Chapter 5

Contact Hypersensitivity in the Rat

1 Introduction

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

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

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

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

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

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

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

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

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

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

2 Materials and Methods

2.1 Chemicals

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

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

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

2,4-Dinitro-fluorobenzene solutions

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

Oxihumate solution

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

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

Brown coal derived humic acid solution

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

Prednisolone solution

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

2.2 Animals

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

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

2.3 Methods

2.3.1 Contact Hypersensitivity

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

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

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

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

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

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

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

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

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

2.3.2 Statistic Analysis

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

3 Results

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

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

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

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

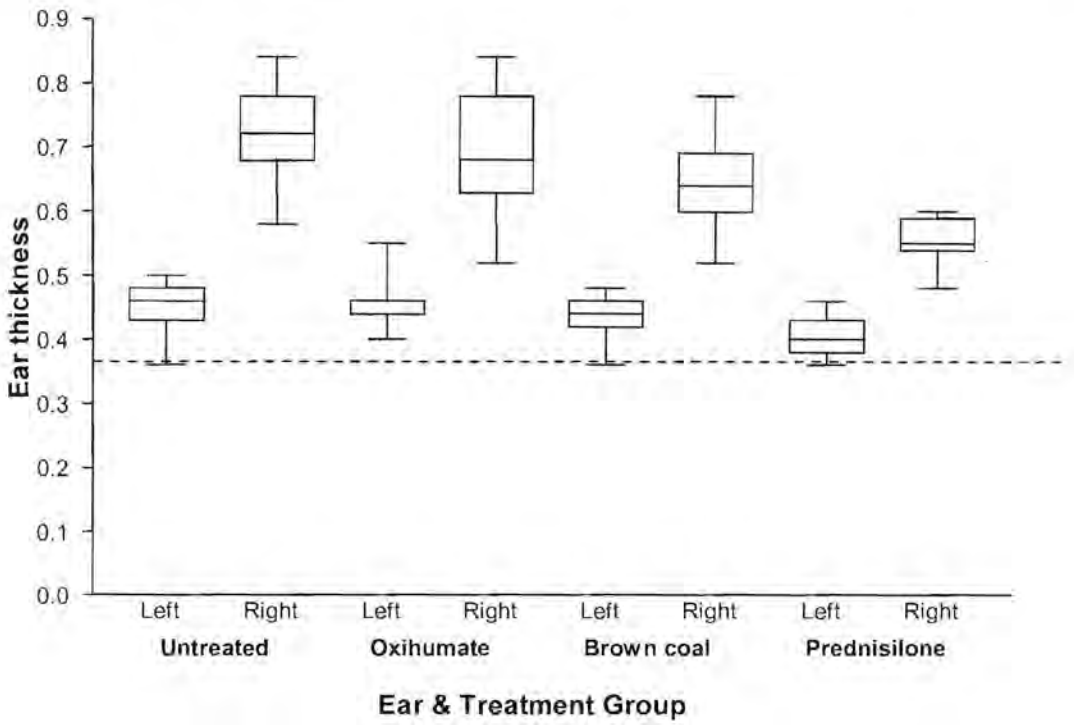

Figure 5-1: A whisker box diagram of the thickness of the left and right ear thicknesses at 3 hours after challenge with vehicle solution or 0.25% DNFB. The boxes bound the 25 to 75 quartile values with the whiskers extending to the minimum and maximum values recorded. Note that both the prednisolone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

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

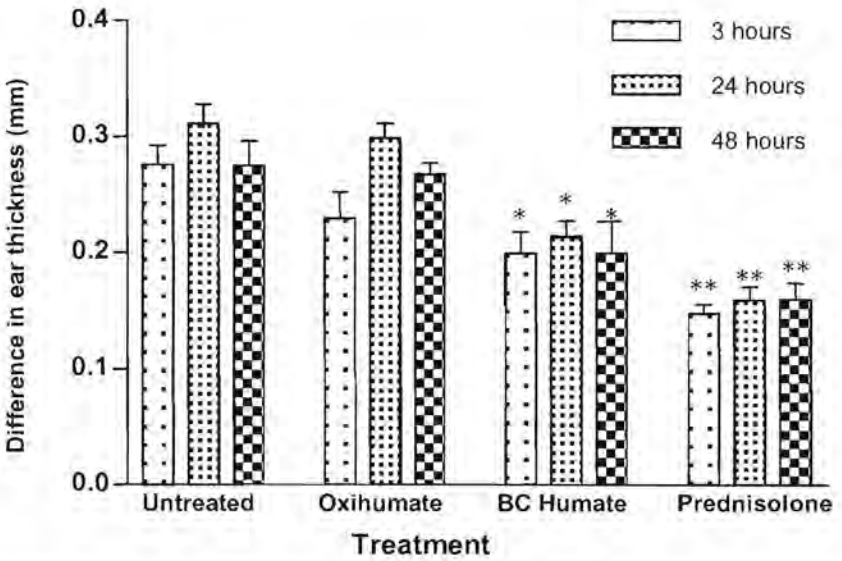

Figure 5-2: The difference in ear thickness (of the combined batches of experimental animals) between left and right ears of DNFB challenged rats after one week of non-treatment or oral treatment with oxihumate (61mg/kg/day), brown coal humate (BC Humate) (61mg/kg/day) or prednisolone (1mg/kg/day). The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

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

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

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

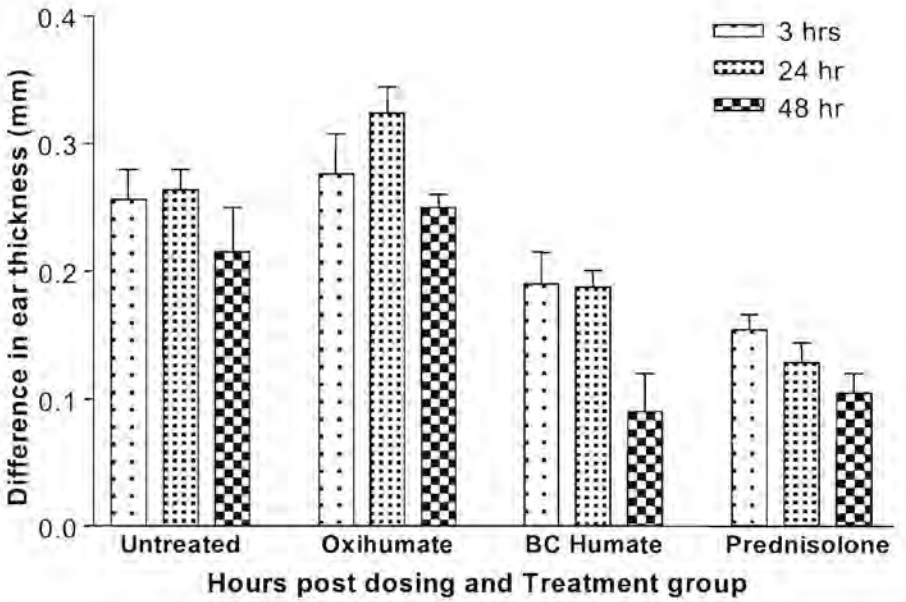

Figure 5-3: The difference in ear thickness between left and right ears of the first batch of DNFB challenged rats after one week of oral treatment as shown below each group. The three columns represent the differences in ear thickness with SEM bars at 3 hours; 24 hours and 48 hours post challenge. Although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

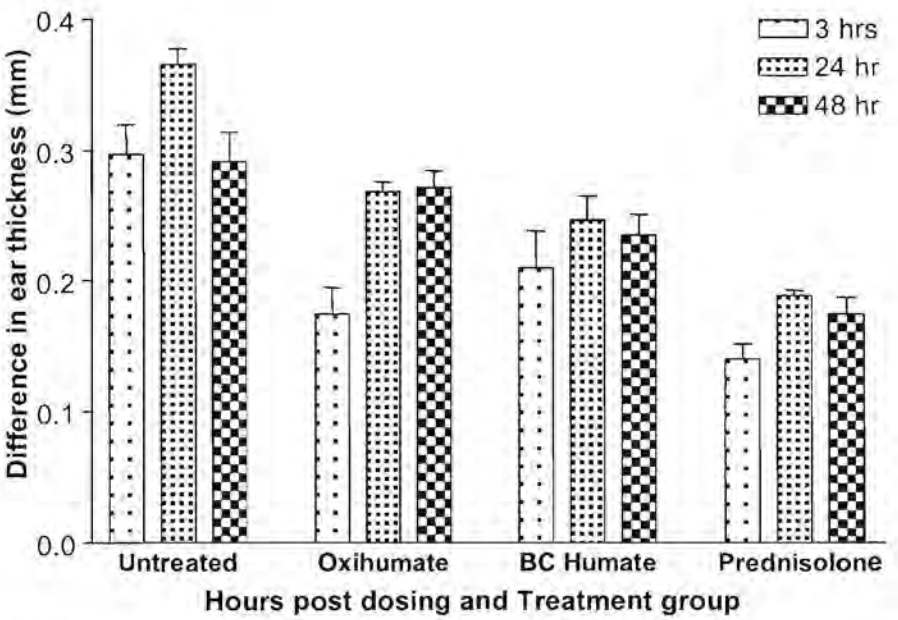

Figure 5-4: The difference in ear thickness between left and right ears of the second batch of DNFB challenged rats after one week of oral treatment as shown below each group. The three columns represent the differences in ear thickness with SEM bars at 3 hours; 24 hours and 48 hours post challenge.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

