THE LYMPHATIC ABSORPTION OF THE RETINOIDS.

by

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ABSTRACT.

An understanding of the criteria governing lymphatic absorption of drugs from the gastrointestinal tract may lead to selective uptake of drugs *via* this route. Lymphatic absorption may have potential advantages over the portal absorption of drugs; first pass elimination may be avoided as the drug reaches the systemic circulation before it reaches the liver, the absorption of some poorly absorbed drugs may be improved and it may provide a means of targeting anticancer agents directly to the lymphatics.

In the present study, the lymphatic uptake of a series of retinoids was investigated after oral administration. The most important factors which were found to affect lymphatic absorption were, the chemical nature of the compound and the nature of the oily vehicle in which the retinoid was administered.

It was found that the greater the lipophilicity of the retinoid, (as defined by the logarithm of its octanol : water partition coefficient, log. P), the greater was its lymphatic absorption. Temarotene, Ro 15-0778, (log. P = 8.7) exhibited a maximum dose adjusted lymphatic absorption rate of 4100 ng/h compared with the less lipophilic Ro 04-3780 (log. P = 6.8) which showed a maximum rate of only 150 ng/h.

The oily vehicle in which the retinoid was orally administered strongly influenced the rate of absorption *via* the lymphatic route. Ro 04-3780 demonstrated a 150 fold difference between the selected oils giving the maximum and the minimum lymphatic absorption rate. The best oils in this role appeared to be those in which the retinoid showed the least solubility.

Mesenteric lymph flow rate was also shown to vary depending upon the oil. Basal lymph flow rate in the fasted rat, after dosing with saline, was 1.6 ml/h/kg. Cottonseed oil and soyabean oil promoted an increase in this flow rate to greater than 3.0 ml/h/kg ($p < 0.01$) when orally administered with Ro 04-3780. Conversely, linoleic acid suppressed the mean lymph flow rate to 0.8 ml/h/kg ($p < 0.01$) after oral administration with Ro 04-3780.

Lymph turbidity was evaluated as an indication of chylomicron formation and transport in the lymph. Since the chylomicron is the particle in which dietary fats enter the lymphatic system, it was thought that lipophilic drugs, which are soluble in dietary lipid, may be carried into the lymphatic system *via* this pathway. The mixed long chain fatty tri-acyl glycerol oils, cottonseed oil, soyabean oil and peanut oil, when orally dosed to rats, produced the most turbid lymph (25 - 48 times greater than

the turbidity produced after an oral dose of saline). These type of oil also promoted the highest lymphatic uptake rate for the retinoids. Other oils including, oleic acid, linoleic acid and MTS (a Miglyol 812 based self-emulsifying oil system), demonstrated much wider ranging extents of lymphatic absorption, but produced lymph with similar but lower turbidity (10 - 12 times greater than the turbidity produced after an oral dose of saline).
A self-emulsifying oil system was developed for use in the oral administration of a retinoid. This system (MTS), produced a stable emulsion with a particle size of 500 nm after gentle mixing with an aqueous solvent and contained 80 % Miglyol 812 and 20 % surfactants. MTS increased both the lymphatic and portal absorption rates for Ro 15-0778 by three fold compared with Miglyol 812 alone, improving the overall bioavailability but without selective promotion of lymphatic uptake.
The effect of feeding, prior to orally dosing with an oil (linoleic acid) containing Ro 10-9359, was to suppress greatly the portal absorption rate of the retinoid from 310 ng/h to less than 25 ng/h.

A number of factors, which were believed to be important in the lymphatic absorption of the

retinoids, have been investigated here, using the rat as an animal model. The data obtained in this work suggest that lymphatic absorption is a very complex process and the factors which govern this absorptive pathway vary depending upon the nature of the drug being studied and the nature of the orally dosed vehicle.

INTRODUCTION.

i. **Historical background to the lymphatic system.**

Historical records show that there has been interest in the anatomy and physiology of the lymphatic system since antiquity. While the phenomena of lymphoedema and elephantiasis were mentioned in some of the early Hebrew and Greek literature, the first direct references to the lymphatic vessels were made by the Greeks in the fourth century B.C. At this time Aristotle may have observed lymphatics, since he described vessels containing a colourless fluid, while Hippocrates referred to "white blood" (Barrowman, 1978). More accurate descriptions of lymph vessels however, came from the Alexandrian school of medicine. In the fourth and third centuries B.C., Herophilus and Erasistratus of that school described mesenteric lymph vessels and glands. Erasistratus is quoted by Galen: "For on dividing the epigastrium and along with the peritoneum we may see arteries in the mesentery of sucking kids, full of milk" (Cruickshank, 1786). Little progress was made in the investigation of the lymphatic system until the great experimental work of Arsellius (1581-1625), Professor of Anatomy and Surgery in Milan. In 1622 he demonstrated an extensive system of ramifying white vessels, "venae albae et lactae", in the mesentery of a dog which had been fed shortly before its death. Incising one of these with a scalpel, he observed the leakage of

white fluid. These vessels were not readily demonstrated in a second animal which had been starved, but Arsellius correctly assumed that the vessels would be best seen in the post-prandial state. He confirmed this in an experiment on a third animal. Arsellius' thesis "De lactibus sive lacteis venis", was published (Arsellius, 1627) by two of his friends two years after his death. Arsellius believed that mesenteric lymphatics ran to the liver conveying absorbed nutrients for the "concoction" of blood in that organ.

Between 1650 and 1653, Olaf Rudbeck, a Swedish Professor of Medicine studied lymph vessels of the liver, abdominal wall and thoracic viscera, and traced the course of vessels lying on the posterior surface of the rectum to the cisterna chyli (a large collecting vessel in the abdomen). In 1652 he gave a famous demonstration of his findings to Queen Christina of Sweden. Rudbeck showed that the lymphatic vessels constituted a system comparable with the blood vascular system and he was able to show that lymph in the cisterna chyli does not flow to the liver as had been supposed by Arsellius. He later showed that lymph flowed from the thoracic duct to the venous circulation.

Along with increasing understanding of the anatomy of the lymphatic system came the acceptance of the concept of the absorptive function of the

lymphatics. Much of the work supporting this idea came from the work of Hewson (Hewson, 1774) and Cruikshank, (1786). Hewson observed the motility of lymphatic vessels, referring to "peristaltic contraction of the coats" of the vessels and considered that this activity, together with the transmitted pulse from neighbouring arteries would account for the lymph propulsion, the valves observed in the lymphatic vessels contributing a directional component to the flow. Cruikshank (Cruikshank, 1786) made the interesting observation that certain dyes introduced into the intestinal lumen were taken up by mesenteric lymphatics.

The now commonly accepted definition of the lymphatic system is a system composed of channels having the principal function of maintaining blood volume by returning to the general circulation, fluid and protein molecules that leak from the capillaries into the interstitial space. In addition, the lymphatic system includes circulating lymphocytes and lymphoid organs, which are important in defence against infection and tumour growth.

The structure of the lymphatic system is very similar to that of the vascular system. In the lymphatic system, there are innumerable small vessels that flow into larger, thicker-walled collecting vessels. These vessels in turn flow into the major lymphatic ducts.

The peripheral vessels, which have been termed lymphatic capillaries, or initial lymphatic ducts, undertake the primary function of the lymphatic system, removal of material from the tissues. The larger vessels transport the collected material to the lymph nodes, and from there to the large veins at the base of the neck.

The initial lymphatic (lymphatic capillary) forms a blind-end tube or saccule in the interstitium. The vessels are located near the site of cellular metabolism and are situated near to the blood capillaries and smaller venules. This one-way drainage system is strategically placed and is constructed to permit a continuous and rapid removal of transient interstitial fluids, plasma proteins and cells.

The initial lymphatics are characterised by several properties. They have a wider and more irregular lumen than the corresponding blood capillaries. Their endothelial lining is attenuated, with the exception of the perinuclear region. Their intercellular junctions are loosely opposed and easily separate to form patent junctions. Anchoring filaments, which provide a strong connection between the lymphatic wall and surrounding connective tissue, reside in a dense section of the thin outer layer of the plasma membrane.

Lymph drained from the interstitium by the initial

lymphatics is received by the collecting vessels which flow to the lymph nodes. The size and shape of endothelial cells of the collecting vessels is similar to those of the initial lymphatic duct, however, the basement membrane is well developed, often continuous, and elastin and collagen fibres are frequently observed in close contact with the basement membrane. Smooth muscle cells, comprising the tunica media, are arranged in a spiral around the wall of the collecting vessel. Surrounding these smooth muscle cells are bundles of collagen, small blood vessels and non-myelinated nerve fibres.

The structure of the major lymphatic trunk resembles the structure of the muscular arteries and large veins. There are three distinct layers, the tunica intima, the tunica media and the tunica adventitia. The tunica intima consists of an endothelium having tight intercellular junctions, a continuous basement membrane and small bundles of elastic fibres between the basement membrane and the tunica media. The tunica media is comprised of smooth muscle cells arranged in three to six layers around the vessel, whilst the tunica adventitia, the outermost layer, is comprised of a connective-tissue complex of veil cells (flattened, fibroblast-like cells), collagen fibres, nerves and a ramifying series of blood capillaries.

Lymph from most tissues filters through at least one

lymph node in its passage from the tissue interstitium to the blood. The lymph nodes are ovoid or rounded structures and in the human, vary in size from 1 mm to 25 mm in diameter. Fibrous tissue surrounds the node and an internal stroma of reticular endothelial tissue forms the basic outline of the nodes. The node contains two types of cells, the primitive (reticular) cells that produce lymphocytes and plasma cells, and the macrophages or phagocytes, members of the reticuloendothelial system, which line the walls of the lymph node sinuses.

The lymph node filters the lymph, retaining particulate antigens and senescent and faulty cells by phagocytosis. The node produces antibodies, provides a site for exchange between blood and lymph, supports the proliferation of lymphocytes and related cells and is an important area for the recirculation of lymphocytes.

This description of the lymphatic system is limited to its structural elements eg. cells, vessels and organs and to the function of these elements within the system eg. lymph protein transport. However, the lymphatic system serves the whole living organism by maintaining the physiological environment of individual cells in the non-lymphoid tissues of the body. This cellular environment includes the fluid and interstitial matrix surrounding the cells.

Therefore, lymphology can be defined as the biomedical discipline dealing with regulation of the cellular micro-environment. The principal tasks of the lymphatic system are (1) maintenance of the most favourable composition of the mobile intracellular fluid and matrix for the integrity and function of the tissue cells; (2) removal and processing of chemical products released from cells as well as their shed subcellular substances eg. membrane receptors; (3) elimination of dying or mutant cells; and (4) removal of foreign organic particles eg. bacteria, viruses and inorganic matter that have entered the intercellular space (Olszewski, 1985).

ii. <u>Dietary fat absorption.</u>

Dietary fats are mixed with gastric juices in the stomach and produce small fat globules during mechanical mixing of the chyme (blended food material produced by the action of gastric secretions). The fat droplets are further emulsified within the lumen of the small intestine by interaction with bile acids and the activity of lipase, carboxylic acid hydrolase and phospholipase A_2 from pancreatic juice. These pancreatic enzymes catalyse the splitting of dietary triglyceride into long chain fatty acid (LCFA) and mainly 2-mono-acyl glycerols (Tso, 1985). The enzymes also hydrolyse cholesterol esters, and are responsible for the

formation of biliary phospholipids, of which lysolecithin (lysophosphatidylcholine) accounts for 90% of total biliary phospholipid (Borgstrom, 1976). Bile salts are known to be amphiphilic compounds ie. they have a hydrophilic group at one end and a hydrophobic group at the other end. This property enables bile salts to form, together with 2-mono-acyl glycerols, LCFA's, phospholipids and cholesterol, the so-called mixed micelles in which the polar hydrophilic groups of bile salts are directed towards the aqueous environment, whereas the non-polar hydrophobic ends are facing towards the interior of the micelle (Friedman & Nylund, 1980). These micelles are typically less than 500 nanometres in diameter (Carey & Small, 1972).

It is widely accepted that the lipid digestion products (fatty acids, 2-mono-acyl glycerols, lysolecithin and free cholesterol) pass into the absorptive cell membrane from aqueous dispersions by a carrier-mediated, passive absorption process. This fact is clearly demonstrated by the absence of an effect on lipid absorption of metabolic inhibitors (oligomycin, potassium cyanide) and an uncoupler of oxidative phosphorylation (2,4-dinitrophenol) (Hollander, 1981).

Once digestion products have penetrated the cell membrane, they are transported through the cytoplasmic matrix to the site where resynthesis of

triglyceride occurs. The major site of metabolism of absorbed lipid is the endoplasmic reticulum (Schiller, 1970). However, the mechanism by which fatty acids and other lipid fractions are transported to the endoplasmic reticulum is unknown. Ockner (1972) have described the presence of a fatty acid binding protein in the intestinal mucosa, and have characterized this protein.

Two pathways of triglyceride resynthesis are possible; the importance of each depends upon the lipid mixture absorbed. The reconstitution of triglycerides through the monoglyceride pathway (Figure 1) becomes important when monoglycerides and fatty acids are absorbed (Tso, 1985). The enzymes involved are acyl coenzyme-A synthetase, monoglyceride transacylase and diglyceride transacylase. These enzymes are present together as an enzyme complex called "triglyceride synthetase" (Johnston, 1968). The L-α-glycerophosphate pathway (Figure 2) is important when LCFA's are absorbed (Tso, 1985). This pathway involves five major reactions; the activation of fatty acid to an acyl CoA derivative, formation of L-α-glycerophosphate from glycerol, conversion of L-α-glycerophosphate to phosphatidic acid, conversion of the phosphatidic acid to a diglyceride and finally conversion of diglyceride to triglyceride (Tso, 1985).

Figure 1. The monoglyceride pathway for triglyceride synthesis.

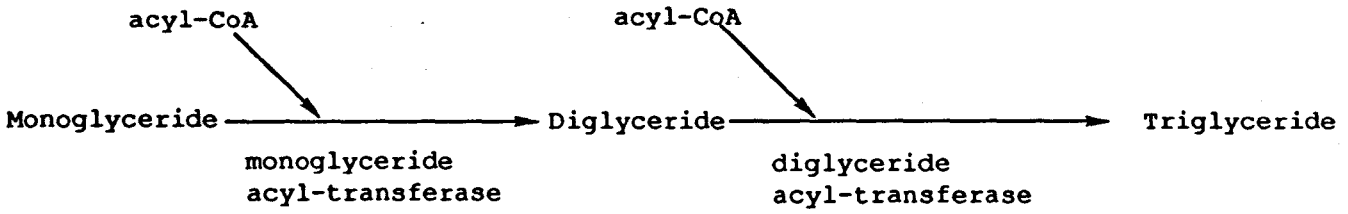
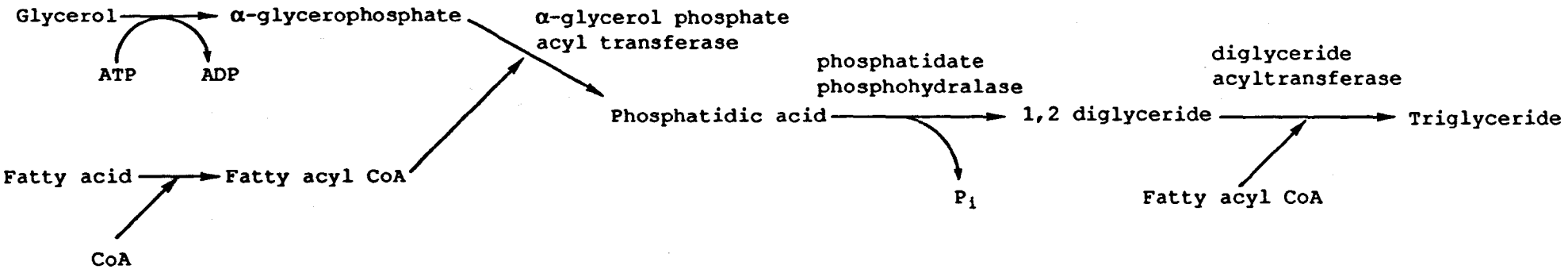


Figure 2. The α-glycerophosphate pathway for triglyceride synthesis



iii. Oil in water emulsion systems as delivery vehicles for lipid soluble drugs

Oil in water emulsion systems have been investigated as formulation vehicles which may deliver lipid soluble drugs to the site of absorption from the gastrointestinal tract after oral administration (Hashida et al., 1977, Myers & Stella, 1989). These lipid emulsions range in lipid content and particle diameter but resemble the emulsified dietary lipids which are formed in the stomach and which exist as droplets of less than 5 μm in diameter (Carey et al., 1983). The mechanisms, rate and extent of drug absorption from these emulsions is strongly dependent on the oils and surfactants used in each formulation. Absorption may be influenced by the ability of the intestinal enzymes to digest the oil and by the partitioning of the drug between the oil and water (Armstrong & James, 1980). In addition, the surfactant may modify absorption by a variety of mechanisms (Attwood & Florence, 1983). Excipients used for such formulations need to be non-irritant and free from other acute or toxicity problems. The choice of oils is likely to be restricted to vegetable oils and their derivatives. These may be used in combination with non-ionic surfactants which are generally less toxic than ionic surfactants (Attwood & Florence, 1983), examples may include the

Tweens which are pharmaceutically acceptable (Varma et al., 1985).

iv. Chylomicron formation.

