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.

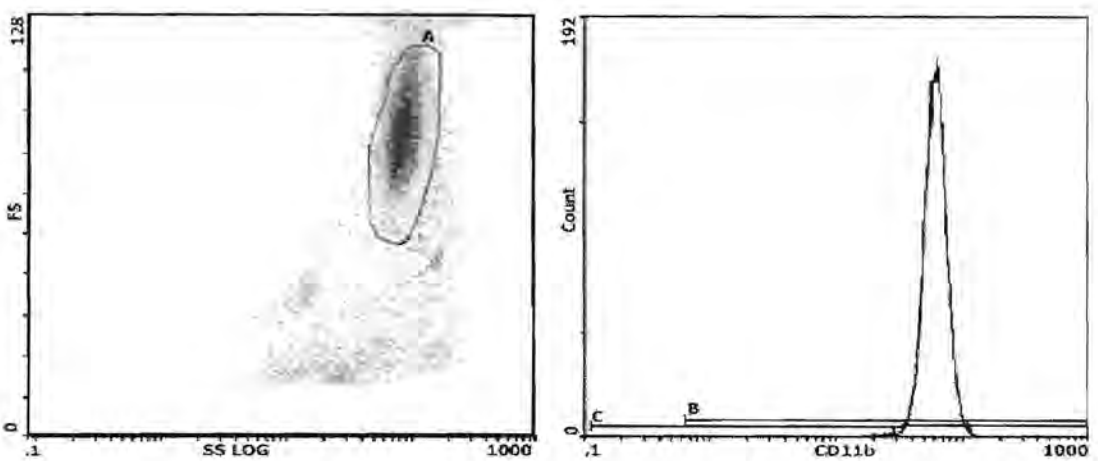

Figure 3-1: A typical scattergram and histogram of a control sample of isolated neutrophils. The scattergram on the left has been gated for the neutrophil population, which is indicated by the area enclosed by the line marked "A". The histogram on the right indicates the fluorescent intensity of the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

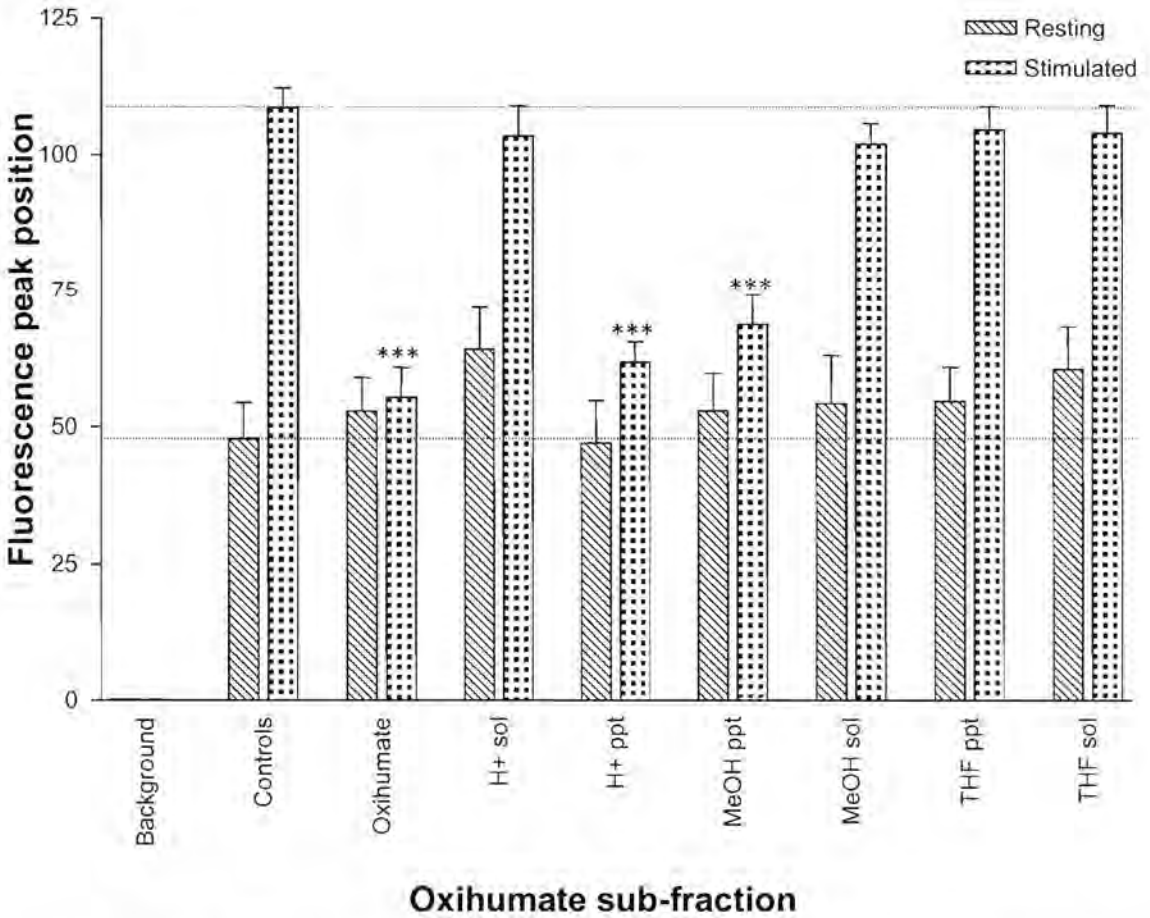

Figure 3-2: The effect of different isolated sub-fractions of oxihumate on the expression of CR3 on the surface of neutrophils. The sub-fractions were added to the neutrophils at the relative concentrations that match the mass of the sub-fractions in the oxihumate. The resting cells were pre-incubated 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.

