestinal Tract

1 Introduction

As *in vitro* effects could be demonstrated on isolated cells of the immune system by humic acid products (Jooné, 2002) it was attempted to ascertain whether potassium oxihumate could be absorbed from the alimentary canal of rats. The vascular system responsible for receiving and distributing the absorbed compounds from the GIT penetrates as deep as the submucosal layer of the alimentary canal and allows compounds that have crossed only as far as the submucosa to be distributed by the blood or lymphatic systems.

In this study an *in vitro* experiment using isolated segments of the gastrointestinal tract dissected from the rat (*Rattus norvegicus)* was performed. The study was performed in accordance with the guidelines set out in for the use of experimental animals (Department of Agriculture, 1990; Medical Research Council, 2002a; Medical Research Council, 2002b; Medical Research Council, 2002c) and approved by the Animal Use and Care Committee of the University of Pretoria Biomedical Research Centre under Project number 8.1 of 2002 (See Appendix I).

As the vascular system that normally transports the absorbed compounds from the GIT was removed during dissection, the compounds in the lumen of the gut had to cross all the cell layers of the GIT segments to confirm that absorption had taken place. There are eight distinct cell layers in the GIT i.e. mucosa, mucosal muscular layer, submucosa, circular muscle layer, intermuscular stroma, longitudinal muscles, subserosal connective tissue and finally the serosa.

The technique used in this experiment is a standard technique to determine whether compounds can cross the GIT and is often used to determine the *in vitro* rate of uptake of drugs and nutrients. The experiments described here were performed using radioactively labelled oxihumate to circumvent the analytical problem of not having a well-defined single compound or a known compound to analyse in the suspending medium.

The object of this experiment was to perform *in vitro* experiments to determine whether oxihumate can cross the mucosal membranes of rat GIT and if possible to determine the pharmacokinetics of the absorption. Use was made of ^{125}I-labelled oxihumate.

2 Materials and Methods

2.1 Materials

Iodide-125 was purchased from Perkin Elmer Life Sciences Incorporated (Boston, USA) as carrier free isotope, in the sodium iodide form, dissolved in 100µl of a 0.1N sodium hydroxide solution. The specific activity was reported to be 643 GBq/mg.

The following analytical grade reagents were purchased from Merck Chemicals, (Darmstadt, Germany): chloramine-T, sodium bicarbonate, glacial acetic acid, trifluoroacetic acid, chloroform, ammonium hydroxide solution as well as TLC plates (5 x 20 cm silica gel 60 TLC plates without fluorescent indicator) and universal pH indicator strips.

HPLC grade methanol (a Sigma product) and acetonitrile (a Fluka product) were both purchased from Sigma-Aldrich (St Louis, MO, USA).

Fujifilm Super RX 100NIF X-ray film sheets (180 x 240mm) and the recommended developing chemicals for these plates as concentrated solutions that required only dilution were purchased from Africa X-ray Industrial and Medical (AXIM) (Midrand, South Africa).

Oxihumate was supplied as a formulated dry powder as a generous gift from Enerkom (Pty) Ltd, Pretoria.

Hanks balanced salt solution (HBSS) containing calcium and magnesium but without phenol red indicator was purchased from Highveld Biological (Johannesburg, South Africa).

2.2 Animals

As only the possibility of absorption of humic acids from the GIT was being studied, no difference was expected between the sexes. Male rats are larger and more available, therefore adult male Sprague Dawley rats (200g and more) were used for the experiments. These were provided by the University of Pretoria Biomedical Research Centre.

2.3 Methods

2.3.1 Labelling of oxihumate fraction with ^{125}I

For the rat gut absorption studies a single large batch of ^{125}I labelled oxihumate was prepared to avoid inter-experimental variation in specific labelling and of the concentrations of oxihumate added into the GIT. The labelling procedure used was that of Hunter and Greenwood where the radioactive iodide was oxidized by chloramine-T to produce iodine, which rapidly reacts with the active phenolic groups in the oxihumate (Hunter & Greenwood, 1962).

A solution of 1 mg/ml Chloramine-T was freshly prepared in deionized water just before starting the labelling procedure. Fifteen microlitres of the ^{125}I sodium iodide solution equivalent to 55.5MBq (1.5mCi) was carefully removed from the vial with a HPLC injection syringe and diluted to 200µl with distilled water in a 10ml glass vial with a rubber septum. 200µl of the freshly prepared chloramine-T solution was added and vortexed for at least 30 seconds. The labelling was then initiated by adding 8ml of a 1.6% solution of potassium oxihumate and again vortexing for at least 30 seconds to ensure proper mixing.

The mixture was allowed to stand for 30 minutes in a lead pot at ambient temperature with occasional mixing by vortexing. During this time the total activity was determined to be 56.6 MBq (1.53mCi) by measurement in a CMC-10 gamma dose calibrator. As the calibrator does not have long half-life isotopes as a standard selection, the energy was set to 139 in the "other" setting that was the required set-up for measuring ^{125}I according to the instrument instruction manual.

After the 30-minute incubation time, 1.0ml glacial acetic acid was added to the mixture, the vial vortexed again, placed inside a 50ml centrifuge tube with a cotton wool plug as support and centrifuged for 10 minutes at 3500g. The vial was gently removed from the centrifuge tube to prevent disturbing the fine black precipitate, carefully uncapped and the supernatant transferred to a 30ml urine bottle by means of a syringe fitted with a long blunt needle. The activity of 100µl aliquots of the supernatant were measured in a LKB Wallac 1261 multiwell gamma counter. The precipitate was washed again by adding a further 1.0ml glacial acetic acid and 5ml deionized water, recapping the vial and vortexing for 30 seconds. The washing procedure was repeated three times in all, with the activity of the washing supernatant being measured before combining with the previous acid wash supernatant fractions.

The final activities of the washed precipitate as well as the combined supernatants were measured and used to calculate the labelling efficiency. The final precipitate was redissolved in 6.0ml water to which 100µl of 1.0 M NaOH had been added.

Thin layer chromatography was run on 5 x 20 cm silica gel 60 TLC plates (without fluorescent indicator) with a layer thickness of 0.25mm. 5µl spots were applied approximately 1cm from the bottom of the plate and developed with mobile phase of the following compositions:

Mobile phase I

MeOH : $CHCl_3$: nBuOH : H_2O : CH_3CO_2H **9 : 9 : 3 : 3 : 0.5**

Mobile phase II

Acetone : nBuOH : NH_4OH(25%) : H_2O **65 : 20 : 10 : 5**

Mobile phase I is a mixture found to be suitable for separating iodide from humic acid compounds.

Mobile phase II is suitable for separating halide ions (Seiler, 1969).

Autoradiography of the TLC plates was done in Okamoto X-ray plate cassettes of appropriate size using Fujifilm Super RX 100NIF X-ray film sheets. In a dark room with a safe light, a sheet of X-ray film was placed directly onto the amplification surface of the cassette. The TLC plates were covered with a layer of clingwrap plastic to protect the X-ray film from the silica gel layer and secured to the film with the coated surface against the X-ray film using suitable tape. The cassette was closed and locked down under the safe light. The exposure times depended on the activity of the separated compounds but were generally in the 2-5 day period if the activity per applied sample was approximately 6000 cpm.

After exposure the X-ray film was removed and developed under a safe light at 21°C for 6 – 8 minutes in developer solution, rinsed in stop solution for 3 minutes, briefly rinsed in water and fixed for 10 minutes in fixing solution. The plates were then washed for at least 5 minutes under running water then finally rinsed in distilled water and dried.

2.3.2 Absorption of oxihumate by rat gastrointestinal tract

Adult male rats (± 200g) were given no solid food for a period of 24 hours but had glucose added to the drinking water. Individual rats were euthanased by cervical dislocation, the abdomen dissected

open and the entire abdominal section of the gastrointestinal tract was removed, from where the oesophagus penetrates the diaphragm to the rectum. The entire omentum and peritoneal membranes together with the vascular network was carefully removed from the GIT and discarded.

The short section of oesophagus was left connected to the stomach and the duodenum cut just caudal to the pyloric valve. Due to the gall duct and the pancreatic duct entering the duodenum and it not being possible to seal off these ducts effectively the duodenum was cut just caudal to the pancreatic duct and discarded.

The small intestine (the combined jejunum and ileum) from the pancreatic duct to the 5mm from the caecum was kept intact.

The colon was cut from the caecum about 5mm after the caecum narrowed to form the ascending colon.

The contents of each of the above segments of the gastrointestinal tract was cleared by gentle pressure mimicking peristalsis, starting by clearing approximately 3cm from the caudal end and then working back towards the mouth end. After the bulk of the solid contents were cleared, each segment was gently flushed with about 50ml of HBSS (containing calcium and magnesium but not phenol red indicator) that was introduced into the organ segments by means of a 60ml syringe and a size 14 Jelco catheter.

The washed organ segments were suspended in cold HBSS while one the ends of each segment were sealed by tying off with suture silk at two points approximately 2mm apart at approximately 4 mm from the ends.

Into each segment 1 - 5ml of HBSS was introduced followed by 0.5ml ^{125}I labelled oxihumate solution equivalent to 250 – 300KBq (± 7 - 8μCi) by means of a 1ml tuberculin syringe. The gut segments were then sealed with suture silk ensuring that there would be no leakage from the segments. In the case of the small intestine (combined jejunum and ileum) the volume of the labelled oxihumate solution was 1ml with a specific activity of 600 – 750KBq (± 16 - 20μCi).

Each organ was quickly rinsed in warm PBS (37°C) to ensure that there was no activity on the outside of the segments and especially at the ends outside of the closures. The organ segments were each placed in a sealed plastic 30ml urine vial containing 20ml HBSS (the combined small intestine

was incubated in a 100ml glass reagent bottle with 75ml HBSS) and allowed to incubate at 37°C with gentle shaking every 5 minutes for a total of 2 hours. The gut segments were agitated every 15 minutes by raising each end alternatively using two tweezers to hold the silk closures at each end. Aliquots (2ml) of the suspending HBSS were removed from the incubation tubes and transferred into suitable sealed tubes for counting in a LKB Wallac 1261 multiwell gamma counter at 10 minutes, 15 minutes 30 minutes, 45 minutes, 1 hour and 2 hours. Two millilitres of fresh HBSS was added to each incubation tube to replace the volume removed for counting. The first experiment was continued for 22 hours with additional aliquots being removed at 4 hours, 8 hours, 18 hours and 22 hours.

After the two hours incubation period, the organs were removed from the incubating medium and each of the suspending HBSS solutions evaporated to dryness in Petri dishes under a draught of air. The dark sticky residues contained many salts and residual sugars that could interfere with further analysis. To avoid the interferences the residues were redissolved in a minimum volume of ammonium hydroxide and then adjusted to 40% methanol. After standing for at least 60 minutes the suspension was centrifuged at 3500*g* for 10 minutes and the supernatant removed and dried as before. The new residue was solubilized in 80% methanol. Addition of acetone to this methanol solution to give a 60% acetone solution resulted in an "acetone soluble" fraction and a precipitate that was collected by centrifuging as before. The precipitate was resolublized in 5% ammonium hydroxide solution. These fractions were analysed by HPLC in an attempt to determine which compounds in oxihumate were being absorbed from the lumen of the GIT.

2.3.3 Precipitation of radioactivity in absorbed fractions

In the first experiment the aliquots that were removed for counting after 22 hours were used to determine what the effect of acidification and of silver nitrate precipitation was on the distribution of activity found in the 22 hour supernatant.

The 2 ml aliquots of each of the organ segments collected at 22 hours incubation time were counted twice for 60 seconds in a LKB Wallac 1261 multiwell gamma counter to ensure accuracy of the counts of the aliquots. To each aliquot was added 400µl of glacial acetic acid and the tube vortexed well. After standing for 30 minutes the tubes were centrifuged at 3500*g* for 10 minutes. The acidic supernatant was carefully removed by removal of the liquid phase with an auto-pipette fitted with a long thin disposable tip. The supernatant and the precipitate of each organ were again counted in the multiwell gamma counter to determine the percentage of the original activity in each phase.

The acidic supernatant was then treated with 400µl of a 20% solution of $AgNO_3$ to precipitate any free iodide, vortexed to ensure homogenous mixing and allowed to stand for 30 minutes before centrifuging at 3500*g* for 10 minutes to remove the silver nitrate precipitate. The supernatant fraction was again carefully removed as before and the new supernatant and precipitate for each organ counted for ^{125}I activity in the multiwell gamma counter. The activity of each phase after each treatment was calculated as a percentage of the original activity of the 22-hour HBSS aliquot.

The absorbed fractions were analysed by TLC to determine which compounds found in the oxihumate were absorbed from the lumen of the gut

2.3.4 High Pressure Liquid Chromatography analysis of the absorbed fractions

The HPLC system was a Hewlett Packard 1050 HPLC system with a quaternary high-pressure pump, a multi-wavelength UV/Visible detector, a 1046A fluorescence detector coupled in series to the UV/Visible detector and an electronically activated Valco fixed loop injector. The separation was performed on 50µl samples on a 150 mm x 4.6 mm Cosmosil $5C_{18}$ MS-II column (Nacalai Tesque Inc, Japan) using the following mobile phases and gradient program:

Mobile phase A: 0.1% trifluoroacetic acid in deionized water.
Mobile phase B: 0.1% trifluoroacetic acid in HPLC grade acetonitrile.

0.0 – 3.5 minutes	92% A
3.5 – 20 minutes	linear gradient to 40% A
20 – 25 minutes	isocratic at 40% A
25 – 30 minutes	linear gradient to 92% A
35 minutes	end of analysis

The eluent was monitored at 230 nm (bandwidth of 20nm), at 270nm (bandwidth of 36 nm).

The HPLC system was controlled by a Hewlett Packard Chemstation software package that was also used to accumulate and store the chromatographic data.

For selected samples that were analysed using labelled oxihumate, the HPLC eluent was collected with a fraction collector by manually advancing the tubes to collect the eluting peaks into separate tubes that were subsequently counted in the LKB Wallac 1261 multiwell gamma counter to indicate the percent activity in each of the eluted oxihumate peaks in the chromatogram.

3 Results

3.1 ^{125}I Labelling of Oxihumate

The added activity for the labelling experiment was determined to be 56.6MBq (1.53mCi) in the dose calibrator. Of this total activity, 38.5MBq (1.1mCi) remained in the oxihumate acid precipitate after the labelling procedure that meant that a 68% labelling efficiency was achieved.

The supernatant fraction did however show an orange-brown colour indicating that there was some solubilized oxihumate or perhaps oxifulvic acid in the acidic wash solution. This activity was not investigated any further but discarded.

The labelled acid-precipitated oxihumic acid that was used for the gut absorption experiments was further analysed by thin layer chromatography and autoradiography to establish the radioactive free iodide content. Thin layer chromatography on silica gel 60 developed with the methanol-butanol-water-chloroform-acetic acid mobile phase system described above followed by autoradiography, using an exposure time of five days indicated that the labelled oxihumate contained insignificant free radioactive iodide. Figure 4-1 below shows an autoradiogram of the TLC plate of the labelling solution of oxihumate before the acid wash, ^{125}I labelled oxihumate after washing with acid, the acidic washings of the labelled oxihumate and an aliquot of NaI spiked with radioactive ^{125}I. The final lane was the starting oxihumate that had no radioactivity and was therefore not visualized in the autoradiogram.

The labelled oxihumate fraction had very little activity at *Rf* of 0.86, the *Rf* of the iodide but the unwashed oxihumate and the acidic washings both showed significant activity at this *Rf*.

3.2 Absorption of radio-labelled oxihumate by rat GIT

The small intestine (combined jejunum and ileum), the caecum and the colon showed rapid "absorption" of oxihumate from the lumen of these GIT segments into the HBSS solution in which they were suspended, with significant activity already evident after only 10 minutes of incubation. The radioactivity of the HBSS solutions in which these organ segments were suspended increased steadily for the first hour after which the increase appeared to plateau and increased progressively slower for the remaining duration of the experiment. In the experiment where the time of incubation was extended to 22 hours it was evident that a plateau was reached after approximately 80% of the dose (less that 4 hours) had been absorbed and further absorption was very slow.

Figure 4-1: An autoradiogram of ^{125}I labelled oxihumate for rat gut absorption study. The silica TLC was run in the MeOH:$CHCl_3$:nBuOH:H_2O:CH_3CO_2H solvent system. Lane 1 is ^{125}I labelling oxihumate solution, 2, acid washed ^{125}I labelled oxihumate; 3, acid washings of the labelled oxihumate; 4, ^{125}I as NaI; 5, unlabelled oxihumate.

In the case of the stomach, the total absorption was significantly lower than that of the other organ segments. Initially the absorption rate was much slower but this rate did begin to increase after approximately thirty minutes incubation. This increasing rate of absorption in the stomach continued even after the absorption by the other organs had reached their plateau although it only attained about 35% of the total administered activity being absorbed compared to more than 50% by the other GIT segments.

The absorption results are graphically summarized in Figure 4-2 where the "absorbed" oxihumate for each organ segment is shown as a percentage of the total dose that was introduced into the respective organ segment.

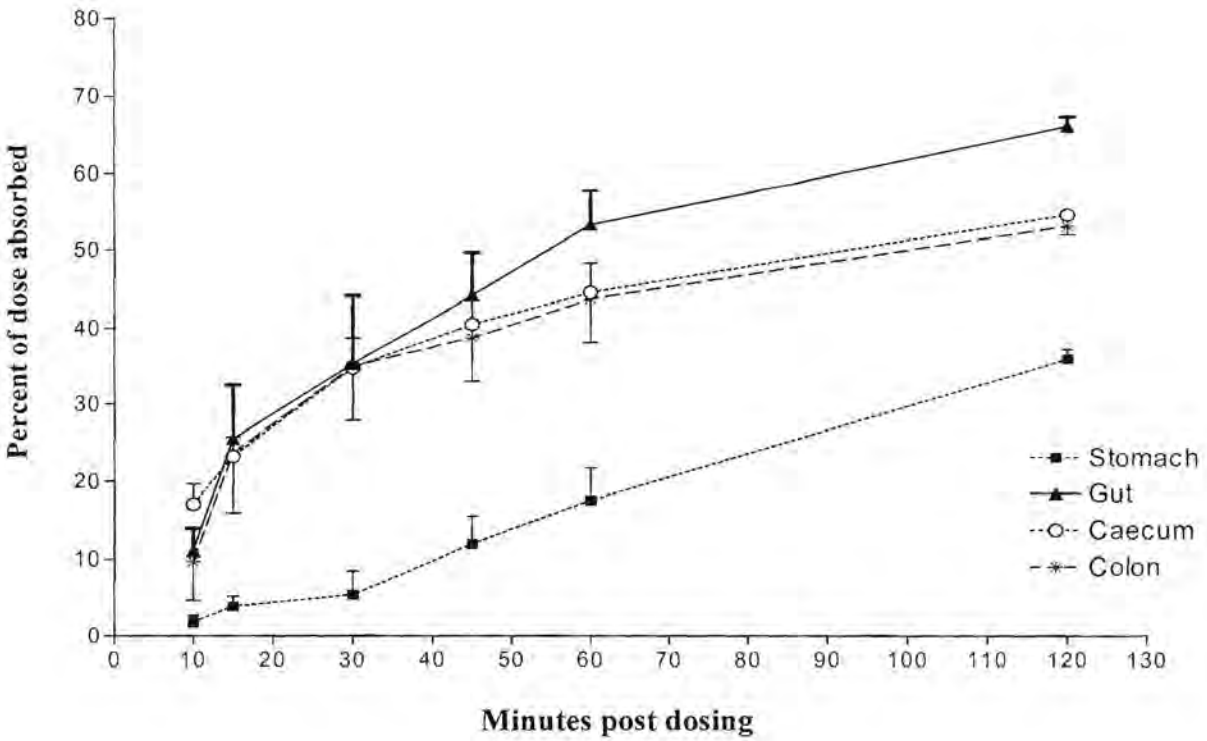

Figure 4-2: The *in vitro* absorption of ^{125}I labelled oxihumate from isolated rat GIT segments. GIT segments containing labelled oxihumate were suspended in HBSS and incubated for the indicated times. The ^{125}I activity of 2 ml aliquots the suspending HBSS were counted to determine the "absorbed" activity. The data is the average of three separate experiments where the same amount of oxihumate was used per segment for each experiment.

3.2.1 Precipitation of radioactivity in absorbed fractions

In the first experiment the 2ml aliquots that were removed for radioactivity counting at 22 hours were further used to determine what the effect of acidification and of addition of silver nitrate solution was on the distribution of activity found in this 22 hour supernatant.

If the activity of each GIT segments supernatant is normalized to 100% and the activity present in the different sub-fractions expressed as percentages of the activity of these supernatants it was found

that acid precipitation had a minimal effect on the distribution whereas the silver nitrate precipitated the activity almost quantitatively. These results are summarized in Figure 4-3 below. It can also be seen that the different GIT segments showed no real differences from each other with respect to the distribution of activity.

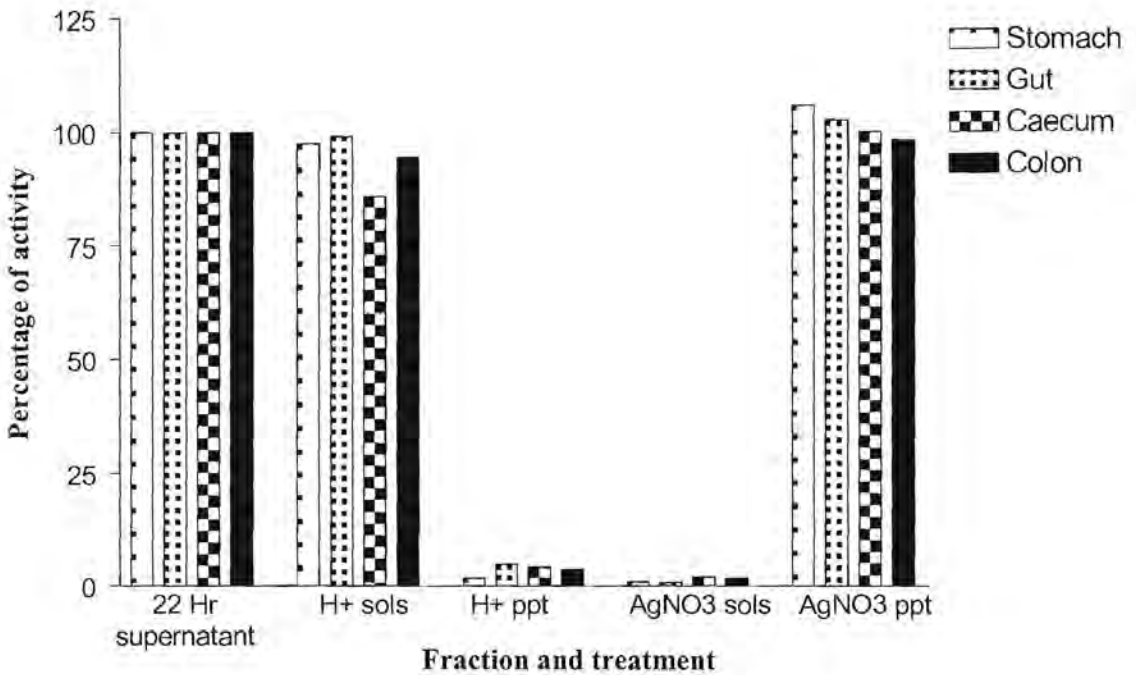

Figure 4-3: Sub-fractionation of the 22-hour supernatant of the rat GIT absorbed oxihumate. The supernatant was treated consecutively with glacial acetic acid then $AgNO_3$, solution, centrifuging between steps to remove any precipitate. The ^{125}I activity of each sub-fraction was calculated as a percent of starting material activity.

The absorbed fractions were analysed by TLC to determine which compounds found in the oxihumate were absorbed from the lumen of the gut. The compounds absorbed appeared to remain close to the origin implying that they were very polar.

3.2.2 HPLC analysis of absorbed compounds

HPLC analysis of the gut-absorbed oxihumate was not as reproducible as expected and the possibility that some of the compounds in the "absorbed fraction" are irreversibly absorbed onto the C_{18} HPLC column stationary phase cannot be excluded. Initially the 40% methanol solutions were analysed but later analyses were done on the methanol, acetone and ammonium hydroxide soluble

fractions prepared as described above. The methanol soluble fraction and the acetone soluble fraction appeared to have larger peaks as well as more later eluting peaks than the ammonium hydroxide solubles. These later eluting peaks generally elute after 10 minutes during the acetonitrile gradient. Figure 4-4 is a staggered overlay of HPLC chromatograms of these three different fractions from the combined jejunum and ileum segment of the GIT.