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

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

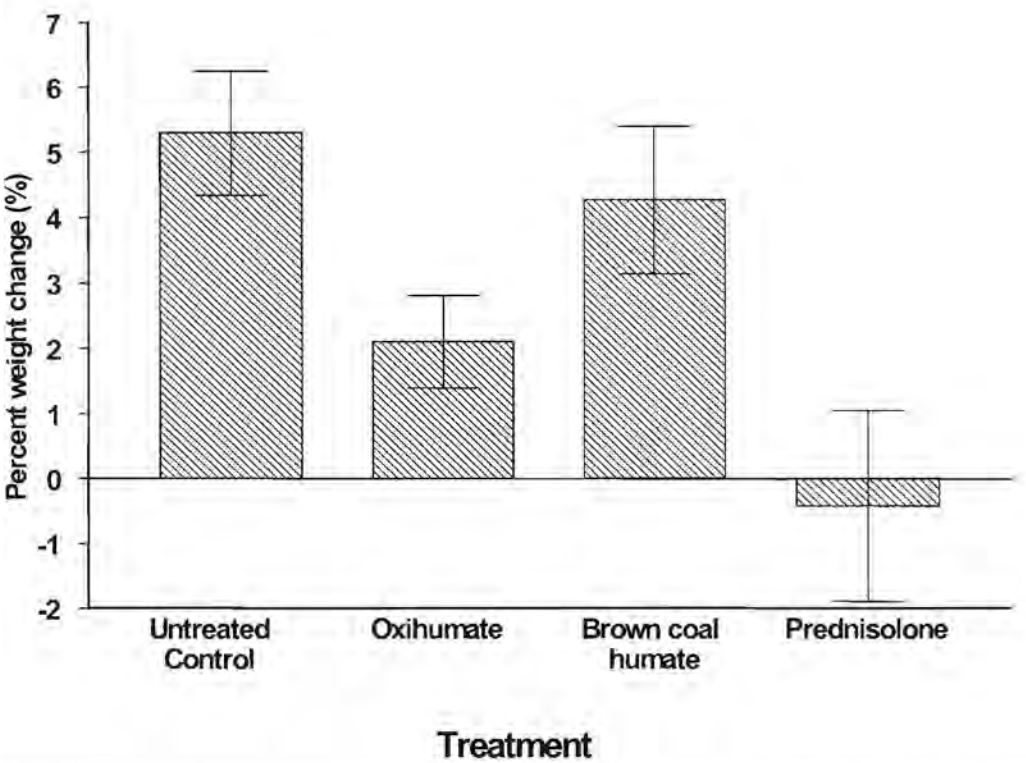

Figure 5-5: A bar graph illustrating the percentage changes in the body weight of the rats in each of the experimental groups. The error bars depict the SEM. The untreated control was dosed with distilled water whereas the two humic acid treated groups were each dosed with 61 mg/Kg/day of the respective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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In conclusion it can be confirmed that

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

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

Chapter 6

Pharmacokinetics of Oxihumate in the Baboon Model

1 Introduction

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

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

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

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

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

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

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

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

2 Materials and Methods

2.1 Materials

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

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

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

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

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

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

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

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

2.2 Methods

2.2.1 Labelling of Oxihumate and sub-fractions

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

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

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

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

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

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

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

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

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

2.2.2 Binding of labelled oxihumate to blood components

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

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

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

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

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

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

2.2.3 Distribution of Radioactivity in the baboon model

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3 Results

3.1 Labelling of Oxihumate

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

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

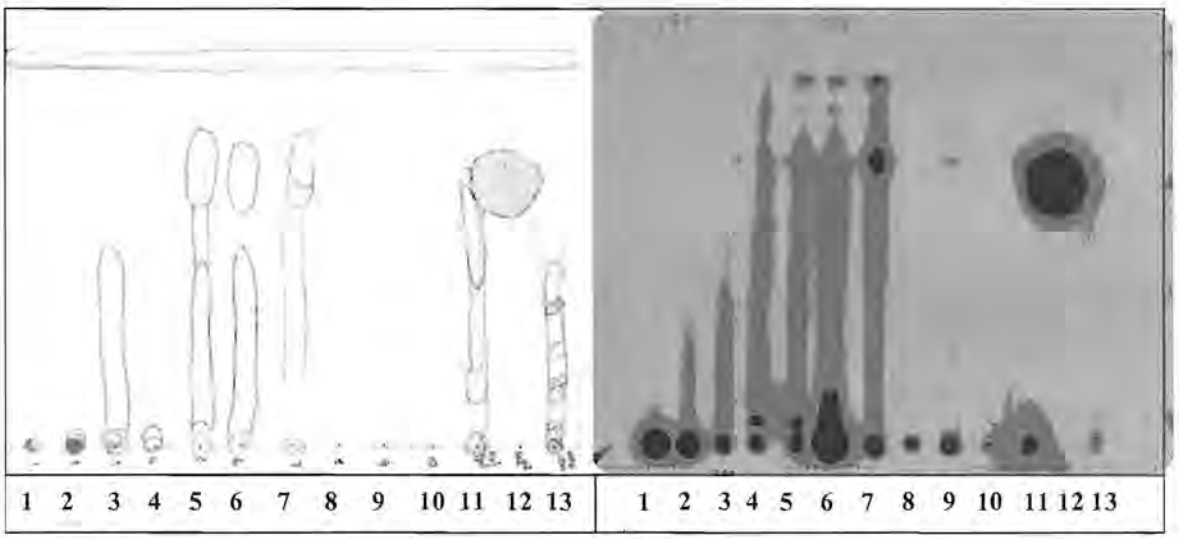

Figure 6-1: A TLC separation of ^{123}I labelled oxihumate. Lanes 1 to 10 are 1ml fractions eluted from a P-10 desalting column. The pencil marks indicate where fluorescent compounds were detected. Fraction 7 corresponds to the elution of iodide from the same column under the same conditions. Lane 11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

3.2 Binding of labelled oxihumate to blood components

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

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

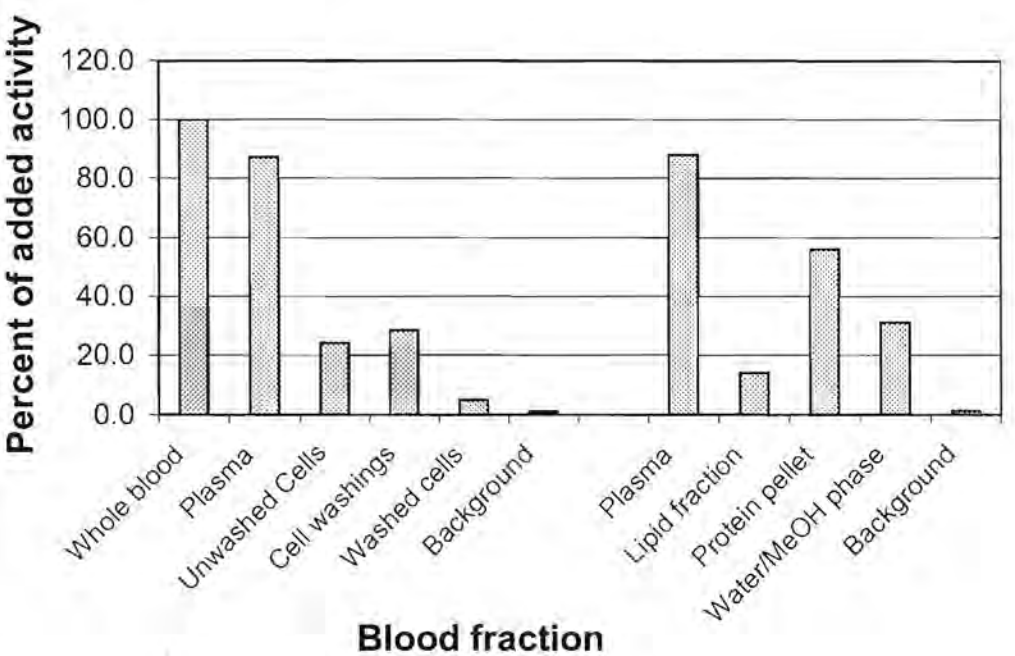

Figure 6-2: The average distribution of radioactivity of two different experiments between various blood fractions after *in vitro* addition of ^{123}I labelled oxihumate to whole human blood and incubating for 30 minutes. Temperature appeared to play no role in the binding kinetics. The data set on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

3.3 Distribution of Radioactivity in Baboons

3.3.1 Control baboon 1 (free iodide by intravenous injection)

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

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

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

Dynamic and static scans.