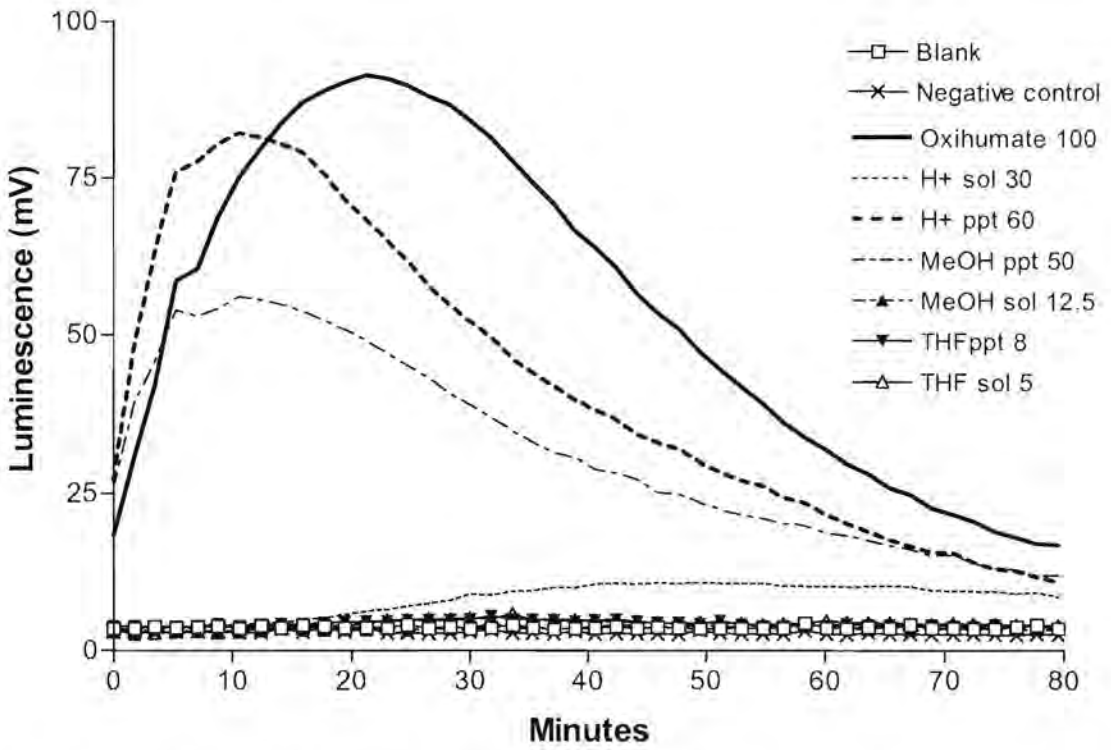

Figure 3-3: Overlaid plots of chemiluminescence against time for human neutrophils in HBSS containing 10% FCS and lucigenin. No stimulants were added to the cell suspensions so the release of reactive oxidation species was due to the isolated sub-fraction added to the cells when monitoring was initiated. 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.

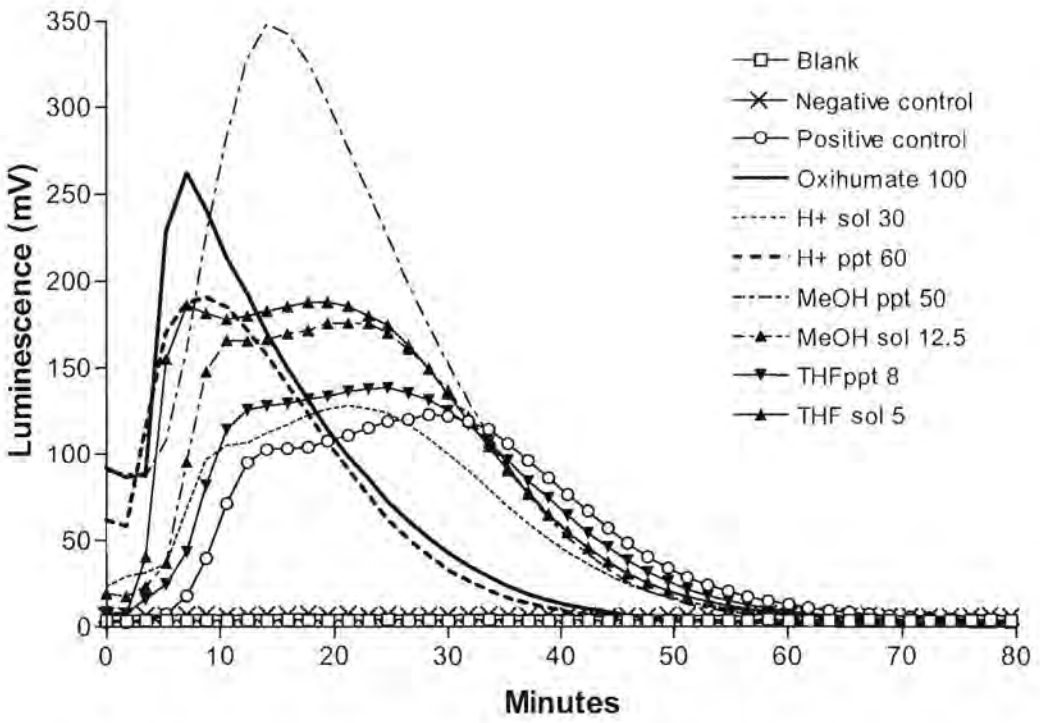

Figure 3-4: Overlaid luminescence response curves of neutrophils that were pre-incubated with the different fractions of oxihumate for 20 minutes prior to stimulation with PMA. The water soluble, acid precipitated and MeOH precipitated sub-fractions showed a single peak whereas the other sub-fractions 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.