Dietary fats are absorbed almost entirely into intestinal lymph in the form of chylomicrons and very low density lipoproteins (VLDL). The process of the assembly of chylomicrons in the enterocytes is poorly understood, however, certain aspects of the process are now quite well documented. Chylomicrons, are synthesised mainly from tri-acyl glycerols produced from the L-α-glycerophosphate or monoglyceride pathway and irrespective of the fat in the diet, consist of about 70-90% long chain triglycerides (more than 14 carbon atoms in the aliphatic chain), 4-8% phospholipids, 4% cholesterol esters, 3% cholesterol and about 2% protein and other triglyceride rich lipoproteins (Gurr & James, 1971). The chylomicrons are secreted from the Golgi complex into the lateral intracellular spaces where they then enter the lacteals and the intestinal lymphatics through the lamina propria. The movement of chylomicrons across the lymphatic endothelium occurs both by passive diffusion and active transport within pinocytotic vesicles (Friedman & Nylund, 1980., Gangl & Ockner, 1975). Since dietary fats enter the intestinal lymphatic system by this pathway, it is believed that lipophilic drugs, which

are highly soluble in the dietary lipid, may be carried from the gut lumen into the lymphatic system *via* this chylomicron pathway.

v. Lymphatic absorption.

It is now generally accepted that many, mainly lipophilic, substances can be transported in the lymph chylomicrons after absorption from the gut. These include endogenous compounds such as; lipid molecules (Morgan, 1966., Ockner *et al.*, 1969., Barrowman, 1978); triglycerides (Mansbach *et al.*, 1985., Mansbach & Arnold, 1986), fatty acids (Bloom *et al.*, 1951., Bernard *et al.*, 1990), cholesterol (Watt & Simmonds, 1984), steroid hormones such as testosterone (Horst *et al.*, 1976) and testosterone esters (Noguchi *et al.*, 1985), the fat soluble vitamins; A, D, E & K (Forsgren, 1969., Yeung & Veen-Baigent, 1972., Hollander, 1980., Blomhoff *et al.*, 1984) and many lipophilic drugs (DeMarco & Levine, 1969., Levine *et al.*, 1969., Olszewski, 1985., Stella & Charman, 1990) and pharmacological agents are also reported to be transported *via* this route, these include; chemotherapeutic agents; interferon (Yoshikawa *et al.*, 1984., Yoshikawa *et al.*, 1986, Bocci *et al.*, 1989., 5-fluorouracil (Hashida *et al.*, 1977) and bleomycin (Yoshikawa *et al.*, 1983), antifungal agents and immuno-suppressants; griseofulvin (Noguchi *et al.*, 1977),

cyclosporin A (Ueda et al., 1983)., Takada et al., 1985., Hirate & Ueda, 1989., Yangawa et al., 1989), analgesics and anxiolytics; diazepam (Lamka et al., 1990), naproxen (Sugihara et al., 1988) and the novel analgesic compound, LY108964 (Ho et al., 1989), the antiepileptic agent; phenytoin (Gowan & Stavchansky, 1986), the β-blocking agent; propanolol (White et al., 1991) and carcinogens (Kamp & Neumann, 1975) including the highly lipophilic DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane) (Sieber, 1976., Palin et al., 1982., Noguchi et al., 1985., Charman et al., 1986b., Charman & Stella, 1986). These data are summarised in Table 1.

Work in the early part of this century addressed the problems of the absorption of fat soluble substances. Drummond et al. (1935) questioned how chemical substances, sharing with the fats the one common property of showing similar solubilities in certain solvents, are absorbed from the intestine to an extent which was unsuspected. Two main theories were suggested by the then existing knowledge. There was good reason for considering the view that specific absorption from the gut may occur. Alternatively, a more conventional path may be followed by trying to picture an intermediate conversion of these "fat soluble" substances into "water soluble" and "diffusible" forms. In thinking of "water soluble" complexes the possible function

Table 1. Pharmacological agents absorbed via the lymphatic route

Author	Substance studied	Animal	Result
Yoshikawa et al., 1984	Human interferon (mixed micelle infused into large intestine)	Rat	0.050% of dose absorbed via thoracic lymph after 6h.
Bocci et al., 1989	Human interferon (intramuscular dose in saline)	Rabbit/Pig	Lymph/plasma ratio = 4.3.
Hashida et al., 1977	5-fluorouracil (Gelatin in oil microspheres)	Rat	Intraduodenal administration of 5-FU microspheres in oil gave a concentration of 5-FU in lymph nodes of 12 μg/g but only 0.1 μg/ml in plasma.
Yoshikawa et al., 1983	Bleomycin (FITC labelled)	Rat	0.33% of dose absorbed via thoracic lymph after 3h.
Noguchi et al., 1977	Griseofulvin (oil in water emulsion)	Rat	Approximately equal peak concentrations found in plasma and mesenteric lymph after infusion into in-situ gut loop.
Ueda et al., 1983	Cyclosporin-A (oral dose in olive oil)	Rat	2% of total absorption via thoracic lymph.
Takada et al., 1985	Cyclosporin-A (mixed micelle into duodenum)	Rat	0.78% of dose absorbed via thoracic lymph after 6h. Lymph/plasma concentration ratio = 25.

Table 1. continued

Author	Substance studied	Animal	Result
Yanagawa et al., 1989	Cyclosporin-A (in a lipid microsphere)	Rat	Thoracic lymph/plasma concentration ratio = 16.
Lamka et al., 1990	Diazepam (intra-duodenal infusion in olive oil)	Rat	0.032% of of dose absorbed via thoracic lymph. Lymph/plasma concentration ratio = 2.20.
Sugihara et al., 1988	Naproxen (glyceryl octyl-ester)	Rat	Thoracic lymphatic absorption rate = 28.3% of dose.
Ho et al., 1989	LY108964 (corn oil emulsion)	Mouse	Thoracic lymph/plasma concentration ratio = 50.
Gowan et al., 1986	Phenytoin	Rat	Thoracic lymph/plasma concentration ratio approx. = 1.
White et al., 1991	Propanolol (in a novel delivery system)	Pig	Hepatic portal vein concentration of propanolol were initially lower than those observed in mesenteric lymph.
Kamp et al., 1975	3-Methyl-cholanthrene	Rat	9% methylcholanthrene was recovered in thoracic lymph.

Table 1. continued

Author	Substance studied	Animal	Result
Palin *et al.*, 1982	DDT (in Arachis oil)	Rat	Thoracic lymph/plasma concentration ratio approx. = 4.
Noguchi *et al.*, 1985	DDT (in oleic oil)	Rat	21.9% - 16.6% of dose appeared in mesenteric lymph after 24h.
Charman *et al.*, 1986	DDT (intra-duodenal infusion in peanut oil over 2h)	Rat	Approx. 42% of dose collected from mesenteric lymph in 24h.
Charman *et al.*, 1986	DDT (intra-duodenal infusion in oleic acid over 2h)	Rat	Approx. 36% of dose collected from mesenteric lymph in 12h.

of bile was implicated. Observations by Channon & Collinson, (1929) questioned whether solubility in bile might be the chief factor in determining the absorption of certain fatty substances.

Vitamin A (retinol), is necessary for the development and maintenance of human life, but cannot be synthesized by the body and must therefore be supplied in the diet. It is necessary for the support of growth, differentiation of epithelial tissues, the synthesis of protein, and the preservation of vision (Hollander, 1980). Vitamin A alcohol (retinol) and its esters (β-carotene, a pro-vitamin which is cleaved in the intestine to vitamin A) are the major dietary sources (Lucek & Colburn, 1985). Vitamin A is metabolised to vitamin A esters, vitamin A aldehyde (retinal) and vitamin A acid (retinoic acid) which are stored in the liver. (Lucek & Colburn, 1985).

Drummond et al. (1935) studied two fat soluble substances, vitamin A and β-carotene. These molecules were found to have hydrophobic properties, and were therefore thought to exist in a form which would be transported through the intestinal wall in a way similar to other fats.

Studies were carried out on a patient suffering from a chylothorax, in which a considerable proportion of the chyle (a milky fluid produced from food after digestion and emulsification by gastric, biliary and

pancreatic fluids) was diverted from the thoracic lymph duct and collected in the pleural cavity. It was necessary to remove this fluid every few days for the relief of the patient. During the course of these studies, it was determined to what extent, and in what form, the β-carotene and vitamin A administered orally in the course of treatment appeared subsequently in the chyle. Approximately 125 milligrams of vitamin A and 100 milligrams of β-carotene were given orally divided over 60 and 7 days respectively. In the case of vitamin A, 100 milligrams (80%) were recovered in the chyle after correcting for an estimated daily chyle production of 3000 ml, however, of β-carotene less than 20 milligrams (<20%) was recovered. From these data the conclusions drawn were that vitamin A is almost exclusively absorbed via the lymphatic route, β-carotene, however, shows poor absorption via this route.

Studies have also been performed on oxen, sheep and rats (Eden & Sellers, 1949) which had been orally dosed with halibut liver oil (containing approx. 17 mg/ml (5000 iu./ml) of vitamin A). Vitamin A levels were estimated in systemic blood, portal blood and in the lymph glands from various regions of the body. The samples were collected as soon as possible after slaughter of the animals. The results confirmed that vitamin A was absorbed almost

exclusively *via* the lymphatic route, mainly from the upper part of the intestine. The differences observed between the concentration of vitamin A in portal and systemic blood were not significant, further supporting the conclusion that the extent of portal absorption was small.

Although the methods used to assay the vitamin A levels in these early studies were crude, more recent studies (Blomhoff *et al.*, 1984) generally agree that the intestinal absorption process of the fat soluble vitamins closely follows the pathways of lipid absorption. It should be noted however, that an increased amount of retinol may be absorbed *via* the portal route when the thoracic lymph duct has been ligated (Yeung & Veen-Baigent, 1972).

The close association of the absorption of fat soluble compounds with fat absorption has prompted many workers to search for other possible associated features of these absorptive processes and in particular the effects of oily vehicles, including food, and oil emulsion systems.

Noguchi *et al.* (1977a), have studied the absorption from the small intestine of the model oily compounds: sudan blue, oil red-O and vitamin A acetate from emulsions containing oleic acid and triolein. The amount absorbed decreased as the ratio of oleic acid:triolein in a mixed micelle increased, suggesting selectivity for certain types of oil

emulsion systems.

Hashida (Hashida et al., 1977), evaluated the efficiency of water in oil and gelatin microspheres in oil emulsions as a delivery system for 5-fluorouracil (5-FU) into the lymphatics of the rat. After oral gavage, both emulsion systems increased the area under the concentration time curve (AUC) for 5-FU in the regional lymph nodes and cumulative amount transported into thoracic lymph compared to aqueous solutions of 5-FU.

Myers (Myers & Stella, 1989), studied the effect of various emulsion systems on the absorption and bioavailability of the lipophilic compound penclomidine. Their results showed that soyabean and triolein emulsions gave the greatest cumulative lymphatic transport of penclomidine because chylomicron formation was increased by the LCFA triglycerides.

There is a general belief that the lipophilicity of an administered molecule, as estimated by its partition coefficient, e.g. between octan-1-ol and water, or between the chylomicron fraction and infranatant phases of lymph is a major determinant of the eventual degree of lymphatic transport of a lipophilic molecule (Sieber et al., 1974., Sieber, 1976., Kamp & Neumann, 1975., Palin, 1985). This hypothesis is based on the fact that most drug molecules which are transported in significant

quantities in the intestinal lymph are highly lipophilic, and reside in the chylomicron fraction (as opposed to the infranatant fraction) of intestinal lymph (Sieber, 1976., Kamp & Neumann, 1975., Vost & Maclean, 1984).

Chylomicrons are the major lipid-transporting lipoproteins of intestinal lymph and are composed of a triglyceride core which is stabilised in the aqueous environment of the lymph by a surface coating of protein and phospholipid (Sabesin, 1976., Zilversmit, 1978). An apparent relationship between the partition coefficient of a drug molecule and its lymphatic transport has been demonstrated within an homologous series of DDT analogues (Sieber, 1976), and a series of testosterone esters (Noguchi et al., 1985a).

Most lymphatically transported lipophilic drugs are present in the chylomicron fraction of the intestinal lymph (Charman & Stella, 1986a). There are therefore two major factors which can influence the amount of drug transported by this intestinal absorptive pathway. Firstly, the quantity of lipid transported in the lymph in the form of chylomicrons and secondly, the amount of drug per chylomicron. Both of these factors can be manipulated in order to optimise or promote the lymphatic absorption of a particular drug (Charman & Stella, 1986a). The chylomicron flux can be influenced by factors such

as the type of lipid vehicle which is co-administered with the drug and also by the diet. Colburn et al. (1983a) have demonstrated a 1.5 to 2 fold increase in AUC in groups of healthy human volunteers when a synthetic retinoid, isotretinoin (Ro 04-3780) was given 1 h after food, with food or 1 h before food compared with the AUC measured in a fasted group. Holazo et al. (1990) also showed that food increased the AUC for the highly lipophilic, Temarotene (Ro 15-0778) 12 fold, and greatly increased the maximum plasma concentration (C_{max}). It has been suggested that such increases in AUC and C_{max} are due to transient changes in gastrointestinal motility; the slowing of stomach emptying and an increase in gastric and intestinal secretions, resulting in more complete dissolution and prolonged residence time at the site of absorption (Welling (1980). Food may also increase the absorption of fat soluble vitamins by the formation of bile salt emulsion systems after increasing bile flow (Holazo et al., 1990). The loading of drug per chylomicron will be affected by a combination of partition coefficient and lipid solubility considerations, as well as the nature of lipid vehicle in which the drug is administered (Charman & Stella, 1986a). The lipid vehicle may include variable chain length free fatty acids, a mono-acyl or mixed tri-acyl glycerol containing long, medium or short chain fatty acid

esters, oil emulsions, or some combination of these oil systems.

vi. The retinoids.

Since the first synthetic production of retinoids in the early 1970's much work has been carried out studying their pharmacology, pharmacokinetics and toxicological effects.

The number of similarities between the clinical manifestations of hypervitaminosis A syndrome (Silverman et al., 1987), such as hyperkeratosis and squamous metaplasia, and skin alterations observed in various disease states (keratinisation disorders and certain precancerous conditions) have provided a basis for the investigation of the role of vitamin A in dermatology and oncology (Bollag, 1970., Bollag & Hanck, 1977., Mayer et al., 1978., Bollag, 1979), Sporn & Newton, 1979., Bollag & Matter, 1981., Bollag, 1989., Lippman et al., 1989., Orfanos et al., 1987., Warren & Khanderia, 1989., Shealy, 1989, Natruzzi et al., 1990).

Clinical investigations of the antikeratinising properties of vitamin A in such diseases as acne, ichthyosis, lichen ruber planus, Dariers disease and pityriasis rubra pilaris have met with only moderate success. Furthermore, the doses required for therapeutic effect were accompanied by severe adverse reactions, such as changes in skin and

mucous membranes, and the bone, liver and neurological complications (Biesalski, 1989., Silverman et al., 1987). In an attempt to separate the numerous side effects associated with use of the retinoids (including vitamin A) from clinical efficacy, more than 1500 retinoids have been synthesized as a result of the chemical manipulation of the vitamin A molecule.

The retinoids possess three main structural features; a cyclic ring, a tetraene side chain and a terminal group which may be polar or non-polar. Modifications to these three regions have enabled the development of three generations of retinoids (Bollag, 1981). These show a range of physicochemical and pharmacological properties. The first generation of retinoids includes vitamin A (retinol) and naturally occurring compounds (metabolites of vitamin A) which have been found in the body. These show similarity in structure to retinol but have modified terminal polar groups. This class includes the two acidic compounds, all-trans-retinoic acid (Ro 01-5488) and 13-cis retinoic acid (Ro 04-3780).