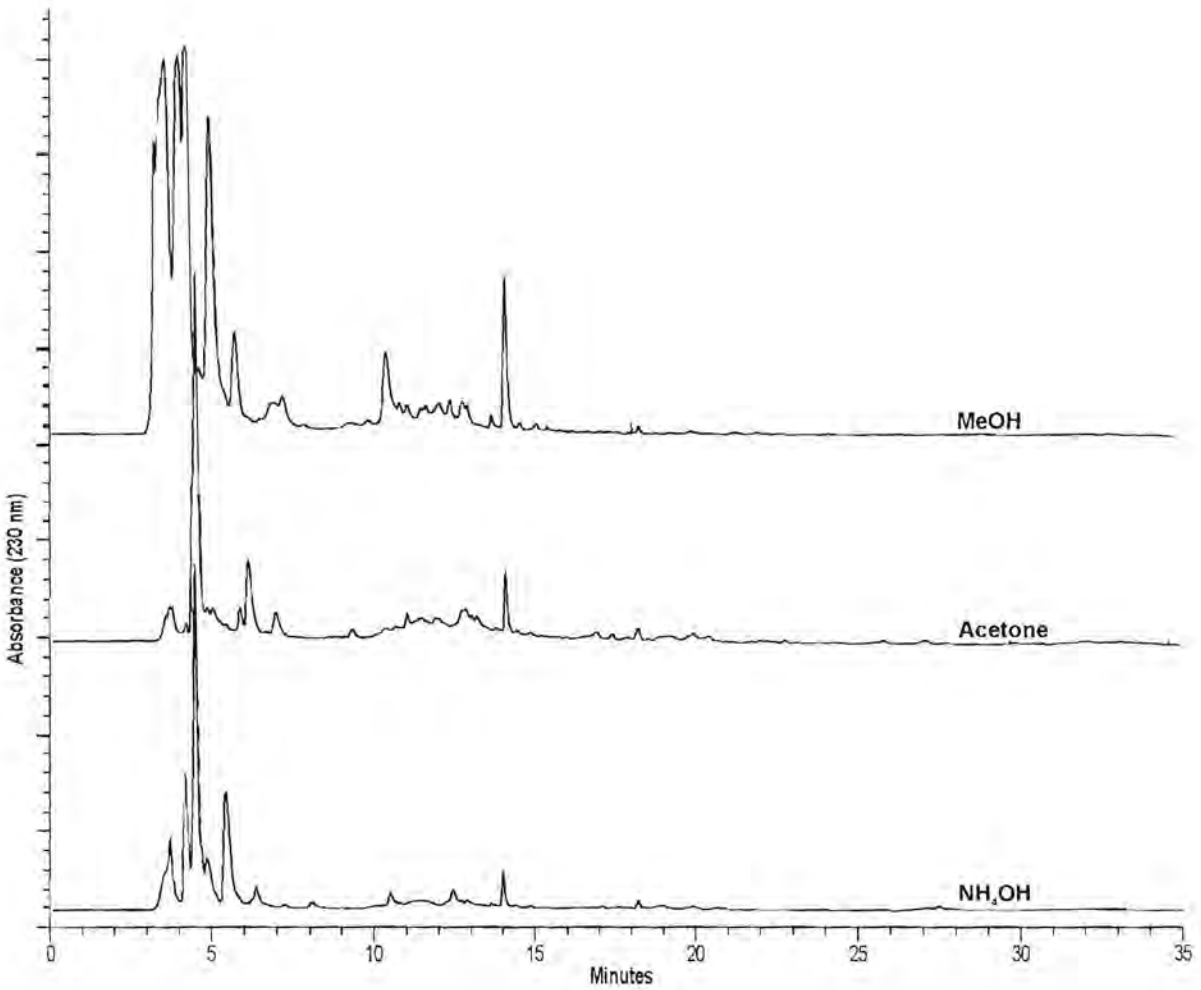

Figure 4-4: Overlaid chromatograms of the different fractions of the same small intestine absorbed oxihumate fraction. The dried "absorbed oxihumate fraction" was fractionated as described in the materials and methods section to remove inorganic salts and glucose derived from the incubation medium then analysed by HPLC using an acidic eluent system. The methanol soluble fraction showed the most peaks including a significant group of later eluting peaks. The acetone fraction showed the most late-eluting peaks although they were small compared to the first three eluted peaks. Most of the acetone insoluble but ammonium hydroxide soluble fraction was found to elute in 4 early peaks.

From the HPLC analysis it could be seen that the bulk of the compounds absorbed by the stomach elute very early in the chromatogram with essentially all the compounds represented in the pass-through peak and two further smaller peaks eluting within 7 minutes. There were small minor peaks eluted later in the chromatogram but these were insignificant. Figure 4-5 illustrates the HPLC chromatogram of a stomach absorbed fraction where it can be seen that the bulk of the compounds are eluted within 7 minutes and that the remainder of the chromatogram appears almost like noise on the baseline.

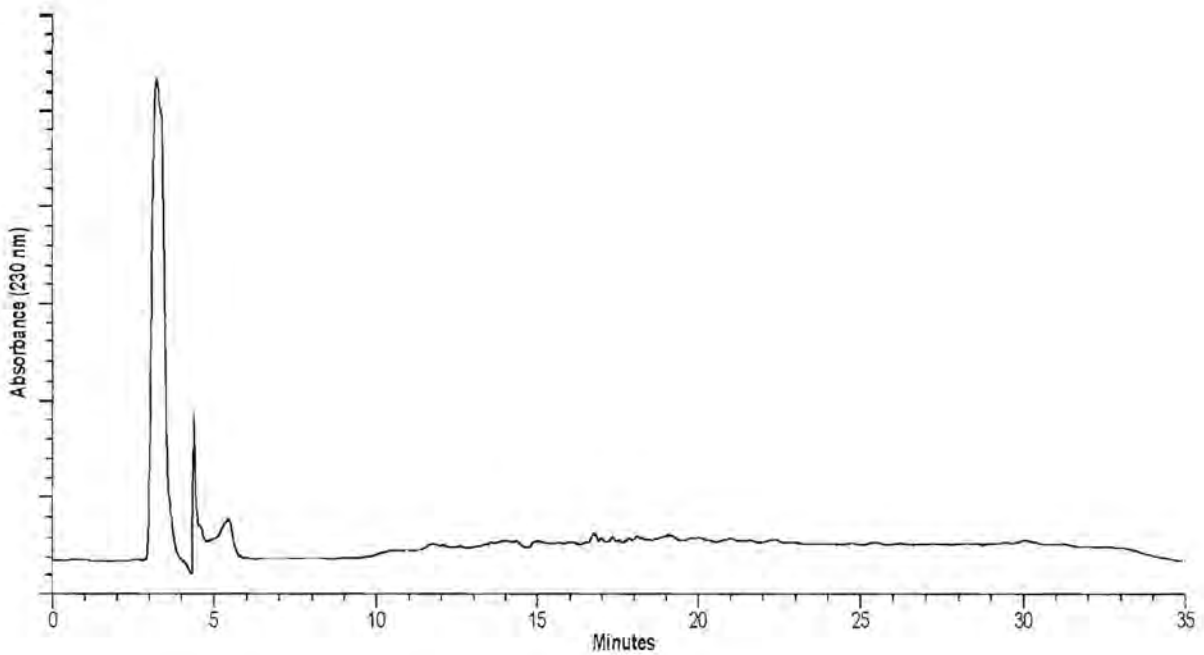

Figure 4-5: A HPLC chromatogram of the oxihumate absorbed from the stomach. Two unresolved peaks rapidly followed a large pass-through peak, one sharp the other a broad squat peak. The rest of the chromatogram shows very small insignificant broad peaks eluting throughout the gradient program.

More of the oxihumate appeared to have been absorbed from the combined jejunum and ileum in terms of mass and colour. The peak profile in the chromatograms was different from that of the stomach and the colon. A very early eluting peak often occurred although it was not reproducible and may have been a carryover peak or a residue left in the injector from the previous injection and released into the mobile phase during loading the sample. The pass through peak and the next two peaks were similar to the profile seen with the stomach but several large unresolved and closely eluting peaks were eluted between 10 minutes and 16 minutes. Several small insignificant peaks

eluted after these major peaks. A typical chromatogram of the small intestine absorbed fraction is shown in Figure 4-6.

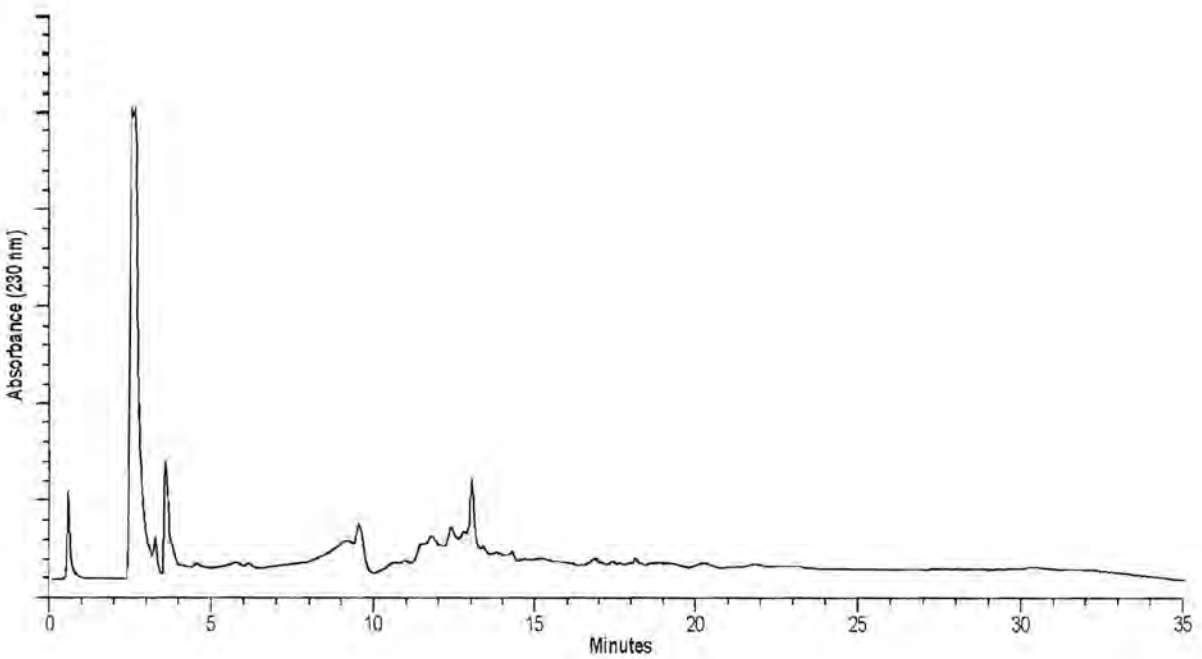

Figure 4-6: A HPLC chromatogram of the oxihumate absorbed from the combined jejunum and ileum. A single sharp peak rapidly follows the large pass-through peak. Between 8 and 17 minutes several unresolved peaks elute. The peak eluting at 0.8 minutes appears to be a ghost peak as it elutes before the void volume of the column.

The absorption from the caecum most closely resembled that of the stomach. The largest peak was the pass-through peak followed by two fairly large sharp peaks with a few small late eluting peaks present in the chromatograms at about 12 minutes. Several small peaks eluted later between 17 minutes and 21 minutes. A distinct peak (sometimes two) eluted during the re-equilibration of the column at the change of the gradient. This peak appeared to be due to a milky appearance of the eluting mobile phase but did not represent any significant mass or spot on TLC when collected and analysed further. A typical chromatogram of an oxihumate fraction absorbed from the caecum is shown in Figure 4-7.

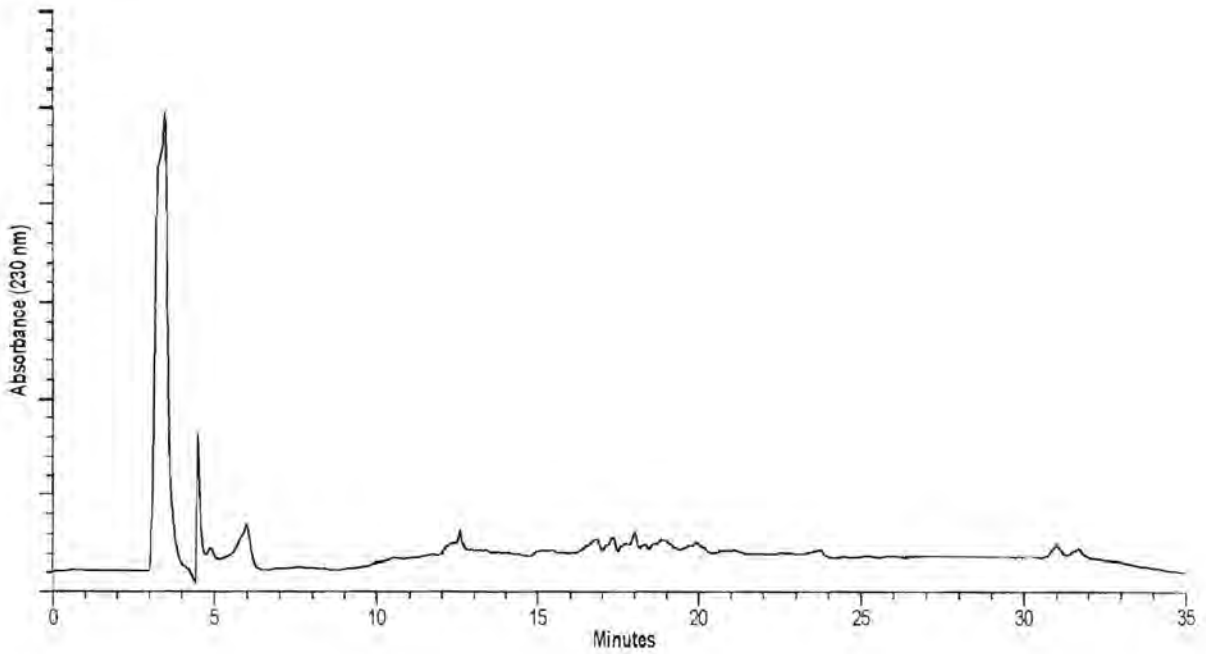

Figure 4-7: A HPLC chromatogram of the caecum absorbed fraction of oxihumate. The pass-through peak is large and followed by two peaks as in the case of the stomach-absorbed fraction. Later in the chromatogram there are several small peaks eluted by higher concentrations of acetonitrile. The two small peaks eluting at 31 minutes appear to be due to the gradient re-equilibration.

The oxihumate fraction absorbed from the colon again showed a large pass-through peak followed by a sharp second peak. The third peak that was seen in the stomach and caecum absorbed fractions was present but was very much smaller than in these fractions. Several small closely resolved and fairly broad peaks eluted between 16 and 22 minutes, which closely matches the peak profile of the caecum. A typical colon absorbed fraction chromatogram is presented in Figure 4-8.

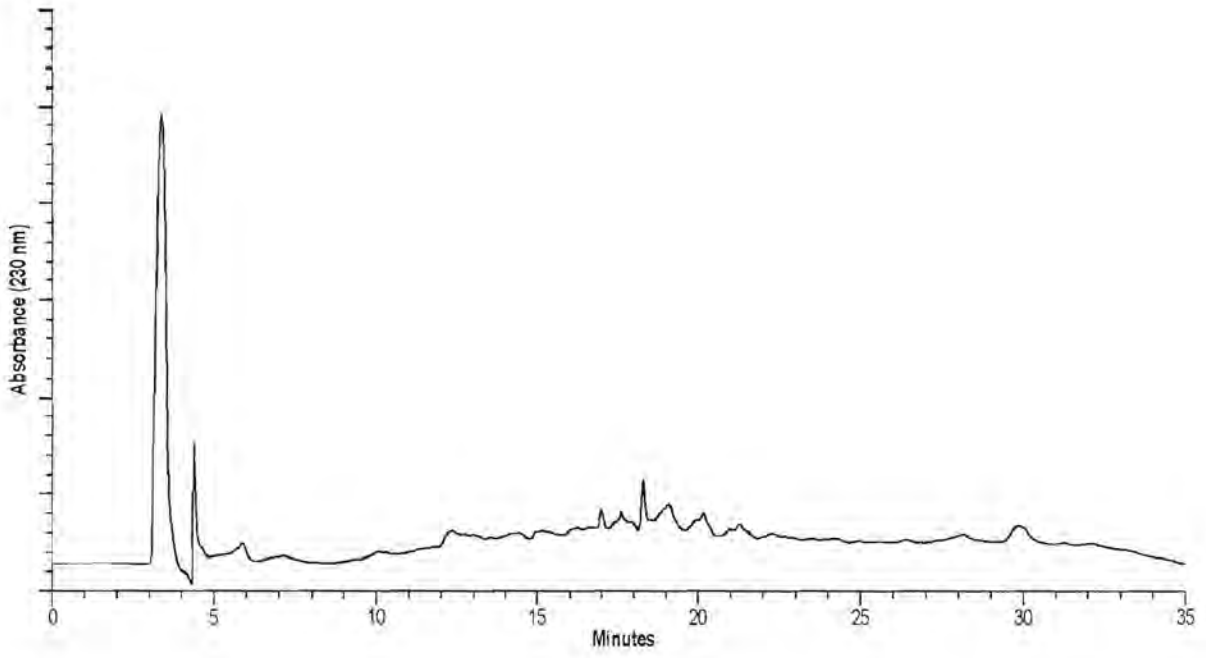

Figure 4-8: A HPLC chromatogram of the colon absorbed fraction of oxihumate. The pass-through peak is again large and followed by one sharp peak and one small broad peak. Several small peaks, which account for much of the dark coloured compounds in the fraction elute between 12 and 23 minutes. The small peak eluting at 30.5 minutes appears to be due to the gradient re-equilibration.

In the HPLC separations where ^{125}I labelled oxihumate was used for the absorption assay it was found that the radioactivity was associated with the pass-through peak and the peak immediately following it. Between 80% and 100% of the injected activity was recovered from the column, with most of this activity in the first two peaks to elute from the column. The results of the separation of the radiolabelled oxihumate are summarized in Table 4-1 below. The activity of the sample was determined before injection and the entire eluent was collected using a fraction collector to determine the recoveries.

Table 4-1: Summary of the GIT absorbed activity eluted from an HPLC column using the solvent gradient described in the text. The second eluting peak eluted at 4.2 minutes and was the most radioactive peak for the acetone soluble and ammonium hydroxide soluble extracts of the absorbed oxihumate. The first eluting peak at 3.4 minutes in the MeOH soluble fraction was the most radioactive. The recovery of activity from the HPLC column was generally greater than 80% with almost all the activity in the early eluting peaks.

	MeOH solubles		**Acetone soluble**		**Acetone ppt/NH4OH soluble**	
Peak number	**% of total activity**	**Total % recovered**	**% of total activity**	**Total % recovered**	**% of total activity**	**Total % recovered**
P1	72.4		0.0		12.4	
P2	22.1		69.4		67.8	
P3	1.0	95.5	2.8	72.2	7.3	87.5
P4	0.9		1.2		4.2	
P5	0.2		0.1		3.3	
P6	0.2		1.4		0.7	
P7	0.2		0.8			
P8	0.2		0.2			
P9	0.0		0.2			
P10	0.1		0.4			
Residual	0.1	97.6	3.0	79.4	4.2	100.0

4 Discussion

A labelling efficiency of 68% was achieved using the chloramine T oxidation technique for iodide and with the acidic sample washing managed to remove essentially all the residual free iodide. It was obvious that there were several fulvic acid compounds present in the mixture and that these were also removed from the humic acid fraction by the acid washing process. The oxihumate showed some activity on the solvent front of the TLC plates, which would indicate that there are some very lipophilic compounds being labelled and that these compounds remained in the oxihumate complex after the acid wash. These lipophilics could not be very basic nor amines as they would then not have moved with the solvent front in the acidic medium used to develop these TLCs.

The bulk of the radioactivity remained on the origin indicating that they are either not soluble or carries a charge at acidic pH. This was also the spot that had most of the colour. An almost black spot was left on the origin with a dark brown area streaking for about 5mm up the plate. Some of the spots above *Rf* of 0.5 did show some colour although when using the acid mobile phases these were very light brown.

In the rat gut absorption studies it was obvious that coloured compounds were crossing the gut membranes and that this was clearly visible within 15 minutes and became progressively darker during the incubation period. The radioactivity due to ^{125}I that had crossed the gut membranes was also measurable within 10 minutes, which indicates that at least some compounds in the oxihumate were crossing the membranes fairly rapidly. The kinetics of the absorption follows a typical first order uptake curve from the gut, approaching a plateau within 60 minutes although the absorption still continues. In the 22-hour absorption study it was found that about 90% of the administered dose was absorbed indicating that not all the labelled oxihumate compounds could cross the GIT membranes.

The uptake from the stomach was, as expected, much slower and did not reach a plateau within two hours. This is due to the limited surface area and the mucous that protects the mucosal layer of the stomach. It was interesting to note that any absorption occurred from the stomach because the pH of the stomach content is low and although the content had been flushed out the parietal cells would have continued producing HCl normally once the incubation began as the tissue was still viable. The humic acids however would be expected to precipitate at the low pH expected in the stomach. The actual pH of the stomach was not measured during or after any of the experiments in this study so it is open to speculation as to how far the pH does drop. What was observed is that the content of the stomach did form a thick almost black flocculated gel during the incubation. At the end of the experiment only about 37% of the activity had been absorbed from the lumen of the stomach. One observation that was made is that the majority of the stomach-absorbed compounds appear to be polar and perhaps even charged. This is evident from the HPLC analysis (Figure 4-5) where the only significant peaks in the chromatograms were the pass-through and two further peaks that eluted in less than 7 minutes. The radioactivity was associated with the first peak only and the precipitation of this activity was almost quantitative after adding silver nitrate. This would point to the activity being in the form of free iodide although it is possible that the activity could be bound to an organic molecule that is also precipitated by the silver. In support of this was that all four GIT segments showed almost the same silver nitrate precipitation and that the activity was in all cases associated with the first few peaks eluting from the HPLC column when using an acidic mobile phase.

The radio-activity that was detected on TLC plates when running GIT absorbed fractions under acidic conditions did not move from the origin, which implies that the activity was not free iodide but that it must be bound to some very polar or insoluble molecule, possibly even a protein.

Absorption from the small intestine (Figure 4-6), caecum (Figure 4-7) and colon (Figure 4-8) all had HPLC pass-through peaks similar to that of the stomach absorbed fraction, but the compounds eluting later during the acetonitrile gradient, i.e. the more lipophilic compounds, varied considerably from the stomach absorbed fraction and also from each other. The volume of incubation buffer was three time more for the small intestine than for the other GIT segments, which resulted in a 3 times more inorganic salts and glucose in the dried residue. These compounds derived from the media could interfere with the HPLC chromatography and were therefore partially eliminated by solublizing the dried residue in a minimum volume of ammonium hydroxide solution and adding methanol up to 40%. The soluble fraction was separated by centrifugation and dried in a draft of air. The residue from the 40% methanol solution was redissolved in either methanol or acetone. The acetone insoluble portion was collected by centrifugation and redissolved in 2.5% ammonium hydroxide solution. These three sub-fractions of the small intestine absorbed fraction were analysed by HPLC that revealed that there are many lipophilic compounds in the absorbed fraction and that the dark compounds are generally lipophilic eluting at more than 30% acetonitrile when using acidic mobile phases. Although a subjective observation, it would appear that a substantial percentage of the dark colour is not eluted from the column under acidic conditions.

The caecum absorbed fraction appeared to have more of the late eluting peaks although these were minor peaks in the chromatogram. The first three peaks to elute appeared to be the same as those seen in the stomach absorbed fraction and would be polar type compounds. The colon-absorbed fraction had the same three polar compounds elute in less than 7 minutes similar to the other GIT segment fractions, but there were many more lipophilic compounds eluted. The lipophilic compounds were however not well resolved and some peaks were fairly broad despite being small.

The small intestine fraction absorbed over 22 hours was subjected to acidic conditions that normally precipitates humic acids, but no precipitation of radioactive compounds occurred despite the fact that the oxihumate fraction had been acid washed before being used for the absorption experiment. This could indicate that the activity is being stripped from the oxihumate and that the activity is associated with free iodide and not humic acids. To test this the acidic supernatant was further treated with silver nitrate to precipitate free halide ions. It was found that the radioactivity was essentially 100% precipitated by the silver nitrate treatment that would imply that the activity was

due to free iodide. However TLC analysis of the same absorbed fraction showed only one radioactive spot at the origin in a solvent system that develops free iodide to an *Rf* of 0.7 – 0.85.