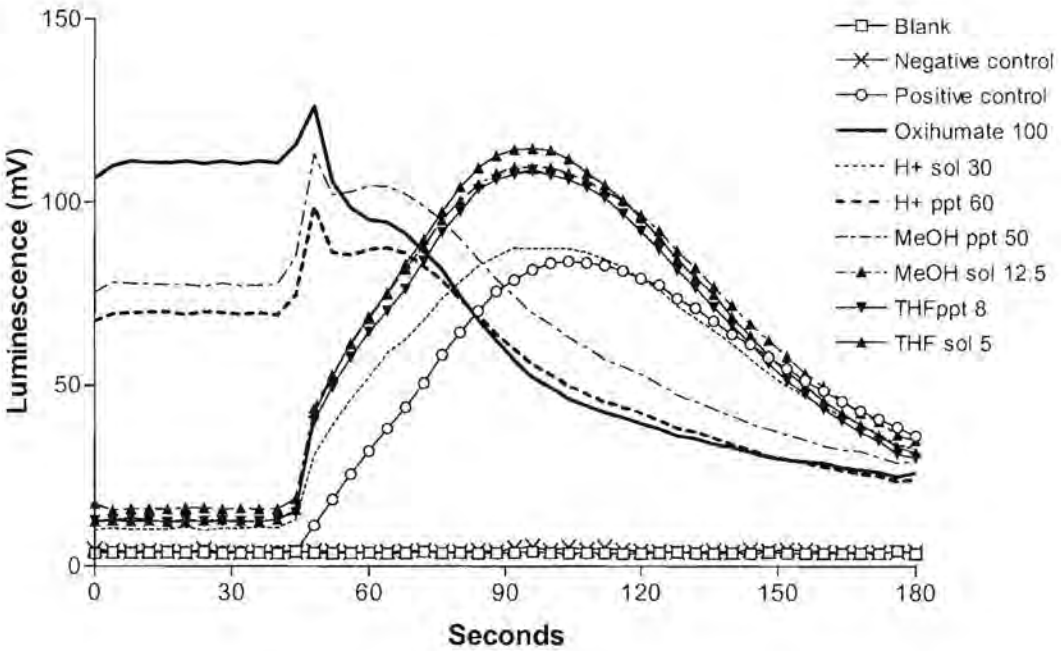

Figure 3-5: Overlaid chemiluminescence response curves of neutrophils that were pre-incubated with the different fractions of oxihumate for 20 minutes prior to stimulation with FMLP. The water soluble, acid precipitated and MeOH precipitated sub-fractions showed significant background activity and gave 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.

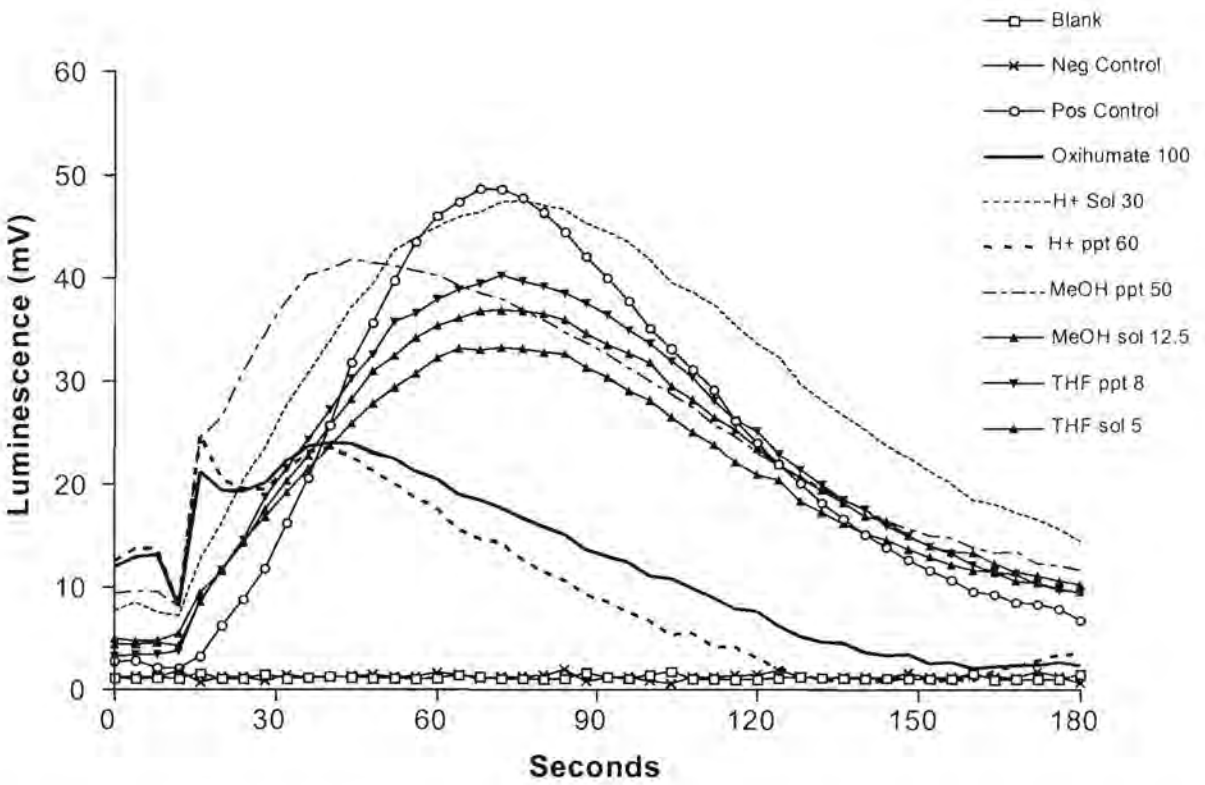

Figure 3-6: Overlaid chemiluminescence response curves of neutrophils that were pre-incubated with the different sub-fractions of oxihumate for only 3 minutes prior to stimulation with FMLP. The blank and negative control show no release of reactive oxidants while the positive control is the response 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.