The second generation of retinoids contain an aromatic ring which forms a conjugated system with the tetraene side chain and this therefore increases the lipophilicity of these compounds. This group includes compounds such as Acitretin (Ro 10-1670,

Etretin), its ethyl ester Etretinate (Ro 10-9359) and Ro 11-5036.

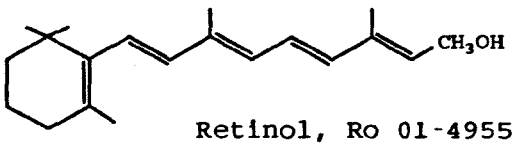
The third generation of retinoids are essentially non-polar and are commonly termed arotinoids. They possess both an aromatic ring and a non-polar terminal group produced by the cyclisation of the tetraene side chain. Examples include Temarotene (Ro 15-0778) and its sulphone derivative Etarotene (Ro 15-1570). The structures of the some of these compounds are displayed in Figure 3.

A deficiency of vitamin A in man, has been shown to enhance the susceptibility to carcinogenesis (Bjelke, 1975). Symptoms include a marked hyperplasia and enhanced synthesis of DNA by basal cells of various epithelia and reduced cellular differentiation. The administration of vitamin A and other retinoids to animals reverses these changes in the epithelium of the respiratory tract, mammary gland, bladder and skin (Mandel et al., 1980). The exact mechanism of this anticarcinogenic effect remains unclear but is clearly of great interest.

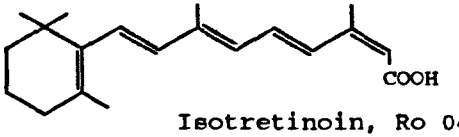
vii. The pharmacokinetics of the retinoids.

Pharmacokinetic studies performed by Khoo et al. (1982) demonstrated that after oral administration of 100 milligrams isotretinoin (Ro 04-3780) to 12 healthy human volunteers, the drug appeared in the blood after a lag time of 0.5 to 2 hours. Peak blood

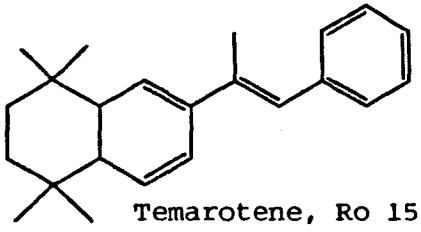
Figure 3. Chemical structures of some of the retinoids.



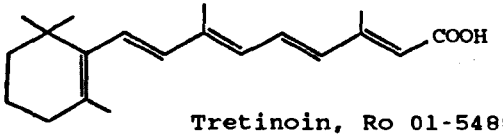
Retinol, Ro 01-4955.



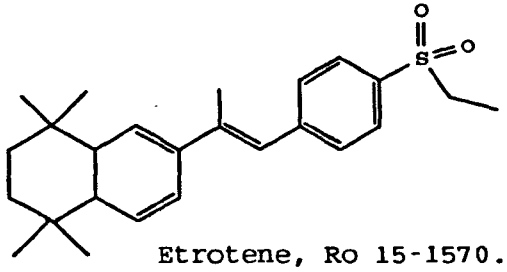
Isotretinoin, Ro 04-3780



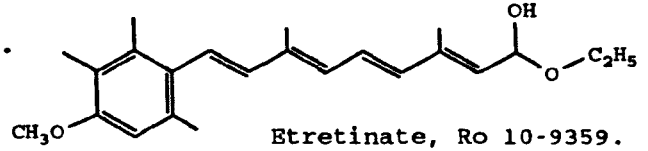
Temarotene, Ro 15-0778.



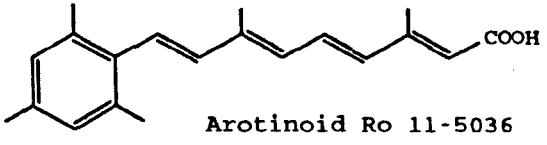
Tretinoin, Ro 01-5488.



Etrotene, Ro 15-1570.



Etretinate, Ro 10-9359.



Arotinoid Ro 11-5036

concentrations (74 to 511 ng/ml) were found at between 1 and 4 hours after administration, falling to below 5 ng/ml at 72 hours; secondary peaks were observed between 6 and 24 hours after dosing. The mean half life was approximately 20 hours.

Following a single 80 mg dose of Roaccutane (a gelatin capsule containing Ro 04-3780 dissolved in oil) to 15 healthy human male volunteers, peak blood concentrations of 436 to 950 ng/ml were found at between 1 to 4 hours after dosing. Ro 04-3780 exhibited mean half lives of 1.3 and 17.4 hours for distribution and elimination respectively (Colburn et al., 1983b).

Whilst the studies by these two groups reported similar results, some marked differences were evident; Khoo et al. (1982) have reported an absorption time lag which was not seen by Colburn et al. (1983) this difference has been purported by Colburn (Colburn et al., 1983b) to be due in part to differences in the dosage forms used, the drug being absorbed more rapidly from an unencapsulated oral suspension than from the soft gelatin capsule formulation (Roaccutane).

Secondary peaks in Ro 04-3780 blood concentrations were observed by Khoo et al. (1982) which were said to reflect enterohepatic circulation of the drug. These peaks were not seen by Colburn et al. (1983b).

Low bioavailability and high intersubject variation

in blood concentrations in humans after oral administration was reported in both studies. This may have been the result of degradation of the retinoid in the gut lumen, extensive first pass metabolism or poor absorption (Brazzell & Colburn, 1983).

The bioavailability of Ro 04-3780 has also been shown to be influenced by food intake, the relative bioavailability being between 1.5 and 2.0 times greater when the dose is administered between 1 hour before and 1 hour after or concomitantly with food, than when given after a complete fast (Colburn et al., 1983a).

Following a single oral dose of 3 or 5 mg/kg Ro 04-3780 to patients with advanced cancer of the colon, peak plasma concentrations of 200 - 800 ng/ml and 190 - 1500 ng/ml, respectively, were found at 3.2 $\pm$ 1.1 hours and 4.5 $\pm$ 2.4 hours (Goodman et al., 1982). The mean distribution phase half lives observed were 2.0 $\pm$ 0.7 hours and 2.4 $\pm$ 1.6 hours, these values were not significantly different. The almost eight fold range in peak plasma concentrations and AUC values have been explained as a result of malabsorption due to the advanced disease condition (Lucek & Colburn, 1985).

In a similar study in which advanced cancer patients received 0.5 mg/kg/day Ro 04-3780 initially, increasing stepwise over 4 weeks to 8 mg/kg/day, a

linear correlation of plasma concentration with dose was observed with large inter-individual variation in peak plasma concentrations (Kerr et al., 1982). The pharmacokinetics of Ro 04-3780 during a multiple dose regimen were studied in 10 patients with cystic acne (Brazzell & Colburn, 1982, Brazzell et al., 1983). Each patient received an initial oral dose (80 mg) of Ro 04-3780 (two 40 mg gelatin capsules), followed by 25 days of 40 mg twice daily and then a final single 80 mg dose. Peak blood concentrations of Ro 04-3780 following the initial dose were found between 2 and 4 hours (mean 2.9 hours) post dosing and ranged from 98 to 535 ng/ml (mean 262 ng/ml), whereas, following the final dose, peak blood concentrations were 188 to 473 ng/ml (mean 310 ng/ml) occurring also at between 2 and 4 hours (mean 2.5 hours). No significant difference in the pharmacokinetics of Ro 04-3780 between the first and last dose was found. The areas under the plasma concentration time curves (AUC) and elimination half lives (10.4 and 9.2 hours) were similar for the first and last 80 mg dose. Thus, neither enzyme induction nor drug accumulation were evident. Isotretinoin (Ro 04-3780) is used widely in the treatment of acne and acneform conditions. However there is a wide discrepancy between the doses applied by European dermatologists (0.5 to 1.0 mg/kg/day) and the very high doses (up to

3 mg/kg/day) used in the USA (Saurat, 1989b).
Dose ranging studies have only been performed in acne patients. It has been shown that doses as low as 0.1 mg/kg decreased sebum excretion and brought clinical improvement. Relapse rates were higher in patients treated with 0.1 mg/kg/day than in those treated with 1 mg/kg/day. Peck (Peck et al., 1979), reported the complete remission of acne in 13 of 14 patients undergoing treatment with a maximum dose of 2 mg/kg/day. The 14th patient was reported to show 89% improvement after 3 years post treatment. Lucek (Lucek & Colburn, 1985) have suggested a therapeutic dose of 0.5 mg/kg/day for Ro 04-3780 but also noted acute side effects at doses above 1.0 mg/kg/day.
The aromatic retinoid, Etretinate (Ro 10-9359), is less effective than Ro 04-3780 in the treatment of acne (Goldstein et al., 1982), but is currently marketed for the treatment of psoriasis and disorders of keratinisation.
Oral administration of Ro 10-9359 has been shown to produce an excellent response in up to 80% of psoriatics (Ehmann & Voorhees, 1982). Ro 10-9359 has a narrow therapeutic range, with a recommended dosing regimen of 0.75 to 1.0 mg/kg/day in divided doses. It is poorly bioavailable (less than 25%) in the dog (Cotler et al., 1983) and moderately bioavailable (40%) in humans (Paravicini, 1981). Plasma concentrations after oral administration have

been shown to be highly variable. Wills (Wills et al., 1982) studied the dose proportional absorption of Ro 10-9359 given to 12 healthy male volunteers. Intersubject variation in peak plasma concentration was large, 45 - 229 ng/ml, 111 - 292 ng/ml, 115 - 492 ng/ml and 173 - 1120 ng/ml following doses of 25, 50, 75 and 100 mg. Dose proportional increases in maximum concentration (3.3 to 3.9 hours) and AUC(0-12 h) ($p < 0.05$) were observed. After oral administration the drug was quickly absorbed with a time lag of 1.7 to 2.3 hours and the peak plasma level was found at 2 to 3 hours. The elimination half life was estimated to be 7.3 hours (Paravicini et al., 1981). Using the graphical method of residuals, three phases of decline of drug concentrations following a single i.v. dose were apparent (half life of the phases: 5 - 10 minutes, 30 - 60 minutes, 6 - 12 hours). Evidence of at least one other longer phase was indicated but the sensitivity limits of the assay method did not permit visualization, however the presence of significant plasma levels of etretinate 140 days after cessation of chronic therapy suggested a long elimination phase (half life approx. 100 days) (Bollag, 1981, Paravicini et al., 1981).

Ro 15-0778 is an arotinoid and in contrast to the other retinoids has no polar terminal carboxyl group. In animal experiments using Ro 15-0778, no

hypervitaminosis A was induced and in contrast to Ro 04-3780, the inhibition of sebum production in rats was superior (Boris et al., 1988). After oral administration of 96 mg of Ro 15-0778 in an oil filled gelatin capsule to fasted healthy human subjects the mean peak plasma concentration was achieved after 6.9 hours, with a harmonic mean elimination half life of 24.1 hours (Holazo et al., 1990).

viii. Aims of the project.

The lymphatic route of drug delivery offers several potential advantages over portal absorption of drugs including the retinoids. First pass elimination may be avoided as the drug reaches the systemic circulation before the liver, absorption of some poorly absorbed compounds may be improved and it may also provide a means of targeting anticancer agents directly to the lymphatics (Hashida et al., 1977). Since it is now generally accepted that vitamin A is absorbed from the gut via the lymphatic system, it was decided to investigate the molecular and formulation factors which influence the absorption of the retinoids (vitamin A analogues) via the lymphatic route following oral administration. These investigations may be divided into a number of phases to include determination of the lipophilicity and solubility of the retinoids, since these

properties would appear to be important factors for compounds selectively absorbed *via* the lymphatics eg. cholesterol, octadecanoic acid, octadecanol and DDT, (Sieber *et al.*, 1974., Kamp & Neumann, 1975., Ueda *et al.*, 1983). It was also intended to assess whether there was a relationship between these parameters and the extent of lymphatic absorption of a particular compound. The effect of a formulation vehicle on the delivery of selected lymphatically absorbed retinoids into the venous system was to be considered. Previous studies have shown some selective absorption into the general circulation of drugs from oily emulsions, therefore the effects of different oily formulations and self-emulsifying oil systems on the lymphatic and venous absorption were to be studied. A self-emulsifying system is a mixture of oil and surfactant which emulsifies in water under conditions of gentle agitation. Such a system may have potential uses for the administration of lipophilic drugs by the oral route. There may be a need for formation of small particles, less than 500 nanometres in diameter, by analogy with chylomicrons (Barrowman, 1978). Such a formulation will present a lipophilic drug in oily solution with a large interfacial area across which diffusion can take place.

MATERIALS AND EQUIPMENT

a) Equipment and chemicals.

- LKB model 2150 hplc pump and model 2151 ultraviolet detector. LKB, Bromma, Sweden.
- Spectra Physics model SP4290 computing integrator.

Spectra Physics, Palo Alto, California, USA.

- Gilson model 231 sample injector and model 401 sample dilutor.

Gilson Medical Diagnostics, Villiers le-Bel, France.

- Rheodyne model 7010 sample loop injector.

Rheodyne, Cotati, California. USA.

- Chromacol, hplc autosampling vials and caps.

Anachem, Luton, Bedfordshire. LU2 0EB

- 5 μm Spherisorb S5ODS2 (125 mm x 4.6 mm) and 3 μm Spherisorb S3CN (150 mm x 4.6 mm) hplc columns.

Hichrom, Theale, Reading. RG7 4AA

- MSE Centaur 2 and MSE Microcentaur.

Fisons PLC., Loughborough, Leicestershire.

- "Gyrovap" centrifugal vacuum evaporator.

V A Howe & Co., London. SW18 2LS

- Gas-liquid chromatograph, model 104.

PYE Unicam Ltd., Cambridge, Cambridgeshire.

- Gold/red fluorescent safelights.

Thorn EMI, Birmingham.

- "Microman" 250 μl, positive displacement pipette.

"Pipettman" P200 & P1000, air displacement pipette.

Gilson Medical Diagnostics, Villiers le-Bel, France.

- Analytical balance (5 decimal place).

Ohaus, Florham Park, New Jersey, USA.

- Coulter "Nano-sizer", emulsion particle sizer.

Coulter Electronics Ltd., Harpenden, Herts.

- DMS 100, ultraviolet/visible scanning spectrophotometer.

Varian Techtron Pty. Ltd., Australia.

- Ultrasonic water bath.

Decon Ultrasonics Ltd., Conway House, Hove, Sussex.

- Ultrafree, 0.1 μm ultrafiltration units.

Millipore (UK) Ltd, Watford, Herts. WD1 8YW

- Magnetic stirrer

Analytical Supplies, Little Eaton, Derbyshire.

- Rotary tube mixer.

Denley, Billinghurst, Sussex. RH14 9EZ

- MFC Microfluidiser.

Microfluidics Corporation, Newton, Mass., USA.

b) Disposables.

- Polypropylene microcentrifuge tubes (1.5 ml).

Elkay Laboratory Products (UK) Ltd., Basingstoke, Hants RG2 0NA.

- Hypodermic needles; 25 g x 5/8", 23 g x 1¼",

Syringes, polythene disposable 1 ml and 2 ml volume.

Sabre International Products Ltd., Reading, Berkshire RG2 0LQ.

- Sagatal: Sodium Pentobarbitone 60 mg/ml.

RMB Animal Health Ltd., Dagenham RM10 7XS.

- Borosilicate glass tubes (15 ml capacity).

Corning Glass Co., Halstead, Essex.

c) Chemicals.

- Ammonium acetate (Analar),

  Hydrochloric acid (Analar),

  Sodium chloride (Analar).

BDH chemicals Ltd., Poole, Dorset.

- Acetonitrile, HPLC grade.

  Diethyl ether, HPLC grade.

  Methanol, HPLC grade.

May & Baker, Dagenham, Essex.

- Tetrahydrofuran, HPLC grade.

Sigma Chemical Co., Poole, Dorset.

- Octan-1-ol: glass distilled.

Aldrich Chemical Co. Ltd., Gillingham, Dorset.

- Retinoids; Ro 01-4955 (Retinol)

  Ro 01-5488 (All-trans retinoic acid)

  Ro 04-3780 (13-cis retinoic acid)

  Ro 10-9359 (Etretinate)

  Ro 11-5036 (Aromatic retinoid)

  Ro 15-0778 (Temarotene)

  Ro 15-1570 (Etarotene)

Roche Products Ltd., Welwyn Garden City, Herts AL7 3AY

- Softigen 767 (PEG-6 caprylic/capric glycerides)

Dynamit Nobel, Gateway House, Slough, UK.