These two results were contradictory and needs to be studied further to characterize the compound to which the radioactive iodide is bound. It should be noted that there are many organic compounds that could form insoluble compounds with silver salts.

Chapter 5

Contact Hypersensitivity in the Rat

1 Introduction

It has been shown *in vitro* that oxihumate inhibits the expression of the adhesion molecule, CR3 by PMA stimulated neutrophils (Dekker & Medlen, 1999a) and that adhesion to cultured human epithelial cells can be suppressed (Jooné, 2002). Since this CR3 adhesion molecule is associated with the early stages of inflammation the possibility exists that oxihumate would demonstrate *in vivo* anti-inflammatory properties when administered *per os*, the probable route of administration for oxihumate. One concern was that the measured effects have only been demonstrated *in vitro* where the complete mixture of compounds present in oxihumate have been administered directly to cells in culture and that it is possible that the required combination of active compounds in oxihumate may not cross the GIT rendering oxihumate ineffective *in vivo*. The anti-inflammatory properties of oxihumate therefore needed to be confirmed *in vivo* where the compound is administered *per os*.

Several rodent inflammation models are available, which address different types and phases of inflammatory processes. Contact hypersensitivity is an inflammatory response elicited in the skin of sensitized animals and results from the release of pro-inflammatory cytokines by hapten-specific lymphocytes residing in the challenged dermis. A cascade of events occurs during which erythemia (redness), oedema (swelling), vascular leakage of proteins together with other molecules and cellular invasion (neutrophil, monocytes and T cells) occur. All these effects are measurable and if the effect of orally administered humic acid on the immune system is significant one or more of these inflammatory parameters would be suppressed.

Contact hypersensitivity has been described as a delayed-type immune response involving cellular or Th1 type effects to a much greater scale than the Th2 effects although the molecular mechanisms are still not fully understood (Riemann *et al*, 1996; Hauser, 1990). However, the involvements of cytokines and growth factors that are derived from keratinocytes in the epithelium have been implicated. This aspect of contact hypersensitivity was reviewed by Schwarz and Luger (Schwarz & Luger, 1992). During contact hypersensitivity, interleukin-12 (IL-12), which is an important cytokine for the stimulation of a Th1 type response, has been shown to be expressed by the Langerhans cells (Rook *et al*, 1994), which in turn play a critical role in the initial induction process

of the contact hypersensitivity reaction. It was demonstrated *in vivo* that by decreasing IL-12 concentrations by interperitonial injection of antibodies directed against this interleukin, that both the induction and effector phases were inhibited and that a hapten tolerance was induced that suppressed the response to further challenges (Riemann *et al*, 1996). Another IL that is associated with inflammatory processes and in particular the infiltration of eosinophils is IL-5. Peripheral blood cells from atopic dermatitis patients have been shown to produce excessive IL-5 and IL-5 has been found in detectable concentrations in allergic skin lesions (Tanaka *et al*, 1994; Yamada *et al*, 1995). It was also demonstrated that the contact hypersensitive response is accelerated and intensified in transgeneic mice expressing excessive IL-5 (Nagai *et al*, 1999).

There are well documented rodent models available for contact hypersensitivity with varying selectivity of the immunological response (Th1, Th2 or delayed type hypersensitivity) and the type of hypersensitivity reaction (Type I, II, III or IV) that is elicited by the experimental conditions (Akiba *et al*, 2002; Grabbe & Schwarz, 1998; Krasteva *et al*, 1996; Sarnstrand *et al*, 1999; Tang *et al*, 1996). These models all make use of a sensitisation phase, where the animals are treated epicutaneously with a known "sensitising compound" with the most common sensitising agent being dinitro-fluoro-benzene (DNFB), and followed up approximately a week later by a challenge, with a diluted solution of the same sensitising compound, on an ear. DNFB appears to have a stronger sensitising effect than many other agents resulting in the popularity of this agent (Krasteva *et al*, 1996; Hauser, 1990; Klimuk *et al* 1999).

The immune reaction that follows on the challenged ear is a reddening, an oedema (thickening of the ear) and immune cell infiltration into the area of the challenge. The time for maximal swelling, the cell type involved in the oedema and the immunoglobulins (IgE or IgG, IgM) and cytokines released indicate the type of hypersensitivity response. More than one type of hypersensitive response, especially a combination of a rapid response i.e. a Type I response, that reaches a maximal effect within a few hours of the challenge and a delayed type hypersensitive response that occurs between 24 and 72 hours after the challenge can occur.

The variation in the response is partially chemical dependant, partially dose dependant and partially genetically determined as seen by the different reactions to the same protocol by different strains of mice and rats (Woods *et al*, 1996).

The object of this experiment was to determine whether oral treatment with potassium oxihumate or an equivalent concentration of a humic acid derived from brown coal for a period of a week could

elicit an effect on the contact hypersensitivity reaction. Prednisolone treatment was included as a positive treatment control.

This experiment was carried out at the Onderstepoort Veterinary Animal Research Unit with the approval of the Animal Use and Care Committee. The project was approved by this ethics committee under project number 11/2002 (see Appendix II).

2 Materials and Methods

2.1 Chemicals

Acetone was an analytical grade reagent from BDH purchased from Merck, Midrand, South Africa; olive oil was B.P. grade purchased from Tedro (Johannesburg, South Africa).

2,4-dinitro-fluorobenzene (DNFB) was a Fluka product of analytical grade purchased from Sigma-Aldrich, (St. Louis, MO, USA).

Oxihumate was obtained from Enerkom (Pty) Ltd, Pretoria, South Africa as a dry powder. Brown coal humic acid potassium salt was a kind gift from Zylem Pty Ltd, Pietermaritzburg, South Africa as a dry brown powder.

2,4-Dinitro-fluorobenzene solutions

A 2.5% solution of 2,4-dinitro-fluorobenzene (DNFB) in acetone:olive oil (4:1) was prepared by dissolving 1.25g of DNFB crystals in 30ml acetone. Once dissolved 10.0 ml olive oil was added and mixed well. The volume was then adjusted to 50.0ml with acetone. A 0.25% solution was made in the same manner except that 0.125g DNFB was used in a volume of 50ml. These solutions were stored at 4°C protected from light. A vehicle solution of 20% olive oil in acetone was prepared as the control challenge solution by diluting exactly 10.0ml of olive oil to 50ml with acetone and the solution mixed well before storing at 4°C protected from light.

Oxihumate solution

36.5 g oxihumate powder was suspended in 500ml distilled water and vigorously stirred on a magnetic stirrer at ambient temperature overnight. The solution was then allowed to stand for a further 24 hrs before decanting into centrifuge tubes and centrifuging at 3500g for 20 minutes at 10°C. The supernatants were carefully decanted taking care not to disturb the precipitate, and the

concentration of this supernatant solution determined by gravimetric analysis. The concentration of the solution was adjusted to 27.6g/l by dilution with distilled water.

Brown coal derived humic acid solution

As the brown coal derived potassium salt of humic acid was reputed to be completely soluble, only 18.0 g of this powder was suspended in 500ml distilled water and stirred on a magnetic stirrer overnight at ambient temperature. The solution was then allowed to stand for a further 24 hrs before decanting into centrifuge tubes and centrifuging at 3500g for 20 minutes at 10°C. The supernatant was carefully decanted and the concentration of the supernatant solution determined by gravimetric analysis. The concentration of the solution was adjusted to 27.6g/l by dilution with distilled water.

Prednisolone solution

A liquid formulation containing 3.0mg/ml prednisolone was purchased from Adcock Ingram. Just before dosing a dilution of the prednisolone formulation was made by diluting 500µl of the formulation to a final volume of 3.30 ml with sterile distilled water and vortexing to ensure proper mixing. The solution was administered within 30 minutes of dilution and vortexed again just before removal of each aliquot to ensure that no separation had taken place.

2.2 Animals

Female Sprague Dawley rats of 8 to 10 weeks (between 150 and 200g) were purchased from SA Vaccine Production Unit of NHLS, Rietfontein, South Africa. These rats were allowed to acclimatize at the OVARU centre for 10 days in their new environment prior to experimentation starting.

The rats were housed individually under barrier conditions in plastic cages under 12 hour light/dark cycles at 22°C with ad libitum access to water and a standard rat chow.

2.3 Methods

2.3.1 Contact Hypersensitivity

An initial pilot study was done to confirm that the doses of DNFB used for sensitisation and challenge would induce a measurable reaction. During the pilot study, two further rats were treated with 6 daily administrations by gavage of 400µl of a 2.76% solution of oxihumate in deionized water. This was equivalent to 61mg oxihumate per kilogram body weight, the dose calculated by

Enerkom as the daily therapeutic dose for humans (personal communications). On day 6 the right ear was challenged with 0.25% DNFB in acetone:olive oil (4:1) while the left ear was challenged with vehicle alone. The left and right ear thicknesses were each measured at 3, 5, 7.5, 12, 23, 24, 26.5, 28.5, 32 and 48 hours post challenge to determine whether the elicited reaction was measurable and the time post dosing for the maximal reaction. The two oxihumate treated rats were control rats included to confirm that oral administration of oxihumate did not induce sensitivity to DNFB.

In the main experiment each rat was individually marked and weighed. The individual weights were recorded at the time of grouping then ranked by weight and sequentially allocated to one of four groups of 15 rats each. The groups were then randomly allocated to a treatment (two experimental groups to be dosed with the different humic acids, one positive treatment control group to be treated with prednisolone and one untreated control group). Due to daily time constraints the experiment was carried out in two phases. The first phase involved 8 rats from each of the four experimental groups and the second phase started a week later with the remaining 7 rats from each group.

On day 0, the rats from all four groups were weighed then sensitized by shaving the abdomen and painting the shaved area with 400µl of a 2.5% solution of DNFB in acetone:olive oil (4:1). Directly after sensitising the rats, they were treated by gavage with 400µl of the appropriate solution depending on which experimental group they were allocated to.

The 400µl gavage of the appropriate solutions was repeated daily for seven days at the same time until the end of the experiment. The following dosages were administered: distilled water (control group); oxihumate 61mg/kg/day; brown coal humate, 61 mg/kg/day; prednisolone, 1 mg/kg/day

On day 6, all the rats were challenged on the right ear by application of 25µl of a 0.25% solution of DNFB in acetone:olive oil (4:1) to the upper surface of the ear. The left ears were treated in the same way using vehicle solution alone i.e. acetone:olive oil (4:1).

Three hours after challenge, both the left (control) and right (challenged) ear thickness was measured across the ear at a distance of 3mm from the tip using an engineering calliper (Mitutoyo, Japan) with an accuracy of 20µm and recorded. Three measurements were taken for each ear and the average used as the measurement.

The measurement of the ears was repeated in the same manner after 24 and 48 hours. All the rats in each group were weighed 24 hours after the ear challenge. Six of the rats from each of the

experimental groups in the first set were euthanased at 24 hours to perform a histogical evaluation. This ment that only two rats for each group were measured at 48 hours for this set of rats.

2.3.2 Statistic Analysis

As measurements of the same ears were done on more than two different occasions and on four separate groups a general linear model for repeated measures was applied. The statistical differences between the means of each group was measured simultaneously and evaluated as significant when $\rho < 0.05$. In addition to testing the H_0 hypotheses, this test can estimate within-subject and between-subject parameters. The data was analysed using SPSS statistical analysis software (SPSS, 2001).

3 Results

During the pilot study where no treatment regimen was followed but where sensitisation and challenge was performed to confirm that the response was measurable, it was found that the DNFB challenged ears swelled within the first three hours showing a Type I response and that this swelling began to decrease fairly rapidly again after about 5 hours. After approximately 12 hours, swelling would begin increasing again reaching a maximal swelling between 18 and 28 hours, typical of a Type IV response, after which time swelling would slowly start to decrease again. There was minimal swelling of the vehicle solution challenged ears but the DNFB challenged ears swelled significantly.

As part of the pilot study, two rats that were not initially sensitized with DNFB but were administered 6 daily treatments by gavage with 400µl of a 2.76% solution of oxihumate. On day 6, both these rats were challenged on the right ear by application to the upper surface of the ear, of 25µl of a 0.25% solution of DNFB in acetone:olive oil (4:1). The left ears were treated with the vehicle solution only. The right ears of both these rats showed minimal swelling (0.04mm or < 8%), while the left (vehicle challenged) ear showed essentially no measurable change.

In the main experiment where initial DNFB sensitisation was performed, significant differences were observed for the increase in ear thicknesses of the DNFB challenged relative to the vehicle only challenged ears in all four experimental groups. It was found that the left ears of the prednisolone treated group (the positive treatment control group) showed a smaller increase in ear thickness after 3 hours and again at 48 hours than the two corresponding experimental and the untreated control groups at these same times, yet was similar to the other experimental groups at 24 hours.

The thickness of the left ears, challenged with vehicle solution alone, in all four experimental groups never altered by more than 25% whereas the DNBF challenged ears in all four groups were significantly increased (±30% for prednisolone and 50 – 60% for the other experimental groups). The different ear thicknesses are illustrated as whisker box diagrams in Figure 5-1. At no time after the challenge did the left ears exhibit any noticeable erythemia (redness) despite the slight measurable oedema at three hours. This mild oedema began decreasing slowly after about 6 hours.

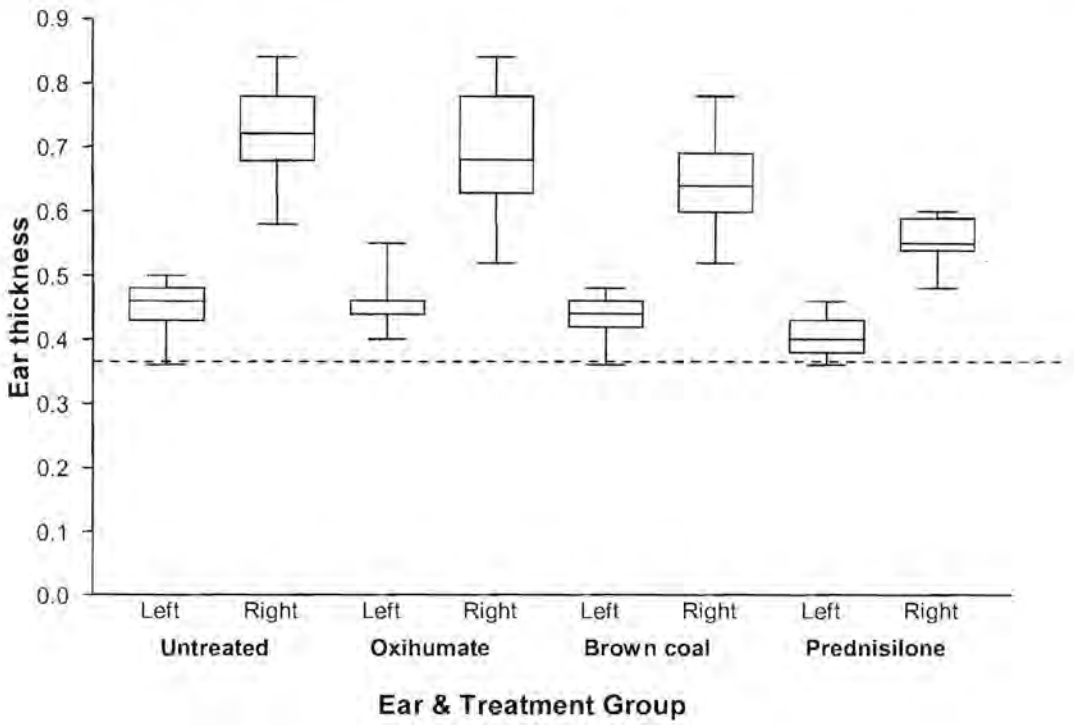

Figure 5-1: A whisker box diagram of the thickness of the left and right ear thicknesses at 3 hours after challenge with vehicle solution or 0.25% DNFB. The boxes bound the 25 to 75 quartile values with the whiskers extending to the minimum and maximum values recorded. Note that both the prednisolone treated animals' ears are thinner. Ear thickness prior to challenge was 0.37±0.22 mm and indicated by the dashed line.

When comparing the DNFB challenged ear thicknesses, differences were observed both intra-groups at the different measurement times as well as inter-group at the same measurement times. Figure 5-2 illustrates the differences between the left and right ear thickness at the times shown for each experimental group. An assumption was made that the untreated control rats (dosed with water) were representative of a normal contact hypersensitivity response, and any change from the response shown by these rats implies a drug-induced effect. There was a general trend in all the experimental groups in that the extent of swelling increased from 3 hours to 24 hours and that by 48 hours the thickness appeared to decrease again slightly, but not as low as the 3 hour measurements.

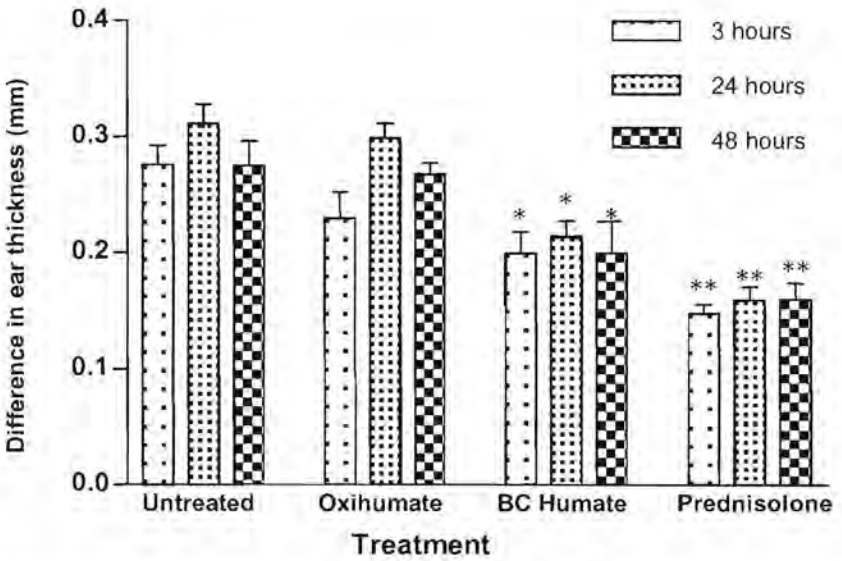

Figure 5-2: The difference in ear thickness (of the combined batches of experimental animals) between left and right ears of DNFB challenged rats after one week of non-treatment or oral treatment with oxihumate (61mg/kg/day), brown coal humate (BC Humate) (61mg/kg/day) or prednisolone (1mg/kg/day). The three columns represent the differences in ear thickness with SEM bars at 3 hours; 24 hours and 48 hours post challenge. * $p < 0.01$ ** $p < 0.001$ compared to the relevant untreated control using the general linear model for repeated measures which compares data from each data set against all the others.

The oxihumate treated group showed a large variation in ear thickness, especially if the first and second batches of rats were compared separately. In the first batch of eight rats, there appeared to be an increase in the ear swelling (Figure 5-3) whereas the second batch appeared to have an inhibiting effect on the ear swelling (Figure 5-4) when these two batches were compared to the corresponding untreated groups.

In contrast to these results, the brown coal derived humate showed inhibition of ear swelling in both batches of rats to the extent that a significant ($p<0.01$) difference between the untreated and brown coal humate group existed and that there was no significant difference between the prednisolone treated and brown coal humate treated groups.

The prednisolone treated rats showed a highly significant ($\rho < 0.001$) smaller difference in ear swelling than the untreated group at all three measurement times. In addition to the smaller difference, both the DNFB and vehicle challenged ears showed a small but significant reduction in the percentage swelling.

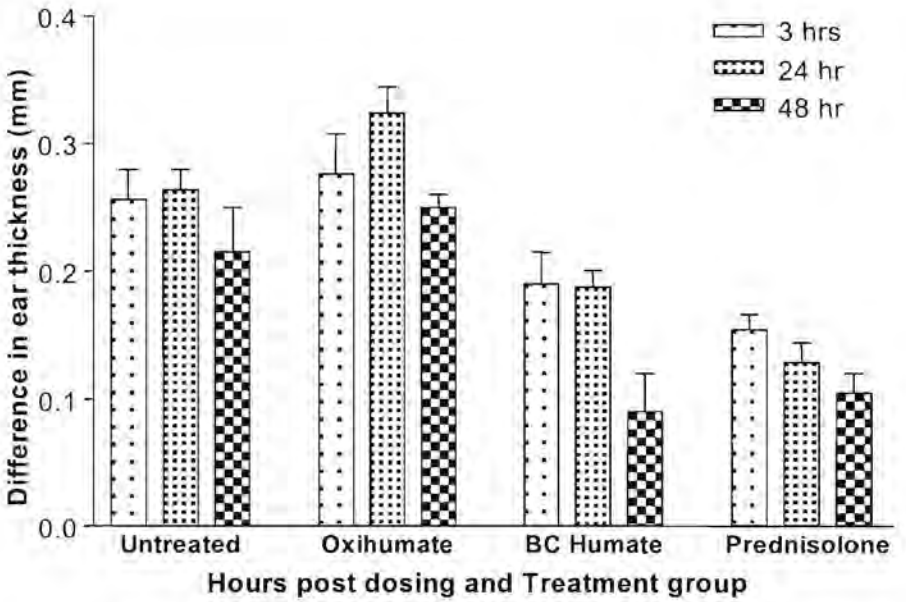

Figure 5-3: The difference in ear thickness between left and right ears of the first batch of DNFB challenged rats after one week of oral treatment as shown below each group. The three columns represent the differences in ear thickness with SEM bars at 3 hours; 24 hours and 48 hours post challenge. Although there were 8 rats in each group only 2 rats remained in the 48-hour groups in this batch.

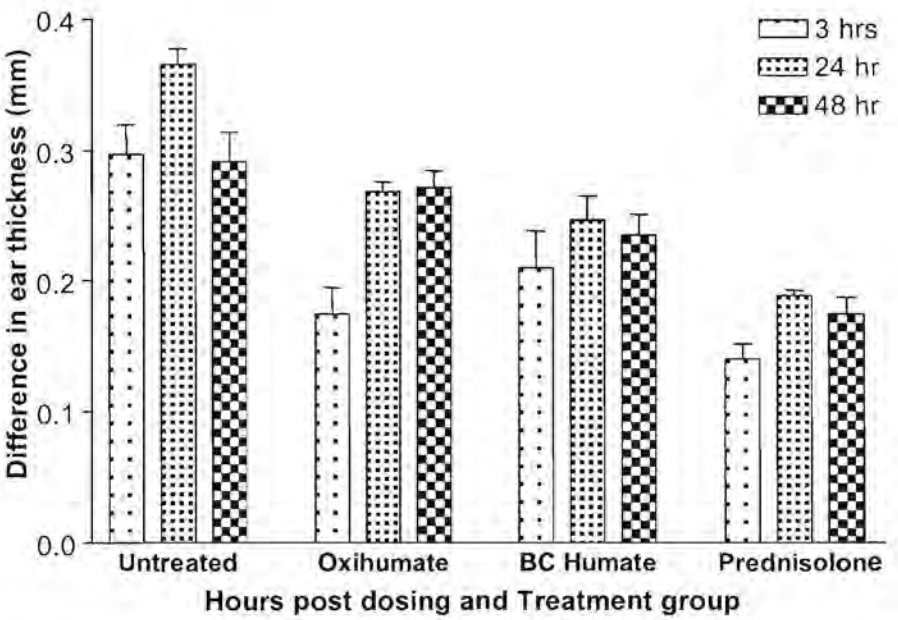

Figure 5-4: The difference in ear thickness between left and right ears of the second batch of DNFB challenged rats after one week of oral treatment as shown below each group. The three columns represent the differences in ear thickness with SEM bars at 3 hours; 24 hours and 48 hours post challenge. There were 7 rats in all the groups.

Each rat was weighed at the end of the experiment and the individual changes in body weight calculated. This change in body weight was then expressed as a percentage of the starting weight of each rat. The results were collated into the experimental groups to which the rats were allocated. These collated results are presented in Figure 5-5.

It was found that the untreated control rats increased in weight by an average of 5.2% while the oxihumate group increase by only 2%. The brown coal humic acid treatment had a smaller effect on the change in weight of the rats with and average increase in weight of ± 4.5 %. The biggest difference in body weight changes was seen in the group that were dosed with prednisolone. This positive anti-inflammatory control group had an average decrease in the body weight of ±0.3% but also exhibited the largest standard error in this difference.