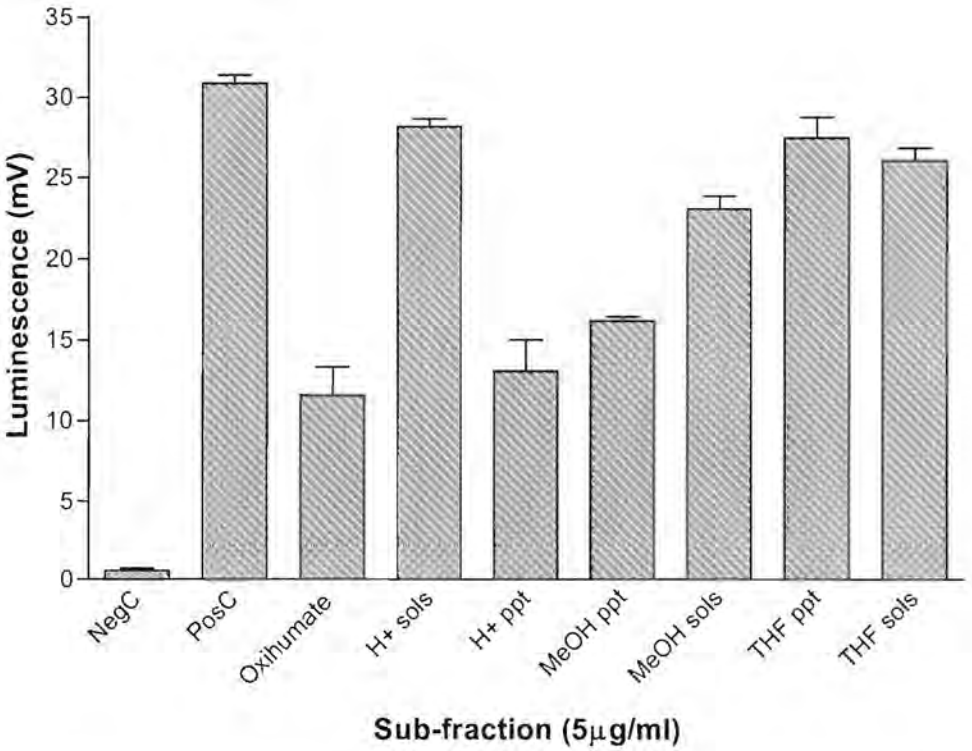

Figure 3-7: The superoxide scavenging effect of a final concentration of 5µg/ml of the various isolated sub-fractions of oxihumate compared to a negative and positive control. The most active sub-fractions appear to be the least refined sub-fractions. The negative control had no enzyme to generate the 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.

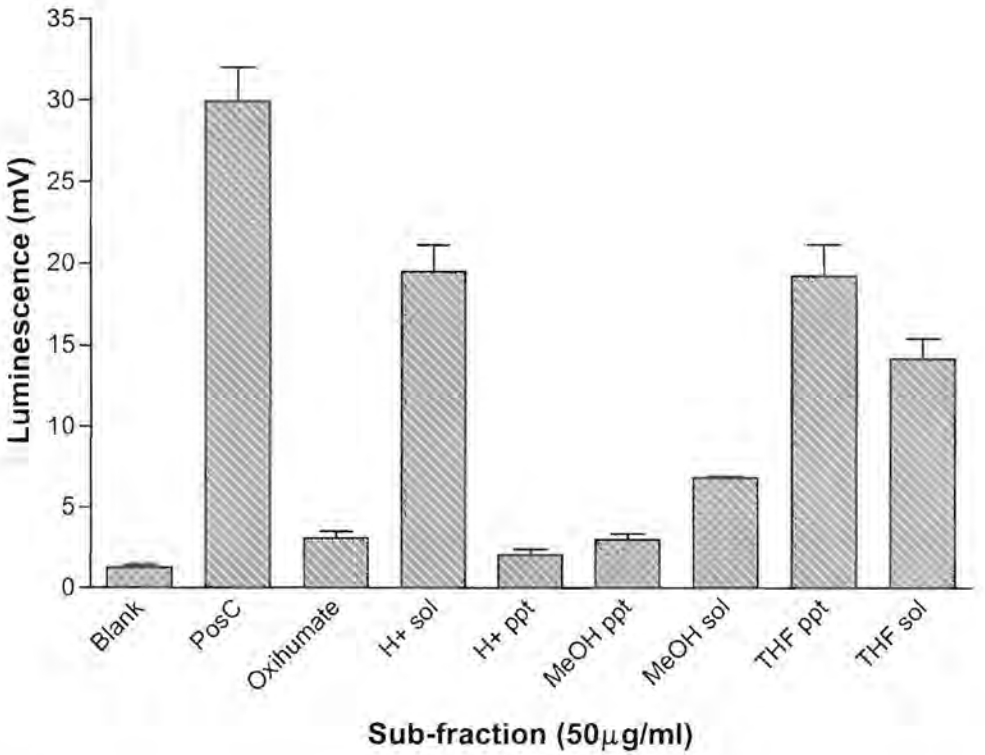

Figure 3-8: The superoxide scavenging effect at a final concentration of 50μg/ml of the various isolated sub-fractions of oxihumate compared to a negative and positive control. The most active sub-fractions still appeared to be the least refined sub-fractions. The negative control had no enzyme to generate 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.