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

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

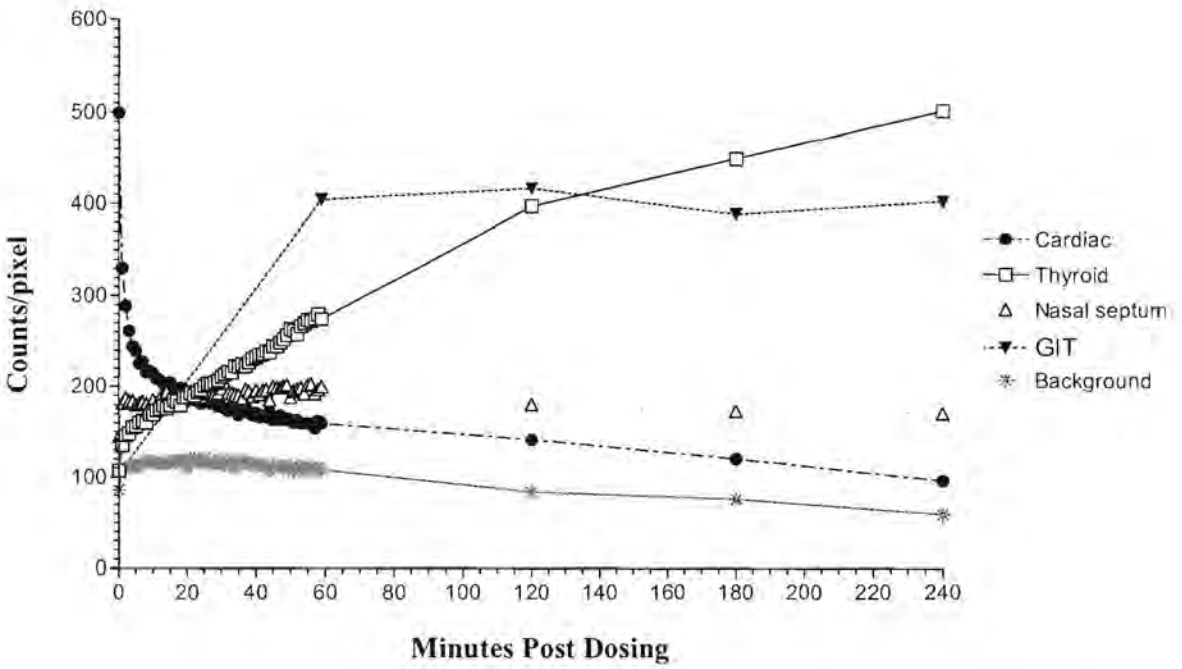

Figure 6-3: Control baboon 1: Decay corrected organ distribution of intravenously administered free iodide-123. Note the rapid decrease in cardiac activity due to the dilution effect, the rapid uptake in the GIT and the continuous uptake by the thyroid gland.

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

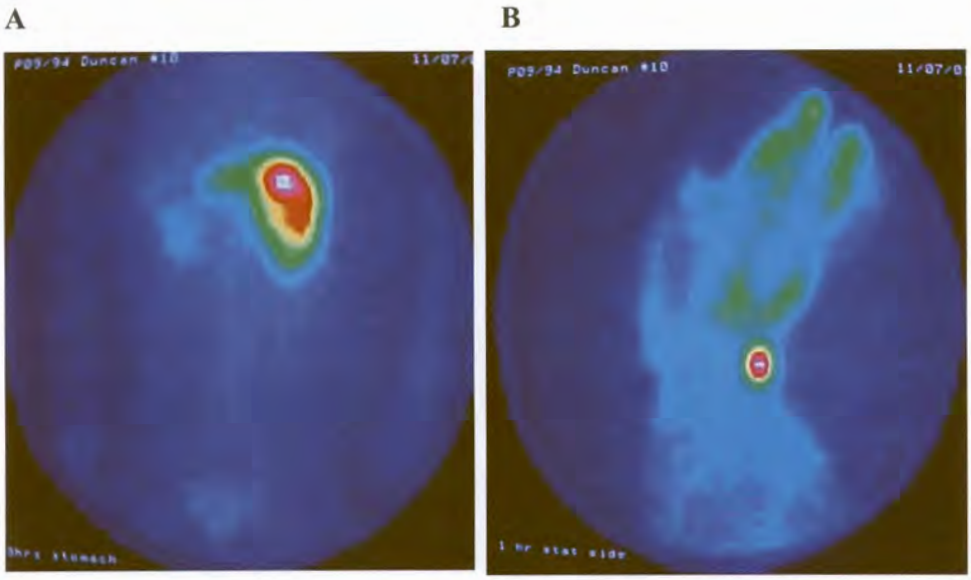

Figure 6-4: Scintigrams of the control baboon taken at 3 hours post administration of an IV dose of free iodide. Panel A shows a scan of the abdominal area where the accumulation of activity in the jejunum (GIT) can be seen clearly. The outline of the baboon can just be made out with the arms on the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

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

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

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

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

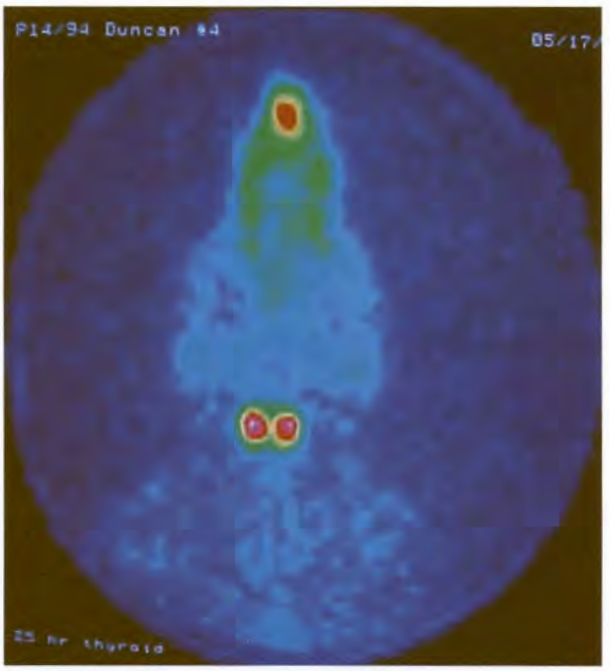

Figure 6-5: The anterior view of a baboons head 25 hours after administering an oral dose of ^{123}I labelled oxihumate. The thyroid glands are clearly visible at the throat with the salivary glands and the nasal septum also showing as areas of accumulation. The outline of the baboon's head is obvious.

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

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

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

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

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

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

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

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

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

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

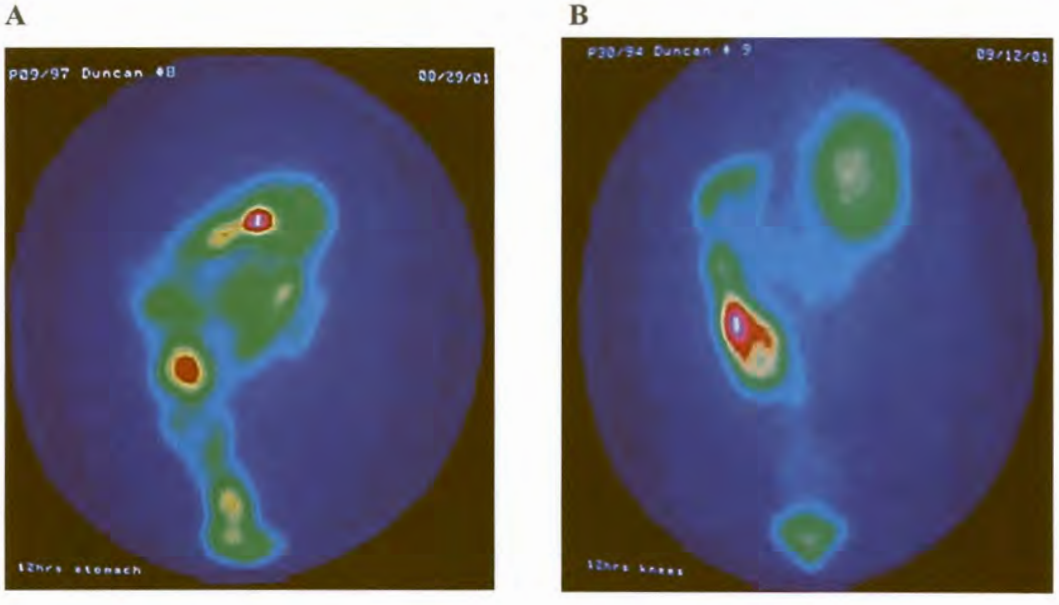

Figure 6-6: Two scintigram images of different baboons showing the distribution of activity in the abdominal region of the baboons 12 hours after oral administration of ^{123}I labelled oxihumate. The section of the jejunum can be seen clearly in both images as the area of accumulation at the top just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