- Tween 20 (Polyethylene sorbitan monolaurate)
  Tween 80 (Polyethylene sorbitan monooleate)
  Tween 85 (Polyethylene sorbitan trioleate)

BDH chemicals Ltd., Poole. Dorset.

- Labrafil M1944CS (Apricot kernel oil PEG-6 complex).

- Labrosol (Glyceryl caprylate/caprate and PEG-8 caprylate/caprate)

Alfa Chemicals Ltd., Bracknell, UK.

- Span 20 (Sorbitan monolaurate)
  Span 80 (Sorbitan monooleate)
  Span 85 (Sorbitan trioleate)

Sigma Chemical Co. Ltd., Poole, Dorset.

- Lipoid E80

BASF, Parsippany, NJ 07054.

- Soyabean oil, Cottonseed oil, Linoleic acid (95%), Oleic acid (95%), Caprylic acid (99%), Trilinolein (99%).

Sigma Chemical Co. Ltd., Poole, Dorset.

- Miglyol 812 (Mixed medium chain tri-acyl glycerol).

Dynamit Nobel, Gateway House, Slough, UK.

- DL Monoolein

Fluka Bio-Chemika, Glossop. SK13 9XE

- Neobee M5 (Mixed medium chain mono-acyl glycerols)

Alfa Chemicals, Bracknell, Berkshire.

- Monocaprylin (Imwitor 308)

Huls (UK) Ltd., Slough, Berkshire

- Arachis oil BP (Peanut oil).

Evans Ltd.,Greenford, Middlesex.

-SDS water soluble diet 821338

Special Diet Services Ltd., Witham, Essex.

d) Surgical equipment and apparatus.

- Clippers.

Wahl Clipper Corporation, Illinois, USA

- Polythene cannula: i.d. 0.5 mm /o.d. 1.0 mm

Portex Ltd., Hythe, Kent.

- Scalpel blades size 22, cotton buds, Surgikos disposable surgical gloves.

Medical School Stores, Queens Medical Centre, Nottingham. NG7 2RD

- Swann-Morton scalpel handle size 4, "Spencer-Wells" artery forceps, round ended scissors, miniature spring scissors (5 mm blades), medium and small curved ended forceps, small rodent artery clip.

H. Wilkinson & Co. Ltd., Nottingham NG1 4EJ

- Electrically heated mat 15 Watts.

IMS, Congleton, Cheshire. CW12 1LA

- Stereo dissecting microscope.

Leitz UK Ltd.,Milton Keynes. MK5 8LB

CHAPTER 1

A STUDY OF THE PHYSICOCHEMICAL PROPERTIES OF THE RETINOIDS.

1.1. Partition behaviour of the retinoids.

1.1.1.1. The octanol-water partition coefficient.

A partition coefficient (P) is defined as the ratio of the equilibrium solution partitioning of a solute between an organic and an aqueous phase, most often octan-1-ol and water (Pow).

$$Pow = \frac{\text{Concentration in the octan-1-ol phase}}{\text{Concentration in the aqueous phase}}$$

Equation 1

"Solutes which display some preference for the octan-1-ol phase (Pow > 1) are designated lipophilic or hydrophobic, those with preference for the aqueous phase (Pow < 1) are labelled as hydrophilic" (Van der Waterbeemd & Testa, 1987).

The simplest and most frequently used technique for the experimental determination of P is to allow a compound to distribute between an aqueous and an organic phase in a glass vessel. Once equilibrium has been reached the concentration of the solute in one or both phases is measured. This is known as the shake flask method (Lyman, 1982).

The range of possible values for P covers several orders of magnitude, therefore it is most convenient to express the partition coefficient in terms of its logarithm to base 10 (log. P).

The precision and accuracy of partition coefficients depend heavily upon numerous experimental factors:

a) The pH and the ionic strength of the aqueous phase may influence the degree of ionisation of the solute if this contains basic or acidic functionalities (Scherrer & Howard, 1977).

b) The nature of any buffer may influence ion-pair formation. An ionic solute may be stabilised by a counter ion present in the buffer which may affect its affinity for the aqueous phase.

c) The purity of the organic phase, which may affect the solubility of the solute and may result in analytical interference.

d) The purity and stability of the solute, solute concentrations, the ideality of the solution (formation of dimers, aggregates, micelles) the problems of centrifugation (formation of emulsions) and the mutual solubility of the two phases may influence the analytical method (Brookes et al., 1986). It has been reported that log. P is a function of the solute concentration for concentrations greater than 0.01 mol/l.

The shake flask method is normally restricted to compounds with log. P values of less than 4 units in order to reduce these effects (Lyman, 1982., Brookes et al., 1986).

1.1.1.2.1. Validation of a slow stir method for measurement of log. P_{ow}.

A novel method for the determination of log. P values for solutes with log. P > 5 has been described by Brookes et al. (1986).

The method describes the difficulties in determining log. P values for chemicals with high values of P when using the traditional shake flask method. Large volumes of equilibrated aqueous phase are usually required for analysis and traces of octan-1-ol in this sample can lead to large errors. Contamination of the aqueous phase is a particular problem in shake flask experiments because emulsion formation in the aqueous layer often occurs during shaking. The technique described to surmount some of these problems was to equilibrate the water and octan-1-ol phases under conditions of slow stirring. The octan-1-ol containing the compound being tested was floated onto the water phase in a small conical flask. A glass sampling tube was dipped into the aqueous phase to allow sampling but preventing contamination of the sampling pipette with the octan-1-ol phase. The flask was stirred slowly using a magnetic bar and equilibration between the two phases was reported to be achieved within 2 to 3 days.

In order to validate this method for determining the lipophilicities of the retinoids, a number of simple

experiments were performed in order to determine the solubility of octan-1-ol in the aqueous phase of the slow stir flask. Slow stir flasks, containing water (90 ml) and octan-1-ol (10 ml) were prepared and allowed to stir for 72 hours. Samples of the aqueous phase were removed from the flasks and analysed for the presence of octan-1-ol using the gas-liquid chromatographic (G.L.C.) method described below.

1.1.1.2.2. G.L.C. method for the analysis of octan-1-ol in water.

The G.L.C. system comprised a PYE 104 gas chromatograph fitted with a 1.5 m Porapak Q column. Nitrogen carrier gas was passed down the column at a flow rate of 85 ml/min. A flame ionisation detector was used with an air flow of 10 ml/min and a hydrogen flow of 17 ml/min.

The column temperature was adjusted to 230°C and the injector and detector temperatures to 250°C.

A stock standard of octan-1-ol (37.00 mg/l) was prepared by weighing 37.00 mg into a 1000 ml volumetric flask and diluting to volume using double distilled water. A second standard (18.50 mg/l) was prepared by diluting the standard with an equal volume of water.

Samples (5 μl) of the aqueous octan-1-ol standard or the aqueous phase from the slow stir flask were injected onto the G.L.C. system.

1.1.1.2.3. Results and discussion.

Using the GLC method described, octan-1-ol eluted at a retention time of 25.8 minutes.

The peak heights of the octan-1-ol peaks were measured on samples taken from the aqueous phase of the slow stir flask and were compared with those measured for the octan-1-ol standard solutions. The octan-1-ol concentration in the aqueous phase of the slow stir flask was estimated to be 1.36 ($\pm$ 0.09) x 10^{-4} mol/l (17.7 p.p.m.). To permit a clearer understanding of the proportion of octan-1-ol in water, concentrations of octan-1-ol have also been expressed in parts per million (p.p.m.).

Previous reports of the concentrations of octan-1-ol in water and water in octan-1-ol have been reported on samples taken from a shake flask (Leo et al., 1971). At equilibration the aqueous phase was reported to contain 4.5 x 10^{-3} mol/l (585 p.p.m.) of octan-1-ol.

An explanation for the difference in concentration estimated using the two methods may be the presence of a micro-emulsions of octan-1-ol in water formed during vigorous shaking in the shake flask method. It seems likely that slow stirring will prevent the formation of such emulsions and these data may be closer to the true solubility of octan-1-ol in water.

These data support the hypothesis that both the slow

stir flask method and the shake flask method would tend to underestimate values of log. P for solutes with log. P values greater than 5. For example, in a slow stir flask containing a solute with a log. P of 6, there will be 17.7 times more solute dissolved in the octan-1-ol which is present in the aqueous phase than the amount of solute dissolved in the water itself. This predicted negative deviation between the log. P data, measured using the slow stir flask technique, and the theoretical log. P would explain some of the discrepancies between experimentally determined and computer predicted log. P values reported in the original text (Brookes et al., 1986).

1.1.2. Other methods for the measurement of log. P.

Many other methods have been used to determine log. P. These include the use of a continuous solvent extraction method (Davis et al., 1976), segmented continuous flow (Kinkel & Tomlinson, 1980), potentiometric titrations, automated log. P measurements using a segmented continuous flow technique (Garst & Wilson, 1984) and liquid membrane electrodes (Staroscik & Blasciewicz, 1985). Various workers have also correlated the lipophilicity of a number of compounds with their respective chromatographic retention parameters and some of these methods are considered below.

1.1.2.1. Chromatographic methods for measurement of log. P.

Many authors have correlated log. P measured using the shake flask method with the logarithm of capacity factors (log. k') using an high performance liquid chromatographic (hplc) method (Bruggeman et al., 1982., Thus & Kraak, 1985., Minick et al., 1987., Miyake et al., 1986., Sabatka et al., 1987) or the retention parameter (R_m) measured from a thin layer chromatographic system (Equation 2 and Equation 3) (Hulshoff & Perrin, 1976).

$$R_m = \log. k' = \log. ((1/R_f)-1)$$

Equation.... 2

$$R_f = \frac{\text{distance travelled by sample spot}}{\text{distance travelled by eluent}}$$

Equation.... 3

R_m = thin layer chromatographic retention parameter.

k' = capacity factor.

These various authors have claimed to demonstrate good correlations between log. P and R_m, but only for congeneric series of compounds such as the n-alkyl and chloro-substituted benzenes (Thus & Kraak, 1985), phenols, anilines and nitroimidazole derivatives (Carlson et al., 1975). None made any

predictions of log. P on the basis of experimentally determined log.k′ values.

1.1.2.2. Experimentally determined log. P values from group contribution or π-fragment constant data.

The partition coefficient for most drugs can be predicted reasonably well by several functional group contribution approaches. Either the Hansch π-system (Leo et al., 1971., Leo et al., 1975), or the Rekker f-system (Rekker, 1977., Rekker & de Kort, 1979).

Both systems are based on the addition of various functional groups or fragmental constants to the overall properties of a molecule and log. P values may be calculated from the Equation 4a (Rekker) and Equation 4b (Hansch).

$$\log. P = \sum f_n \times a_n \qquad \text{Equation .....4a}$$

f_n= fragmental constant for a molecular fragment n

a_n= number of times this fragment appears

$$\log. P_{(RG)} = \log. P_{(RH)} + \pi_G \qquad \text{Equation .....4b}$$

$\log. P_{(RG)}$ = Compound containing a constituent group, G.

$\log. P_{(RH)}$ = Parent compound.

π_G = Constant, which is characteristic for a given atom or group.

The Hansch system has been reported to be more

convenient when log. P of the parent compound is known and a single group is changed from parent to drug (Lyman et al., 1982). The Rekker system is simpler if no data on the log. P are available and calculations are based on the complete chemical structure (Lyman et al., 1982).
These two methods give differences in their predictive values (Van der Waterbeemd & Testa, 1987). For example, DDT and propanolol have experimental log. P values of 5.98 and 3.56, respectively using the shake flask method (Van der Waterbeemd, 1986) but the Rekker and Hansch methods predict values of 7.56 and 3.66, and 6.89 and 2.90 respectively (Leo et al., 1971., Leo et al., 1975, Rekker, 1977., Rekker & de Kort, 1979).
Van der Waterbeemd & Testa (1987), have studied the partitioning behaviour of protonated amines and concluded that, "predictive models of averaging values such as existing fragmental systems are acceptable only as a first approximation, with predictive values ranging from excellent to illusory."

1.1.2.2.1. Calculation of log. P values for the retinoids using a computer database of fragmental data.

A computer modelling program was used (Compudrug ProLog P. version 2.) which contained a database of

the Rekker fragmental constants. After entry of the various constituent groups of a molecule into a spreadsheet the log. P value for the composite molecule was computed. Data could be entered into the program in number of forms:

a) as the constituent atoms in the molecule e.g. carbon (C), hydrogen (H), oxygen (O), sulphur (S).

b) as the main functional groups in the molecule e.g. methyl (CH_3), carboxylic acid (COOH), alkenyl (C=C).

or c) as the larger constituent fractions within the molecule e.g. napthyl, phenyl.

Log. P values were calculated using this program (Rekker system) for a series of six retinoids; retinol (Ro 01-4955), tretinoin (Ro 01-5488), isotretinoin (Ro 04-3780), acitretin (Ro 10-1670), etretinate (Ro 10-9359) and temarotene (Ro 15-0778). These values were compared with log. P data obtained on the same molecules using the Hansch system (Van der Waterbeemd, 1990).

1.1.2.2.2. <u>Results and Discussion.</u>

The calculated values for log. P using the methods of Rekker and Hansch are displayed in Table 2.

It can be seen that each method gave slightly different results for the same retinoids but the general trend for this series of compounds was similar. Retinol (Ro 01-4955) showed the lowest

Table 2. Calculated values of log P for a selection of retinoids using Hansch and Rekker fragmental data.

Retinoid	Log P Rekker method *	Log P Hansch method #
Ro 01-4955	6.908	6.203
Ro 01-5488	6.921	6.614
Ro 04-3780	6.921	6.614
Ro 10-1670	7.718	6.115
Ro 10-9359	8.625	7.020
Ro 15-0778	8.925	8.400

#.. From Van der Waterbeemd (Van der Waterbeemd, 1990))

*.. From Compudrug (ProLog P, Version 2).

values (least lipophilic) of 6.908 and 6.203 for the Rekker and Hansch methods respectively and the arotinoid (Ro 15-0778) gave the highest values (most lipophilic) of 8.950 and 8.481. All the retinoids showed log. P values in excess of 6 and could therefore be classed as highly lipophilic.

A number of points should be stressed with respect to calculated log. P values;

i) Hansch and Leo have been cited by Lyman (1982) as saying that values of log. P greater than 6 are likely to be over-estimates of experimentally determined log. P perhaps by one or more log units.

ii) neither the Rekker or Hansch methods take into consideration the ionisation of polar groups in the molecule and deal with the situation where the molecule is completely unionised. Calculated values for log. P obtained for compounds such as Ro 04-3780 or Ro 10-1670, which contain a ionisable acid group and will partially ionise in an aqueous environment, will be overestimates of experimentally determined log. P. It is however, simple to correct these calculated log. P values if the pH of the solvent and pKa of the molecule is known.

iii) calculation of log. P values using the Rekker fragmental data and using the three methods for entering structural data as described in 1.1.2.2.1. gave identical log. P values for all the retinoids estimated.

The data for log. P calculated here confirmed the predicted increase in lipophilicity in the three compounds selected for the lymphatic absorption studies.

Ro 04-3780 (Isotretinoin - Roaccutane - 13 cis-retinoic acid) was one of the least lipophilic of those studied (log. P = 6.921 or 6.614).

Ro 10-9359 (Etretinate - Tigason) was found to be approximately ten times more lipophilic (log. P = 8.625 or 7.020) than Ro 04-3780. This compound is the ethyl ester of the aromatic acid derivative of retinoic acid.

Ro 15-0778 (Temarotene) is approximately ten times (log. P = 8.925 or 8.480) more lipophilic than Ro 10-9359 and one hundred times more so than Ro 04-3780. These data are displayed in Table 2.

1.2. Solubility studies of selected retinoids in a range of oils, oil mixtures and self-emulsifying oil systems.

The measurements of partition coefficient and oil solubility are often used as estimates of the lipophilicity of a particular molecule. As discussed in section 1, the partition coefficient is defined as the ratio of the equilibrium solution partitioning of a drug between an organic and an aqueous phase (most often octan-1-ol and water). The solubility of a compound in oil is an absolute

measurement of the solubility of a solute in equilibrium with its pure phase. It is possible for a compound to have a large log. P value but not necessarily a high solubility in an oil whilst another compound may be very soluble in an oil but have a relatively low log. P value (Charman & Stella, 1986). In addition a high solubility of a compound in a solvent such as octan-1-ol will not always relate to a high solubility in a poorly solvating, largely hydrocarbon vehicle such as a long chain fatty acid triglyceride (Charman & Stella, 1986b).