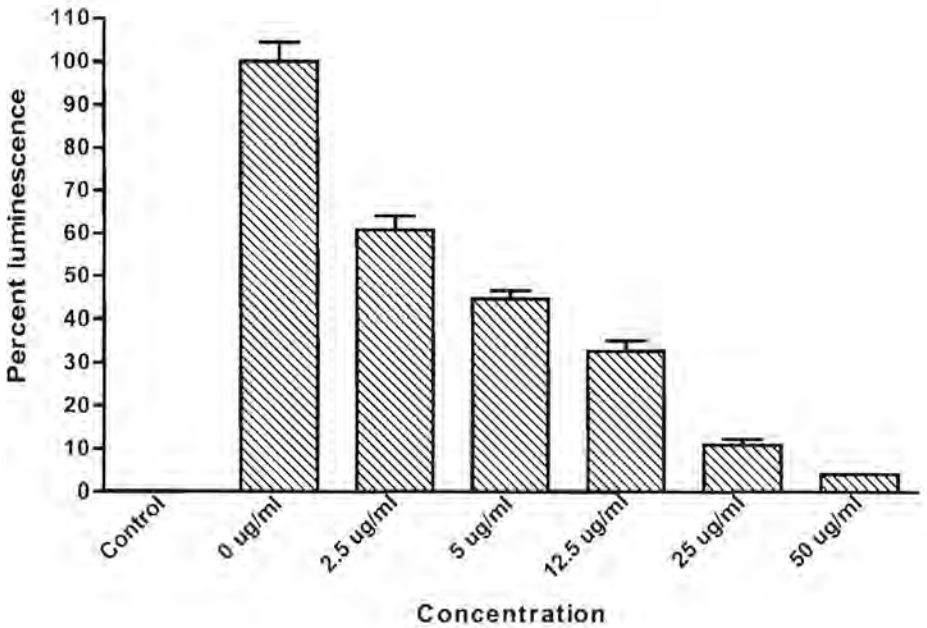

Figure 3-9: Bar graph showing the concentration effect exhibited by the acid precipitated sub-fraction on the superoxide anion scavenging. The control contained no superoxide generating enzyme system and is therefore also used as the background value. Each bar is the average of 5 experiments with SEM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

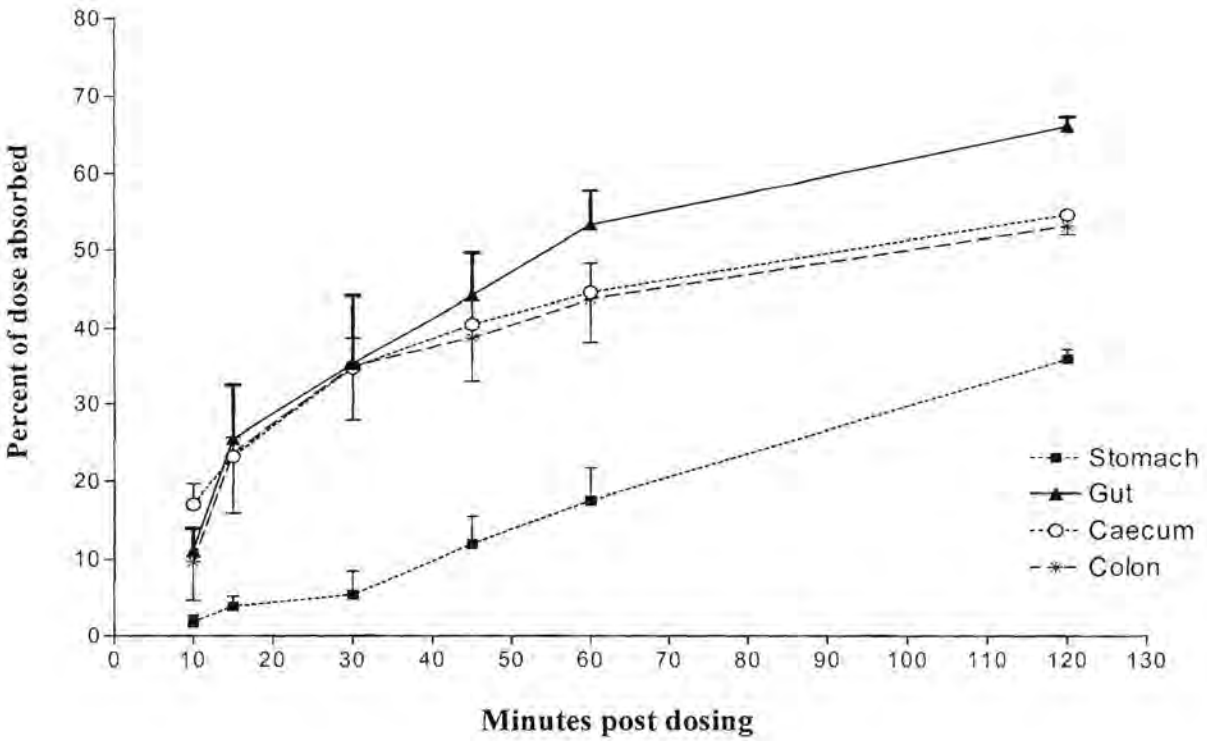

Figure 4-2: The *in vitro* absorption of ^{125}I labelled oxihumate from isolated rat GIT segments. GIT segments containing labelled oxihumate were suspended in HBSS and incubated for the indicated times. The ^{125}I activity of 2 ml aliquots the suspending HBSS were counted to determine the "absorbed" 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.