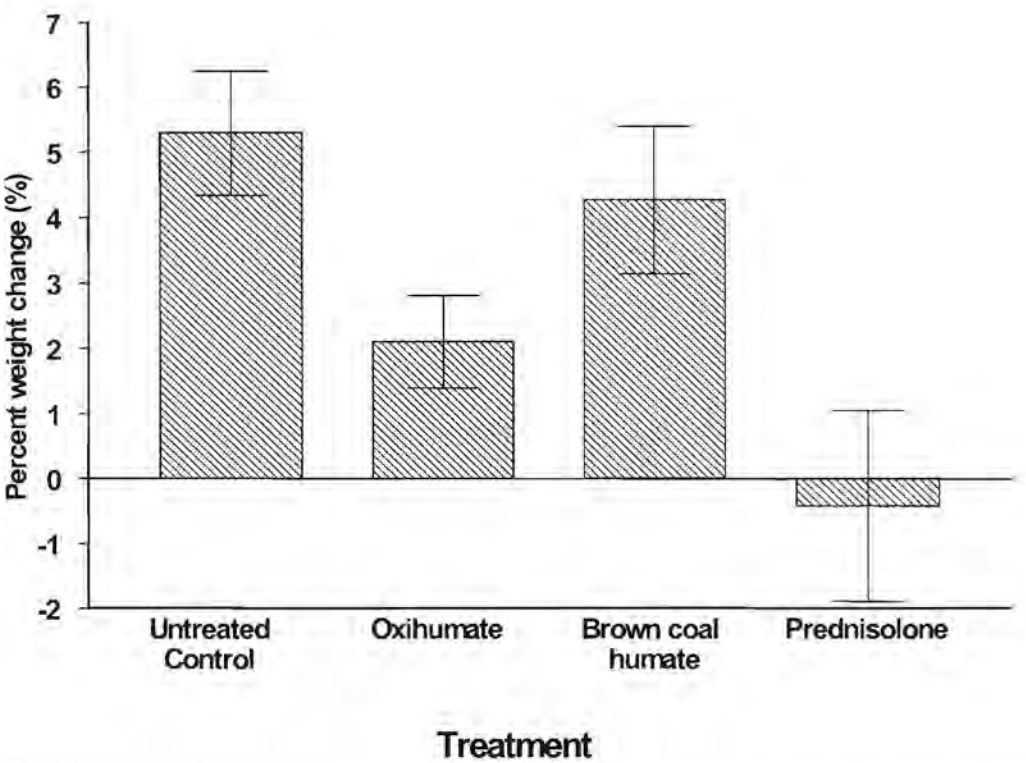

Figure 5-5: A bar graph illustrating the percentage changes in the body weight of the rats in each of the experimental groups. The error bars depict the SEM. The untreated control was dosed with distilled water whereas the two humic acid treated groups were each dosed with 61 mg/Kg/day of the respective humic acid solution while the prednisolone dosage was 1 mg/Kg/day. The rats were in a rapid growth phase so their body mass should have increased during the seven days that the experiment lasted. The rats treated with prednisolone however showed a decline in body weight over the same period.

4 Discussion

Contact hypersensitivity is an inflammatory reaction resulting from a challenge in sensitized animals and involves an initial allergic type response involving IgE but is followed by a stronger and longer-term effect due to increased vascular permeability and cellular infiltration into the oedemic tissue. For immune-type cellular infiltration to take place, these cells must adhere to the endothelium of the vascular capillaries in the area of the stimulus followed by diapedesis (the sequence of events that inflammatory cells carry out to be able to leave the vascular system and enter the surrounding tissue). In the experiment reported here, a single daily administration of prednisolone, a well-known steroidal anti-inflammatory drug, was demonstrated to reduce the extent of the oedema and erythemia, proving that oral dosing with anti-inflammatory drugs can reduce the extent of the contact hypersensitivity response.

Daily dosing with the two humic acid products revealed that there was an effect on the ear swelling although there were differences in the effects shown by the two different products.

Oxihumate treatment demonstrated a small and insignificant effect that also appeared to be variable. In the first batch of experimental animals receiving oxihumate treatment the ear swelling appeared to be slightly stimulated, yet the second batch of animals showed a small inhibitory effect on the swelling. This difference could not be explained as an artefact of the method as the same person measured all four groups at the same time. The experimental group size was too small to determine statistically whether the oxihumate treatment was inhibitory or stimulatory on the swelling response.

In contrast to these results the brown coal humic acid treatment exhibited a more obvious inhibitory response to the challenge with both batches of animals in the group receiving brown coal humic acid treatment giving similar results. The extent of the reduction in the inflammatory response for this treatment was significantly ($\rho < 0.01$) less than for the untreated controls but not as effective as the prednisolone treatment.

It was not the object of the study to determine a mechanism of action, as several mechanisms are possible, but rather to determine whether a modification of the inflammatory reaction could be elicited after oral administration of humic acids. Any modification to the inflammatory reaction would imply that active compounds are present in the humic acids and secondly that these active compounds can be absorbed from the GIT in therapeutically significant concentrations.

The first conclusion that can be drawn from the results of this experiment is that the swelling observed for the challenged ears was due to the DNFB treatment sequence and was not a general reaction. The rats that were dosed orally with oxihumate for a week without prior sensitisation with DNFB showed a very slight insignificant reaction to the DNFB challenge. When comparing the response of these oxihumate treated rats to the sensitized rats it was clear that the reactions were not the same in extent or reaction, which proved that the daily oral oxihumate treatment did not result in a sensitisation of the rats to DNFB. Further support for the DNFB specific response was that the ears that were treated with the vehicle solution only (the left ears of each rat that were used as controls) showed a minimal reaction despite the initial sensitisation with DNFB in the same vehicle solution.

Both the sensitisation and challenge reactions are immune type responses with mechanisms involving various immune-cell types, antigen/hapten presentation and chemical signalling molecules, especially cytokines and adhesion molecules. If the humic acids could not be absorbed from the GIT, the only area of the animals to be exposed to these humic acid compounds would be the lumen of the GIT. The GIT is not normally involved in contact hypersensitivity responses or in the control of immune responses elsewhere in the body. It can therefore be concluded that an effect on the immune response after administration of humic acid products would imply that there are compounds with anti-inflammatory activity being absorbed from the lumen of the GIT.

The oral administration of humic acid had a measurable effect on the response of DNFB sensitized and challenged rats, which leads to the conclusion that there must have been uptake from the GIT of at least one active compound that could modify this response. This observed modification of the immune response was similar, but smaller, than that seen for the positive control where prednisolone treatment was used. This result implies that at least one anti-inflammatory compound exists in the humic acid complex and that this active compound is probably soluble in the environment of the lumen of the gut allowing absorption from the GIT after oral administration. This is despite the fact that humic acids would probably precipitate in the stomach due to the low pH and would not be available for uptake until the solubility increased in the GIT where the pH is high enough to allow resolubilisation of the formed precipitate.

The effect shown by the brown coal humic acid derivative confirms that there is inflammatory suppressant activity in these humic compounds although the efficacy is at least 50 times smaller than prednisolone when comparing the administered doses. The solubility and rate of absorption of the humic acid products would play a role in the total effect observed. This aspect is partially supported by the fact that the oxihumate, which is less soluble, appeared to have a smaller effect than the

brown coal, which was almost twice as soluble as the oxihumate *in vitro*. However, the concentration and number of compounds absorbed could not be determined during these experiments as the identities of the absorbed compounds were unknown. Further experiments (see Chapter 6) using labelled oxihumate indicated that the bulk of the labelled humic acid compounds appear to bind strongly to the plasma proteins. This would have made identifying the absorbed compounds even more difficult as binding to protein would have altered the apparent concentration of the humic acid compounds detected in the plasma.

Another aspect that should be considered is that both the humic acids used in this study were complex mixtures of many different individual compounds, as demonstrated in the previous experiments where sub-fractionation and chemical characterization was done, of which perhaps only a few are active as anti-inflammatory compounds. This would have an effect on the apparent total administered dose of humic acid required to exhibit the same effect as the prednisolone. Some or perhaps many of the humic acid compounds may in fact not be absorbed from the GIT that would further complicate the calculation of the concentration of active compounds.

Another possibility is that a synergistic combination of compounds is required to elicit the observed inhibitory effect. If these compounds were not absorbed together or not absorbed in the required ratio they would not be effective as an anti-inflammatory drug.

An aspect that raised some concern was the dramatic difference in the weight gain of the different experimental groups. As the untreated control group was handled and given water by gavage in the same manner in which the other groups were given the daily treatments, the higher weight gain in this control group could not be ascribed to the experimental procedure. The rats used in this study were in a fairly rapid growth phase and it was observed that the untreated control group increased weight by approximately 5% within the week that the experiment ran. The two humic acid products appeared to affect the weight gain minimally with the brown coal humic acid product having a smaller inhibitory effect on the weight gain than that of the oxihumate. The reason for this is not known as the toxicity of the compounds has been tested previously and no toxic effects were reported. It is possible that the treatment may have had an effect on the appetite of the treated animals or that some essential nutrients were being adsorbed onto the oxihumate residue and excreted via the faeces.

Predisolone treatment was found to be detrimental to the weight gain with several of the rats showing a decline in weight while other showed almost no weight gain over the one week period of

the experiment. This was unexpected, as corticosteroid treatment is generally known to induce water retention and increase the appetite of animals. Many adverse effects are described for long term use of steroids for treating inflammatory conditions and it is interesting to see that one of these effects appears to be reduced weight gain during the growth phase of the rat.

In conclusion it can be confirmed that

- there is at least one active compound in the humic acid products tested
- that the active compounds can be absorbed from the GIT after oral administration of humic acid products
- the anti-inflammatory effect of humic acid products can be demonstrated *in vivo* for the contact hypersensitivity model
- the humic acids do not show the side effects seen for prednisolone.

These observations would make humic acid products relatively safe anti-inflammatory drugs although the active compound(s) still need to be isolated and identified. It is possible that by extracting and concentrating the active compounds a more effective drug could be formulated and if the adverse effects remain minimal, similar to what was seen in this study, could provide a cheap, effective anti-inflammatory with no known adverse side effects

Chapter 6

Pharmacokinetics of Oxihumate in the Baboon Model

1 Introduction

An earlier baboon study in these laboratories using labelled oxihumate showed that uptake of the labelled humic acid from the gut was taking place in high enough concentrations to achieve therapeutic levels. No organs that could be associated with the immune system were seen to accumulate activity in this preliminary study (Dormehl, 1998). No attempt was made to analyse the labelled humic acid for free iodide but it was assumed that any residual free iodide would have been removed from the reaction mixture by the extensive washing procedure used.

One of the objectives of the present study was to repeat an earlier study where radiolabelled oxihumate was administered to baboons per os (Dormehl, 1998). The prime objective was to ascertain whether absorption of humic acid compounds did in fact take place from the gastro-intestinal tract. Additional data that was to be collected was kinetic data on the uptake and elimination of the labelled compounds, to find possible immune system related target organs and to analyse the urine to find excreted labelled compounds.

This previous baboon study using ^{123}I labelled oxihumate and oral dosing revealed that approximately 11% of the activity was absorbed and that distribution appeared to be via the circulatory system. The uptake appeared faster when the dose was delivered directly into the duodenum using gastroscopic delivery. The areas that were reported to show accumulation of radioactivity after administering ^{123}I labelled oxihumate included the thyroid gland (possibly indicating free iodide), salivary glands, and the septum between the nostrils, the liver and the kidneys. Excretion was essentially via the urine and was persistent for up to 48 hours after which time the residual isotope activity became too low to detect. The liver and kidneys would be involved with the metabolism and excretion of the humic acid respectively, and except for the nasal septum these are areas where it could be safely speculated that free iodide would normally accumulate. Apparently none of the lymph nodes or organs of the immune system showed any uptake of activity.

No data could be found in the literature with respect to the normal distribution of free iodide in the baboon but discussions with several pharmacologists and physiologists (personal communications) pointed out that it is generally accepted that any drug that has covalently bound iodide within its structure, would be metabolized intracellularly with the release of the iodide atoms. This is supported by the results of several workers who used various iodide labelled compounds and reported free iodide distributions in addition to the expected target areas of the compounds (Ercan & Senekowitsch, 1991; Sinn *et al*, 1990; Klett *et al*, 2003; Press *et al*, 1996). The thyroid gland and any other areas where iodide is known to accumulate, such as the salivary glands, eyes, testes etc would then scavenge the released iodide.

The target organs where labelled oxihumate accumulates and rate of elimination can give some idea of which physiological systems are involved and therefore an indication of the mode of action.

All potential drugs must be tested on animal models before human clinical trials may be initiated. The pharmacological parameters that are most often determined using animal models are the toxicity, pharmacokinetics and the pharmacodynamics. The most common animal model used in the initial phase of testing are the mouse or rat models. Medical and pharmacological research makes extensive use of inbred or genetically deficient strains of mouse models (Festing, 1979), but the results from these models are often not directly applicable to humans. Despite this shortcoming murine models are popular due to availability, known susceptibilities, fast breeding, similar responses to the experimental procedures, ease of transferring techniques between laboratories and the low cost of maintenance compared to most other animal models. Extrapolation of data from rodent models to humans is not without problems due to anatomical, physiological, immunological and genetic differences. As rodent models are often from closely inbred or specific hybrid models they show no or very little variation to induced responses. This "homogenous" response is not always representative of the effect that would be observed in a general human population where heterogeneous responses can be expected.

In contrast the primates are the animals that are closest to humans with respect to physiology (Redl & Schlag, 1998; Redl *et al*, 1999)(including similarity in the nervous, respiratory, circulatory, digestive, immune and endocrine systems) and gross anatomy. Anatomical similarity is evident although the normal quadruped movement of the primates as opposed to the bipedal motion in the human results in some unique variations in gross anatomy (Swindler & Wood, 1982).

Despite the anatomical differences the baboon model remains the animal model that is closest matched to the human and which is fairly accessible for pharmacokinetic studies (Nyindo & Farah, 1999; Redl & Schlag, 1998; Redl *et al*, 1999). Some differences in the anatomy of the baboon digestive system exist when comparing the human and baboon. The most obvious difference is that the baboon has a well-developed caecum, whereas the human has an insignificant appendix as the equivalent organ. As the caecum is situated distal to the small intestine where most nutrient and drug absorption is expected to take place, this anatomical difference should have little effect on the pharmacokinetics of most drugs.

2 Materials and Methods

2.1 Materials

Iodide-123 was purchased from the National Accelerator Centre (Faure, South Africa) and was produced as a carrier free NaI solution in NaOH at a specific activity of ±3000MBq (80mCi/ml).

Chloramine-T, sodium bicarbonate, trichloroacetic acid, glacial acetic acid, methanol, chloroform and ammonium hydroxide solution were analytical grade reagents as well as pH indicator strips (0 – 14) were all purchased from Merck, (Darmstadt, Germany).

Oxihumate was supplied as a formulated dry powder as a generous gift from Enerkom (Pty) Ltd, Pretoria.

Heparin was purchased from Sigma Diagnostics, (St Louis, MO, USA). 300mg heparin powder was dissolved in 100 ml distilled water and the solution filter sterilized. The solution was stored at 4°C in sealed tubes. 100µl of the solution was equivalent to 5 U.

Glass vials, septum seals and septum caps were purchased from Anatech, (Randburg, RSA).

Disposable syringes, disposable hypodermic needles, Jelco catheters, intravenous lines along with administration sets, stomach tubes, urinary catheters, tracheal tubes and electrocardiogram electrode patches were supplied by the Pretoria Biomedical Research Centre.

Five millilitres heparinised and EDTA blood tubes were purchased from Vacutainer, supplied by Becton Dickinson (Cockeysville, USA).

Deionized water (18 MΩ) was produced from the municipal supply by an Elga Option 4 reverse osmosis, deionisation and carbon adsorption system.

2.2 Methods

2.2.1 Labelling of Oxihumate and sub-fractions

2.2.1.1 Labelling of Oxihumate with 123-Iodine for administration to the baboons.

A solution of 1 mg/ml chloramine-T was freshly prepared in deionized water just before starting the labelling procedure.

For each baboon study an amount of 150-300 µl carrier free sodium iodide-123 with a specific activity of ±3000MBq/ml (80 mCi/ml) was added by syringe to a septum-capped 2ml wide-neck vial containing 100 µl of the freshly prepared chloramine-T solution (1mg/ml). The mixture was vortexed for 20 seconds and immediately after this the labelling reaction started by the addition of 600µl of a 3.5% solution of oxihumate and the mixture again vortexed for 20 seconds. The mixture was allowed to stand for 20 – 30 minutes in a lead pot at ambient temperature with occasional mixing by vortexing.

After the incubation time, 1000 µl glacial acetic acid was added to the mixture and the vial vortexed again, placed inside a 50ml centrifuge tube with a cotton wool support plug and centrifuged for ± 10 minutes at 3500*g*. The vial was gently removed from the centrifuge tube to avoid disturbing the fine black precipitate, carefully uncapped and the supernatant removed by means of a syringe fitted with a long blunt needle. The activity of the supernatant and precipitate were measured in a Capintec CRC-15 gamma dose calibrator.

The precipitate was then washed a further two times by adding 1000µl glacial acetic acid, capping the vial and vortexing for 30 seconds. The washing procedure was usually repeated three times in all, with the activity of the supernatant being measured after each wash before combining with the previous supernatant acid fractions.

The activity of the individual and combined supernatant was measured. The supernatant of the final wash had to exhibit less than 1% of the activity of the labelled oxihumate precipitate, otherwise a further washing step was performed. The final activity of the washed precipitate was measured and calculated to give the activity at reference time.

The labelled oxihumate precipitate was packaged for airfreight and shipped on a same day delivery to the Pretoria Biomedical Research Centre where it was further prepared for dosing by drop wise addition of ±4ml of a 0.5M $NaHCO_3$ solution. Slow addition was essential to avoid excessive effervescence during the resolubilisation of the labelled Oxihumate. Spotting 2µl aliquots onto a pre-wetted pH stick indicated the pH of the solution. When the pH was greater than pH 6 an aliquot of 500±10µCi was removed from the solution using a disposable syringe and counted in a CRC-15 dose calibrator. This aliquot was used to auto-calibrate the gamma camera for the isotope. The remainder of the activity was diluted to about 4ml with deionized water. This neutralized solution of ^{123}I-labelled oxihumate was taken up in a 5ml disposable syringe ready for dosing the baboon.

2.2.1.2 Labelling of the lipophilic sub-fraction of Oxihumate with Iodine-123 for administration to the baboons

For the labelling of the lipophilic fraction, a solution of about 50mg of the THF soluble sub-fraction of oxihumate was dissolved in 650µl of a 1:1 mixture of MeOH and $CHCl_3$. A 2mg/ml solution of chloramine-T in deionized water was prepared and diluted with an equal volume of methanol to give a 1mg/ml chloramine-T solution in 50% methanol. To 100µl of this methanolic chloramine-T solution in a capped and sealed 2ml wide neck vial was added 300µl carrier free ^{123}I sodium iodide with a specific activity of ±3000MBq/ml (80mCi/ml) by syringe and the mixture vortexed well for at least 30 seconds. The above 650µl lipophilic fraction was then added and the mixture vortexed again. During the next 30 minutes the mixture was vortexed at 5-minute intervals to ensure that separation did not take place. At the end of the 30-minute incubation time 100µl water and 500µl chloroform:methanol (1:1) were added, the vial vortexed and centrifuged at 3500g for 10 minutes to separate the phases. The lower chloroform:methanol phase containing the labelled compound was removed with a syringe with a blunt end needle and was washed a further two times with 250µl water to remove any remaining chloramine-T. The washed chloroform phase was counted in a CRC-15 dose calibrator and dried down under a stream of nitrogen. The combined aqueous reaction mixture and washes were counted in the Capintec CRC-15 dose calibrator and the labelling efficiency calculated. The dried labelled lipophilic fraction was either adsorbed onto dried bread crumbs or maize meal for oral administration (Baboons O7, O8, O9 and O10) or dissolved in 300µl ethanol:DMSO (1:1) which was then diluted to 6 ml with 0.1 M $NaHCO_3$ that was pH adjusted to 7.2 for rectal administration (Baboon R1).

2.2.2 Binding of labelled oxihumate to blood components

Fresh human blood was collected by venipuncture into evacuated EDTA blood tubes and divided into 6 x 5.0 ml portions in 12ml glass test tubes with rubber caps. A 100µl dose of labelled oxihumate in sterile PBS (amounting to 240µCi each) was added to each of the blood samples and gently mixed by inversion of the tubes. Two tubes were then incubated at 37°C and the other four at 4°C for 30 minutes with occasional mixing by inversion.

After the incubation period, all the tubes were centrifuged at 500*g* for 10 minutes to separate the blood cells. The plasma was removed and transferred to fresh 12ml test tubes. The activity of the cell pellets were determined in a CRC-15 dose calibrator, then washed with 5ml PBS each and centrifuged at 500*g* for 10 minutes. The radioactivity of the washed cell pellets and the washings were again determined.

A tube of plasma from each incubation temperature was treated with 8 ml methanol to precipitate the proteins, vortexed, incubated for 10 minutes at 4°C and centrifuged at 3500*g* for 10 minutes to remove the precipitated protein. The supernatant was removed and the protein pellet washed 2x with 70% methanol solution. The radioactivity of the pellet and the combined methanolic supernatants were determined.

To determine whether the radioactivity was associated with an acid type compound, an aliquot of 0.5ml plasma was passed through a small column (Pasteur pipette plugged with cotton wool and filled to a height of 2cm with resin, equivalent to 0.52g resin) of BioRad AG MP1 resin – a strong anion exchanger with quaternary amine functional groups. The pass-through fraction and PBS washings were combined and the radioactivity determined. The column was plugged on both ends with Prestick® and the retained activity determined. The column was unplugged, washed with 4ml saturated NaCl solution adjusted to pH 1 with HCl and the column washings and column radioactivity were measured.

To determine the amount of radioactivity associated with lipophilic compounds a second 0.5ml plasma aliquot was passed through an activated and PBS equilibrated ODS (C_{18}) solid phase extraction (SPE) sample preparation cartridge containing 500mg stationary phase. The cartridge was slowly washed with 4ml PBS and 1ml deionized water. The activities of the SPE cartridge and the pass through fraction combined with the washings were determined. Further washings of the SPE cartridge with 4ml each of methanol and acetone were combined and the radioactivity of these eluents determined.

Extraction of the lipid fraction from the plasma was done on the remaining tubes of plasma by extracting 3x with equal volumes of chloroform. The chloroform phases were separated by centrifugation and removed from the bottom of the tubes by a long blunt needle and a glass syringe. The extracts were combined and analysed for radioactivity. The chloroform-extracted plasma was treated with an equal volume of 10% trichloroacetic acid and the precipitated protein removed by centrifugation at 3500g for 10 minutes. The protein pellet and supernatant radioactivity was determined.

2.2.3 Distribution of Radioactivity in the baboon model

All baboon work was performed with the approval of the Animal Ethics Committee of the Pretoria Biomedical Research Centre (PBRC) and the Animal Use and Care Committee of the Onderstepoort Veterinary Animal Research Unit who apply the codes and guidelines for use of experimental animals as set out by the Medical Research Council (Medical Research Council, 2002a; Medical Research Council, 2002b; Medical Research Council, 2002c) and Department of Agriculture (Department of Agriculture, 1990). The project was approved by both these ethics committees under project number 06/2001 (see Appendix II). The work was performed at the PBRC over a period of 14 months. Due to a technical problem with the gamma camera, one baboon was scanned at the Nuclear Medicine Department of MEDUNSA after all preparation of the animal was done at the PBRC.

A total of 13 baboons were used for the determination of the distribution of radioactively labelled oxihumate. Two of these baboons were used for control purposes, one where a dose of 3mCi free iodide was administered intravenously to determine the normal distribution of free iodide in a baboon and the other was dosed with 4.2mCi free iodide that was administered rectally. Three of the baboons were treated with 20µl aliquots of Lugols solution injected into their food for 3 consecutive days prior to the experiment in an attempt to "block" the organs of iodide accumulation, especially the thyroid. The two control baboons and five of the experimental animals did not receive Lugols solution prior to dosing as a blocking agent but Lugols was used to displace the accumulated radioactive iodide directly after the final static scans had been completed. All 13 baboons had food withheld for 16 hours prior to dosing but had free access to water. Table 6-1 below summarizes the different treatment procedures, including the route of administration, oxihumate fraction used and the radioactive dose administered as well as the time of administration of the Lugols solution used to displace radioactivity from the thyroid gland.

Table 6-1: Table summarizing the baboon numbers, route of administration, the treatment, the activity given and when Lugol solution blocking agent was administered.