It was decided to study the solubility of the three chosen retinoids (Ro 04-3780, Ro 10-9359 and Ro 15-0778) in a range of oils, oil mixtures and self-emulsifying oil systems which were thought to be possible candidates for use in the oral dosing studies in the rat. A complete list of oil systems is shown in Table 3.

1.2.1.1. <u>The determination of the solubility of selected retinoids in a series of oil systems.</u>

a) Duplicate measured volumes of each oil (1.0 ml) were transferred by positive displacement pipette into microcentrifuge tubes (1.5 ml). One pair of tubes was used for each retinoid to be tested.

b) Small additions (5 - 10 mg) of the retinoid were accurately weighed into each tube, to give a known

Table 3. **Oil systems used in the solubility studies of three selected retinoids.**

1) Free fatty acids.

Caprylic acid $C_{8:0}$

Oleic acid $C_{18:1}$

Linoleic acid $C_{18:2}$

2) Monoglyceride oils.

Glyceryl monocaprylate

Glyceryl monooleate

3) Mixed medium chain monoglyceride.

Neobee M5 Glyceryl monocaprylate $C_{8:0}$ and Glyceryl monocaprate $C_{10:0}$

4) Mixed medium chain triglyceride.

Miglyol 812 Glyceryl esters of caprylic (approx. 55%) and capric acid (approx. 45%).

5) Mixed long chain triglycerides (Altman & Dittmer, 1972).

	Palmitate $C_{16:0}$	Oleate $C_{18:1}$	Linoleate $C_{18:2}$
Cottonseed	26%	18%	52%
Soyabean	12%	24%	51%
Peanut	12%	53%	26%
Trilinolein	--	--	99%

concentration in the oil.

c) The oil samples were placed in an ultrasonic water bath for 30 minutes (during which time the temperature of the samples rose from 24°C to 55° - 60°C).

d) The samples were allowed to equilibrate at a temperature of 24°C and each tube was examined for visible signs of precipitation.

e) After centrifugation (3000 x g for 10 minutes), small aliquot of the oil was removed from each tube for analysis. In oil samples where no visible signs of precipitation was observed, steps b) to e) were repeated until visible precipitation was observed.

f) Quantitative analysis of the retinoid in each sample of oil was carried out using the hplc method as described below.

1.2.1.2. Method for the quantitative analysis of retinoids in oil samples.

Analysis of the retinoid concentration in the oil samples was carried out using the hplc method described in Chapter 2 and summarised in Table 6. For analysis, standard solutions of the three retinoids were prepared by accurately weighing the pure compounds into a volumetric flask and diluting with THF (tetrahydrofuran, hplc grade). Standard solutions contained 6.88 mg/ml of Ro 04-3780,

9.00 mg/ml of Ro 10-9359 or 12.85 mg/ml of Ro 15-0778.

An aliquot (approximately 0.15 ml) of each oil sample was transferred by positive displacement pipette into a Millipore Ultrafree filter unit (0.1 μm) and centrifuged (10,000 x g for 20 minutes) to remove undissolved retinoid. A small accurately weighed sample (10.0 - 50.0 mg) of the filtered oil was dispensed into a 10 ml volumetric flask and adjusted to volume using THF. THF was found to disperse and solubilise the oil samples completely and was also miscible with the hplc mobile phase. A sample (25 μl) of the retinoid in THF was injected onto the appropriate hplc system using a fixed volume loop injection system. Peak areas were calculated using a Shimadzu CR3-A computing integrator and retinoid concentrations were calculated by comparison of the peak areas of the oil samples with the standard retinoid solutions. Retinoid concentrations measured on oil samples to which accurately weighed additions of retinoid had been made, but in which the retinoid was completely soluble, were used to assess the recovery of retinoid from the oils.

1.2.1.3. Results and Discussion.

From the analysis of the concentration of retinoid measured in a fixed volume of oil (1 ml) containing

accurately weighed additions of retinoid, an estimate of the mean recovery of added retinoid of 95.4% (± 4.25) was achieved after dispersion of the oil in THF (compared with only 77.8% (± 4.28) when oil was dispersed in mobile phase).

The concentration of the retinoid in each oil system was adjusted for the density (obtained from the suppliers' data sheets) of the oil and expressed as milligrams retinoid per millilitre of oil. These data are shown in Table 4.

The oil solubility data for the three retinoids were consistent with the data reported by Roche Products Ltd. The solubility of Ro 04-3780 in peanut oil was 6.1 mg/ml (compared with 3.0 mg/ml (Weber & Felix, 1983)), the solubility of Ro 10-9359 in peanut oil was 22.0 mg/ml (compared with 26.0 mg/ml (Weber & Felix, 1983)) and the solubility of Ro 15-0778 was 144.9 mg/ml (compared with greater than 100 mg/ml (Weber et al., 1983).

The oil solubility data for the three retinoids show a number of common trends.

a) For any oil type, Ro 15-0778 showed the greatest solubility, Ro 10-9359 was the second most soluble and Ro 04-3780 the least soluble. This decrease in solubility relates closely to the decrease lipophilicity as defined by the respective log. P values (Table 2) of these compounds.

b) All retinoids showed poor solubilities in the

Table 4. Solubility (mg/ml) at 24°C. of three retinoids in a range of oils, oil mixtures and self-emulsifying systems.

Ro 15-0778		Ro 10-9359		Ro 04-3780	
Monocaprylin	47.8	Monoolein	6.60	Monoolein	2.16
Monoolein	58.0	Monocaprylin	16.0	Trilinolein	2.51
A.T.S.	79.3	Oleic acid	20.9	Cottonseed	5.10
A.T.L.	89.2	Soyabean	21.2	Soyabean	5.68
M.T.L.	103.8	Cottonseed	21.3	Peanut	6.10
Linoleic	109.5	Peanut	22.0	Neobee	11.1
Soyabean	126.8	A.T.S.	22.8	Miglyol 812	12.0
Trilinolein	139.5	A.T.L.	23.5	A.T.L.	15.8
Peanut	144.9	Linoleic	25.1	A.T.S.	15.9
Oleic	154.1	Mig:Oleic	26.3	M.T.S.	23.8
Mig:Linoleic	155.8	Mig:Linoleic	27.4	Oleic	24.4
Mig:Oleic	165.3	Trilinolein	33.2	M.T.L.	25.3
Cottonseed	165.5	M.T.S.	33.5	Monocaprylin	26.9
Miglyol 812	170.6	M.T.L.	35.2	Linoleic	30.5
M.T.S.	171.7	Mig:Caprylic	36.0	Mig:Linoleic	35.7
Mig:Caprylic	173.2	Neobee	42.1	Mig:Oleic	36.7
Caprylic	196.2	Miglyol 812	44.5	Mig:Caprylic	49.5
Neobee	201.8	Caprylic	68.7	Caprylic	73.3

Notes:
i) Mig:Linoleic, Mig:Oleic and Mig:Caprylic contain equal volumes of Miglyol 812 and either linoleic acid, oleic acid or caprylic acid.
ii) A.T.S., A.T.L., M.T.S. and M.T.L. are self-emulsifying oil systems containing, 16% Tween 85 (T) and either, 80% peanut oil (A) or 80% Miglyol 812 (M) and either 4% Span 85 (S) or 4% Labrasol (L).
iii) A full list of oils is shown in table 2.

long chain unsaturated oil, monoolein and medium chain saturated fatty acyl glycerol oil, monocaprylin. However, in the mixed monoglyceride oil Neobee M5, which contains glyceride esters of both capric or caprylic acid, the more lipophilic retinoids Ro 10-9359 and Ro 15-0778 showed higher solubilities than in the respective medium chain fatty acyl monoglycerol oil.

c) The solubilities of the retinoids in mixed long chain fatty acyl triglycerol oils (peanut, cottonseed and soyabean) were generally low, and consistently lower than solubilities measured in the mixed medium chain fatty acyl triglycerol oil (Miglyol 812).

d) The solubilities of retinoids in the mixed medium chain fatty acyl monoglycerol oil (Neobee M5) and mixed medium chain fatty acyl triglycerol oil (Miglyol 812) were similar. This observation may be anticipated, since these oils contain the same types of medium chain fatty acid esters.

e) The solubilities of the retinoids in the medium chain fatty acid (caprylic) were very high, however solubilities in long chain (unsaturated) fatty acids (linoleic and oleic acid) were variable and generally found to be intermediate in value between caprylic acid and the mixed LCFA triglyceride oils.

f) There is a trend in the relative solubilities of the retinoids in a range of different oil systems as

the lipophilicity of the retinoids increase. For example: Linoleic acid has a solubility of Ro 04-3780 almost six times greater than that found in cottonseed oil (Linoleic/Cottonseed = 5.98). The ratio for the solubility of Ro 10-9359 in these oils was near to unity (Linoleic/Cottonseed = 1.18) and was less than one (Linoleic/Cottonseed = 0.66) for Ro 15-0778.

g) Some of the solubility data shown in Table 4 are surprising. For example; the solubility of Ro 04-3780 in Miglyol:oleic acid is considerably higher than in either component alone, whereas for the other retinoids it lies, as expected, between the two values. This effect may be explained by some specific interaction between the retinoid and the oil. These may include hydrophobic interactions (Van der Waals forces) and hydrogen bonding.

When comparing the solubility of retinoids in the self-emulsifying oil systems (SES) containing 80% peanut oil with peanut oil alone, one might have predicted the solubility to be 20% lower to reflect the reduction in oil content. Ro 15-0778 gave a much lower solubility in this SES, which is difficult to explain, unless the surfactant systems have some desolubilising effect on this compound influenced by the ionic nature of the SES or the retinoid.

The increased solubility of the more polar Ro 04-3780 in SES compared with its solubility in

oil alone, may also be explained by a solubilisation effect.

1.3. The molar absorptivity (ε) and absorbance maximum (λ_{max}) of the retinoids.

The molar absorptivity (ε) and absorbance maximum (λ_{max}) of the retinoids were measured in order to determine their minimum detectable absorbance on the proposed hplc system and to determine their optimum (maximum) analytical absorption wavelength.

1.3.1. Method for the measurement of ε and λ_{max}.

Stock solutions of three retinoids were prepared by accurately weighing small samples (10.0 - 100.0 mg) of each compound into a 100 ml volumetric flask and adjusting to volume using hplc grade methanol.
An appropriate dilution of these stock solutions was prepared in methanol and placed in an ultraviolet spectrophotometer and the absorbance was measured between 250 and 400 nanometres. The absorbance maximum (λ_{max}) was located and the molar absorptivity was calculated from Beer's law (Equation 5).

$$A = \varepsilon.b.c \qquad \text{Equation.... 5}$$

A = Absorbance at a specified wavelength.

ε = Molar absorptivity.

b = Path length of the measuring cuvette (1 cm)

c = Molar concentration of the solution.

In order to avoid any deviation from linearity due to instrumental or chemical factors, the concentration at which measurements were taken was adjusted to give absorbance readings within a linear concentration range of 0.3 to 0.8 absorbance units (Willard et al., 1988).

1.3.2. Results and conclusions.

The calculated molar absorptivity (ε) and λ_{max} of the three retinoids are shown in Table 5.

From these data it can be seen that all three compounds show strong absorbance maxima in the ultraviolet region of the spectrum, with molar absorptivities of 23,800 $l.mol^{-1}.cm^{-1}$. (Ro 15-0778), 43,600 $l.mol^{-1}.cm^{-1}$. (Ro 04-3780) and 51,000 $l.mol^{-1}.cm^{-1}$ (Ro 10-9359), these data are in close agreement with those reported by Groenendijk et al. (1980) for a series of vitamin A analogues, including the all-trans isomers of retinol (52,100 $l.mol^{-1}.cm^{-1}$) and by Szuts & Harosi (1991) for retinal (42,400 $l.mol^{-1}.cm^{-1}$) retinol (38,300 $l.mol^{-1}.cm^{-1}$) and all-trans retinoic acid (49,700 $l.mol^{-1}.cm^{-1}$.). Ro 04-3780 and Ro 10-9359 show a λ_{max} around 350 - 360 nm, whereas the arotinoid, Ro 15-0778 has an λ_{max} value further into the ultraviolet at 279 nanometres correlating with the conjugated aromatic structure of this compound.

Table 5. Molar absorptivities and absorption maxima of the retinoids.

Retinoid	Molar absorptivity ($litre.mol^{-1}.cm^{-1}$)	Absorption maximum (nanometres)
Ro 04-3780	4.358×10^4	349
Ro 10-9359	5.192×10^4	360
Ro 15-0778	2.388×10^4	279

1.4. Effects of light exposure on the retinoids.

It is known that the retinoids undergo photo-isomerisation and decompose in the presence of short wavelength radiation (Brazzell & Colburn, 1982).
In view of this reported photo-degradation, care was taken to protect all retinoid samples from light. For this purpose a darkroom was set up and fitted with "safelights" which did not emit ultraviolet or visible light below 500 nanometres. This room was designated the "yellow room" and all unprotected procedures using retinoids were performed in this room. Procedures performed outside this room were protected from light, the samples being wrapped in aluminium foil.
In order to assess the effect of light on the handling of retinoid samples during in-vivo and in-vitro procedures it was decided to test the effect of various lighting conditions on the stability of a model retinoid, Ro 04-3780.

1.4.1. Method to study the effects of light on the retinoids.

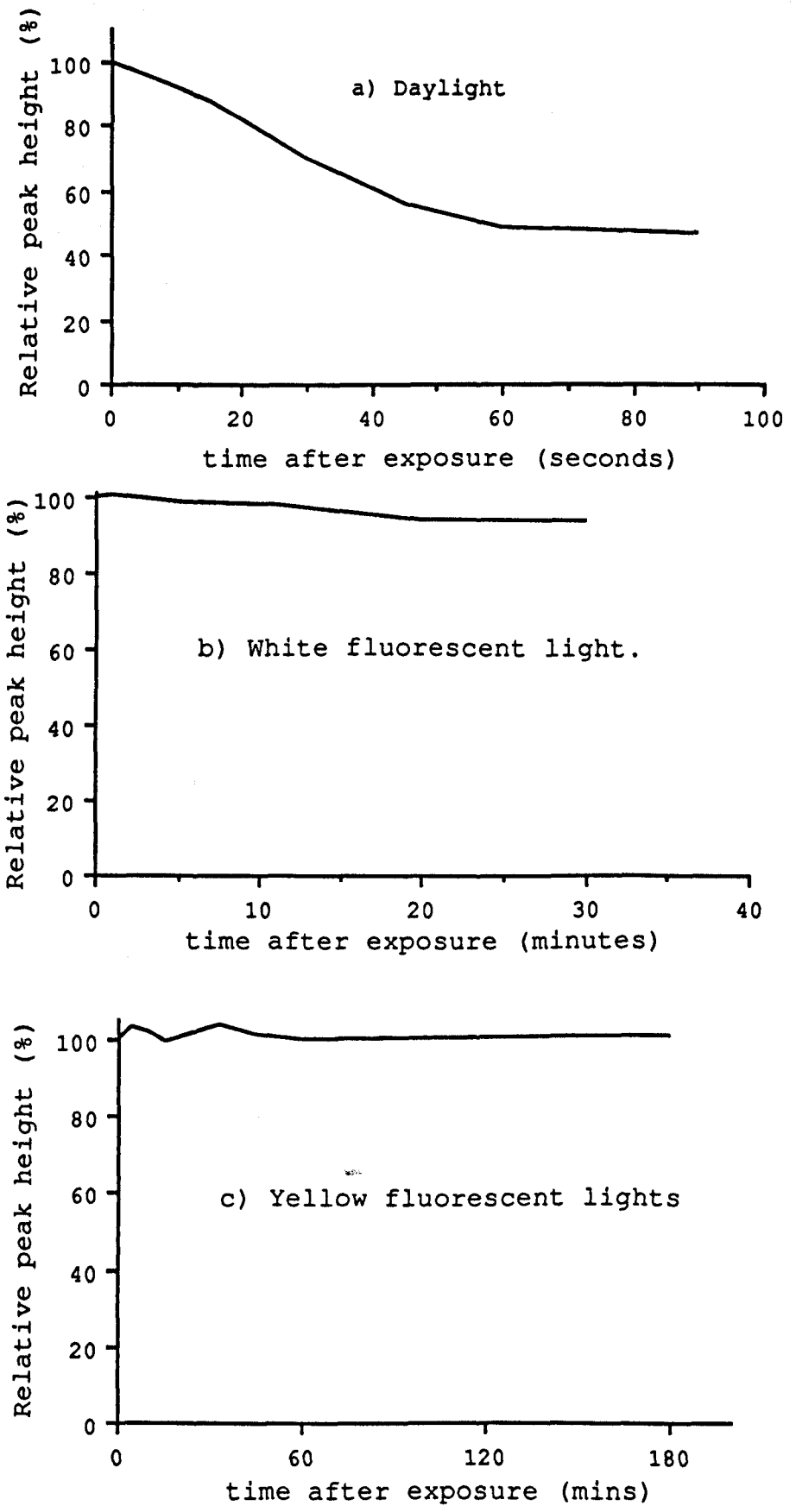
A series of experiments were performed in order to investigate the effects of various lighting conditions on the stability of 04-3780.
A stock solution containing 5 µg/ml of Ro 04-3780 was prepared in methanol, under protective lighting conditions, and was divided into four samples.