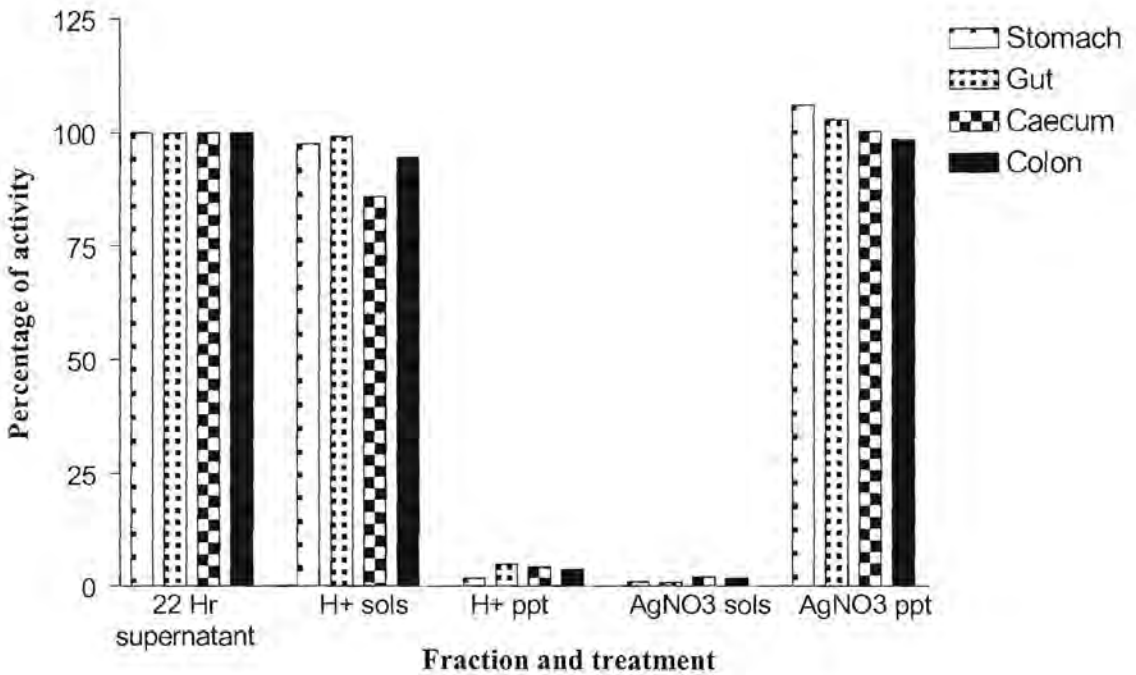

Figure 4-3: Sub-fractionation of the 22-hour supernatant of the rat GIT absorbed oxihumate. The supernatant was treated consecutively with glacial acetic acid then $AgNO_3$, solution, centrifuging between steps to remove any precipitate. The ^{125}I activity of each sub-fraction was calculated as a percent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

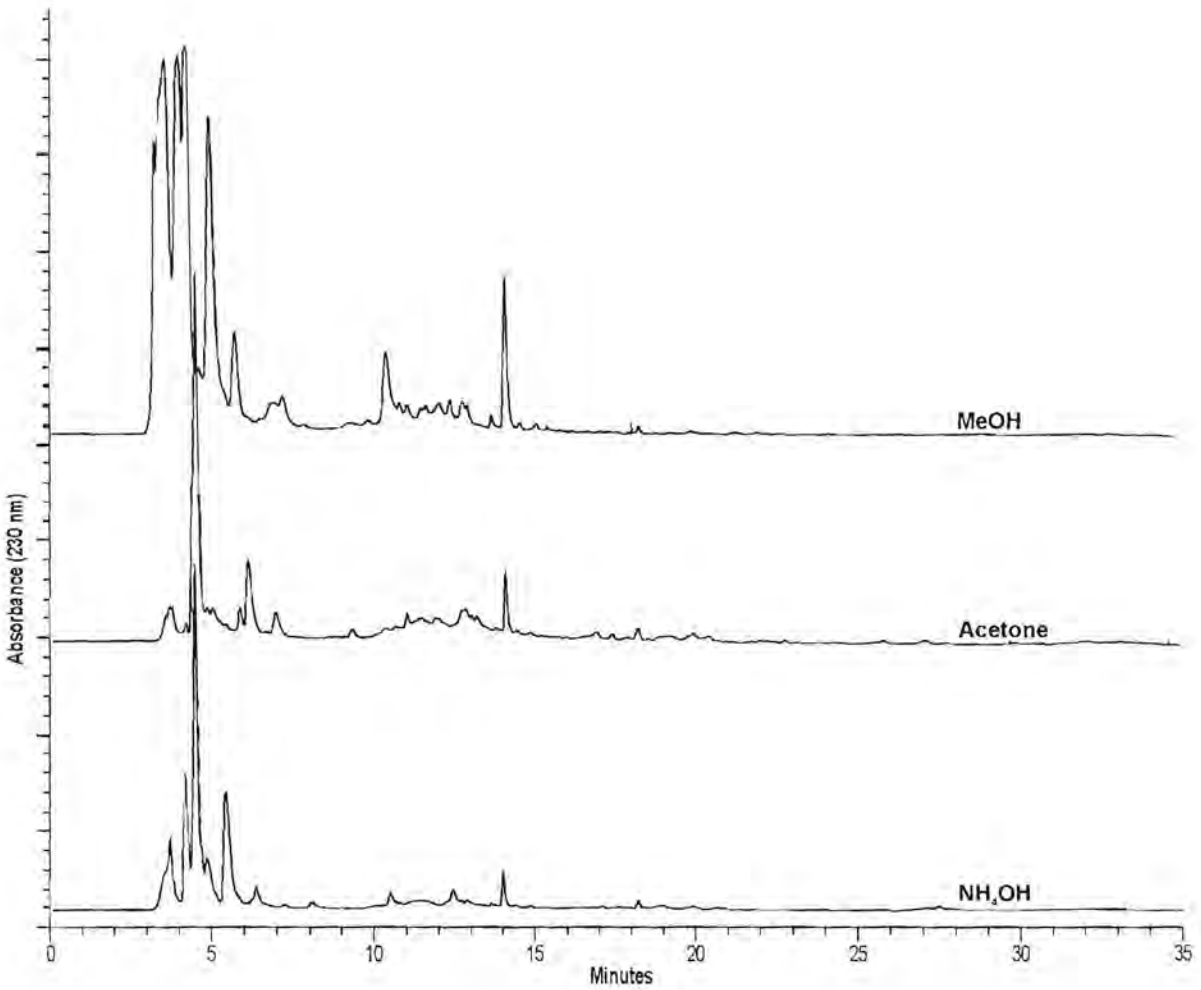

Figure 4-4: Overlaid chromatograms of the different fractions of the same small intestine absorbed oxihumate fraction. The dried "absorbed oxihumate fraction" was fractionated as described in the materials and methods section to remove inorganic salts and glucose derived from the incubation medium then 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.