Baboon number	Route of Admin	Oxihumate fraction	Radioactivity	Scan times	Blocking (time)
Control 1 P09/94	I.V.	NaI	110 MBq (3.05mCi)	0 – 1 - 4 + hrs	After 50hrs
Control 2 P09/97	Rectal	NaI	155 MBq (4.21mCi)	0 – 1 + 2hrs	None
O1W P09/94	Oral	H_2O soluble	300 MBq (8.10mCi)	0 + 12 hrs	After 12hrs
O2W P16/95	Oral	H_2O soluble	121 MBq (3.27mCi)	0 + 18hrs	6 days before
O3W P82/90	Oral	H_2O soluble	284 MBq (7.69mCi)	0 – 1 - 4 + 25hrs	After 24hrs
O4W P26/95	Oral	H_2O soluble	289 MBq (7.81mCi)	0 + 17-23 + 42hrs	Before
O5W P08/91	Oral	H_2O soluble	310 MBq (8.40mCi)	0-4 + 24 + 48hrs	After 48hrs
O6W P14/94	Oral	H_2O soluble	232 MBq (6.29mCi)	0 + 23-27 + 48hrs	Before
O7L P09/97	Oral	Lipophilic	334 MBq (9.04mCi)	0 + 1 + 12hrs	After 12hrs
O8L P30/94	Oral	Lipophilic	331 MBq (8.95mCi)	0 + 12hrs	After 12hrs
O9L P14/94	Oral	Lipophilic	57 MBq (1.55mCi)	0 + 5 + 24hrs	None
O10L P09/97	Oral	Lipophilic	311 MBq (8.42mCi)	0 + 5 + 24hrs	After 24hrs
R1L P17/94	Rectal	Lipophilic	278 MBq (7.51mCi)	0-4 + 24hrs	After 24hrs

At the required time a male baboon (weight 24.0 - 30.0kg) was anaesthetized by an intra-muscular injection of a Ketamine cocktail (20mg Anaket and 0.04mg Dormicum per kg) injected by blowpipe dart.

The anaesthetized baboons were weighed and brought to the gamma camera facility where they were dosed with between 110MBq and 335MBq (3 – 9mCi) of the I-123 labelled Oxihumate by means of a thin open-ended stomach tube while supported in the sitting position. The volume of solution for dosing the baboon was approximately 4ml and was "chased" with 2 x 10ml distilled water and 10ml air to ensure that the dose was delivered into the stomach and to prevent adhesion of the dose to the stomach tube. Four baboons were given lipophilic sub-fraction of oxihumate by applying the labelled compound to some bread or maize meal. This was done to avoid using any initial anaesthetics when the animals were to be monitored statically several hours later. The two baboons that received rectal administrations were set up completely for dynamic scans prior to dose administration via a 36mm long plastic gauge 14 catheter tube. Care was taken to introduce the dose slowly to reduce the possibility of introduction into the upper rectum in an attempt to avoid portal vein transport after absorption.

In all cases the distribution data of the radioactivity was collected using a Siemens ZLC Digitrac 75 Dual Integrator Gamma Camera fitted with a medium energy 140KeV parallel hole general-purpose collimator and sent directly to a computer operating a dedicated Sophapharm data acquisition and analysis software package. Data collection was initiated directly after administration of the dose. Data analysis was performed after all data for a particular baboon had been collected and the dynamic and static data corrected for both variation in collection times and for isotope decay using software calculated correction factors.

Two different scanning methods were used:
A one-hour dynamic scanning sequence of 60 consecutive one-minute interval scans was initiated directly after dosing and on completion was followed by two-minute static scans of the head/neck region and abdominal region which were performed on the hour every hour after dosing (in which case the baboon was kept under anaesthetic for a total of four hours).
Static two-minute scans were done to confirm single bolus delivery into the stomach and to obtain a time zero background scan of the thyroid region.

Two-millilitre blood samples were drawn and urine samples collected by emptying the bladder via the urinary catheter directly before each static scan set were completed.

In the cases when the one-hour dynamic scan sequence was performed, the baboon was placed on a scanning bed in the supine position with the head back. An arterial line was set up in the *medial femoral circumflex* artery to accommodate a blood pressure monitor and a "T" piece for taking blood samples. Electrocardiograph electrode patches were connected to the hands and one foot and coupled to a heart monitor. An intra-tracheal tube was inserted and connected to a breathing rate and CO_2 monitor. A urinary catheter was inserted to collect urine and prevent over-distension of the bladder during the four-hour anaesthetic period. An intravenous drip with a dosing pump for sodium pentobarbital (Sagatal at 0.9% in saline) was set up in the *saphena parva* vein at the back of the left leg and a metered dose of 30ml/hr for the 1st hour, 15ml/hr for the next 1.5 hours and no further infusion (unless the baboon showed signs of waking, in which case a single 4ml bolus dose was introduced), administered during the 4 hour period of anaesthesia.

If static data collection scans were to be performed only, the baboons were dosed and scanned quickly for two minutes over each of the regions of interest to obtain time-zero reference data for later comparison. Ventral and lateral scans of the head and thyroid area as well as a scan of the abdominal region (including the liver, stomach, GIT, kidneys and the bladder) was done to confirm that the activity was delivered into the stomach as a single bolus, that regurgitation had not occurred and to ensure that there were no other areas of activity visible.

In the cases where only the static scans were performed, immediately after administration of the labelled Oxihumate, the baboons were scanned as described above and returned to the recovery room where they were placed in a metabolic cage without any monitors being used or catheterisations taking place. After the required waiting time (12 – 24 hours) the baboons were again anaesthetized by darting and the static scan repeated together with collection of a 2ml blood sample.

The baboons generally recovered fully within 30 minutes after darting or within an hour after the 4-hour anaesthetic period, but depended on the total time under anaesthetic and whether any additional bolus dose of anaesthetic was required before completion of the procedures. After administration of the radioactively labelled oxihumate, the baboons had free access to their normal diet and water.

The bottom of the metabolic cage was equipped with a funnelled pan for collecting urine and faeces samples. Urine and faeces samples were collected as soon after urination or defecation as possible. Overnight samples were collected as early as possible the following morning. Fifty millilitre aliquots of urine were measured in a CRC-15 dose calibration meter. The total faeces sample was measured in the dose meter and weighed to determine the wet weight.

3 Results

3.1 Labelling of Oxihumate

Routinely a labelling efficiency of ± 60% could be achieved for the humic acid fraction of oxihumate when starting with a 3.5% oxihumate solution. The discarded supernatant fraction containing the balance of the activity was fairly brown in colour indicating the presence of either fulvic acids or soluble humic acid components. TLC separations of the labelled oxihumate indicated that only a very small quantity of free iodide remained in the oxihumate precipitate after the acetic acid washing procedure.

Figure 6-1 shows a typical TLC separation and the corresponding autoradiogram of ^{123}I labelled oxihumate run on silica gel 60 F254 using the methanol-chloroform-n butanol-water-acetic acid solvent system. The free iodide had an *Rf* of about 0.72 while the bulk of the oxihumate remained near the origin. The illustrated TLC is of a "desalting" of labelled oxihumate to ascertain the amount of free radioactive iodide still in the post labelling mixture. As can be seen there was generally very little free iodide left in the solution.

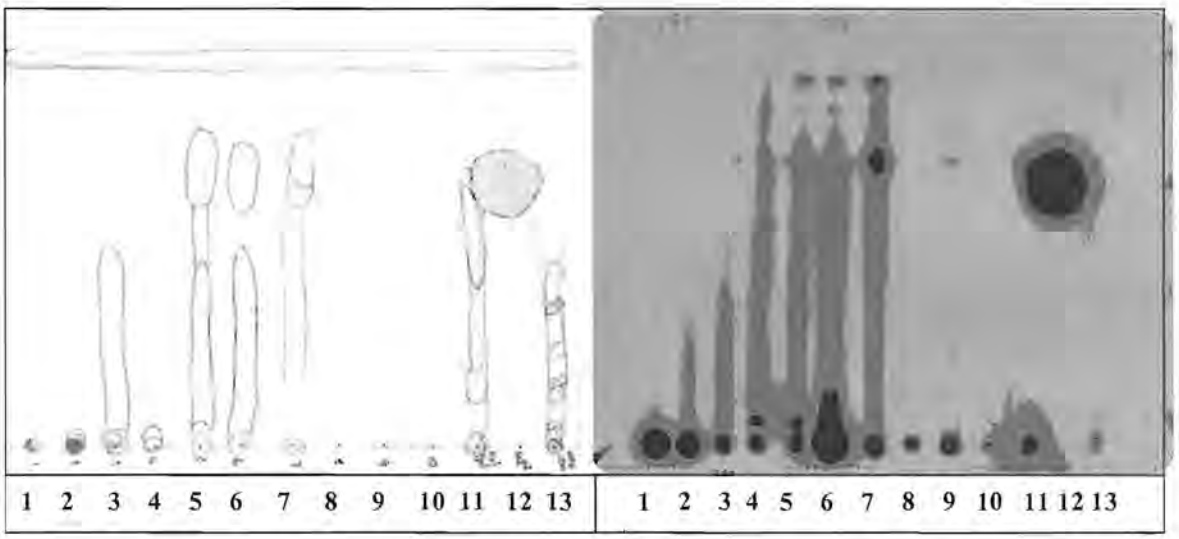

Figure 6-1: A TLC separation of ^{123}I labelled oxihumate. Lanes 1 to 10 are 1ml fractions eluted from a P-10 desalting column. The pencil marks indicate where fluorescent compounds were detected. Fraction 7 corresponds to the elution of iodide from the same column under the same conditions. Lane 11 is a rat gut "absorbed" fraction that is a concentrated aliquot of the suspending solution of an incubated rat gut segment. The large spot in lane 12 is iodide with ^{125}I added and lane 13 is ^{125}I labelled oxifulvic acid from the acetic acid washing of oxihumate during the labelling procedure. The image on the right is the autoradiogram of the same TLC plate and indicates the areas of radioactivity. The dark areas show highest activity.

3.2 Binding of labelled oxihumate to blood components

The binding of oxihumate to the blood fractions revealed that the radioactivity was bound mostly to the protein fraction (±56%) of the plasma and that less than 15% of the activity was associated with the lipid fraction. The activity associated with the cells was even lower (at ca. 5%) after the cells had been washed with PBS indicating that the labelled oxihumate was not taken up by the blood cells. The balance of about 30% of the added activity remained in the protein free plasma.

The small quaternary amine column removed approximately 65% of the activity and only about one third of this activity could be recovered from the column under the very harsh elution conditions applied. On the contrary the C_{-18} reverse phase solid extraction column only removed about 29% of the applied activity, of which about half could be washed from the column with aqueous methanol. The activity still retained (±17%) however required solvents stronger than 100% acetone to elute the activity from the column. Figure 6-2 highlights the distribution of activity within the different blood fractions.

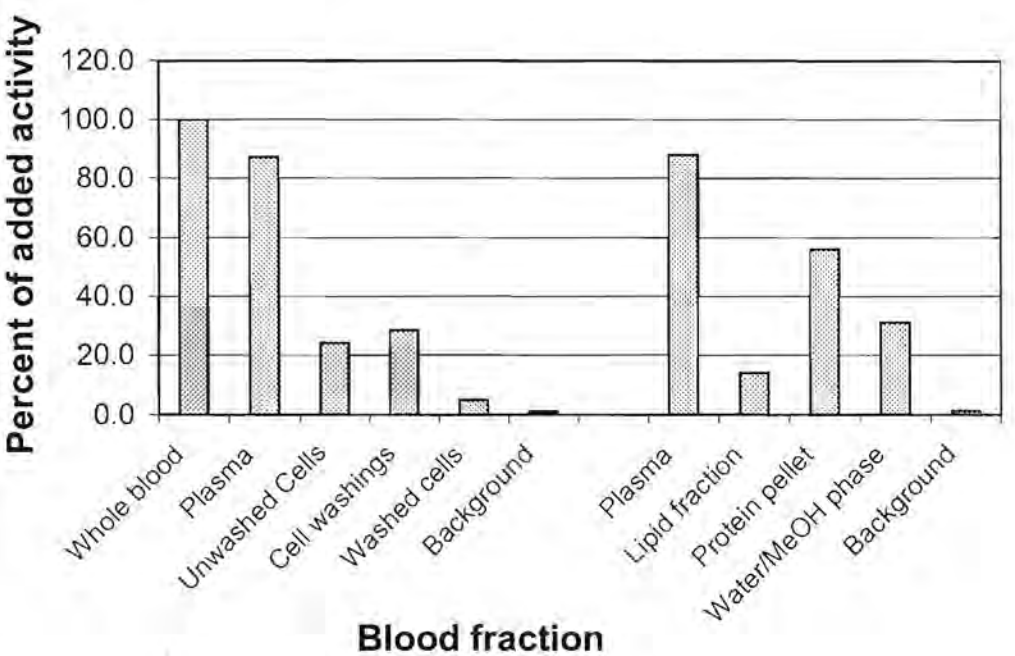

Figure 6-2: The average distribution of radioactivity of two different experiments between various blood fractions after *in vitro* addition of ^{123}I labelled oxihumate to whole human blood and incubating for 30 minutes. Temperature appeared to play no role in the binding kinetics. The data set on the left is the basic separation into cell and plasma fractions while the right hand data set is the further sub-fractionation of the plasma fraction into lipid, protein and aqueous fractions.

3.3 Distribution of Radioactivity in Baboons

3.3.1 Control baboon 1 (free iodide by intravenous injection)

The control baboon had free iodide-123 administered by intravenous injection. The activity was rapidly distributed throughout the circulatory system (within 1 minute) with almost immediate uptake by the salivary and thyroid glands and an area of the jejunum close to the stomach. The concentration of the accumulated activity initially increased steadily in the known iodide target organs (thyroid gland, salivary glands, eyes and testes) as well as in two unexpected target areas – the GIT and the nasal septum. The eyes and testes accumulated very low percentages of activity (< 0.2%) and were scarcely visible above the background although still detectable. As these organs are known to accumulate iodide and that the accumulated activity was very low these areas were not included in the percentage distribution.

Urinary excretion was predictably preceded by an accumulation in the bladder although the activity in the kidneys remained very low, comparable to that of the background at all the times that the kidneys were scanned.

After the initial distribution of the free iodide (which lasted about 25 minutes) there was a slow decrease in the accumulated activity in all the target organs except for the thyroid where the accumulation continued throughout the study and the segment of the jejunum adjacent to the stomach where the activity appeared to be maintained at a constant concentration.

Dynamic and static scans.

The decay corrected changes in absolute activity measured as counts per pixel for the different target areas monitored are illustrated in Figure 6-3. The initial 1-hour dynamic scan of the head and thorax of the control baboon administered free iodide intravenously revealed that there is a rapid distribution and dilution effect of free iodide. This was expected, as a dose introduced by intravenous injection is at its highest concentration directly after administration and before dilution and compartmentalisation can occur. The observed activity in the cardiac pool increased very rapidly (within seconds) after injection and then dropped logarithmically during the next 7 minutes due to the dilution and distribution of the activity. This was followed by a slower rate of decrease over the following 45 minutes after which time it appeared that the distribution had reached a steady equilibrium and that the further decrease in activity in the cardiac pool was due to excretion via the kidneys with the total activity reducing at close to 8% per hour. Over the next 3 hours the cardiac activity decreased steadily until the activity was very close to that of the background.

The salivary glands were the first target areas to show uptake with the activity increasing rapidly during in the first few minutes but this was superseded by the accumulation of the GIT and thyroid gland within 20 minutes. At about 20 minutes the salivary glands began to show a very slow progressive decrease in activity.

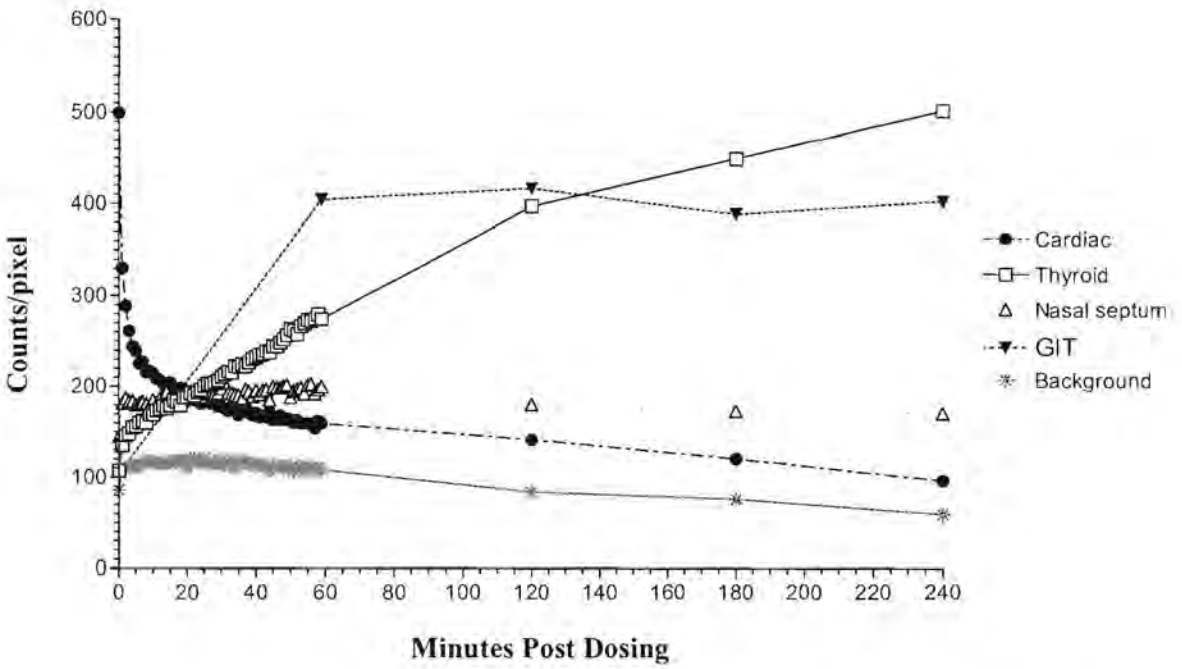

Figure 6-3: Control baboon 1: Decay corrected organ distribution of intravenously administered free iodide-123. Note the rapid decrease in cardiac activity due to the dilution effect, the rapid uptake in the GIT and the continuous uptake by the thyroid gland.

The initial accumulation in the GIT was rapid and appeared to reach saturation within an hour of dosing, after which time the activity was maintained for at least 4 hours. Panel A in Figure 6-4 shows a scintigram of the abdominal region of the control baboon clearly showing the accumulation of activity in the GIT despite the dose being administered by IV injection. During the following 20 hours there was a drop to approximately 40% of the peak activity seen during the initial period of constant activity. This activity was the second highest accumulation in the body. The duodenum and caecum did not show activity at any stage during the control baboon study.

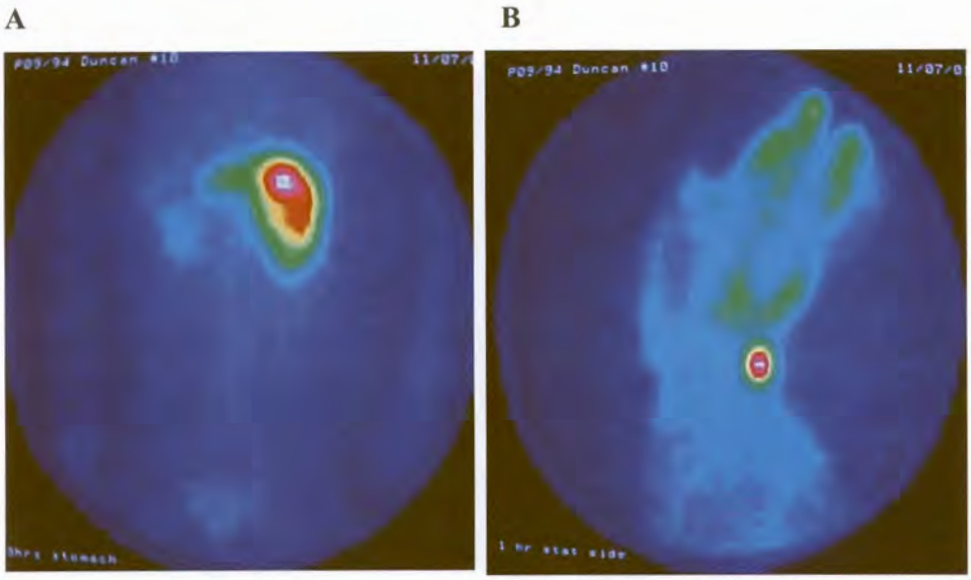

Figure 6-4: Scintigrams of the control baboon taken at 3 hours post administration of an IV dose of free iodide. Panel A shows a scan of the abdominal area where the accumulation of activity in the jejunum (GIT) can be seen clearly. The outline of the baboon can just be made out with the arms on the outside edges of the image. The baboon's head and chest are off the top of this image. The visible area at the lower centre is the bladder and the area to the left of the intense area of accumulation is the baboons right kidney. Panel B shows the lateral view of the head of the baboon taken at the same time post dosing. The thyroid (the small intense accumulation at the throat), salivary glands, eye and the nasal septum are clearly visible. An obvious shadow appears in the cranium due to exclusion of the activity from brain.

The thyroid accumulation started immediately after administration of the dose and this accumulation continued throughout the study period. The activity in the thyroid exceeded that of all other target organs from 2 hours onward. This trend of a high accumulation rate continued for more than 24 hours, after which time the activity in most organs was no longer accurately detectable. The intense accumulation of activity in the thyroid can be seen clearly in the scintigram in Panel B of Figure 6-4.

The cranium showed a "shadow", with less activity seen in the cranium than for the background counts due to exclusion of free iodide from the brain by the blood brain barrier. Panel B in Figure 6.4 illustrates this phenomenon very clearly.

The nasal septum, far forward of the bone structures and in the soft tissue of the nostrils, accumulated some activity although it did not account for a large percentage of the activity. In the lateral view of the head it was not that obvious due to the area being observed but in the anterior

view of the head this accumulation was distinct. The activity was fairly persistent with the septum still visible after 24 hours in most cases. Figure 6-5 shows an anterior view scintigram of the head and throat of an experimental baboon 25 hours post dosing with labelled oxihumate. The scintigram was almost the identical pattern seen for the control baboons.

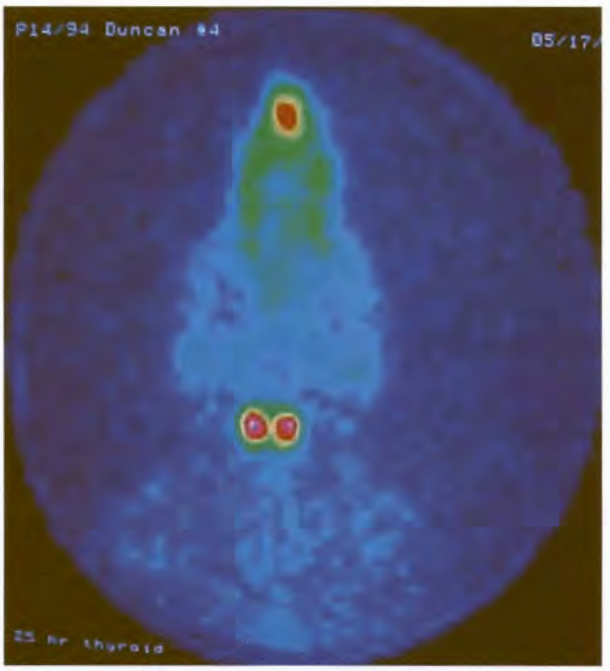

Figure 6-5: The anterior view of a baboons head 25 hours after administering an oral dose of ^{123}I labelled oxihumate. The thyroid glands are clearly visible at the throat with the salivary glands and the nasal septum also showing as areas of accumulation. The outline of the baboon's head is obvious.

The background activity showed a short initial period of increasing activity that lasted for about 25 minutes and was followed by a slow progressive decrease for the rest of the scanning time at a rate similar to that of the cardiac, salivary glands and nasal septum activities indicating a dynamic equilibrium.

The static scans collected at one hour intervals revealed that the thyroid continued to accumulate activity continuously for at least the first 4 hours while most of the other organs showed a trend of decreasing activity. After the initial accumulation, the GIT maintained approximately the same activity throughout the initial 4 hours. In this time approximately 30% of the administered dose was excreted via the urine. Surprisingly, almost no activity was seen in the kidneys although the bladder

showed accumulation that was related to the volume of urine retained in the bladder. Ten percent of the administered dose was found to accumulate in the bladder during a single hour in the first 4 hours. This accumulation in the bladder could not increase further due to the bladder being emptied via the catheter each hour after dosing. The average rate of excretion during the first 4 hours was almost constant at about 8% of the administered dose being excreted per hour.