The first sample was wrapped in foil and kept in complete darkness for the duration of the procedure. This acted as the control sample.
The second sample was exposed to light in the "yellow room".
The third sample was exposed to the light from standard "daylight" fluorescent tubes in the laboratory.
The fourth sample was exposed to daylight.
At timed intervals after exposure to light, samples from each of the test groups were injected onto the hplc system described in Chapter 2.
The chromatographic peak height was measured for each sample and compared with the peak height for the control sample (sample one). The data showing the change in relative peak heights against time of exposure to light are displayed in Figure 4.

1.4.2. Results and conclusions.

The three different lighting conditions used in this procedure were selected to represent typical conditions which might apply during the handling of retinoids.
Under protective yellow lighting conditions over a 180 minute period, changes in the peak height for Ro 04-3780 were not significantly ($p > 0.05$) different from the control sample.
After exposure to standard laboratory fluorescent

Figure 4. Effect of light exposure on the hplc peak height of a Ro 04-3780 standard solution.



lighting conditions for 30 minutes, there was a decrease in the peak height for Ro 04-3780 of 6.2% compared with the control sample. This change in peak height was significantly different ($p < 0.05$) from the control sample.

After only 60 seconds exposure to daylight (not direct sunlight) more than 50% of the peak height in the sample was lost ($p < 0.001$).

Although the peak height of Ro 04-3780 decreased in the presence of light, any conversion to the all-trans isomer, Ro 01-5488, could not be detected and decomposition to any other metabolites which could be detected on the hplc system was not observed (Brazzell & Colburn, 1982).

The following conclusions can be drawn from the results of exposure to lights of various types:-

a) The retinoids are extremely sensitive to the effects of light.

b) The retinoids must be protected from exposure to daylight. All procedures involving retinoids and samples containing retinoids, should be performed under safelights and samples should be protected from light during storage by the use of aluminium foil wrapping.

c) A brief exposure of samples to laboratory lighting was deemed as being acceptable. However, when samples are to be stored for long periods, such as when large batches of extracted retinoid samples

were being analysed using the hplc autosampling injector, the sample rack was darkened using a wrapping of aluminium foil and repeated standard solutions were interspersed within the batch to allow correction for photo-decomposition during the analysis period.

CHAPTER 2.

ANALYTICAL METHODS FOR THE QUANTITATIVE ANALYSIS OF RETINOIDS IN LYMPH, PLASMA AND OIL SAMPLES.

2.1. Introduction.

All retinoids are essentially lipophilic in nature and they may be therefore analysed using either normal phase; (De Leenheer et al., 1990., McNamara & Blouin, 1990., Rhys-Williams, 1985) or reversed phase; (Vane et al., 1982., Curley et al., 1987., Kalin et al., 1984., Cotler et al., 1983., Bugge et al., 1985., Cavina et al., 1988., Bhat et al., 1988) high performance liquid chromatography (hplc).

Normal phase chromatography uses a polar stationary phase (often hydrophilic) such as a silica or a cyano-bonded silica packing and a less polar mobile phase. To select an optimum mobile phase, it is common to start with a pure hydrocarbon mobile phase such as heptane or hexane. If the sample is strongly retained, the polarity of the mobile phase increased, normally by the addition of small amounts of a polar solvent such as methanol or dioxan. In the normal phase mode, water is the strongest eluant and care must to be taken to protect the column from aqueous solvents.

Reversed phase chromatography uses a hydrophobic column packing, usually with a octadecyl (C_{18}) or octyl (C_8) functional group and a polar mobile phase, often a partially or fully aqueous mobile phase. Polar substances are more soluble in the mobile phase and elute first. As the hydrophobic character

of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher is its eluant strength. Water is the weakest eluant. Methanol and acetonitrile are popular solvents because they have low viscosity and are readily available with excellent purity. Eluants intermediate in strength between these solvents and water are usually obtained by preparing mixtures (Willard et al., 1988).

A reversed phase chromatographic system is now the system of choice for the analysis of compounds in biological fluids. The use of such a system removes the problems associated with the need for conditioning of a normal phase column to remove polar materials which may become attached to the column packing. The reversed phase system also offers the advantages of using less organic solvents, faster equilibration with the mobile phase and their more established use in analyses of this kind.

The hplc methods which were to be developed for the analysis of retinoids had to be fulfil the following criteria;

a) Allow the extraction and quantitation of the retinoid in a biological sample such as plasma or lymph.

b) Allow the extraction and quantitation of the retinoid from the oil systems which were to be used

in the in-vivo and in-vitro studies.

c) Since the plasma and lymph sample volumes and the concentrations of the retinoid in the samples were expected to be small, the assay must therefore have a low limit of detection (10 ng/ml in 20 - 100 μl samples)

d) Be suitable for the extraction and analysis of large batches of samples.

e) Be able to resolve the parent compound from any of its possible isomers or metabolites and also from the internal standard.

2.1.1. The hplc assay for Ro 04-3780.

The first system to be developed (Table 6) was for the analysis of Ro 04-3780. Ro 11-5036 was chosen as an internal standard because it was structurally similar to Ro 04-3780 (Figure 3), had a similar absorption maximum to Ro 04-3780 and was readily available in a pure form. It was therefore expected to elute with a similar retention time to Ro 04-3780 on the proposed hplc system.

2.1.1.1. Preparation of standard solutions.

It has been established (Section 1.4) that the retinoids are sensitive to the effects of light. All unprotected procedures involving the use of retinoids were therefore performed in the "yellow" room.

Table 6. **HPLC systems used for the quantitative analysis of retinoids in oils, rat plasma and lymph samples.**

Retinoid:	**Column:**	**Mobile phase:**
Ro 04-3780	Spherisorb S5ODS-2	Acetonitrile : 0.1 M Ammonium acetate pH 6.0 (70 : 30) **Detection:** 350 nm
Ro 10-9359	Spherisorb S3CN	Hexane : Methyl benzoate : propionic acid (991.5 : 6 : 2.5) **Detection:** 360 nm
Ro 15-0778	Spherisorb S5ODS-2	Acetonitrile : 0.1 M Ammonium acetate pH 6.0 (90 : 10) **Detection:** 280 nm

a) Ro 04-3780 standard solution.

A stock standard solution of Ro 04-3780 (approximately 100 µg/ml) was prepared by dissolving an accurately weighed sample of the compound into a volumetric flask and diluting to volume using hplc grade acetonitrile. This stock solution was further diluted in acetonitrile to give a range of working standards from 50 ng/ml to 1000 ng/ml.

b) Internal standard.

The aromatic retinoid, Ro 11-5036 was weighed into a volumetric flask and diluted in acetonitrile to give a working internal standard solution of 1000 ng/ml.

c) Other retinoids.

An acetonitrile solution (approx. 1000 ng/ml) of the all-trans isomer of Ro 04-3780 (Ro 01-5488) was also prepared. This standard solution was used to assess any interference from this compound resulting from isomerisation of Ro 04-3780 to Ro 01-5488 during either in-vivo or in-vitro procedures.

2.1.1.2. Selection of analytical column.

A reversed phase column (5 µm Spherisorb S5ODS) was chosen for the analysis. This stationary phase material consisted of silica which was covalently bonded to an aliphatic (octadecyl) hydrocarbon molecule. Residual sites on the silica were end-capped to decrease the retention of polar components of a sample. End capping is a secondary reaction of

silanol groups with trimethyl silyl chloride to block these polar positions.

2.1.1.3. Selection of mobile phase.

The mobile phase was based on that used by Jakobsen *et al.* (1987) and Curley *et al.* (1987) and contained acetonitrile and 0.01 M ammonium acetate buffer pH 6.0. The relative proportions of the two components were adjusted using a solvent scouting technique. A gradient hplc system was employed to deliver acetonitrile through pump-A and 0.01 M ammonium acetate buffer pH 6.0 through pump-B. With a combined flow rate of 1 ml/min the proportions of the two solvents were adjusted between 60% and 80% acetonitrile to optimize the retention time and peak area of the Ro 04-3780 and Ro 11-5036 peaks.

2.1.1.4. Selection of detection system.

The retinoid compounds have been shown to absorb strongly in the ultraviolet region, hence UV detection was the hplc method of choice. Ro 04-3780, Ro 01-5488 and Ro 11-5036 were shown to have a broad absorbance peak around 350 nanometres and this wavelength was used in the subsequent investigations.

2.1.2. Extraction methods for Ro 04-3780 in biological samples.

2.1.2.1.1. Acid-ether extraction method.

An accepted method for extracting weakly acidic compounds such as Ro 04-3780 (Ro 01-5488) or Ro 11-5036 from an aqueous environment is to acidify the sample in order to convert the compound into the un-ionised form and to subsequently extract the compound into a non-polar, organic solvent.

The initial extraction method was similar to that used by Vane et al. (1982).

i) Plasma (100 µl) was transferred into a borosilicate glass tube (15 ml) using a positive displacement pipette. Acetonitrile (200 µl) containing the internal standard (Ro 11-5036, 1000 ng) was added to the tube.

ii) The sample was acidified with 1 molar hydrochloric acid (1 ml) and extracted into diethyl ether (5 ml) by mixing on a rotary mixer (10 minutes at 30 r.p.m.)

iii) Samples were centrifuged (2,200 x g for 10 minutes) and a sample of the upper organic phase (4 ml) was transferred into a borosilicate glass tube (15 ml) and dried under reduced pressure at 30°C in a vacuum centrifuge.

iv) Mobile phase (500 µl) was added to each sample tube using a positive displacement pipette and the

tube was rotated gently to dissolve the extract. The extracted plasma samples were transferred into hplc autosampling vials.

2.1.2.1.2. Direct protein precipitation extraction method (1).

A second method for the extraction of retinoids from plasma and lymph samples involved a direct protein precipitation method using acetonitrile.

i) Plasma samples (100 µl) were transferred into polypropylene microcentrifuge tubes (1.5 ml) using a positive displacement pipette. The whole of a lymph sample (25 - 100 mg) was analysed in the tube into which it had been collected.

Acetonitrile (100 µl) was added to each sample.

ii) Blank rat plasma samples (100 µl) to which had been added acetonitrile (100 µl) containing the appropriate concentration of retinoid standard (0, 50, 100, 200, 400, 800 or 1000 ng/ml) were extracted for use as calibration standards.

iii) To all lymph samples, plasma samples and standard tubes, acetonitrile (100 µl) containing the internal standard (Ro 11-5036, 150 ng/ml) was added.

iv) The tubes were mixed on a vortex mixer (20 seconds) and centrifuged in a microcentrifuge (13,000 x g, 10 minutes). The clear supernatant was transferred into an hplc autosampling vial.

2.1.2.1.3. Direct protein precipitation extraction method (2).

It has been reported that low ratios of organic phase to plasma volume in direct protein precipitation methods (Kabra et al., 1977., Stafford et al., 1980) result in microprecipitation of proteins which may cause column blockage (Chu et al., 1980). The organic solvent to aqueous sample ratio suggested by Kabra and Stafford (Kabra et al., 1977., Stafford et al., 1980) was one part acetonitrile to one part plasma. Chu et al. (1980) suggested the use of three parts acetonitrile to two parts plasma was necessary to prevent microprecipitation.

Acetonitrile to plasma extraction ratios of 2:1 and 3:1 were used to measure the recovery of retinoid from plasma and lymph samples. With these ratios it was hoped to improve recovery and assay sensitivity despite the increased dilution effect on the sample. Plasma and lymph samples were extracted as described above. (section 2.1.2.1.2.) except that a larger volume of acetonitrile was added to the samples in the form of 200 µl or 300 µl of internal standard (Ro 11-5036, 75 ng/ml or 50 ng/ml).

2.1.2.2. Analytical method evaluation for Ro 04-3780.

A series of analytical procedures were performed in order to validate the hplc system for the analysis of Ro 04-3780.

Samples of rat plasma or human plasma were pooled and extracted using each of the three extraction procedures described above. Analysis of these samples was performed using the hplc system and the hplc traces were examined for the presence of interfering peaks at the retention times of Ro 04-3780, its isomer (Ro 01-5488) or the internal standard.

Using duplicate blank plasma samples (100 µl) to which had been added acetonitrile (100 µl) containing 25, 50, 100, 200, 500 or 1000 ng/ml of Ro 04-3780, extractions were performed using each of the three extraction methods described above.

A parallel set of duplicate control samples were also prepared containing the standards and extraction solvent but replacing the plasma volume with water. In the acid-ether extraction method, the aqueous phase was omitted completely.

Control samples for the acid-ether standards were dried and dissolved in mobile phase (500 µl). All plasma samples and control sample extracts were injected onto the hplc system and peak areas for the Ro 04-3780 and Ro 11-5036 plasma extracts and

controls were compared to assess recovery after extraction (Equation 6).

$$\text{Recovery (\%)} = \frac{\text{Peak area (plasma extract) x 100}}{\text{Peak area (acetonitrile extract)}}$$

Equation.....6

As the concentration of an analyte approaches zero, the signal disappears into the noise and the detection limit is exceeded. The detection limit is defined as the concentration of solute which gives a signal which is significantly different from the blank or background signal. In practice, the signal obtained from a solute which differs by approximately two standard deviations from the magnitude of the background signal is used as an indication of limit of detection for the assay. The average peak height of the background signal, recorded on the hplc trace was measured. Three times the average baseline peak height was compared with the standard calibration curve from each hplc method to give an estimate of the limit of detection concentration.

2.1.3. Results.

In general, solute bands broaden gradually as they migrate through a chromatographic column. Resolution of individual solutes into discrete bands occurs only if the bands broaden to a lesser extent than

their peak maxima separate. Values of the baseline bandwidths of adjacent bands are almost constant. Since baseline bandwidth is equal to four standard deviations (σ) for a given band, resolution can be expressed using Equation 7.

$$R = \frac{t_2 - t_1}{4\sigma} \qquad \text{Equation.....7}$$

R = resolution

t_1 = retention time for band 1

t_2 = retention time for band 2

σ = standard deviation

Resolution may be improved by increasing the separation time for the bands or decreasing the bandwidth of the bands, which is a function of column selectivity or efficiency.
Using a solvent scouting technique (Section 2.1.1.3), the optimum mobile phase system was found to contain 70 parts of acetonitrile to 30 parts ammonium acetate buffer (0.01 M, pH 6.0). At a flow rate of 1 ml/min this mobile phase gave baseline separation (Table 7) of the internal standard (Ro 11-5036), Ro 04-3780 and Ro 01-5488 (resolution of Ro 01-5488 and Ro 04-3780; R = 1.384).