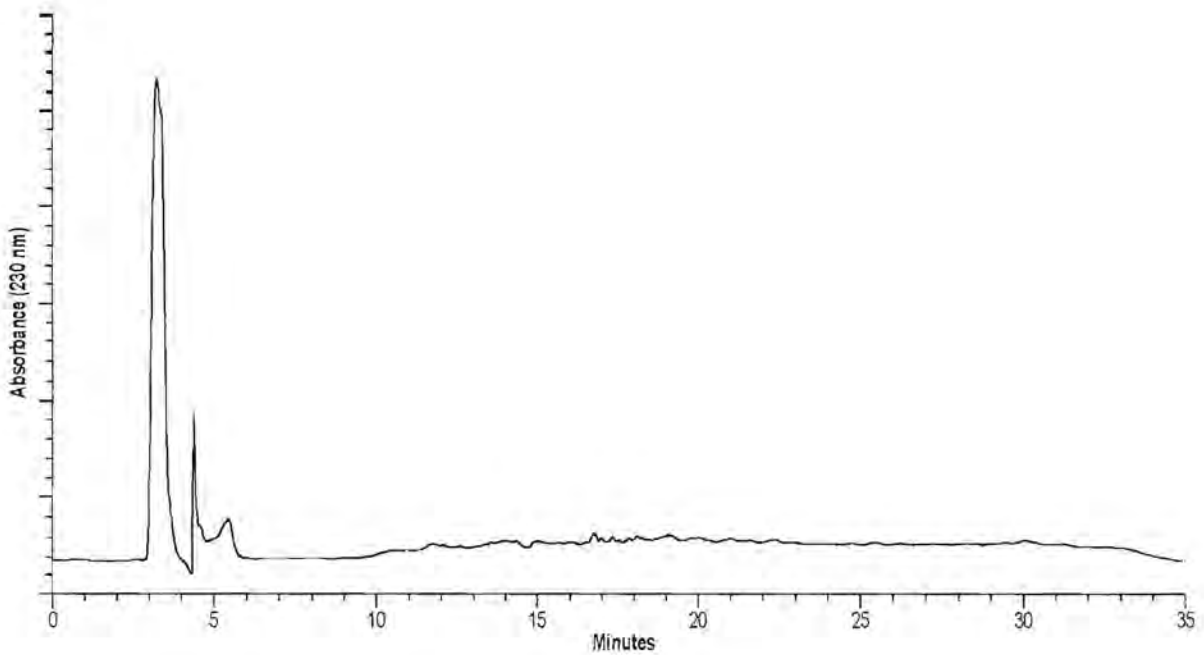

Figure 4-5: A HPLC chromatogram of the oxihumate absorbed from the stomach. Two unresolved peaks rapidly followed a large pass-through peak, one sharp the other a broad squat peak. The rest of the chromatogram shows very small insignificant broad peaks eluting throughout the gradient program.

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.

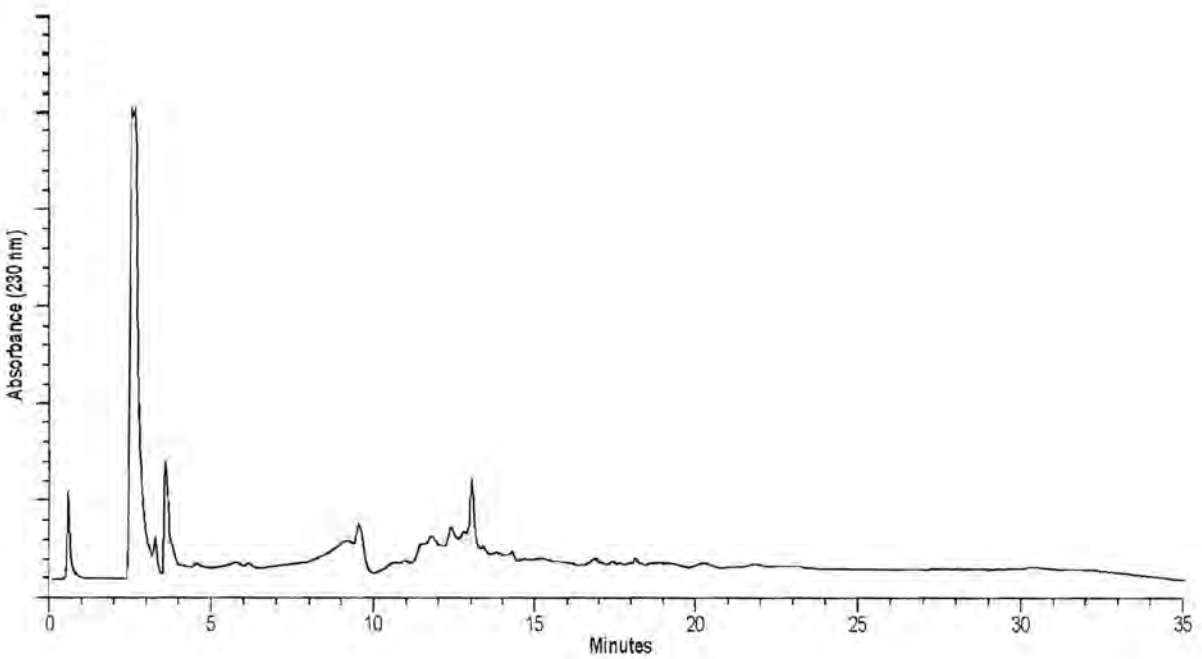

Figure 4-6: A HPLC chromatogram of the oxihumate absorbed from the combined jejunum and ileum. A single sharp peak rapidly follows the large pass-through peak. Between 8 and 17 minutes several unresolved peaks elute. The peak eluting at 0.8 minutes appears to be a ghost peak as it elutes before the void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