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

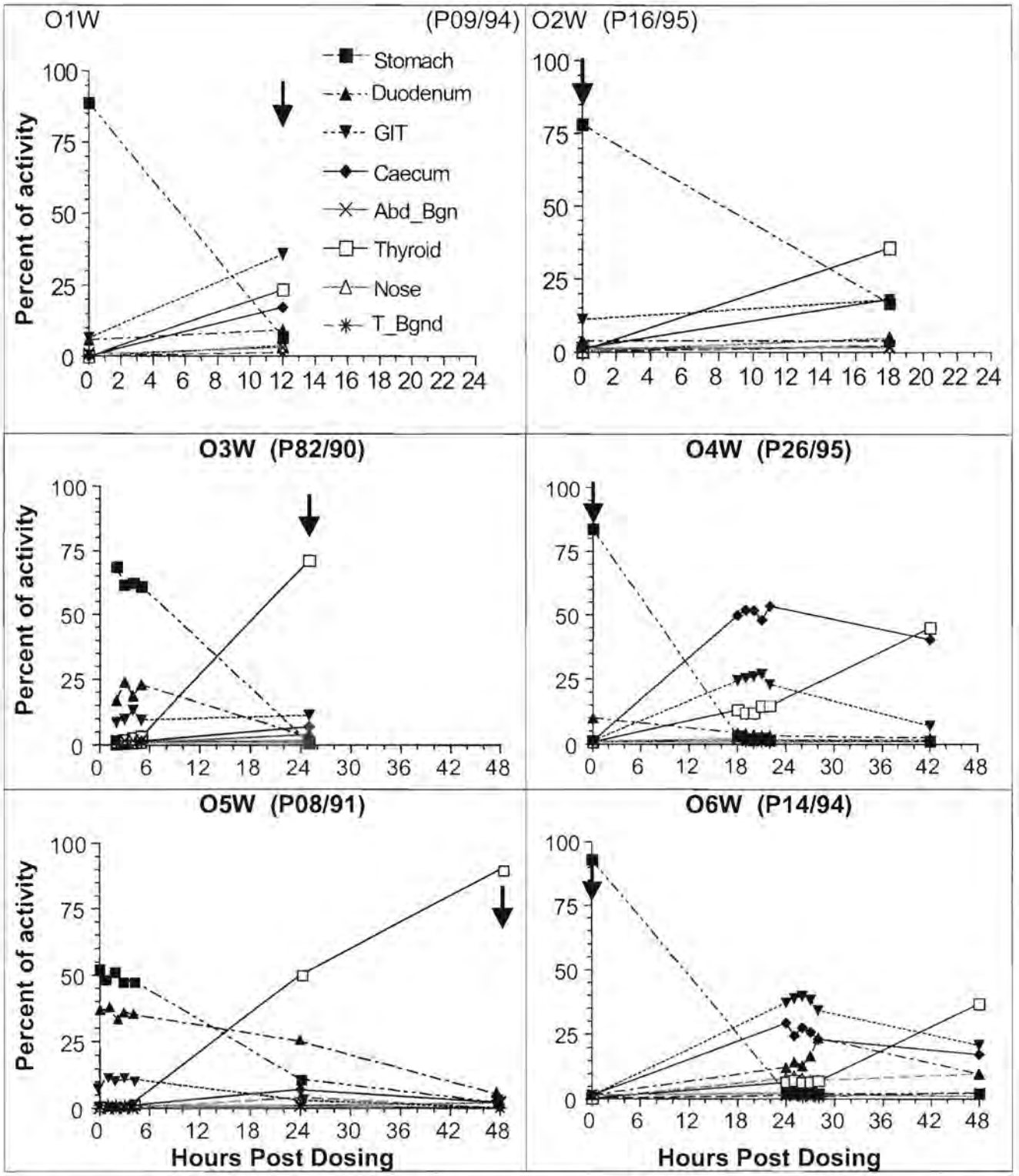

Figure 6-7: Graphs summarising six separate baboon experiments where the animals were dosed orally with ^{123}I labelled water-soluble oxihumate. The graphs present the percentage change in the activity of the target organs against time. The values have been normalized for the target organs shown. Note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

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

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

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

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

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

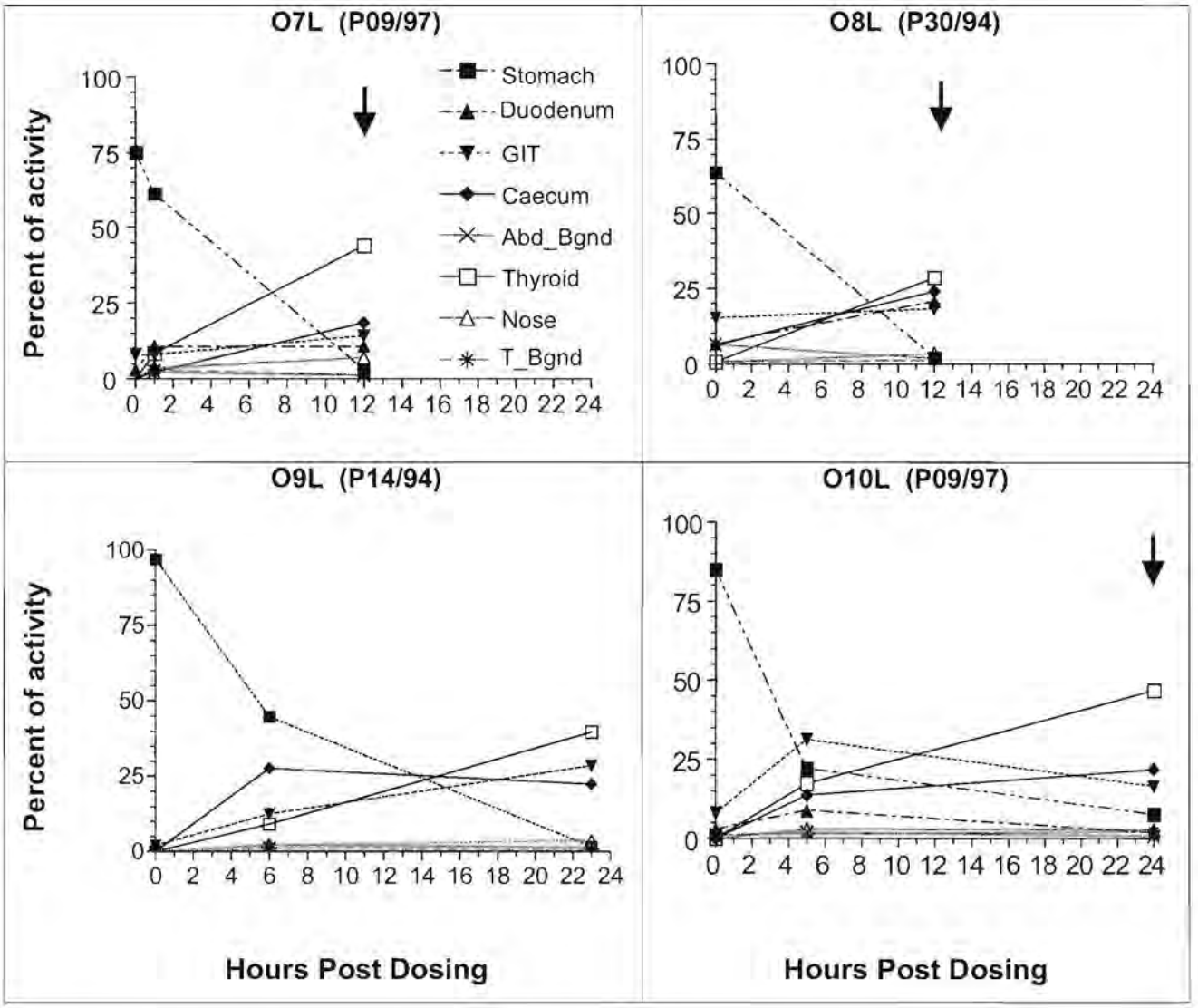

Figure 6-8: Graphs summarising four separate baboon experiments. These graphs present the changes in absolute percentage radioactivity of the target organs of orally administered ^{123}I labelled lipophilic sub-fraction of oxihumate against time. The values have been normalized for the target organs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