Table 7. **Evaluation of a series of mobile phase systems for resolving Ro 01-5488, Ro 04-3780 and Ro 11-5036.**

Acetonitrile	Ammonium acetate	Resolution Ro 01-5488/Ro 04-3780
60	40	0.773
65	35	0.803
68	32	0.952
69	31	1.282
70	30	1.384
72	28	0.751
74	26	0.672
80	20	0.583

Note: Resolution has been calculated using Equation 7 (Section 2.1.3.). Baseline resolution occurs when R > 1.000

The elution times of these compounds were;

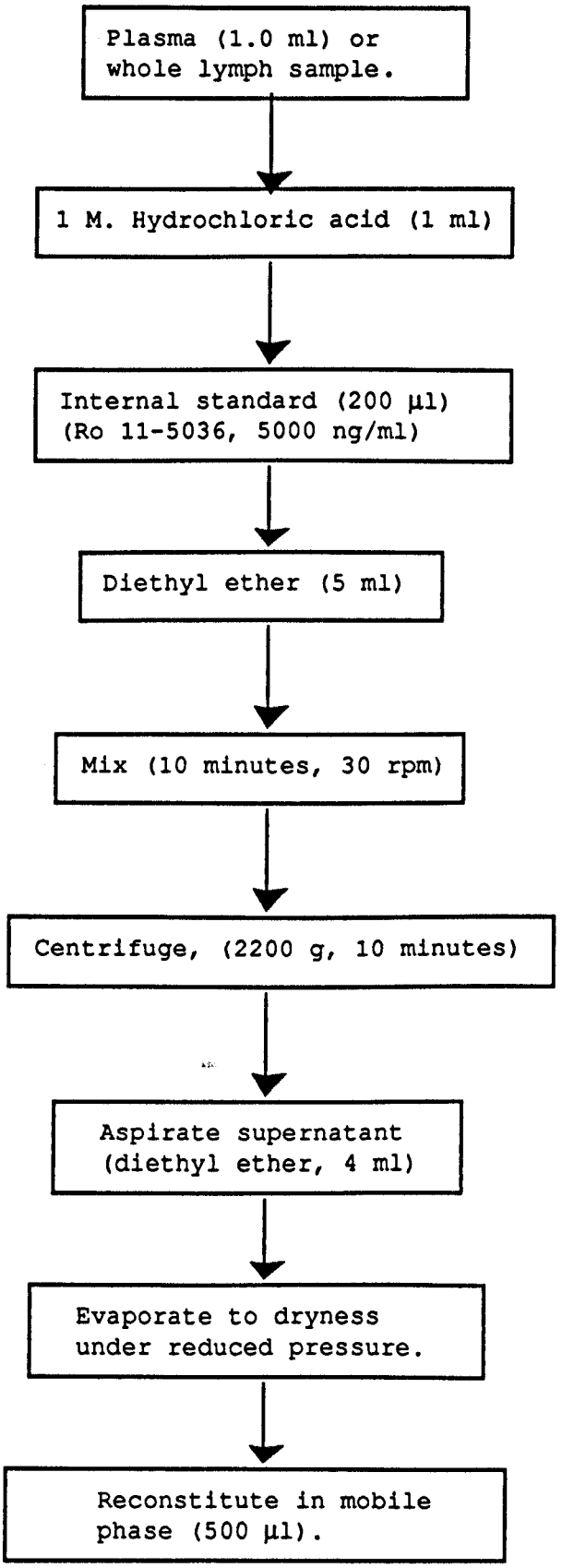
Compound	Elution time (minutes:seconds)
Internal standard.	2:50
13-*cis* Retinoic acid.	4:20
All-*trans* retinoic acid.	4:58
(Retinol.	7:50)

Using mobile phase compositions of between 60% and 80% acetonitrile (the remaining volume made up using 0.01 M ammonium acetate buffer pH 6.0) similar retention times were achieved for the three retinoids but baseline separation was not achieved. A summary of the hplc extraction methods is shown in Figure 5. For each extraction method, the recovery of Ro 04-3780 was estimated by comparison of the peak area obtained for extracted standard samples with the area obtained for direct standard injections after correcting for dilution and sample size (Table 8). A typical standard calibration curve for Ro 04-3780 is shown in Figure 6. A fixed volume sample loop injection of 100 μl was used for all the analyses.

The acid-ether extraction method gave a moderate mean recovery of 78.0 % ($\pm$ 8.3) over the range 100 to 1000 ng/ml. The high degree of variation was thought to be due to the effect of the large dilution factors when small samples of plasma (100 μl) were extracted into large volumes (5 ml) of

Figure 5. Extraction methods for Ro 04-3780 in plasma and lymph.

5.i Acid-ether extraction method.



5.ii Direct protein precipitation extraction method (1).

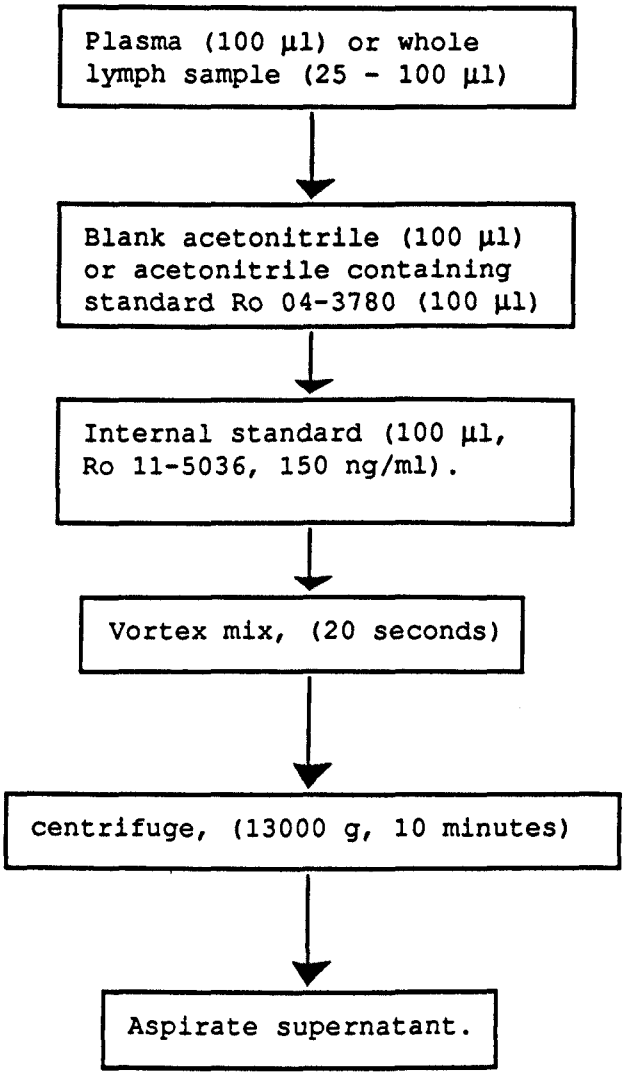


Figure 5.iii. Direct protein precipitation extraction method (2).

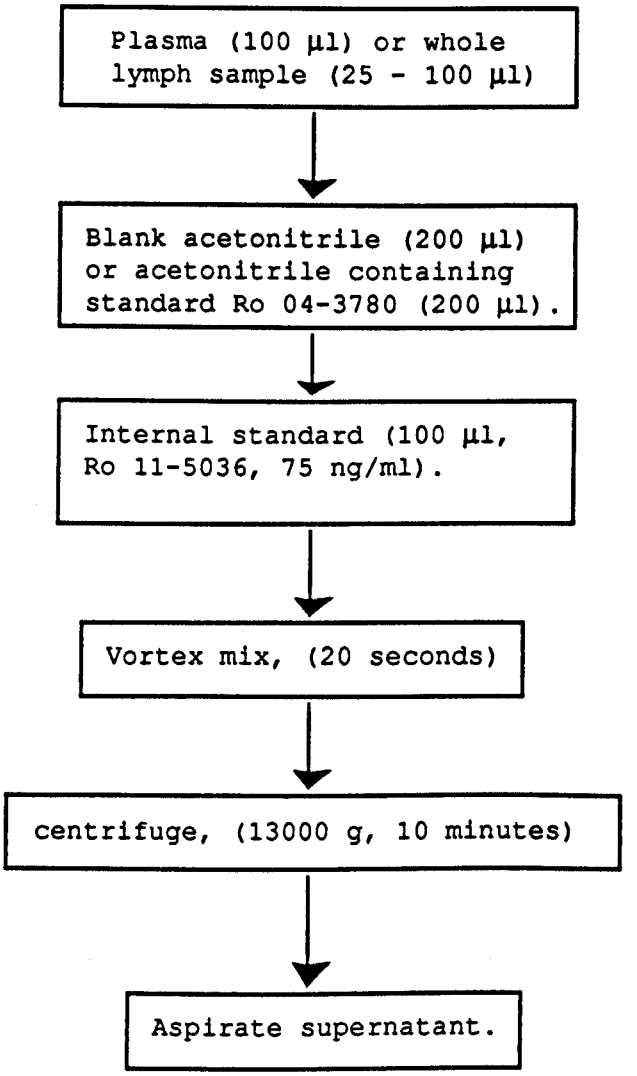


Table 8. Recovery experiments for Ro 04-3780 after extraction of spiked plasma samples.

a) Acid-ether extraction method.(Section 2.2.1)

Added	Recovered	
ng/ml	ng/ml	%
50	20.8	42%
100	66.6	66%
200	158.5	79%
500	413.2	83%
1000	838.3	84%
	Mean recovery =	70.7%

b) Direct acetonitrile extraction 1.(Section 2.2.2)

Added	Recovered	
ng/ml	ng/ml	%
25	16.9	68%
50	35.8	72%
100	73.8	74%
200	197.2	99%
500	489.5	98%
1000	989.2	99%
	Mean recovery =	84.9%

Table 8. continued

c) Direct acetonitrile extraction 2.(Section 2.2.3)

Added	Recovered	
ng/ml	ng/ml	%
25	22.8	91%
50	49.1	98%
100	102.3	102%
200	197.8	99%
500	489.0	98%
1000	1006.9	100%
	Mean recovery =	99.6% (± 3.42%)

a) Coefficients of the linear regression line of the form:

Peak area ratio = (B x Concentration (ng/ml)) + A,

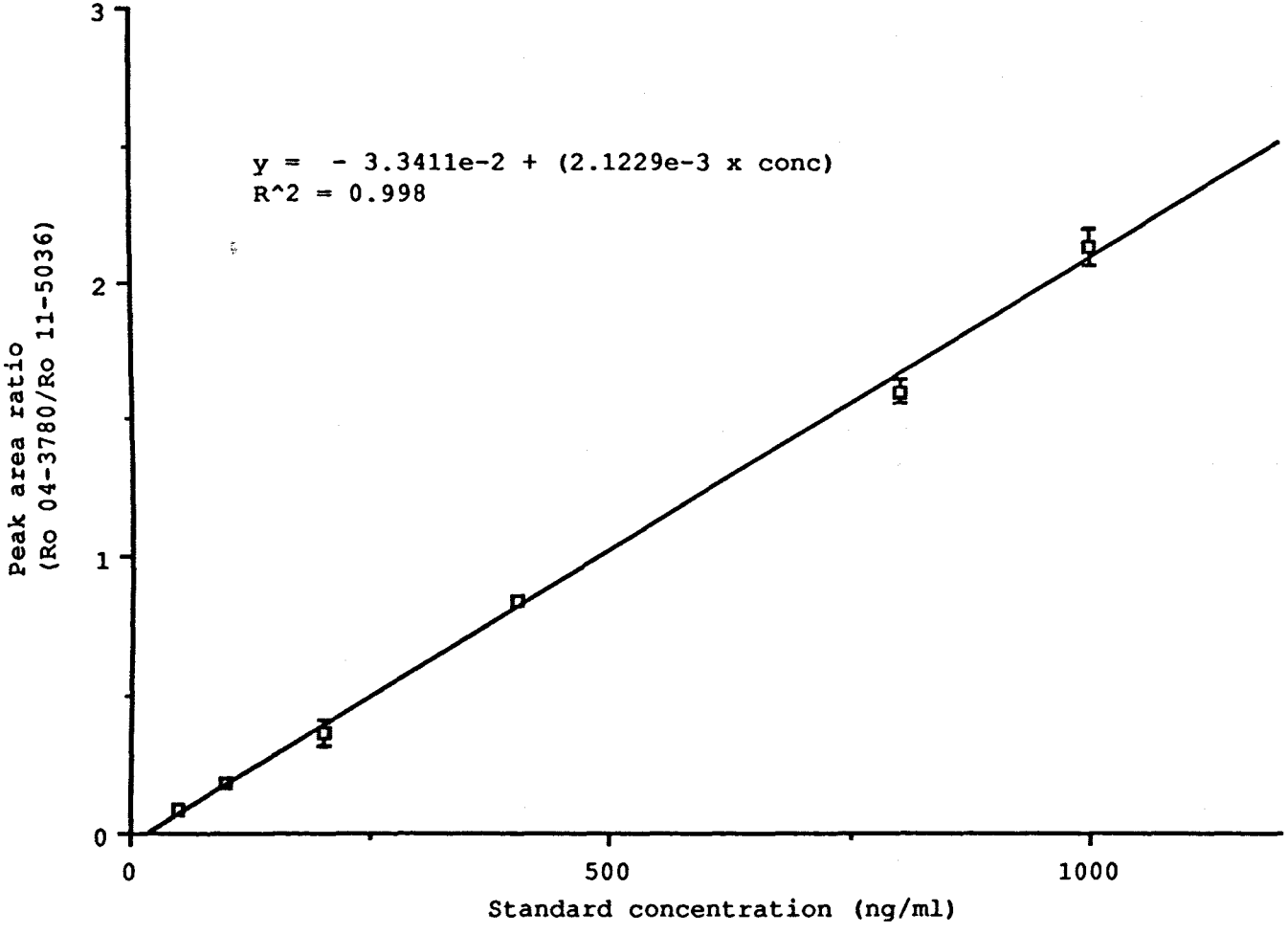
obtained from extracted plasma standards were calculated using the method of least squares;

Intercept (A) = -3.3411×10^{-2}

Slope (B) = 2.1229×10^{-3}

Correlation coefficient (r^2) = 0.991

Figure 6. Calibration curve for the analysis of Ro 04-3780 by hplc.



organic phase.

The first direct extraction method which used two parts of acetonitrile to one part plasma showed slightly improved recovery of extraction (85.0 % ± 15.1) over the range 25 to 1000 ng/ml compared with the acid-ether extraction, however, it was thought the poor recovery might still present some problems with the overall sensitivity of the assay. On further increasing the acetonitrile to plasma phase ratio from two parts to one to three parts to one, overall mean recovery was further improved to 98.0% (± 3.7). Recovery at a retinoid concentration of 25 ng/ml dramatically improved from 68% to 91%. Although the increased volume of organic phase would dilute the sample, the overall increase in recovery was a more favourable effect and all further assay extractions were performed with a dilution of at least three parts acetonitrile. If the concentration of retinoid was very low the limit of detection could be further improved by reducing the acetonitrile volume by evaporation using a vacuum centrifuge. This was however, rarely necessary.

2.2. Development and validation of hplc methods for the analysis of Ro 10-9359 and Ro 15-0778.

2.2.1. Hplc methods for the analysis of Ro 10-9359.

Freshly prepared standard solutions (2000 ng/ml) of Ro 10-9359 were prepared in acetonitrile or n-hexane for the evaluation of a series of hplc systems. A number of reversed phase and normal phase systems were studied.

2.2.1.1. Reversed phase hplc methods.

Initially the system which had been developed for the analysis of Ro 04-3780 (section 2.1.1.) was investigated in order to establish its applicability to the analysis of Ro 10-9359.

The system comprised a 5 μm Spherisorb S5ODS column (100 x 4.6 mm) eluted with a mobile phase containing 70% acetonitrile and 30% 0.01 M ammonium acetate buffer (pH 6.0) at a flow rate of 1 ml/min. Detection was by UV absorbance at 360 nanometres (λ_{max} for Ro 10-9359).

A sample of the Ro 10-9359 standard in acetonitrile (2000 ng/ml) was injected onto the hplc using a fixed volume loop injector (100 μl). The retention time, peak height and peak width of the eluted Ro 10-9359 peak were measured from the hplc integrator trace.

The mobile phase of the initial hplc system was modified to decrease the retention time of the

Ro 10-9359 peak and to produce a peak with a narrower peak width and therefore more acceptable peak shape. The acetonitrile content of the mobile phase was increased from 70% to 80% and the 0.01 M ammonium acetate buffer (pH 6.0) was decreased from 30% to 20%. The retention time, peak height and peak width of the eluted Ro 10-9359 peak were measured from the hplc integrator trace.

2.2.1.1.1. Internal standard for the reversed hplc system.

In order to assess the suitability of potential internal standards in the assay of Ro 10-9359 using this system, a series of retinoids (Ro 01-5488, Ro 04-3780, Ro 10-1670, Ro 11-5036) solutions were prepared at concentrations of approximately 2000 ng/ml in acetonitrile. These compounds were injected onto the hplc system and their retention times were recorded.

2.2.1.2. Investigations of normal phase hplc methods for Ro 10-9359.

A series of normal phase hplc systems were evaluated for the analysis of Ro 10-9359. The column of choice was a cyano-bonded silica column. This offered the advantages of being capable of separations in either the normal phase or reversed phase modes, thereby making the column more versatile in its application

and also less susceptible to the effects of traces of polar solvents when used in the normal phase mode. The column was also faster in equilibrating with organic solvents than traditional normal silica columns. A 3 µm Spherisorb S3CN (CN) column (150 x 4.6 mm) was used for these analyses.