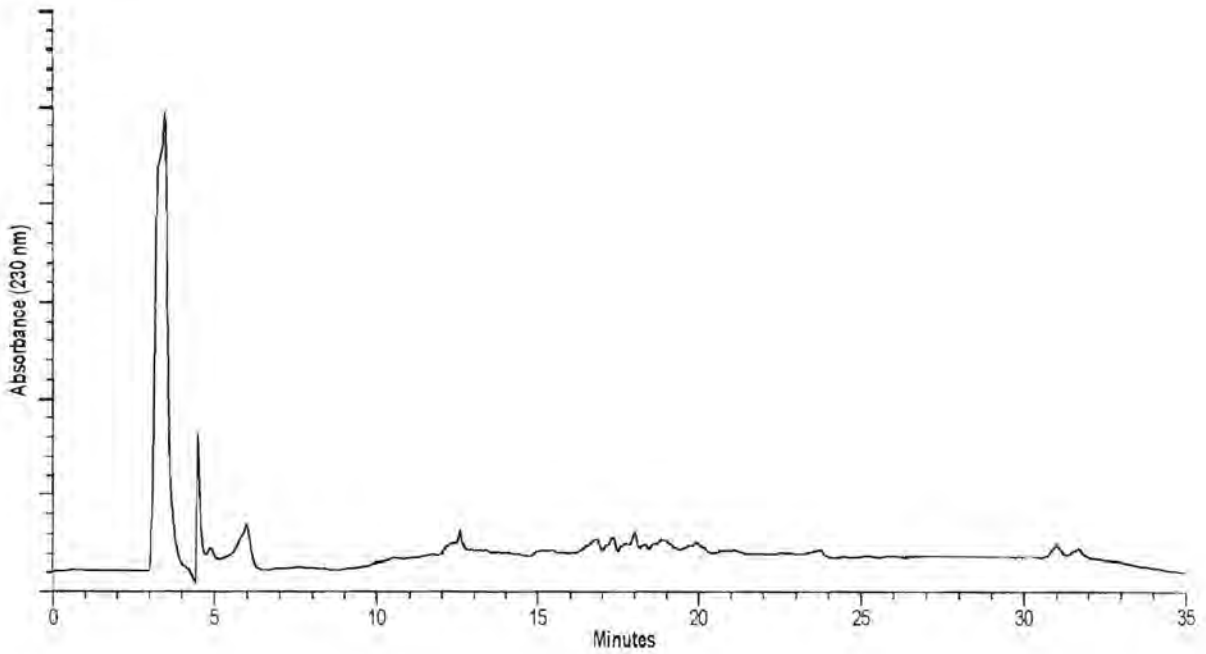

Figure 4-7: A HPLC chromatogram of the caecum absorbed fraction of oxihumate. The pass-through peak is large and followed by two peaks as in the case of the stomach-absorbed fraction. Later in the chromatogram there are several small peaks eluted by higher concentrations of acetonitrile. The two small 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.

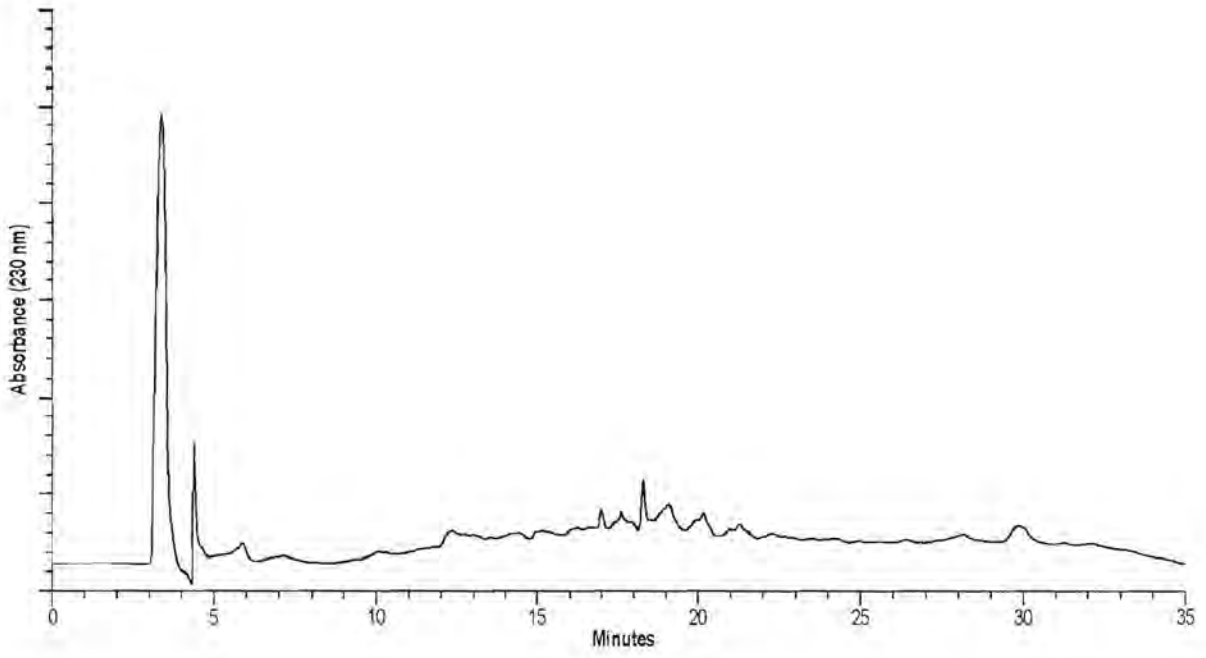

Figure 4-8: A HPLC chromatogram of the colon absorbed fraction of oxihumate. The pass-through peak is again large and followed by one sharp peak and one small broad peak. Several small peaks, which account for much of the dark coloured compounds in the fraction elute between 12 and 23 minutes. The small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