3.3.2 Experimental Baboons (water soluble sub-fraction of oxihumate *per os*)

The distribution of radioactivity seen in the experimental baboons dosed with labelled water-soluble oxihumate varied with respect to the absolute percentage of the dose accumulating in each target organ, yet the trends and changes of activity in the target organ distributions were similar (Figure 6.6).

The two baboons that were anaesthetized for four hours directly after administration of the dose showed an extended stomach and duodenum transit time in the initial scans. When the animals were initially anaesthetized for only 30 - 40 minutes, the stomach activity decreased fairly rapidly. Approximately 50% of the initial activity appeared to remain in the stomach after about 6 hours although it was later found that an accumulation in the jejunum adjacent to the stomach could have accounted for the apparent lack of uptake from the stomach. The duodenum in most of the baboons was not visible against the background.

The accumulation in the eyes and testes was extremely low with the combination of these two areas amounting to less than 0.2% of the normalized activity and is therefore not shown on the graphs. The cranium showed a radioactivity shadow implying that the activity cannot cross the blood-brain barrier.

Activity was detectable in the body of the animals within 5 minutes of administration but the uptake was generally fairly slow.

The distribution of activity that was seen after dosing with labelled water-soluble oxihumate fraction was very similar to that seen with the free iodide control baboon. The greatest accumulations were seen in the thyroid and GIT as well as the unabsorbed fraction that remained in the gut for the duration of the study. There was a significant amount of activity that progressed down the gut as far as the caecum but onward movement from the caecum was delayed. Typical distribution of the activity after 12 hours showing the abdominal accumulation in the areas adjacent to the stomach is illustrated in Figure 6.6. In panel A of Figure 6.6 the small hot spot is probably due to the jejunum

positioned such that the image was collected looking down a short length of the gut. It can also be seen that the areas of accumulation in the GIT are close to the area in which the stomach is positioned, but do not show the typical shape nor size of the stomach. In one animal the region of accumulation in the GIT was closer to the baboon's right side, almost at the position of the duodenum or gall bladder

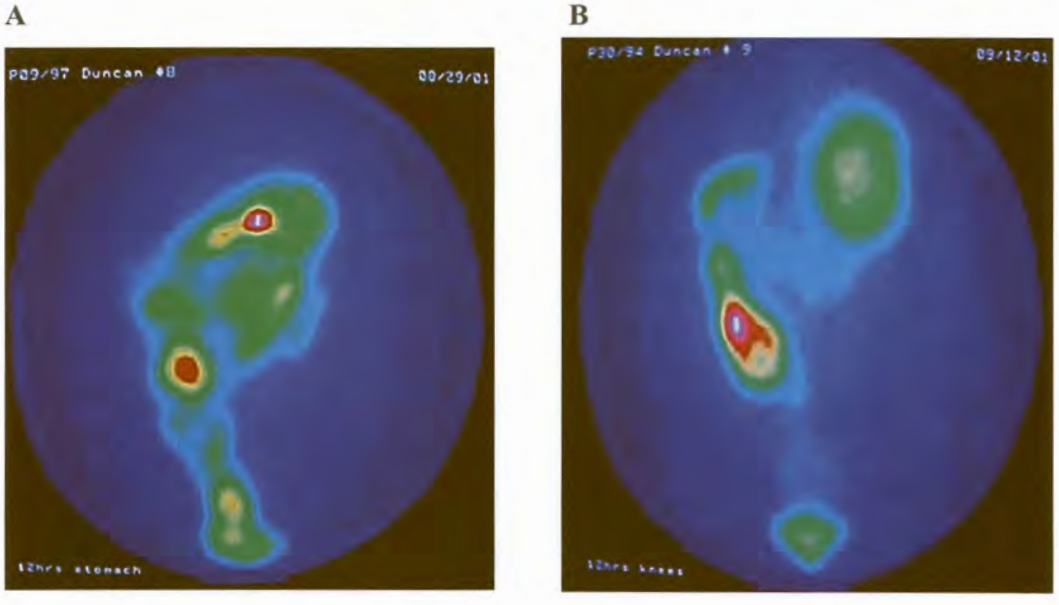

Figure 6-6: Two scintigram images of different baboons showing the distribution of activity in the abdominal region of the baboons 12 hours after oral administration of ^{123}I labelled oxihumate. The section of the jejunum can be seen clearly in both images as the area of accumulation at the top just right of centre. The accumulation at middle left is in the caecum and the accumulation at the bottom centre is the bladder. The rest of the activity is at various points along the colon and small intestine

What was of particular interest was that the activity accumulating in the thyroid was affected by the use of Lugols iodine solution as a pre-treatment to block the thyroid glands. It is clear that the baboons that were given Lugols before the administration of the radioactively labelled oxihumate (Figure 6.7: panels O4W and O6W) had much lower percentages of accumulation of activity in the thyroid at 24 hours than the equivalent baboons that were treated with Lugol solution only after the scanning had been completed (Figure 6.7: panels O3W and O5W). As the activity was shown as percentage accumulation normalized for the selected target organs this lower accumulation in the thyroid caused an apparent increase in the percentage accumulation in the GIT and caecum.

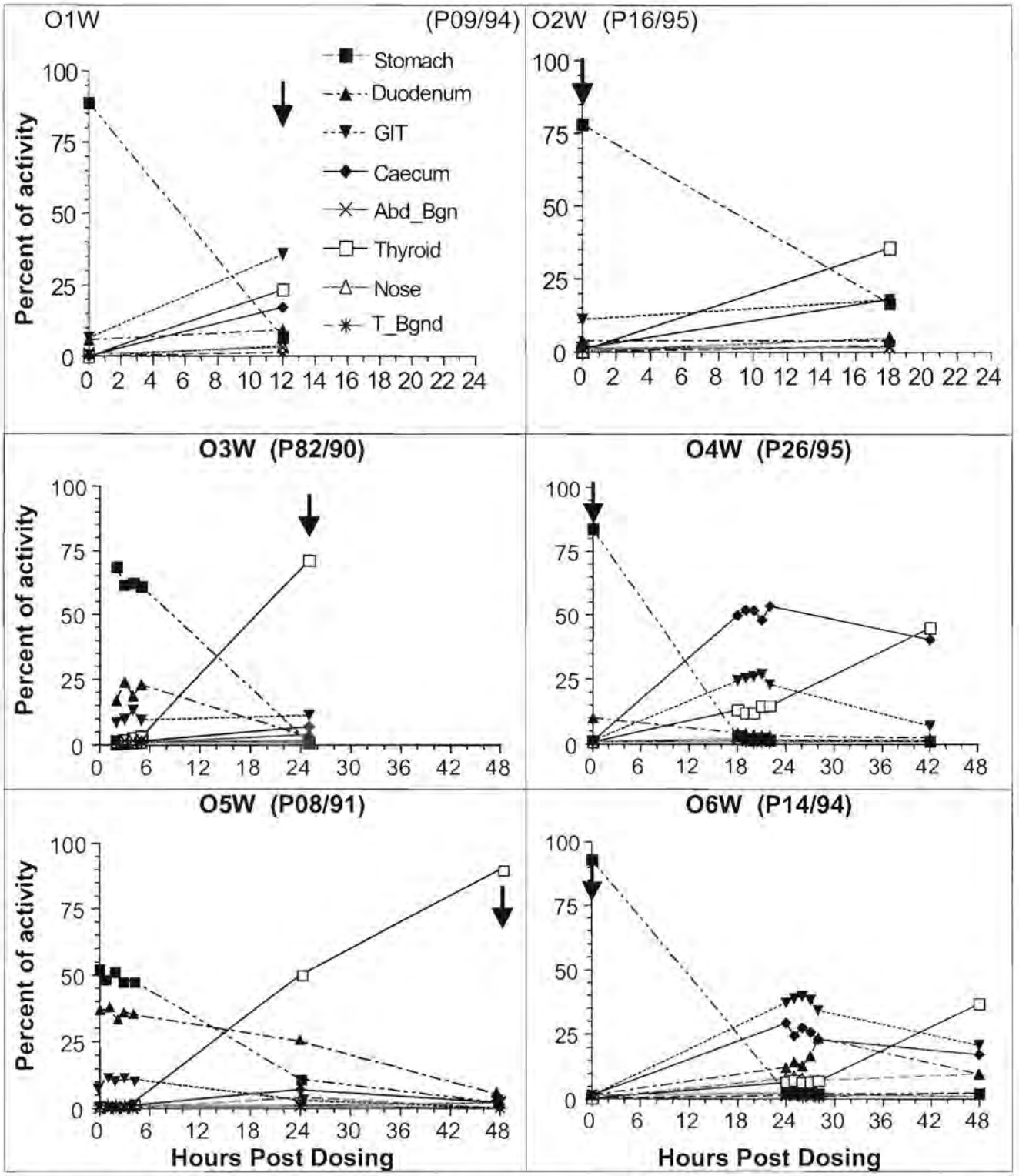

Figure 6-7: Graphs summarising six separate baboon experiments where the animals were dosed orally with ^{123}I labelled water-soluble oxihumate. The graphs present the percentage change in the activity of the target organs against time. The values have been normalized for the target organs shown. Note that the abscissas are not all the same. Legend: Shown in Panel O1W. The vertical arrow marks the time of Lugol solution administration.

Even in the case where the Lugol solution treatment was given five days before the administering the radioactively labelled oxihumate (Figure 6.7: panel O2W) the final percentage accumulation in the thyroid was still less than 40% of the total dose.

3.3.3 Experimental Baboons (lipophilic sub-fraction of Oxihumate *per os*)

The lipophilic sub-fraction of oxihumate was isolated and labelled as described above and administered to the baboons by absorbing the solvent free lipophilic sub-fraction onto either bread or a maize meal block. This technique does have a certain risk in that the baboons can smell that the food is adulterated and in the case of baboon O9L only consumed about 25% of the activity in the maize meal block containing the labelled lipophilic sub-fraction. The advantage however was that there was no period during which the baboon was under anaesthetics prior to the time to monitor the distribution of activity for the first time. None of the baboons that were dosed with lipophilic sub-fraction were scanned dynamically but were scanned at both 5 hours and again at 24 hours post dosing or only once at 12 hours post dosing.

The baboons dosed with lipophilic sub-fraction appeared to absorb the dose faster than the complete oxihumate but this trend cannot be proved statistically due to the different method of administering the dose, the different time intervals used between the scans of the different baboons and the small number of experimental animals in this group of the study.

The distribution of the activity was very similar to that of the water-soluble oxihumate fraction with the same regions accumulating activity and the rate of decrease in the activity was also similar to the water-soluble oxihumate fraction if the effect of the anaesthetic is taken into consideration. None of these animals were anaesthetized for the administration of the dose, which resulted in a more rapid stomach emptying. By scanning the animals 5 hours after dosing it could be established that approximately 25% of the total dose remained in the stomach. Figure 6-8 panels O9L and O10L show the accumulated activity at 5 and 24 hours post dosing. The maximum time that any of these baboons were under anaesthetic was 35 minutes. The jejunum had by this time already accumulated a significant percentage of the dose and accounted for the highest accumulation of activity at five hours post dosing. This was higher than the accumulation in the thyroid at that time but the thyroid exceeded this GIT accumulation of the activity within 12 hours as can be seen from Figure 6-8 panels O7L and O8L.

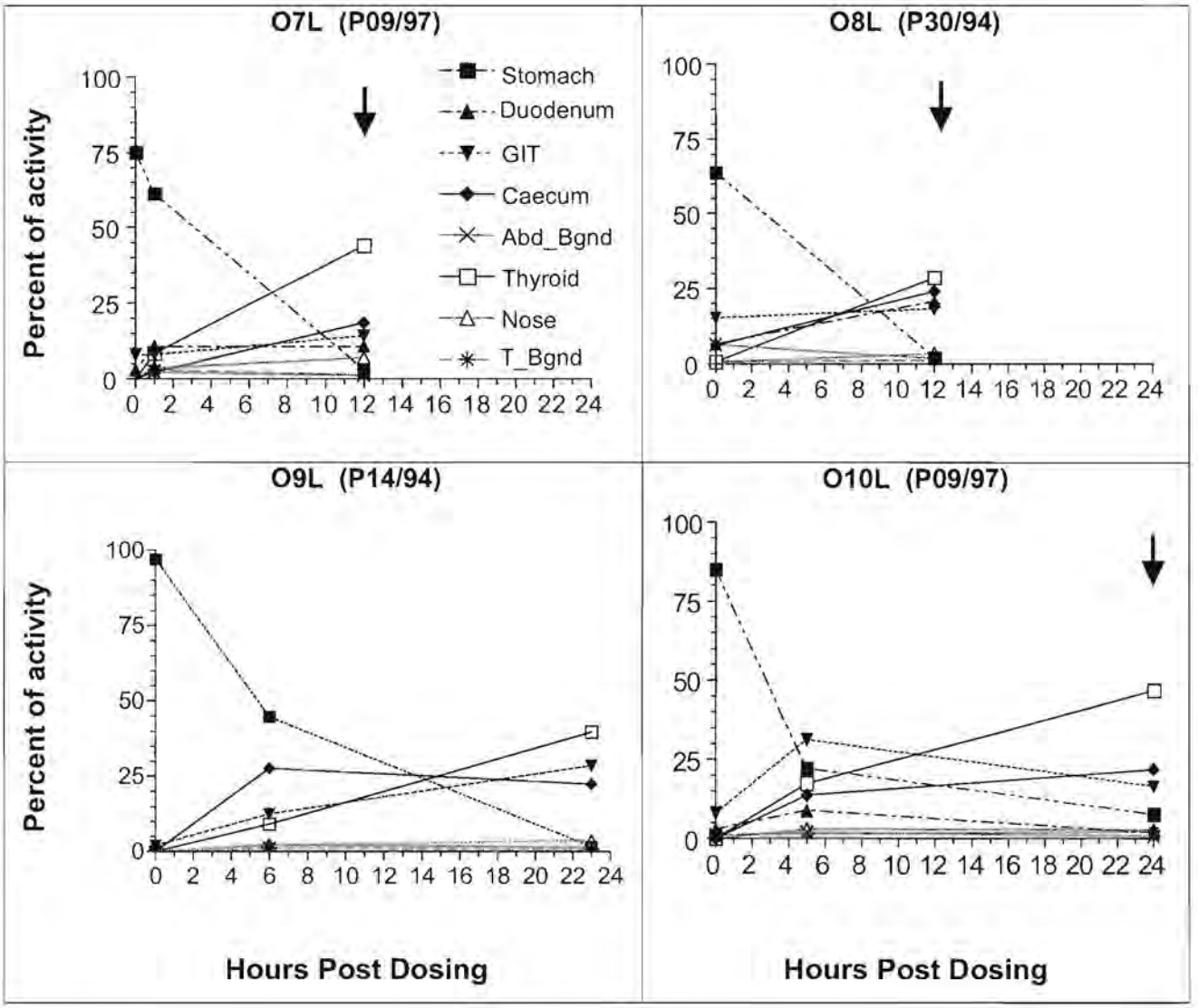

Figure 6-8: Graphs summarising four separate baboon experiments. These graphs present the changes in absolute percentage radioactivity of the target organs of orally administered ^{123}I labelled lipophilic sub-fraction of oxihumate against time. The values have been normalized for the target organs shown. Note that the abscissa does not change. Legend: Shown in Panel O7L. The vertical arrows indicate the time of blocking with Lugol solution. O9L was not dosed with Lugol solution.

3.3.4 Control baboon 2 (free iodide by rectal administration)

This control baboon was scanned in the Nuclear Medicine Department of MEDUNSA due to a technical problem with the gamma camera at the PBRC which meant that the baboon had to be transported to this facility under anaesthetic. This resulted in the scan time being shortened to only two hours and that the baboon had been under anaesthetic for approximately one hour prior to the dosing taking place.

The control baboon for the rectal dosing was prepared and scanned dynamically in the same way as the first control baboon that was dosed intravenously. The dose for the rectal administration was formulated by adding 150µl DMSO and 150µl ethanol to the radioactive NaI in the vial and diluting to 6.5ml with PBS solution. The volume was limited to 6.5ml to avoid anal leakage and was administered slowly by syringe fitted with a plastic gauge 14 catheter of 40mm length. Slow administration avoided administering the dose high into the rectum where portal vein drainage occurs. The formulation included DMSO and ethanol to mimic the formulation to be used for the rectal administration of the lipophilic fraction

The first hour of scanning was a dynamic series of 1-minute scans taken over the thoracic region that included the thyroid glands, the liver and the upper GIT areas but excluded the head. This dynamic series of images was immediately followed by static scans of the head, thorax and abdominal regions at 1 hour and again at 2 hours.

The uptake of the radioactive iodide was rapid but not as fast as in the case of the intravenous injection. A graph of the various organ accumulations of activity after rectal administration of free iodide is illustrated in Figure 6-9. There was a continuous increasing accumulation in the thyroid glands but in contrast to what was seen in the control dosed by intravenous injection there was no plateau effect seen in this accumulation. An accumulation of activity in the GIT adjacent to the stomach was observed and this accumulation appeared to be slower than in the case of IV administered iodide but continued to accumulate until the end of the study. This accumulation in the GIT became visible within 30 minutes and increased steadily until the end of the 2-hour scanning period.

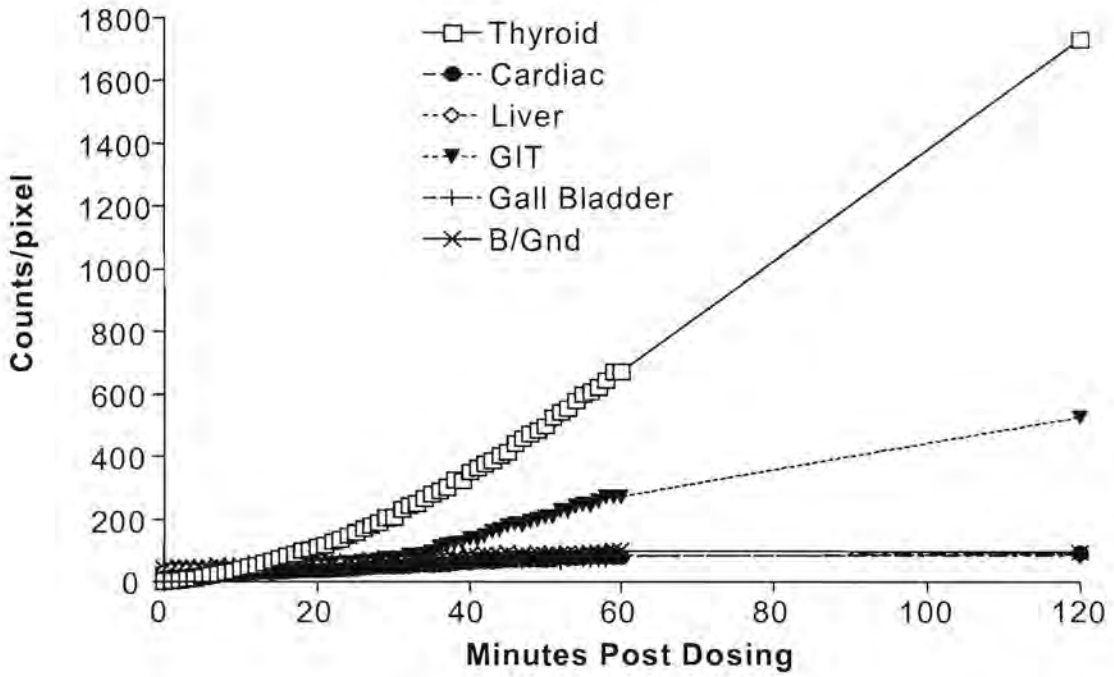

Figure 6-9: Control baboon 2: Decay corrected organ distribution of the abdominal region after rectal administration of free iodide-123. The first hour was monitored dynamically at 1-minute intervals. Note the rapid onset and continuous uptake by the thyroid gland. The cardiac, liver and background regions did not accumulate activity whereas the GIT showed uptake although much slower than the thyroid. The lines are extrapolations between the data points.

Figure 6-10 shows a series of the sixty 1-minute scans taken during the dynamic scanning starting immediately after dosing rectally. Despite the route of administration there was an accumulation of activity in the thyroid within 5 minutes of administration. This activity is seen in the images as the butterfly shape at the centre top of each separate image. This accumulation of activity continued increasing until the end of the 2-hour study period. Within 25 minutes of dosing the accumulation in the jejunum was evident (the horizontal line of activity just below the centre of the images) and this area also increased in activity throughout the study. The pulmonary and cardiac region had a slight transient accumulation between 10 and 35 minutes but this activity was insignificant compared to that of the thyroid and GIT. The bottom edge of the image shows scatter between 23 and 35 minutes resulting from the combined activity in the bladder and rectum but by emptying of the bladder this scatter was eliminated.

The activity in the cardiac pool, gall bladder and liver showed no significant accumulation during the first two hours after administration having similar activity to the background. As the head was

not in the area being monitored dynamically no information was collected in the first hour with respect to the salivary glands and nasal septum.

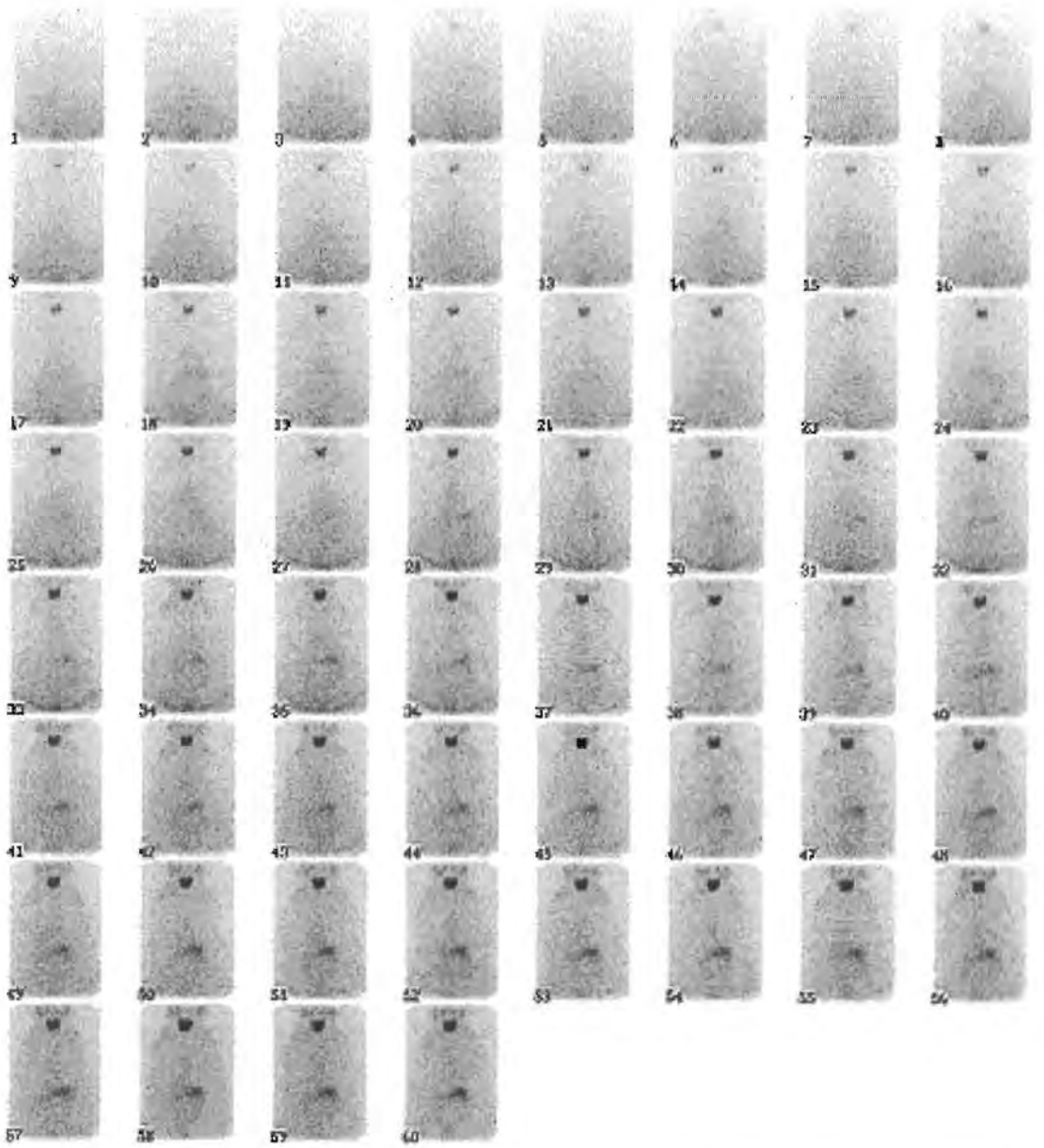

Figure 6-10: A sequence of 60 one-minute images of the initial 1-hour dynamic scan of the control baboon dosed rectally with free iodide. Note that there is accumulation of activity in the thyroid as early as 5 minutes post dosing and that the uptake in the GIT is visible within 15 minutes. The double spot at the centre top of each image are the thyroid glands and the oblong just right of the centre of the images is the GIT. A pulmonary background is visible between 10 and 35 minutes. The outline of the baboon becomes obvious after approximately 30 minutes.