3.3.4 Control baboon 2 (free iodide by rectal administration)

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

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

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

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

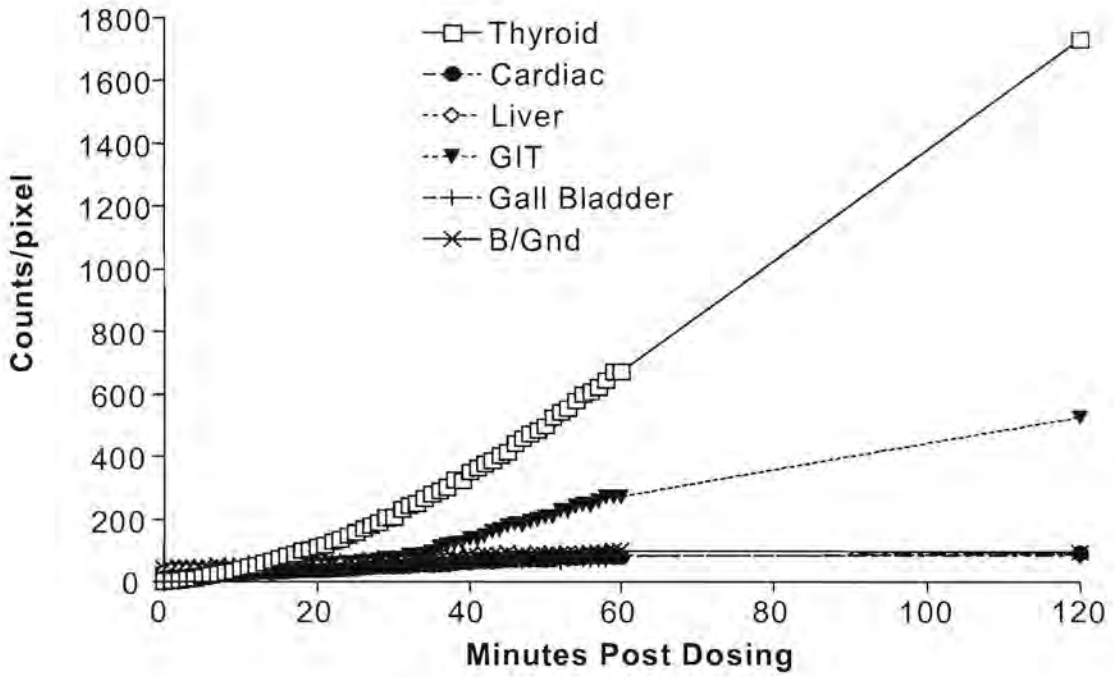

Figure 6-9: Control baboon 2: Decay corrected organ distribution of the abdominal region after rectal administration of free iodide-123. The first hour was monitored dynamically at 1-minute intervals. Note the rapid onset and continuous uptake by the thyroid gland. The cardiac, liver and background regions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

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

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

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

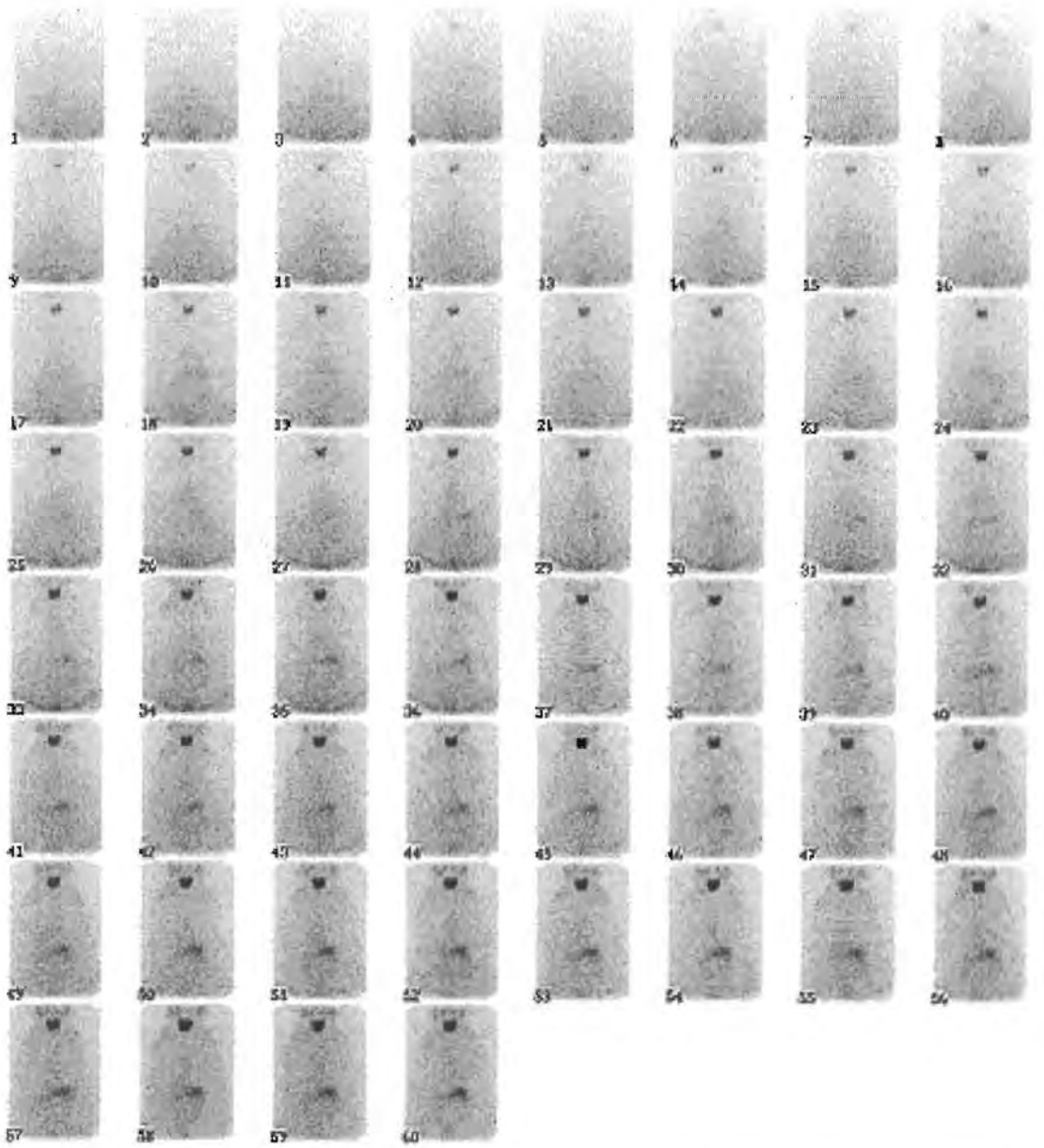

Figure 6-10: A sequence of 60 one-minute images of the initial 1-hour dynamic scan of the control baboon dosed rectally with free iodide. Note that there is accumulation of activity in the thyroid as early as 5 minutes post dosing and that the uptake in the GIT is visible within 15 minutes. The double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