3.3.5 Experimental Baboons (lipophilic sub-fraction of Oxihumate by rectal administration)

One study was performed using lipophilic sub-fraction of oxihumate that was administered rectally. The lipophilic sub-fraction was labelled as described above and formulated into 150µl DMSO and 150µl ethanol. This solution was diluted to 6.5 ml using PBS just before administration to avoid any possible separation of the lipophilics from the aqueous solution. The baboon was prepared for a dynamic scan and the dose slowly administered by syringe fitted with a plastic gauge 14 catheter of 40mm length. Slow administration avoided the introduction of the dose into the region of the rectum drained by the portal vein system.

Dynamic and static scans.
The first hour of scanning was a dynamic series of 1-minute scans taken over the thoracic region that included the thyroid glands, the liver and the upper GIT areas but excluded the head. This dynamic series of images was immediately followed by static scans of the head, thorax and abdominal regions at 1 hour and again at hourly intervals up to 4 hours. A further set of static scans was performed 24 hours after dosing.

Figure 6-11 illustrates the absolute counts per pixel of different regions of accumulation during the twenty-four hour period that the baboon was monitored. For the first time it was observed that there was an accumulation in the liver and in the gall bladder. These accumulations in the liver and gall bladder were rapid, with the accumulations evident within two minutes after dosing and only began decreasing again after 1 hour. A further observation was that the thyroid was initially slow to accumulate activity and reached a plateau level after about 4 hours. The amount of the dose that was excreted via the urine was less than 10% within the first 4 hours. The gall bladder showed a greater accumulation in the initial 28 minutes and reached a peak within the first hour. This activity then decreased slowly to reach a new lower plateau level after 3 hours and thereafter remained almost constant for the next 20 hours.

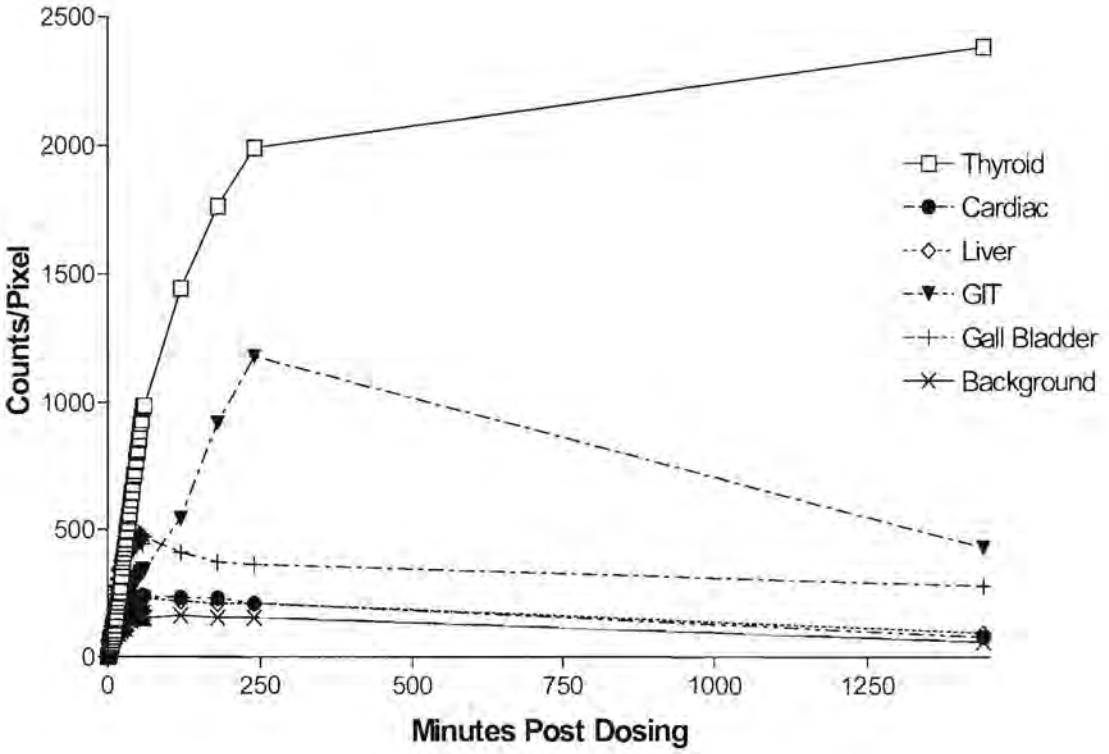

Figure 6-11: The absolute radioactivity distribution in a baboon treated by rectal administration of ^{123}I labelled lipophilic oxihumate sub-fraction. The liver and gall bladder showed the most rapid increase in activity. The thyroid activity surpassed that of the gall bladder 28 minutes after administration and continued to increase until the end of the study. An area of the GIT close to the stomach also showed a transient accumulation of activity.

Figure 6-12 is a more detailed graph of the changes in the accumulation of activity during the first hour after dosing. The accumulation in the liver was very rapid but the accumulation in the gall bladder exceeded that of the liver within 4 minutes. The gall bladder activity was then the area of greatest accumulation until the thyroid gland superseded this activity at 28 minutes. The gall bladder activity plateaued after about 40 minutes and reached a maximum at about 1 hour post dosing after which a slow progressive decrease occurred over the next two hours. After this time a new plateau level appeared to have been reached and this level was maintained for 24 hours. In contrast to the gall bladder activity, the liver reached a plateau level within 25 minutes that was almost identical to the cardiac pool and thereafter showed a slow constant decline in accumulation until the end of the study.

The GIT accumulation became noticeable after about 30 minutes and then increased continuously up to 4 hours. By 24 hours this activity had decreased substantially but still exceeded that of the gall bladder, liver and cardiac pools.

The thyroid showed an obvious lag time of about 5 minutes before increasing its accumulation. The increase in accumulation was then constant from approximately 10 minutes to 60 minutes after which time a slow decrease in the accumulation rate was evident and a plateau level appeared to have been be reached within 4 hours. The thyroid glands were the prime area of accumulation of the activity during the study.

The static scans revealed that not all the activity had been absorbed from the rectum within the 4 hours post dosing but that by 24 hours only a small fraction remained. Other organs that showed any accumulation of activity at 24 hours were the caecum, bladder, testes, nasal septum and salivary glands.

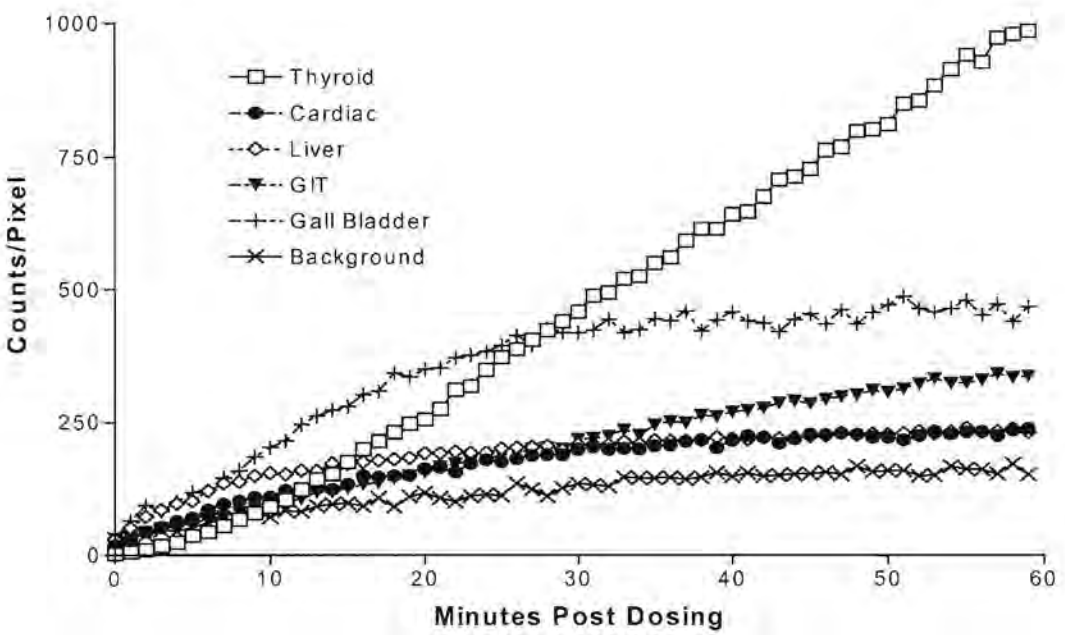

Figure 6-12: A more detailed graph of the first hour of Figure 6-11 showing the dynamic changes in absolute radioactivity distribution in the target organs of a baboon dosed rectally with a ^{123}I labelled lipophilic fraction of oxihumate. The liver and gall bladder showed rapid uptake with the greatest activity in the gall bladder for the first 28 minutes of the study after which time a 30-minute plateau was reached. The area of accumulation in the GIT close to the stomach superseded the liver activity after 30 minutes. The activity accumulation of the thyroid glands demonstrates a distorted sigmoidal curve.

3.3.6 Blood activity

The radioactivity occurring in the blood after administration of the labelled oxihumate was never more than 1μCi/ml. As this was below the lower limit of the CRC-15 dose calibrator, the 2ml blood samples were counted in a LKB Wallac 1261 multiwell gamma counter to obtain more accurate results. All the samples were measured simultaneously to eliminate any differences due to isotope decay. These results were used to indicate the changes in activity and to show the trend in the blood activity. The 60ml blood samples were used to try to identify the compound carrying the activity in the blood and to identify the absorbed humic acid compounds. The majority of this activity was found in the serum and found to be free iodide by precipitation by silver nitrate, PAGE electrophoresis using silver nitrate as an additive to the anode buffer and TLC followed by autoradiography. Approximately 5% of the activity in serum could be extracted using chloroform and analysed further by TLC. The compound remained on the origin when analysed by silica gel TLC using either acidic or alkaline mobile phases. As the quantity of this compound was very small and the activity fell below the detection limits before chemical analyses could be done it was not possible to determine the identity of this extractable compound.

3.3.7 Urine activity

The radioactivity in the urine was determined as soon after urination as possible and the recorded values corrected for isotope decay using a spreadsheet program. The decay-corrected values and urine volumes were used to calculate the total activity excreted per urination and this was then reported as a percentage of the administered doses, and illustrated in Figures 6-13, 6-14, 6-15 and 6-16.

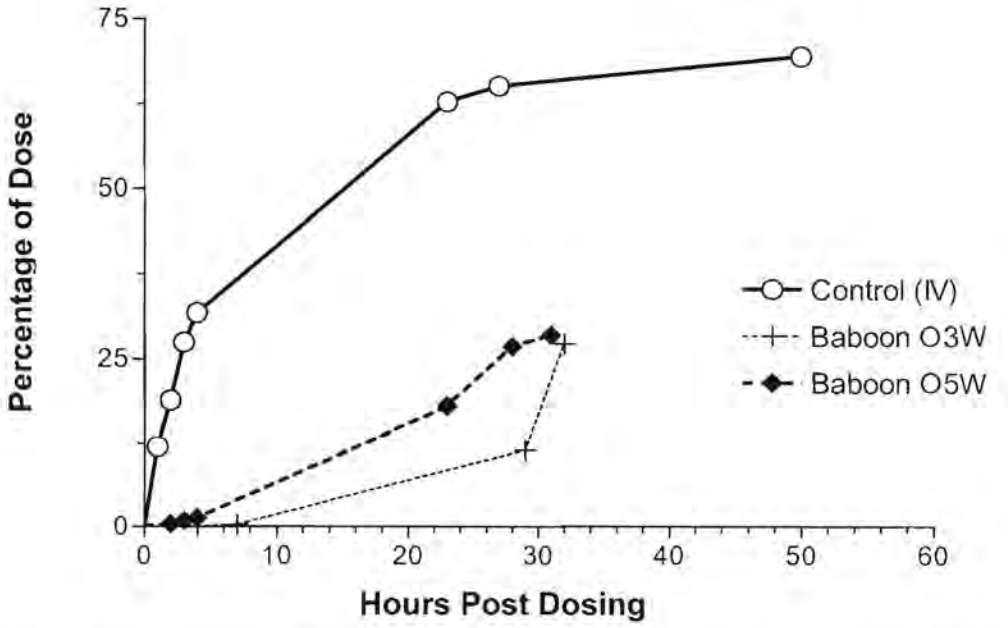

Figure 6-13: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. These baboons were all scanned during the first four hours post dosing. The control baboon had free iodide-123 administered by intravenous injection therefore the excretion of activity started immediately.

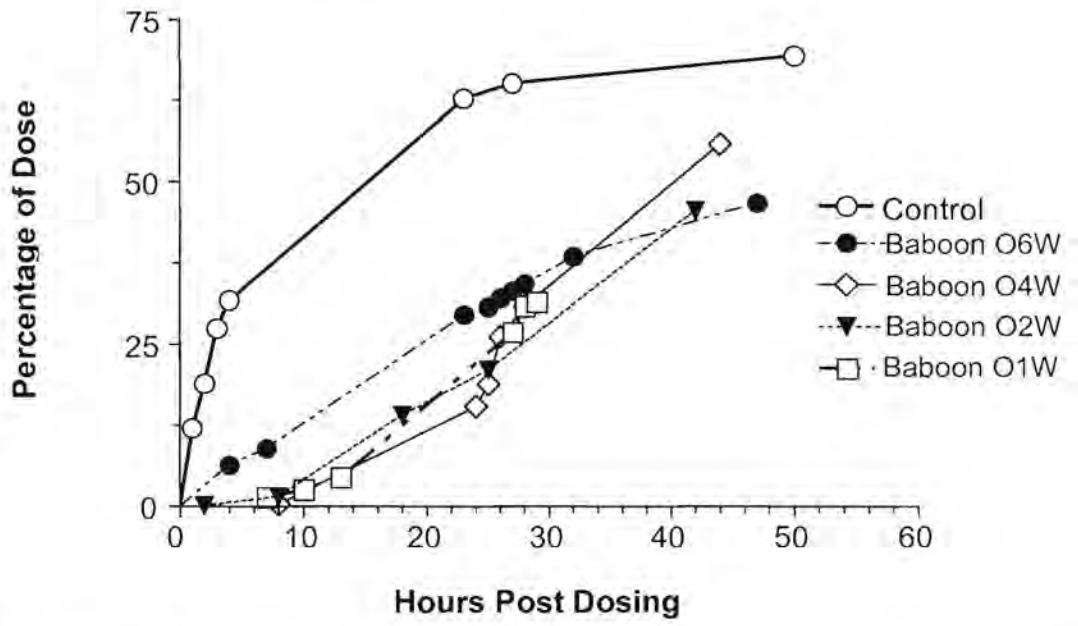

Figure 6-14: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. The four experimental baboons were dosed and scanned at set times after administration without an initial long period under anaesthetics. The control baboon had free iodide-123 administered by intravenous injection resulting in excretion of activity starting immediately.

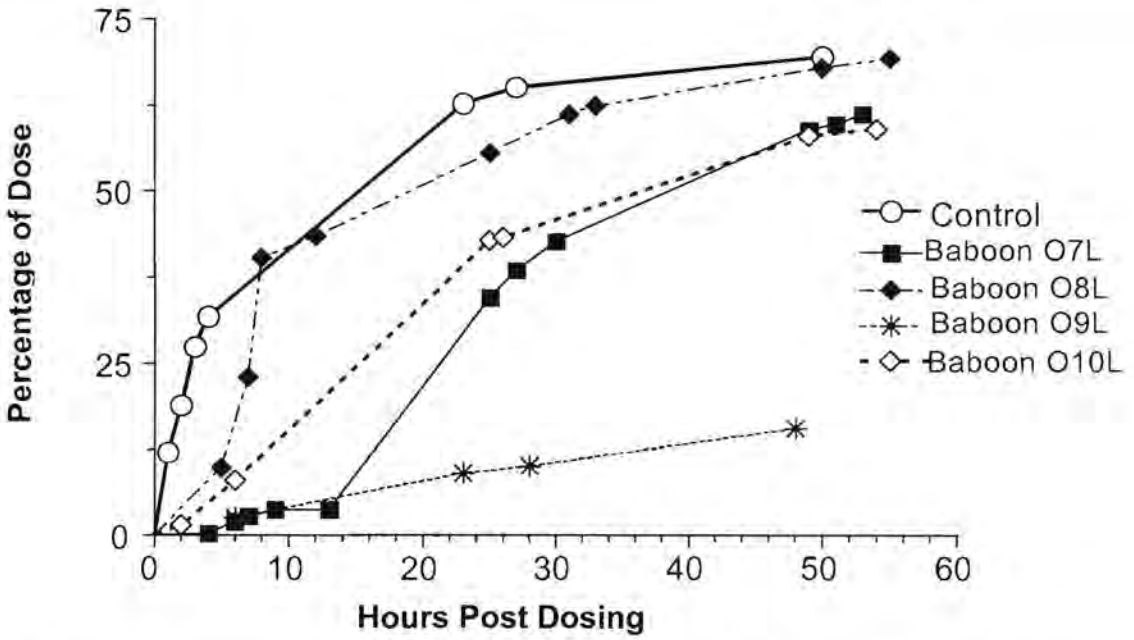

Figure 6-15: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. All the experimental baboons in this set were dosed with the lipophilic sub-fraction of oxihumate. The control baboon had free iodide-123 administered by intravenous injection resulting in excretion of activity starting immediately.

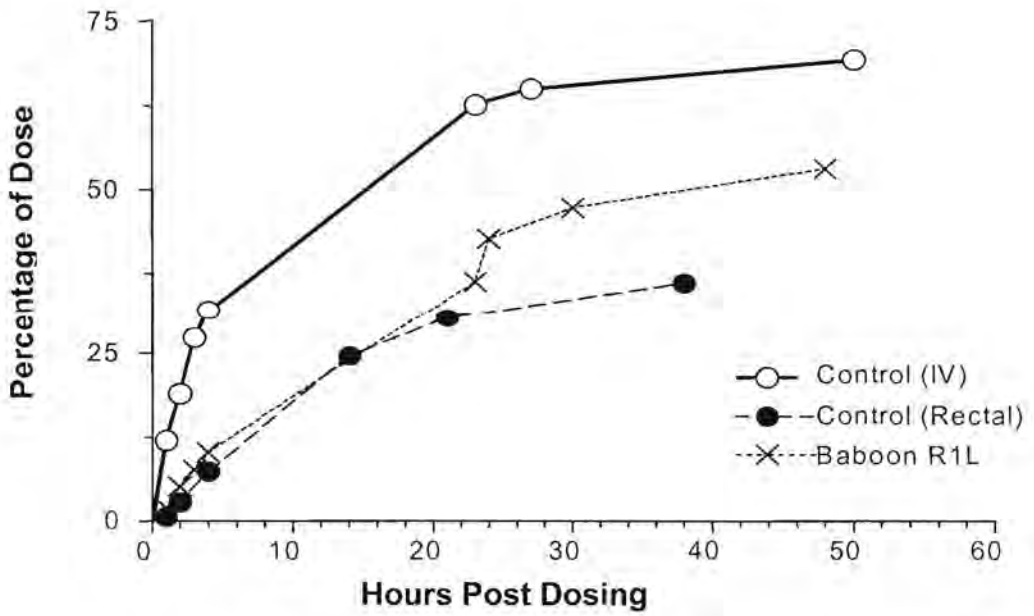

Figure 6-16: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. The experimental baboon was dosed rectally with the lipophilic fraction of oxihumate the second control was dosed similarly with free iodide-123. The IV control baboon had free iodide-123 administered by intravenous injection therefore the excretion of activity started immediately.

3.3.8 Faeces activity

Radioactivity of the faeces was generally low, seldom reaching more than 6μCi per defecation, this amount accounting for less than 0.2% of the administered dose. The exception was Baboon O1W where a high count was excreted (124μCi) at 26 hours post dosing. The faeces still appeared normal and there was no difference in eating or behaviour of this baboon compared to any of the others.

4 Discussion

This study was the first study to attempt to determine the pharmacokinetics and target organs of humic acid in the baboon model. In contrast to synthesized pharmaceuticals, the complexity of humic acids make it difficult to determine the concentration of humic acid in blood or urine, as it is not known which specific compounds are in fact being taken up. To circumvent this problem the humic acid was labelled with a radioactive iodide isotope and this labelled humic acid used to track the uptake and distribution. The approach was novel in that an attempt was being made to determine the complete distribution in the baboons throughout the expected transit time of the humic acids. To achieve this humic acid had to be effectively labelled with a gamma-emitting isotope.

Oxihumate can be efficiently and rapidly labelled with radioactive iodide using the chloramine-T method (Hunter & Greenwood, 1962). The cleanup procedure is simple, quick and complete but some of the acid soluble compounds – fulvic acids by definition - are lost. The quantity of free iodide left in the labelled oxihumate is negligible which makes this method suitable for use with short half-life isotopes of iodide as well as for the longer half-life isotopes for *in vitro* work. The same method for labelling could be used for the lipophilic sub-fraction of oxihumate but the cleanup procedure needed to be adapted to suit the lipophilic nature of the final sample. The residual iodide in the lipophilic sub-fraction was negligible and the procedure was quick, again making it suitable for short half-life isotopes. What must be considered is that only compounds with appropriate binding sites, like conjugated double bonds, phenolic and other activated condensed aromatic compounds would react with the iodine formed during the chloramine-T oxidation of iodide in the first phase of the labelling reaction. This could result in some biologically active compounds in oxihumate not being labelled with iodide yet being absorbed and exerting its effect on target organs. These unlabelled compounds would not have be detected by the technique used in this study.

The binding of ^{123}I labelled oxihumate to blood components revealed that the bulk (50 – 60%) of the activity bound to the plasma proteins. This confirms the results reported by Klocking *et al.* (Klöcking *et al*, 1967) who reported that the major portion of humic acids was found bound to *in*

vivo serum proteins after IV administration. The cells and lipophilic fraction of blood accounted for less than 20% percent of the added activity. There is also a substantial fraction (30%) that was not associated with the cells or with either the protein or lipid fraction. This fraction appears to be dissolved in the serum itself and would therefore represent the bio-available active fraction of the oxihumate.

The fact that almost 66% of the labelled oxihumate could be extracted from cell free plasma by a strong anion exchange resin column would imply that the compounds being extracted carry a net negative charge and are hence acidic, an expected result. The harsh conditions required to remove any of the activity from the column indicates that the extracted compounds are either chemically bound, carry multiple negative charges or have pKa values equivalent to strong mineral acids. The C_{18} extractable fraction accounts for only about 30% of the total activity, implying that about 30% of the activity is bound to lipophilic compounds in the oxihumate. Some of these compounds are very lipophilic requiring solvents like THF or chloroform to elute them from the C_{18} column. These could be the compounds that are retained by C_{18} HPLC columns and which affect the resolution of these columns (see Chapter 2).

As no literature could be found with respect to the normal distribution of free iodide in a baboon model, control studies had to be performed to provide a basis for comparison of where the activity of the labelled oxihumate was accumulating relative to that of the iodide. Two control baboon studies using free iodide were performed. The first control was dosed by administering $Na^{123}I$ as an intravenous injection and the second control was dosed by rectal administration of the radioactive iodide.

The control baboon where the iodide was administered by intravenous injection showed that redistribution of iodide is very rapid with accumulation in the target organs starting within seconds after injection. Initially the cardiac activity was very high due to the concentrated bolus of activity introduced into the blood but this reduced very rapidly as a result of the dilution effect and the reduction of total circulating activity resulting from uptake by target organs and excretion via the kidneys. This cardiac effect was not seen in any of the other baboons including the rectally dosed control animal and was a direct result of the method of administration. The salivary glands and thyroid were the initial target organs with the eyes and testes showing a small but insignificant accumulation.

These are areas that are known to be iodide target organs in the human. An area of the jejunum adjacent to the stomach accumulated a substantial portion of the administered dose within the first hour then appeared to remain fairly constant for the next 3 hours. This accumulation was unexpected as the dose was administered intravenously completely remote from the gut. Initially it was thought to be the stomach, which can accumulate iodide in a similar way that chloride is accumulated. A second area of accumulation, being the nasal septum directly behind the nostrils also accumulated activity although this was fairly slow. This is an unexpected area of accumulation as there are no known organs or glands in this area. One possible explanation is that the area has a very high blood supply and that this could account for the accumulation, but comparison to other areas of high blood flow (cardiac, renal, pulmonary) did not reveal an equivalent accumulation relative to the surrounding tissue.