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

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

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

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

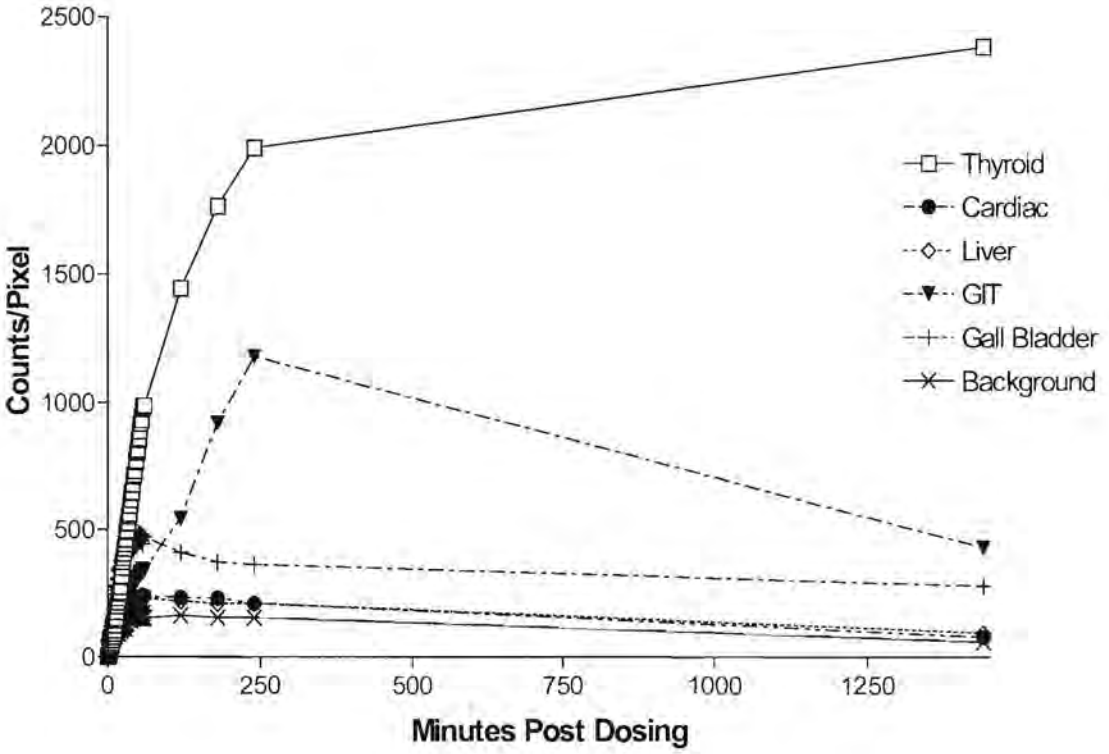

Figure 6-11: The absolute radioactivity distribution in a baboon treated by rectal administration of ^{123}I labelled lipophilic oxihumate sub-fraction. The liver and gall bladder showed the most rapid increase in activity. The thyroid activity surpassed that of the gall bladder 28 minutes after administration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

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

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

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

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

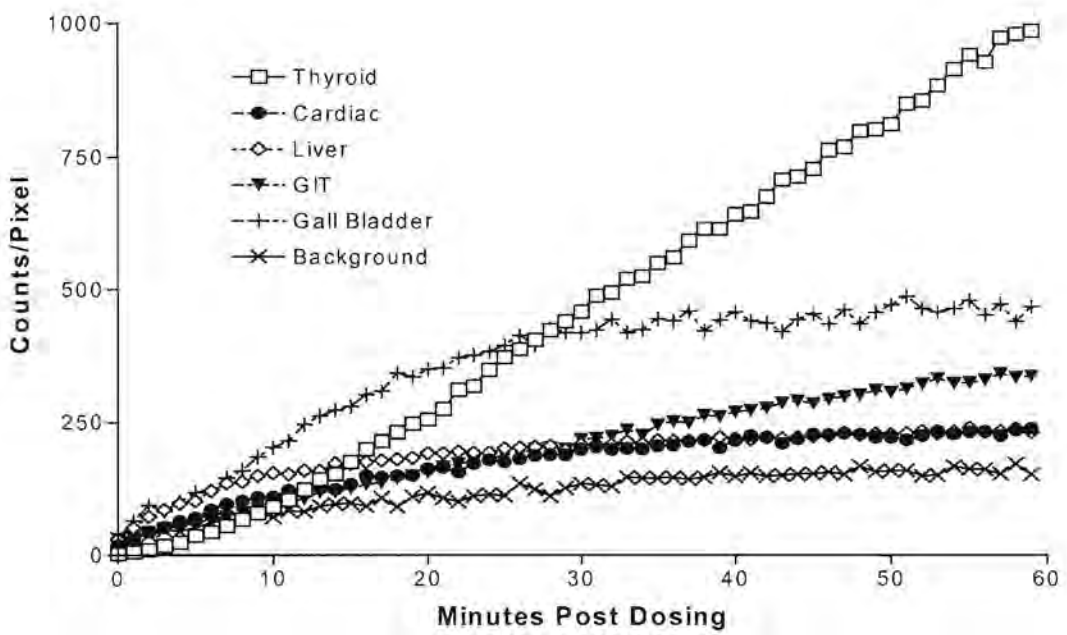

Figure 6-12: A more detailed graph of the first hour of Figure 6-11 showing the dynamic changes in absolute radioactivity distribution in the target organs of a baboon dosed rectally with a ^{123}I labelled lipophilic fraction of oxihumate. The liver and gall bladder showed rapid uptake with the greatest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

3.3.6 Blood activity

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

3.3.7 Urine activity

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

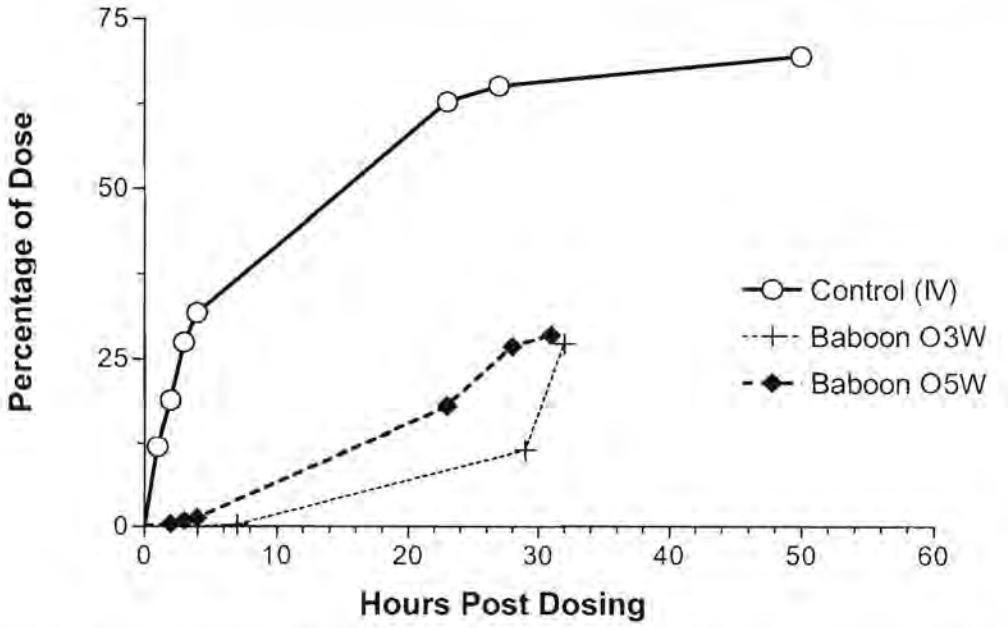

Figure 6-13: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. These baboons were all scanned during the first four hours post dosing. The control baboon had free iodide-123 administered by intravenous injection therefore the excretion of activity started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

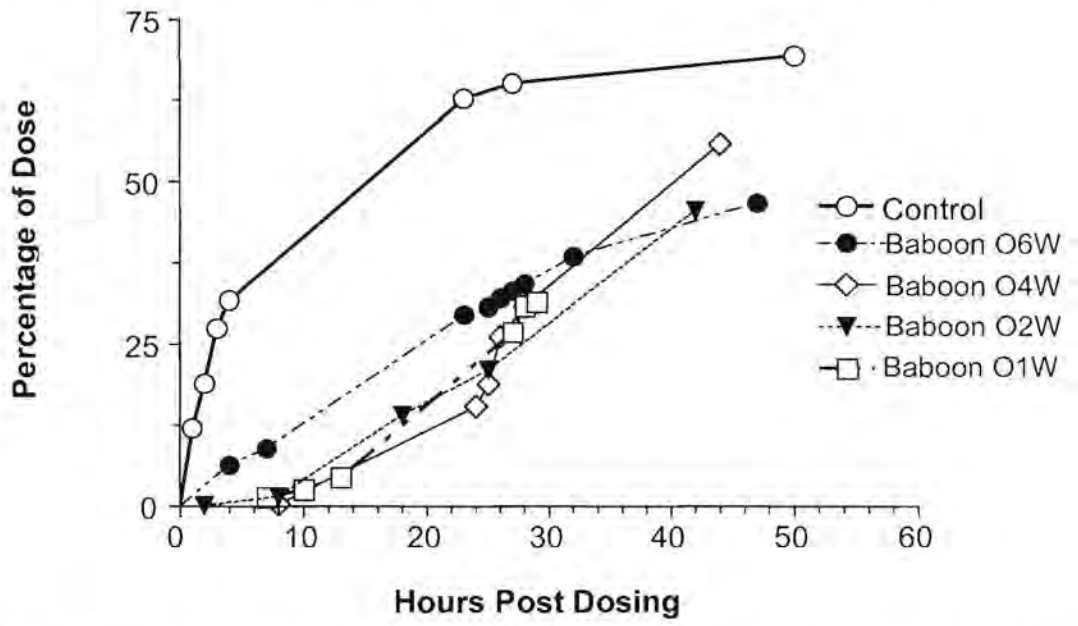

Figure 6-14: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. The four experimental baboons were dosed and scanned at set times after administration without an initial long period under anaesthetics. The control baboon had free iodide-123 administered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