Renal excretion of activity was evident within minutes of administration although the kidneys did not show any significant accumulation of activity during the study. This could be due to the fact that the kidneys are well drained and that some of the excreted iodide may rapidly be reabsorbed and returned to the circulatory system.

Although this route of administration affected the rate of uptake and distribution it allowed determination of the target organs and the normal distribution of iodide using a small dose of radioactivity (Figure 6-7 and 6-8).

The distribution of radioactivity after oral administration of ^{123}I labelled oxihumate was determined and compared to that of a control baboon dosed with free iodide in an attempt identify areas of accumulation that were due to the oxihumate

The bladder however showed increasing accumulation with time and reduced during emptying which confirmed that the activity was associated with the urine and that the bladder is not a target organ of the accumulated activity. The activity in the urine was analysed and found to be mostly iodide by precipitation with silver nitrate and PAGE electrophoresis in a buffer system that contained silver iodide.

In the experimental baboons, the distribution of activity appeared to be fairly slow, especially in the case where the baboons were initially anaesthetized for four hours. This would be due to the slow uptake of compounds from the stomach and the fact that the anaesthetic used temporarily paralyses peristalsis in the GIT. A further reason could be that the acidic pH of the stomach results in the

oxihumate precipitating in the stomach and that the oxihumate then remains insoluble for some distance into the small intestine before the increase in pH enables the precipitated humic acid to become sufficiently soluble to be absorbed from the lumen of the gut.

Accumulation of activity in the thyroid gland and other normal iodide target organs (as seen in the control baboon dosed with free iodide) would imply that the iodide (radioactivity) was being removed from the absorbed compound, probably in the liver or that cleavage of the activity from the labelled oxihumate was taking place in the gut prior to absorption and that free iodide was then being absorbed and distributed resulting in accumulation of activity in the normal iodide target areas.

The lipophilic sub-fraction appears to be taken up faster and this was confirmed by the excreted urine radioactivity curves. The lipophilic sub-fraction appeared to have a faster onset of excretion of activity as well as a larger percentage of administered dose excreted via the urine. This implies that in the case of the lipophilic sub-fraction a higher concentration of activity was circulating in the blood, that the activity was not bound to the plasma proteins or this sub-fraction was being metabolized faster resulting in a higher concentration of free iodide in the blood. The latter scenario is unlikely as the rate of thyroid gland uptake is not significantly faster for the lipophilic sub-fraction than for the oxihumate.

The graphs showing the normalized percent activity per target organ (Figures 5-7 and 5-8) revealed that the stomach empties fairly slowly, but this may also be due to the area of the jejunum where iodide appears to accumulate being very close to the stomach, and that this area was being initially misinterpreted as being the stomach. Generally the activity of the stomach dropped to less than about 50% within 6 hours, more than the expected time for complete gastric emptying. In the cases where the peristalsis stopped initially due to anaesthetics it would be expected that the time for the decrease of activity would be extended but this could not be confirmed due to the times post dosing at which data was collected.

The duodenum showed activity due to passage of the activity through this portion of the gut in the baboons that were initially anaesthetized for 4 hours. This was probably due to limited residual movement in the gut before peristalsis came to a stop. The remainder of the baboons showed no measurable accumulation in the duodenum, which indicates that the activity seen in this segment of the GIT is due to the transit of labelled oxihumate through this area.

The labelled oxihumate that was not absorbed from the small intestine had a prolonged transit through the caecum and up to 50% of the normalized activity could be seen in the caecum between 18 and 30 hours. Activity was detected in the caecum as early as 5 hours post dosing but when peristalsis was affected by long periods of anaesthesia, the time before activity was seen in the caecum was prolonged.

The brain barrier is not crossed by the free iodide or the labelled oxihumate. A shadow could be seen in the cranium against the normal background counts when the head was viewed laterally. This was true for all experimental baboons including those that were dosed with the lipophilic sub-fraction of oxihumate. This would be expected as the blood-brain barrier prohibits passive uptake of ionic compounds into the CSF.

The experimental baboons all showed a very similar pattern of uptake and distribution and the only major differences seen were in the rate of accumulation and excretion.

The obvious conclusion to be drawn from these results is that the activity seen in these baboons is due to redistribution free iodide and not labelled oxihumate compounds.

The second control baboon was dosed with free iodide by administered rectally and during the initial dynamic scan the same target organs showed accumulation of activity as with the intravenously dosed control baboon. The rates of accumulation were similar for both control baboons if the differences in the radioactive dose are taken into account. The initial thyroid accumulation is slower for the rectally dosed baboon but this is due to the route and the fact that the iodide was absorbed from the rectum into the bloodstream prior to distribution and this slower initial accumulation can be explained by the lower initial concentration in the blood. The accumulation from the GIT was slower but did not reach a plateau level during the study as occurred in the IV dosed control baboon. Cardiac and liver accumulation was not evident in the second control baboon.

In contrast to all orally administered doses (water soluble and lipophilic sub-fraction), the rectally administered lipophilic sub-fraction showed a rapid accumulation and transit through the liver with high activity being seen in the gall bladder within a few minutes of dosing. These observations would support the hypothesis that there are some compounds being rapidly absorbed and that they are metabolized in the liver with the metabolite to which the iodide is bound accumulating in the gall. This would be released into the duodenum where further reactions could take place. This accumulation in the liver and gall bladder exceeded the uptake of the thyroid gland for the first 16

and 28 minutes respectively. This may indicate that free iodide was released into the circulatory system by the liver while metabolising absorbed radioactive compounds. This released iodide is then rapidly sequestered by the thyroid gland similar to the way in which the free iodide administered IV was sequestered. The fact that the thyroid accumulation shows a sigmoidal curve in the first hour points to the fact that there is probably an initial rate-limiting step that is overcome within 15 minutes. (See Figure 6-12). The gall bladder retains significant levels of activity for up to 24 hours indicating that there is a replacement of the activity that is slowly secreted into the duodenum. There is a possibility that these compounds are involved in the enterohepatic circulation, as the total absorbed dose would have been taken up within the first few hours, the rest being excreted with the faeces. This is a very positive result with respect to the absorption of humic acid compounds from the GIT as there is positive proof of absorption and it appears as though the liver would rapidly metabolize these absorbed compounds.

If the same lipophilic compounds were absorbed from the stomach or small intestine it would be expected that the same pattern of distribution should have been observed, however this was not the case. The equivalent lipophilic sub-fraction that was dosed *per os* resulted in an accumulation of between 40% and 50% of the normalized activity in the thyroid glands and showed no liver or gall bladder accumulation. Approximately 60% of the radioactive dose administered *per os* as labelled lipophilic sub-fraction was excreted via the urine within 60 hours.

In the urine studies it was found that the radioactivity excreted in the urine accounted for at least 50% of the administered dose. This activity was excreted via the urine within 60 hours with excretion of the lowest percentage of activity shown in the baboons that received smaller doses. This should be true in any baboon where the thyroid and other iodide target organs are not saturated with iodide and therefore sequester a larger percentage and retain this sequestered iodide for a longer time. The intravenously dosed control baboon however showed the highest excretion with almost 75% of the administered dose being excreted via the urine within 60 hours despite the total administered activity being lower than all the experimental baboons. This could have been due to the thyroid being close to saturated as it was later found that this control baboon had received Lugol solution 5 days prior to the study.

One baboon that was dosed with the lipophilic sub-fraction of oxihumate also excreted about 75% of the administered dose with an excreted urine activity curve that closely resembled that of the control baboon (Figure 6-14). This result hints at the possibility that the iodide was completely removed from the lipophilic compounds before distribution could take place and that the iodide, then being

present in the blood as free iodide, followed the normal iodide distribution. Baboon O9L that was dosed with lipophilic sub-fraction in a maize-meal porridge block consumed only about 40% of the total dose resulting in low activity. The excreted urine activity followed an almost linear curve as opposed to the sigmoidal curves observed with all the other baboons and the total activity excreted via the urine accounted for only 27% of the administered dose.

This study is the first study to be performed to determine the pharmacokinetics of humic acids in a baboon model. The choice of this animal model was to mimic the human as closely as possible with respect to the physiology, immunology and anatomy. It was hoped that therapeutic levels of oxihumate would be found in the baboon and that the pharmacokinetics could be determined as well as the target organs which could have given some indication of the mode of action of the humic acid compounds. It is obvious that the rate of absorption from the GIT was slower than the metabolism of the absorbed compounds which is an indication that the enzymes involved in this metabolism are not being induced after the first exposure but are already present and active. It was unfortunate that the target organs could not be determined but it is possible that other routes of administration or a different radioisotope may overcome the problem of iodide removal experienced in this study

The conclusions that can be drawn from this study is that the distribution of radioactivity that was seen in the baboons was that of free iodide and that the iodide was probably removed from absorbed humic acid compounds by the liver. The rate of absorption from the GIT is slower than the rate of metabolism in the liver so the activity could not be seen in the liver before being redistributed throughout the body via the blood. As the humic acid compounds were then no longer detectable by gamma camera their distribution could not be followed and no data with regard to $T_{½}$, T_{max}, C_{ss}, AUC and Vd could be calculated.

In the case of the rectal administration of the lipophilic compounds, the rate of uptake was substantially faster than from the stomach and an accumulation could then be seen in the liver and the gall bladder within a few seconds of administration. The accumulation in the gall bladder appeared to reach a plateau after about 30 minutes at which time the thyroid activity superseded that of the gall bladder and continued to accumulate as would be expected if the activity were present as free iodide.

Furthermore, the rate of uptake in the thyroid was similar to the uptake that was seen in the control baboon where free iodide was administered intravenously which implies that iodide was present in the blood in free iodide form within a very short time after administration.

Chapter 7

Final Conclusions

Despite the widespread occurrence of humic acids and the many studies performed on humic acids from different sources, the structures of these compounds are still not known. Many researchers now accept that humic acids are likely to be a tight association of many organic molecules, derived from plant and other organisms, and includes mineral salts that are complexed within the "supra-molecular" structure.

This study has proved that oxihumate, which is a semi-synthetic humic acid having all the typical characteristics of humic acids isolated from different natural sources, could be fractionated into at least seven sub-fractions. The fractionation was easily achieved by a sequential stepwise increase in the concentration of organic solvents and removing the insoluble sub-fraction by centrifugation.

Each of the isolated sub-fractions had different characteristics. The most lipophilic and oily compounds were found in the sub-fraction that was soluble in 50% tetrahydrofuran (THF soluble sub-fraction) while the methanol precipitated sub-fraction was found to be the darkest coloured sub-fraction, having almost 30 times higher absorbance at both 465nm and 665nm than the acid soluble sub-fraction, which was in turn the least coloured sub-fraction. The THF soluble sub-fraction was the only sub-fraction that was not normally a solid and had a light brown colour like honey and darkened on standing for prolonged periods. The THF precipitated sub-fraction was a dark brown solid whereas the water soluble, acid precipitated, methanol precipitated and methanol soluble sub-fractions were all black solids.

The acid soluble sub-fraction was a dark oily sub-fraction that could be further sub-fractionated by washing with methanol to give a crystalline sub-fraction and a dark brown oily sub-fraction. This last mentioned oily sub-fraction and the THF soluble sub-fractions had the highest fluorescent intensities. However, all the sub-fractions exhibited at least some fluorescent emission centred around the wavelength 430nm, despite using a wide range of excitation wavelengths.

Observations that would indicate that oxihumate was in fact a mixture of different chemical compounds are the following:

- The oxihumate could be sub-fractionated on the basis of differential solubility
- Chromatography on silica gel resulted in several distinct fractions being eluted although most fractions collected were still mixtures of several compounds as determined by TLC
- Solid phase extraction columns used for sample preparation appeared to retain some compounds very strongly and these could be washed from the cartridges after they had stood for at least 16 hours during which time a new equilibrium would establish
- There were at least two major classes of compounds eluting from a reverse phase HPLC column, being a fraction close to the solvent front at 2 – 4 minutes and a fraction eluting between 10 and 16 minutes when using an acetonitrile gradient in an alkaline aqueous eluent. Each of these two major fractions consisted of several very closely eluting individual compounds. The early eluting fraction would be the more polar and/or completely excluded compounds while the later eluting fraction would be the more lipophilic compounds.
- On TLC the seven isolated sub-fractions could be separated into at least a further 30 distinct spots of different R*f* values of which many could be visualized, but this required different techniques or visualising agents.
- Infrared spectra of the various sub-fractions were different from each other although the differences between the water soluble, acid precipitated and methanol precipitated sub-fraction was subtle.
- UV/visible spectroscopy of the various sub-fractions showed similar featureless curves above 350nm but exhibited subtle differences that appeared to be small broad peaks superimposed on the absorbance trend between 200 and 350 nm.
- The ash content of the various sub-fractions varied greatly indicating different ability to complex inorganic salts
- Labelling of oxihumate with radioactive iodide resulted in several different compound being labelled radioactively which indicates that not all the compounds in oxihumate had reactive chemical bonds available
- The sub-fractions had different UV and fluorescence spectra especially in specific absorbance and fluorescent quantum yields

There are reports of humic acids having a molecular mass in excess of 10^6 Dalton that would mean that humic acid was unlikely to be absorbed from the lumen of the GIT. If absorption from the gut cannot take place, it would follow that no therapeutic activity for orally administered humic acid was possible. To test whether oxihumate could be absorbed from the GIT a study was performed on

isolated segments of rat GIT making use of ^{125}I labelled oxihumate. It was found that there was a relatively rapid uptake of oxihumate from the GIT *in vitro* and that the absorption followed typical concentration and surface area dependant kinetics with an initial rapid rate of uptake becoming progressively slower with time. Dark coloured compounds in addition to several different labelled but colourless compounds were found to be absorbed. Furthermore the radioactivity that had been absorbed could be almost quantitatively precipitated by adding silver nitrate to the solution implying that the absorbed activity was free radioactive ^{125}I. However analysis of the labelled oxihumate used for the study indicated very little residual free radioactive iodide that could not account for the high percentage of activity precipitated with silver nitrate. A small quantity of the absorbed fraction of oxihumate appeared to be bound to protein although the suspending medium used contained no protein at the beginning of the study.

It therefore appears that the oxihumate complex can be disrupted to some extent in the rat gut and that some of the dissociated compounds then cross the GIT membranes and are transported in the bloodstream. From a small study done to determine in which blood fraction the humic acid would reside, it was found that the majority of the humic acid bound to the plasma proteins. It can therefore be assumed that after absorption from the GIT the plasma proteins would serve as the main transporters of the humic acids. The absorption from the GIT of humic acid after oral administration has been confirmed which means that this is a feasible route of administration of the compound as a therapeutic drug.

The *in vitro* bioassays used in this study included a flow cytometric method to determine the level of CR3 molecules expressed on the surface of activated and resting PMNL from healthy volunteers and chemiluminescent assays of the release of reactive oxygen species also from PMNL cells. These assays were aimed at determining parameters that affect anti-inflammatory properties, either due to inhibition of adhesion or inhibition of the respiratory burst which releases potentially harmful reactants. The bioassays were performed using the isolated sub-fractions at the same relative concentrations at which each occurred in oxihumate, so the highest concentration of 100µg/ml for complete oxihumate would have included the 5µg/ml of the most refined sub-fraction, being the THF soluble fraction.

Generally it was found that the activity was greatest for the water-soluble oxihumate, the acid precipitated and methanol precipitated sub-fractions, becoming significantly less active for the remaining more lipophilic sub-fractionations. The anti-inflammatory activity of the THF sub-

fraction was very low despite containing at least 30 individual compounds. It would therefore appear that the complete complex is required for the highest activity for the oxihumate.

The chemiluminescence assays were expected to be affected by the colour intensity of the test mixtures containing the dark coloured sub-fractions due to quenching of the light emitted by the reaction. However the extent of chemiluminescence quenching did not follow the trend of the colour intensity at 425nm – the wavelength of the emitted light in these assays. What was of interest was that the dark coloured sub-fractions all appeared to cause some activation of the cells resulting in an increase in the background. These sub-fractions if pre-incubated for extended times seem to have exhausted the cells which then only showed a short burst of activity before returning to baseline values when stimulated with known PMNL activators. The lipophilic sub-fractions, the methanol solubles, THF solubles, THF precipitated and acid solubles, all appeared to prime the cells so that the response initiated very rapidly after stimulation and was greater than that of the positive controls implying an enhanced activity following activation.

Simultaneously with the determination of reactive oxygen species the ability of the different isolated sub-fractions to scavenge superoxide was tested, using a cell free system. Superoxide anion was produced enzymatically using the xanthine/xanthine oxidase enzyme system. It could be demonstrated that there was a definite trend in the scavenging ability of the sub-fractions to scavenge the superoxide anion formed by the enzyme reaction. This response was concentration dependant, but the possibility also exists that quenching plays a role in the case of the darkest fractions.

The study performed to determine the effects of oxihumate *in vivo* using the contact hypersensitivity rat model showed that oxihumate had little effect and compared closely to a water treated control. This assay measures the responses of DNFB challenged ears of previously sensitized rats after a weeklong oral treatment with the test compounds. A positive control used in the study was a known steroidal anti-inflammatory, prednisolone. This control exhibited a definite and significant inhibitory effect on the immune response, as did a humic acid extracted from brown coal, indicating that the test system was appropriate for the measurement of the *in vivo* effects of oxihumate.

The fact that there were differences in the contact hypersensitivity response of humic acid treated verses untreated rats would again imply that the humic acid is in fact being absorbed from the gut of the rat confirming the results that were obtained during the *in vitro* studies using isolated rat gut segments. The lack of activity in this assay for oxihumate could be due to variations in the

concentration of active compounds in the two different humic acid samples used in the assay. This would need to be confirmed in future studies.

In an attempt to determine the target organs of oxihumate a study using 10 experimental baboons and orally administered radioactive ^{123}I labelled oxihumate or THF soluble sub-fraction was performed. The later distribution of the absorbed activity showed a very similar distribution to the control animals that were dosed with free radioactive ^{123}I containing NaI solution alone. This would imply that the iodide is removed from the labelled oxihumate and that the free iodide then distributes according to the normal distribution kinetics of iodide. Two control baboons were included in the study, one being dosed intravenously with ^{123}I containing NaI while the second control was dosed by rectal administration of a free radioactive ^{123}I containing NaI solution. The distribution of the activity for the two NaI controls was almost exactly the same after about 40 minutes implying that the route of administration is not critical to the final distribution.

One experimental baboon was treated with a rectal dose of ^{123}I labelled THF soluble sub-fraction of oxihumate. This animal showed different uptake and distribution kinetics and was the only baboon where activity was detected in the liver post dosing. The liver activity appeared to be transient with a significant accumulation of this activity in the gall bladder within minutes of administration of the labelled oxihumate. The activity in the gall bladder was higher than any of the other organs for at least 28 minutes post dosing after which the continuous thyroid gland accumulation exceeded that of the gall bladder. After about one hour the activity in the gall bladder began decreasing and reached a plateau at about 4 hours after which this activity was maintained till the end of the study.

Excretion of activity from the baboons was via the urine and amounted to almost 60% of the total administered dose within 24 hours. The excretion curves varied and showed a distinct increase after treatment with Lugols solution, which would also imply that the activity was in the form of free iodide. Tests on the urine showed that almost all the activity could be precipitated by the addition of silver nitrate again implying that the excreted activity was in the form of free iodide.

The faeces from all the baboons had a very low activity throughout the study.

The activity in the blood was low during the first four hours, as seen in the baboons that were initially scanned dynamically and when these samples were collected hourly post dosing. This is probably due to the peristalsis that is suppressed by the anaesthetics. The absorbed activity detected in the blood appeared to be associated with the protein fraction of the plasma.

Although the actual structure or combination of compounds in humic acid are unknown, a model semi-synthetic humic acid that appears to represent humic acids extracted from natural sources was used in this study in an attempt to find the active compounds involved in the anti-inflammatory activity of these natural organic acids.

In summary it can be stated that oxihumate can be sub-fractionated and that chemically the various sub-fractions proved to be quite different using different spectroscopic techniques and chromatographic techniques. The separations on HPLC were reproducible to some extent but it was obvious that the recovery was low and this affected further separations using the same column.

The highest anti-inflammatory activity was associated with the water-soluble sub-fraction, the least refined of the sub-fractions and for this reason *in vivo* studies were performed on this particular isolated sub-fraction.

Absorption was found to take place from the GIT after oral administration and although this was found *in vitro* and implied *in vivo,* it appears as though the radioactive iodide used to label the oxihumate was being stripped from the humic acid either before or during the absorption process.

The water soluble sub-fraction was found to have no significant effect on the hypersensitivity response in the rat model although a very similar brown coal derived humic acid did show significant anti-inflammatory activity in the same rat model.

Humic acids have potential as immuno-modulating compounds due to the inhibition of expression of adhesion molecules and the ability to scavenge superoxide anion radicals and to stimulate the expression of IL2. The water-soluble oxihumate sub-fraction could reduce the cellular response of neutrophils initiated by known stimulants, yet sub-fractions isolated from this same sub-fraction showed a priming effect on the neutrophils when pre-incubated with the cells. The mechanism of action of humic acids has not yet been determined, and is being confounded by contradictory results and the fact that the humic acids are not well-defined compounds with a known structure.

Further studies of humic acids would be necessary to elucidate a mechanism of action and to determine which molecules are in fact biologically active, singly or in combination.

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Appendix I

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Onderstepoort 0110

Enq. : Mario Smuts
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Mr. A.D. Cromarty
Department of Immunology
Faculty of Health Sciences
University of Pretoria
P.O. Box 2034
Pretoria
0001

Dear Mr. Cromarty,

RE: PROJECT APPROVAL

11/2002 – AN *IN VIVO* INVESTIGATION OF THE ANTI-INFLAMMATORY PROPERTIES OF HUMATE: A COMPARISON OF THE EFFECTS OF OXIHUMATE, A LOCALLY PRODUCED COAL DERIVED HUMATE WITH ZYLEM, A COMMERCIALLY AVAILABLE COAL DERIVED HUMATE PREPARATION. PART I: CONTACT HYPERSENSITIVITY MODEL.

12/2002 – AN *IN VIVO* INVESTIGATION OF THE ANTI-INFLAMMATORY PROPERTIES OF HUMATE: A COMPARISON OF THE EFFECTS OF OXIHUMATE, A LOCALLY PRODUCED COAL DERIVED HUMATE WITH ZYLEM, A COMMERCIALLY AVAILABLE COAL DERIVED HUMATE PREPARATION. PART II: ARTHRITIS MODEL.

8.1 – APPLICATION FOR THE USE OF ANIMALS IN: CONTINUING EDUCATION. THE IDENTIFICATION OF BIO-AVAILABLE AND ACTIVE COMPONENTS IN OXIHUMATE.

Please find herewith notification that the Animal Use and Care Committee at a meeting held on November 25, 2002 authorised the chairman to sign and approve the above-mentioned research protocol applications on receiving clarifications on the matters addressed in the attached copies of the minutes from the meeting. All the relevant protocol applications was subsequently signed by the chairman on January 21, 2003 after receiving your response dated 27 November, 2002.

We regret the delay in feedback and wish you all success in the relevant studies.

Please contact the UPBRC for any further information.

Sincerely,

Mr. M. P. SMUTS
UPBRC: LABORATORY ANIMALS
2003/05/27

Appendix II

GAUTENG PROVINCIAL GOVERNMENT

DEPARTMENT OF HEALTH

CARING FOR OUR ANIMALS

PRETORIA BIOMEDICAL RESEARCH CENTRE
PRIVATE BAG X169, PRETORIA, 0001, SOUTH AFRICA
Tel: 012 - 354 6215, Fax: 012 - 329 8225

Inquiries : Mario Smuts
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Mr. A. D. Cromarty
Department of Immunology
University of Pretoria
P. O. Box 2034
Pretoria
0001

Dear Mr. Cromarty

RE: PROJECT 06/2001: " INVESTIGATION OF THE BIOLOGICAL DISTRIBUTION OF ^{123}I-LABELLED OXIHUMATE OR ITS SALTS IN THE PRIMATE MODEL."

I am pleased to inform you that our Ethics Committee meeting held on Tuesday, March 13, 2001 approved your research application.

Attached please find invoice no. 00078 for the amount of R9 363-92. This invoice must please be paid before the project may continue. Please transfer the amount to the ***H.A. Grové Research Fund***: *(A 2188 –3501)*.

We are looking forward in assisting you with this project.

Please contact us for any further information.

Yours truly

MR. M. SMUTS
CHIEF ANIMAL HOUSE TECHNICIAN