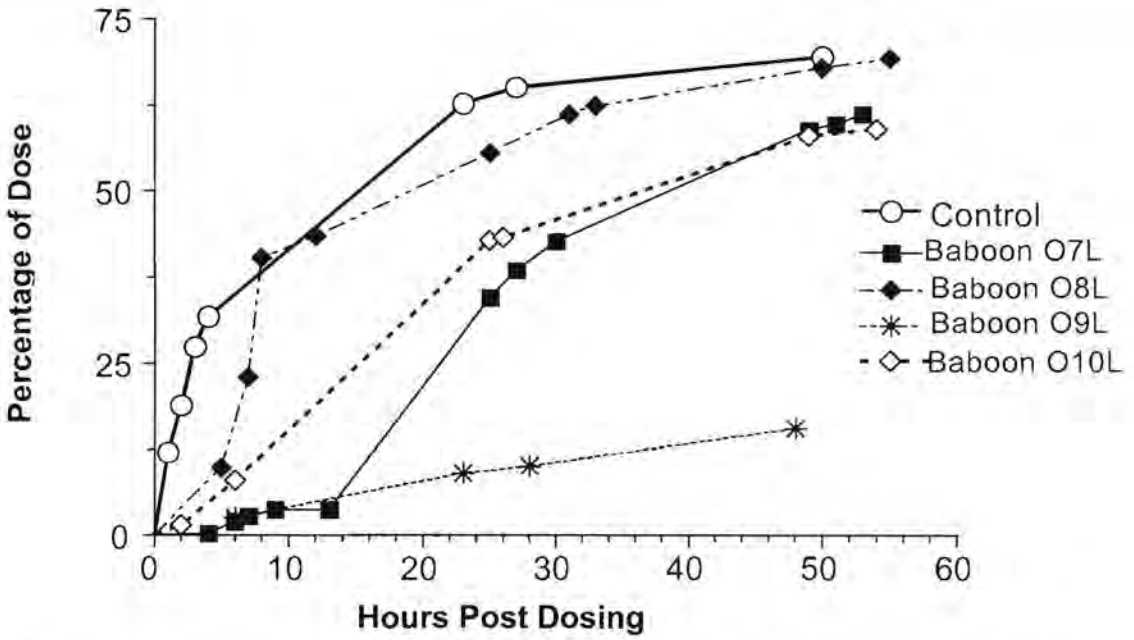

Figure 6-15: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. All the experimental baboons in this set were dosed with the lipophilic sub-fraction of oxihumate. The control baboon had free iodide-123 administered by intravenous injection resulting in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

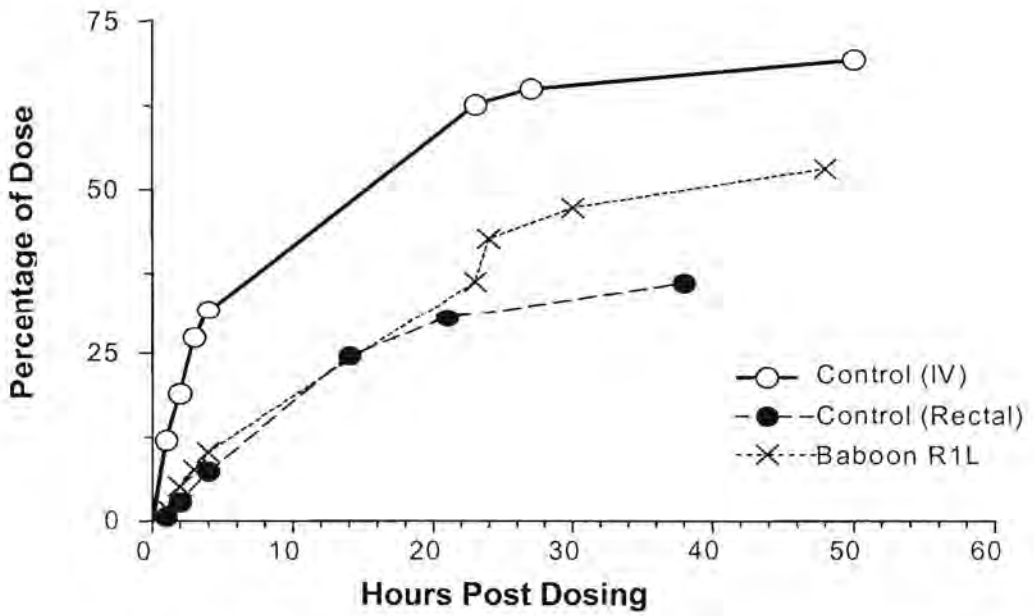

Figure 6-16: Decay corrected radioactivity excreted via the urine shown as a percentage of the administered dose. The experimental baboon was dosed rectally with the lipophilic fraction of oxihumate the second control was dosed similarly with free iodide-123. The IV control baboon had free iodide-123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

3.3.8 Faeces activity

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

4 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In the case of the rectal administration of the lipophilic compounds, the rate of uptake was substantially faster than from the stomach and an accumulation could then be seen in the liver and the gall bladder within a few seconds of administration. The accumulation in the gall bladder appeared to reach a plateau after about 30 minutes at which time the thyroid activity superseded that of the gall bladder and continued to accumulate as would be expected if the activity were present as free iodide.

Furthermore, the rate of uptake in the thyroid was similar to the uptake that was seen in the control baboon where free iodide was administered intravenously which implies that iodide was present in the blood in free iodide form within a very short time after administration.

Chapter 7

Final Conclusions

Despite the widespread occurrence of humic acids and the many studies performed on humic acids from different sources, the structures of these compounds are still not known. Many researchers now accept that humic acids are likely to be a tight association of many organic molecules, derived from plant and other organisms, and includes mineral salts that are complexed within the "supra-molecular" structure.

This study has proved that oxihumate, which is a semi-synthetic humic acid having all the typical characteristics of humic acids isolated from different natural sources, could be fractionated into at least seven sub-fractions. The fractionation was easily achieved by a sequential stepwise increase in the concentration of organic solvents and removing the insoluble sub-fraction by centrifugation.

Each of the isolated sub-fractions had different characteristics. The most lipophilic and oily compounds were found in the sub-fraction that was soluble in 50% tetrahydrofuran (THF soluble sub-fraction) while the methanol precipitated sub-fraction was found to be the darkest coloured sub-fraction, having almost 30 times higher absorbance at both 465nm and 665nm than the acid soluble sub-fraction, which was in turn the least coloured sub-fraction. The THF soluble sub-fraction was the only sub-fraction that was not normally a solid and had a light brown colour like honey and darkened on standing for prolonged periods. The THF precipitated sub-fraction was a dark brown solid whereas the water soluble, acid precipitated, methanol precipitated and methanol soluble sub-fractions were all black solids.

The acid soluble sub-fraction was a dark oily sub-fraction that could be further sub-fractionated by washing with methanol to give a crystalline sub-fraction and a dark brown oily sub-fraction. This last mentioned oily sub-fraction and the THF soluble sub-fractions had the highest fluorescent intensities. However, all the sub-fractions exhibited at least some fluorescent emission centred around the wavelength 430nm, despite using a wide range of excitation wavelengths.

Observations that would indicate that oxihumate was in fact a mixture of different chemical compounds are the following:

- The oxihumate could be sub-fractionated on the basis of differential solubility
- Chromatography on silica gel resulted in several distinct fractions being eluted although most fractions collected were still mixtures of several compounds as determined by TLC
- Solid phase extraction columns used for sample preparation appeared to retain some compounds very strongly and these could be washed from the cartridges after they had stood for at least 16 hours during which time a new equilibrium would establish
- There were at least two major classes of compounds eluting from a reverse phase HPLC column, being a fraction close to the solvent front at 2 – 4 minutes and a fraction eluting between 10 and 16 minutes when using an acetonitrile gradient in an alkaline aqueous eluent. Each of these two major fractions consisted of several very closely eluting individual compounds. The early eluting fraction would be the more polar and/or completely excluded compounds while the later eluting fraction would be the more lipophilic compounds.
- On TLC the seven isolated sub-fractions could be separated into at least a further 30 distinct spots of different R*f* values of which many could be visualized, but this required different techniques or visualising agents.
- Infrared spectra of the various sub-fractions were different from each other although the differences between the water soluble, acid precipitated and methanol precipitated sub-fraction was subtle.
- UV/visible spectroscopy of the various sub-fractions showed similar featureless curves above 350nm but exhibited subtle differences that appeared to be small broad peaks superimposed on the absorbance trend between 200 and 350 nm.
- The ash content of the various sub-fractions varied greatly indicating different ability to complex inorganic salts
- Labelling of oxihumate with radioactive iodide resulted in several different compound being labelled radioactively which indicates that not all the compounds in oxihumate had reactive chemical bonds available
- The sub-fractions had different UV and fluorescence spectra especially in specific absorbance and fluorescent quantum yields

There are reports of humic acids having a molecular mass in excess of 10^6 Dalton that would mean that humic acid was unlikely to be absorbed from the lumen of the GIT. If absorption from the gut cannot take place, it would follow that no therapeutic activity for orally administered humic acid was possible. To test whether oxihumate could be absorbed from the GIT a study was performed on

isolated segments of rat GIT making use of ^{125}I labelled oxihumate. It was found that there was a relatively rapid uptake of oxihumate from the GIT *in vitro* and that the absorption followed typical concentration and surface area dependant kinetics with an initial rapid rate of uptake becoming progressively slower with time. Dark coloured compounds in addition to several different labelled but colourless compounds were found to be absorbed. Furthermore the radioactivity that had been absorbed could be almost quantitatively precipitated by adding silver nitrate to the solution implying that the absorbed activity was free radioactive ^{125}I. However analysis of the labelled oxihumate used for the study indicated very little residual free radioactive iodide that could not account for the high percentage of activity precipitated with silver nitrate. A small quantity of the absorbed fraction of oxihumate appeared to be bound to protein although the suspending medium used contained no protein at the beginning of the study.

It therefore appears that the oxihumate complex can be disrupted to some extent in the rat gut and that some of the dissociated compounds then cross the GIT membranes and are transported in the bloodstream. From a small study done to determine in which blood fraction the humic acid would reside, it was found that the majority of the humic acid bound to the plasma proteins. It can therefore be assumed that after absorption from the GIT the plasma proteins would serve as the main transporters of the humic acids. The absorption from the GIT of humic acid after oral administration has been confirmed which means that this is a feasible route of administration of the compound as a therapeutic drug.

The *in vitro* bioassays used in this study included a flow cytometric method to determine the level of CR3 molecules expressed on the surface of activated and resting PMNL from healthy volunteers and chemiluminescent assays of the release of reactive oxygen species also from PMNL cells. These assays were aimed at determining parameters that affect anti-inflammatory properties, either due to inhibition of adhesion or inhibition of the respiratory burst which releases potentially harmful reactants. The bioassays were performed using the isolated sub-fractions at the same relative concentrations at which each occurred in oxihumate, so the highest concentration of 100µg/ml for complete oxihumate would have included the 5µg/ml of the most refined sub-fraction, being the THF soluble fraction.

Generally it was found that the activity was greatest for the water-soluble oxihumate, the acid precipitated and methanol precipitated sub-fractions, becoming significantly less active for the remaining more lipophilic sub-fractionations. The anti-inflammatory activity of the THF sub-

fraction was very low despite containing at least 30 individual compounds. It would therefore appear that the complete complex is required for the highest activity for the oxihumate.

The chemiluminescence assays were expected to be affected by the colour intensity of the test mixtures containing the dark coloured sub-fractions due to quenching of the light emitted by the reaction. However the extent of chemiluminescence quenching did not follow the trend of the colour intensity at 425nm – the wavelength of the emitted light in these assays. What was of interest was that the dark coloured sub-fractions all appeared to cause some activation of the cells resulting in an increase in the background. These sub-fractions if pre-incubated for extended times seem to have exhausted the cells which then only showed a short burst of activity before returning to baseline values when stimulated with known PMNL activators. The lipophilic sub-fractions, the methanol solubles, THF solubles, THF precipitated and acid solubles, all appeared to prime the cells so that the response initiated very rapidly after stimulation and was greater than that of the positive controls implying an enhanced activity following activation.

Simultaneously with the determination of reactive oxygen species the ability of the different isolated sub-fractions to scavenge superoxide was tested, using a cell free system. Superoxide anion was produced enzymatically using the xanthine/xanthine oxidase enzyme system. It could be demonstrated that there was a definite trend in the scavenging ability of the sub-fractions to scavenge the superoxide anion formed by the enzyme reaction. This response was concentration dependant, but the possibility also exists that quenching plays a role in the case of the darkest fractions.

The study performed to determine the effects of oxihumate *in vivo* using the contact hypersensitivity rat model showed that oxihumate had little effect and compared closely to a water treated control. This assay measures the responses of DNFB challenged ears of previously sensitized rats after a weeklong oral treatment with the test compounds. A positive control used in the study was a known steroidal anti-inflammatory, prednisolone. This control exhibited a definite and significant inhibitory effect on the immune response, as did a humic acid extracted from brown coal, indicating that the test system was appropriate for the measurement of the *in vivo* effects of oxihumate.

The fact that there were differences in the contact hypersensitivity response of humic acid treated verses untreated rats would again imply that the humic acid is in fact being absorbed from the gut of the rat confirming the results that were obtained during the *in vitro* studies using isolated rat gut segments. The lack of activity in this assay for oxihumate could be due to variations in the

concentration of active compounds in the two different humic acid samples used in the assay. This would need to be confirmed in future studies.

In an attempt to determine the target organs of oxihumate a study using 10 experimental baboons and orally administered radioactive ^{123}I labelled oxihumate or THF soluble sub-fraction was performed. The later distribution of the absorbed activity showed a very similar distribution to the control animals that were dosed with free radioactive ^{123}I containing NaI solution alone. This would imply that the iodide is removed from the labelled oxihumate and that the free iodide then distributes according to the normal distribution kinetics of iodide. Two control baboons were included in the study, one being dosed intravenously with ^{123}I containing NaI while the second control was dosed by rectal administration of a free radioactive ^{123}I containing NaI solution. The distribution of the activity for the two NaI controls was almost exactly the same after about 40 minutes implying that the route of administration is not critical to the final distribution.

One experimental baboon was treated with a rectal dose of ^{123}I labelled THF soluble sub-fraction of oxihumate. This animal showed different uptake and distribution kinetics and was the only baboon where activity was detected in the liver post dosing. The liver activity appeared to be transient with a significant accumulation of this activity in the gall bladder within minutes of administration of the labelled oxihumate. The activity in the gall bladder was higher than any of the other organs for at least 28 minutes post dosing after which the continuous thyroid gland accumulation exceeded that of the gall bladder. After about one hour the activity in the gall bladder began decreasing and reached a plateau at about 4 hours after which this activity was maintained till the end of the study.

Excretion of activity from the baboons was via the urine and amounted to almost 60% of the total administered dose within 24 hours. The excretion curves varied and showed a distinct increase after treatment with Lugols solution, which would also imply that the activity was in the form of free iodide. Tests on the urine showed that almost all the activity could be precipitated by the addition of silver nitrate again implying that the excreted activity was in the form of free iodide.

The faeces from all the baboons had a very low activity throughout the study.

The activity in the blood was low during the first four hours, as seen in the baboons that were initially scanned dynamically and when these samples were collected hourly post dosing. This is probably due to the peristalsis that is suppressed by the anaesthetics. The absorbed activity detected in the blood appeared to be associated with the protein fraction of the plasma.

Although the actual structure or combination of compounds in humic acid are unknown, a model semi-synthetic humic acid that appears to represent humic acids extracted from natural sources was used in this study in an attempt to find the active compounds involved in the anti-inflammatory activity of these natural organic acids.

In summary it can be stated that oxihumate can be sub-fractionated and that chemically the various sub-fractions proved to be quite different using different spectroscopic techniques and chromatographic techniques. The separations on HPLC were reproducible to some extent but it was obvious that the recovery was low and this affected further separations using the same column.

The highest anti-inflammatory activity was associated with the water-soluble sub-fraction, the least refined of the sub-fractions and for this reason *in vivo* studies were performed on this particular isolated sub-fraction.

Absorption was found to take place from the GIT after oral administration and although this was found *in vitro* and implied *in vivo,* it appears as though the radioactive iodide used to label the oxihumate was being stripped from the humic acid either before or during the absorption process.

The water soluble sub-fraction was found to have no significant effect on the hypersensitivity response in the rat model although a very similar brown coal derived humic acid did show significant anti-inflammatory activity in the same rat model.

Humic acids have potential as immuno-modulating compounds due to the inhibition of expression of adhesion molecules and the ability to scavenge superoxide anion radicals and to stimulate the expression of IL2. The water-soluble oxihumate sub-fraction could reduce the cellular response of neutrophils initiated by known stimulants, yet sub-fractions isolated from this same sub-fraction showed a priming effect on the neutrophils when pre-incubated with the cells. The mechanism of action of humic acids has not yet been determined, and is being confounded by contradictory results and the fact that the humic acids are not well-defined compounds with a known structure.

Further studies of humic acids would be necessary to elucidate a mechanism of action and to determine which molecules are in fact biologically active, singly or in combination.