In order to remove all traces of polar solvents, especially water, from the column all solvents were dried over anhydrous magnesium sulphate before use.

A series of normal phase hplc systems comprising a cyano-bonded column (3 µm Spherisorb S3CN (150 x 4.6 mm)) and a range of mobile phase systems were studied. The column was stored in methanol and before it could be used with a non-polar mobile phase a range of solvents with decreasing polarity had to be passed down the column. The sequence of solvents was methanol (30 minutes), propan-2-ol (30 minutes), followed by the non-polar mobile phase for several hours. This sequence of solvents was used in reversed order when the column was taken back into methanol for storage.

A standard solution (2000 ng/ml) of Ro 10-9359 was prepared in n-hexane. Samples (100 µl) of this standard were injected onto each hplc system using a fixed volume loop injector. The retention time, peak height and peak width of the Ro 10-9359 and the retention time of the solvent front were recorded and evaluated after elution from the column with a

series of mobile phase systems.

i) Initial studies were undertaken using a mobile phase consisting of 100% n-hexane (hplc grade) at a flow rate of 1 ml/min.

ii) The second group of systems were based on the methods of Hanni (Hanni et al., 1979) who developed mobile phase systems for the determination of etretinate (Ro 10-9359).

The first system comprised n-hexane : THF : acetic acid (980 : 15 : 6). This system was modified by De Leenheer (De Leenheer et al., 1990) to comprise, n-hexane : THF : acetic acid : acetonitrile (895 : 100 : 3 : 2). This system was used by De Leenheer for the determination of etretinate (Ro 10-9359) and its metabolites in human plasma. Further modifications of this mobile phase were made by replacing the THF with di-isopropyl ether or dichloromethane.

The second mobile phase of Hanni (Hanni et al., 1979) comprised hexane : methyl benzoate : propionic acid (872 : 125 : 3.5). A similar system has also been used by McNamara (McNamara & Blouin, 1990) consisting of hexane : methyl benzoate : propionic acid (935 : 62 : 2.5).

Each of the above mobile phases were delivered at 1 ml/min and evaluated using injections of standard solutions of Ro 10-9359, Ro 10-1670, Ro 11-5036 and Ro 04-3780 in n-hexane.

iii) To study the importance of the small quantities

of methyl benzoate and propionic acid on the retention times of the retinoids, the relative proportions of each component was varied.
The proportion of propionic acid in the mobile phase was fixed at 2.5% and the proportion of methyl benzoate was varied, the remaining volume being made up with hexane. The systems shown in Table 9 (proportions by volume) were examined.

2.2.1.2.1. <u>Effect of time of column equilibration on retention time of Ro 10-9359.</u>

In the study of various mobile phase systems on the retention time of retinoids after elution from a normal phase 3 μm Spherisorb S3CN hplc column, it was observed that the retention time varied with time of equilibration with mobile phase. The change in column equilibration time with mobile phase was studied using a mobile phase consisting of hexane : methyl benzoate : propionic acid (991.5 : 6 : 2). Mobile phase at a flow rate of 0.5 ml/min was allowed to recirculate through the hplc column. Daily, the flow was adjusted to 1.0 ml/min and the retention times for the retinoids, Ro 10-9359 and Ro 04-3780 in a standard mixture were recorded. The column was considered to be equilibrated with the mobile phase when consecutive recordings of the retention times for retinoid peaks eluting from the column were found to remain constant.

Table 9. Mobile phase systems used to study the importance of the small quantities of methyl benzoate and propionic acid on the retention times of the Ro 10-9359 on a Spherisorb S3CN hplc system.

	Hexane	Methyl benzoate	Propionic acid
a)	996.5	1	2.5
b)	995.5	2	2.5
c)	994.5	3	2.5
d)	993.5	4	2.5
e)	992.5	5	2.5
f)	991.5	6	2.5
g)	957.5	40	2.5
h)	935	62	2.5
i)	872	125	3.5

2.2.1.2.2. Selection of an internal standard for the normal phase hplc method for Ro 10-9359.

On a reversed phase hplc system the rank order of elution times for a series of compounds is reversed compared with their elution times on a normal phase hplc system. Therefore on the normal phase system a polar compound will elute more slowly than a non-polar compound.

The retention times of Ro 10-9359, Ro 01-5488, Ro 10-1670, Ro 11-5036 and Ro 04-3780 were recorded on an Spectra Physics computing integrator after elution from the 3 μm Spherisorb S3CN column using a mobile phase containing hexane : methyl benzoate : propionic acid (991.5 : 6 : 2.5) (Table 10(h)).

2.2.1.2.3. Extraction procedure for Ro 10-9359.

The extraction of Ro 10-9359 from plasma was investigated using methods identical to those used for the extraction of Ro 04-3780. The method of choice was a direct extraction into acetonitrile as detailed in section 2.1.2.3. which offered the advantages of rapid sample extraction and excellent recovery of retinoid from plasma and lymph. However, it was not believed to be good practice to contaminate the normal phase column with repeated injections of acetonitrile, containing aqueous material from the biological samples. An additional final step was added to the extraction procedure

Table 10. **A summary of the normal phase mobile phase hplc systems validated for the analysis of Ro 10-9359.**

a) **100% n-hexane.**

	k′	Retention time (minutes)
Solvent	-	1.63
Ro 10-9359	0.37	2.24

b) **n-Hexane : Tetrahydrofuran : Acetic acid : Acetonitrile (895:100:3:2).**

	k′	Retention time (minutes)
Solvent	-	1.58
Ro 10-9359	0.12	1.77

c) **n-Hexane : Dichloromethane : Acetic acid : Acetonitrile (895:100:3:2).**

	k′	Retention time (minutes)
Solvent	-	1.62
Ro 10-9359	0.17	1.90

d) **n-Hexane : Di-isopropyl ether : Acetic acid : Acetonitrile (895 : 100 : 3 : 2).**

	k′	Retention time (minutes)
Solvent	-	1.52
Ro 10-9359	0.18	1.79

Table 10. continued.

e) **Hexane : methyl benzoate : propionic acid (872:125:3.5)**

	k′	Retention time (minutes)
Solvent	-	1.60
Ro 10-9359	0.19	1.90
Ro 04-3780	2.09	4.95
Ro 11-5036	Split peaks detected.	

f) **Hexane : methyl benzoate : propionic acid (935:62:2.5).**

	k′	Retention time (minutes)
Solvent	-	1.60
Ro 10-9359	0.55	2.48
Ro 04-3780	1.49	3.98
Ro 11-5036	3.01	6.42

g) **Effect of varying proportions of methyl benzoate (0.1% - 4.0%) in hexane containing 2.5% propionic acid on k′ and retention time of Ro 10-9359.**

Methyl benzoate	k′	Retention time
0.1%	0.80	2.93
0.2%	0.76	2.87
0.3%	0.77	2.89
0.4%	0.79	2.92
0.5%	0.74	2.84
0.6%	0.73	2.82
4.0%	0.68	2.74

Table 10. continued.

h) **Hexane : methyl benzoate : propionic acid (991.5:6:2.5) after extended column equilibration time.**

	k′	Retention time (minutes)
Solvent	-	1.40
Ro 10-9359	2.9	5.40
Ro 01-5488	3.3	6.00
Ro 04-3780	5.6	9.21
Ro 11-5036	11.8	17.9

Note.All systems used a Spherisorb S3CN column and a mobile phase elution rate of 1 ml/min.

which is detailed below.

Plasma samples (100 µl) were transferred into polypropylene microcentrifuge tubes (1.5 ml) using a positive displacement pipette. The whole of a lymph sample (25 - 100 mg) was analysed in the tube into which it had been collected.

Acetonitrile (300 µl) was added to each sample.

Standard extracts were prepared by transferring blank rat (or human) plasma (100 µl) into microcentrifuge tubes (1.5 ml) using a positive displacement pipette followed by the addition of acetonitrile (100 µl) containing the appropriate concentration of standard material (0, 50, 100, 200, 400, 800 or 1000 ng/ml).

To all lymph samples, plasma samples and standard tubes, acetonitrile (100 µl) containing the internal standard (Ro 04-3780, 2000 ng/ml) was added.

The tubes were mixed on a vortex mixer (20 seconds) and centrifuged in a microcentrifuge (13,000 x g, 10 minutes). The supernatant was transferred into a further set of polypropylene microcentrifuge tubes (1.5 ml).

The samples were evaporated to dryness under reduced pressure at 50°C in a vacuum centrifuge.

The samples were dissolved in mobile phase (500 µl) and transferred into hplc autosampling vials.

2.2.1.2.3.1. Validation of the extraction procedure for Ro 10-9359.

A series of experiments were performed to validate the direct protein precipitation extraction procedure.

Blank rat and human plasma samples were analysed using the method described in section 2.2.1.2.3. and using the hplc system (Table 6). The hplc integrator traces were examined for the presence of interfering peaks at the elution times for Ro 04-3780 or Ro 10-9359.

Acetonitrile (100 µl) containing Ro 10-9359 at a range of concentrations from 25 ng/ml to 1000 ng/ml was transferred to a series of rat (or human) plasma samples (100 µl) using a positive displacement pipette. A parallel set of standards samples was prepared in which acetonitrile (100 µl) replaced the plasma volume. Each set of samples was diluted using acetonitrile (300 µl) and acetonitrile (100 µl) containing the internal standard (Ro 04-3780, 2000 ng/ml). The samples were mixed using a vortex mixer (20 seconds) and centrifuged (13,000 x g, 10 minutes). Aliquots of the supernatant from each sample were evaporated to dryness using a vacuum centrifuge and the samples were dissolved in mobile phase (500 µl). Samples were injected onto the hplc system (Table 6) using a fixed volume loop injection (100 µl). The peak areas obtained for the plasma

extracts were compared with the parallel set of extracted control samples from which plasma had been omitted. Recovery of the added Ro 10-9359 was calculated using the Equation 6.

2.2.2.1. Results and Discussion.

The capacity factor (k′) is a quantity used in column chromatography. It relates the equilibrium distribution of the sample within the column to the thermodynamic properties of the column and to the temperature. For a given set of operating parameters, k′ is a ratio of the time spent by the analyte in the stationary phase relative to the time spent in the mobile phase. It is defined as the ratio of the amount of solute in the stationary phase to the amount in the mobile phase (Equation 8).

$$k' = \frac{C_s \times V_s}{C_m \times V_m} \qquad \text{Equation.....8}$$

C_m = Molar concentration in mobile phase.

V_m = Volume of mobile phase.

C_s = Molar concentration in stationary phase.

V_s = Volume of stationary phase.

Capacity factor may be calculated from hplc retention time data (Equation 9).

$$k' = \frac{t_r - t_m}{t_m}$$ Equation.....9

t_r = Retention time for the sample peak.

t_m = Retention time for un-retained solute.

It can be shown that a k′ value of 2 is the optimum value for maximum resolution in unit time and k′ values greater than 10 can waste valuable analytical time (Willard et al., 1988).

The capacity factors have been calculated and used along with retention time data for comparison of the hplc systems studied here.

The hplc system which was developed for the analysis of Ro 04-3780 showed a retention time of 17.7 (± 0.01) minutes for Ro 10-9359 (k′ = 10.1). This time was considered unacceptable since it would have resulted in a total analytical time for some larger batches (100 - 130 samples) in excess of 36 hours. On increasing the percentage of acetonitrile in the mobile phase from 70% to 80% the retention time was decreased to 11.0 (± 0.01) minutes (k′ = 7.0). This was considered to be a much more acceptable run time. However, the peak obtained for Ro 10-9359 on this system was of a poor shape, showing peak broadening, some evidence of peak splitting and peak asymmetry.

The retention times for the retinoids, Ro 01-5488, Ro 04-3780, Ro 10-1670 and Ro 11-5036 were found to

be 3.50, 3.13, 1.8 and 2.07 minutes and the capacity factors 1.5, 1.2, 0.3 and 0.5 respectively.

Since the peaks for Ro 10-1670 and Ro 01-5488 did not show baseline resolution and also Ro 10-1670 was a metabolite of Ro 10-9359 neither of these compounds were considered suitable as internal standards in the determination of Ro 10-9359.

The peaks obtained for Ro 04-3780 and Ro 11-5036 did not show baseline resolution from each other and also eluted very close to the solvent front with capacity factors well below unity. These compounds were also considered to be unsuitable for use as internal standards.

The poor resolution of the retinoid peaks and the poor peak shape for Ro 10-9359 together with the inability to find a suitable retinoid which would act as an internal standard for the assay of Ro 10-9359 had the result of rendering the reversed phase hplc system unsuitable for the analysis of Ro 10-9359.

A number of normal phase systems were therefore investigated as a potential alternatives for the analysis of Ro 10-9359.

The following observations were made based on the peak retention times and k′ values of Ro 10-9359 (Table 10) using the various normal phase solvent systems described in section 2.2.1.2. and using a

3 μm Spherisorb S3CN column (150 x 4.6 mm) in the normal phase mode;

a) **100% n-hexane.**

This mobile phase gave only a slight separation of the Ro 10-9359 peak from the solvent front (k′ = 0.4). Baseline separation was not achieved.

b) i) **n-Hexane : Tetrahydrofuran : Acetic acid : Acetonitrile (895 : 100 : 3 : 2)** or

ii) **n-Hexane : Dichloromethane : Acetic acid : Acetonitrile (895 : 100 : 3 : 2)** or

iii) **n-Hexane : Di-isopropyl ether : Acetic acid : Acetonitrile (895 : 100 : 3 : 2).** Each of these three mobile phases failed to resolve the Ro 10-9359 peak from the solvent front to any extent (k′ < 0.2) and were therefore unsuitable for the analysis of Ro 10-9359.

c) **Hexane : methyl benzoate : propionic acid (872 : 125 : 3.5)** and **Hexane : methyl benzoate : propionic acid (935 : 62 : 2.5).** Each of these two systems were unable to sufficiently resolve the Ro 10-9359 peak from the solvent front, however, the second system, which contained a lower proportion of methyl benzoate, gave a slightly higher value of k′ for this compound (0.6 compared with 0.2).

f) **Varying proportions of methyl benzoate (0.1% - 4.0%) in hexane containing 2.5% propionic acid.** None of these systems gave k′ values above unity, the

lowest percentages of methyl benzoate gave the highest value (k′ = 0.8).

g) **Hexane : methyl benzoate : propionic acid (991.5 : 6 : 2.5) with extended column equilibration time.**

After recirculating the mobile phase through the column at a flow rate of 0.5 ml/min for 72 hours the retention time for Ro 10-9359 increased slowly to give a k′ value of 2.9 with a retention time of 5.40 minutes. This value was considered acceptable and gave good resolution from the solvent front.

This mobile phase system was selected as a suitable system for the analysis of Ro 10-9359 and is summarised in Table 6.

A number of retinoid standards were injected onto normal phase hplc system (mobile phase system g) in order to assess their suitability as an internal standard;

Ro 10-1670 could not be used since it is a metabolite of Ro 10-9359 and there are reports of metabolic interconversion of these two compounds in-vivo (Jensen, 1991).

Ro 11-5036 gave a k′ value of 11.8 from a retention time of 17.9 minutes which would have increased analysis time to an unacceptable figure. Ro 01-5488 gave a retention time of 6.0 minutes (k′ = 3.3), however, this peak was not adequately resolved from Ro 10-9359. Ro 04-3780 exhibited a retention time of 9.21 minutes (k′ = 5.6) and was therefore ideally

positioned with respect to Ro 10-9359 for use as an internal standard.

The direct protein precipitation extraction method, described in section 2.2.1.2.3. and summarised in Figure 7, proved successful in extracting both Ro 10-9359 and the internal standard, Ro 04-3780. The method gave a good recovery of the two retinoids with minimal interference with other extracted compounds.

After extraction of blank rat or human plasma samples;

a) No interfering substances were eluted with elution times near to those of either Ro 10-9359 or Ro 04-3780.

b) The overall recovery of Ro 10-9359 in the concentration range 25 - 1000 ng/ml in spiked plasma was 93.8% $\pm$ 4.93 (Table 11). Ro 04-3780 was recovered with 93.2% $\pm$ 4.66 efficiency at an internal standard concentration of 2000 ng/ml.

c) The limit of detection was estimated to be approximately 10 ng/ml after extraction of a 100 μl sample of plasma, based on the concentration equivalent to three times the level of baseline noise. Variability in background noise from the detector caused some variation in the limit of detection for Ro 10-9359 but never exceeded 25 ng/ml.

Figure 7. Method for the extraction of Ro 10-9359 from plasma and lymph.

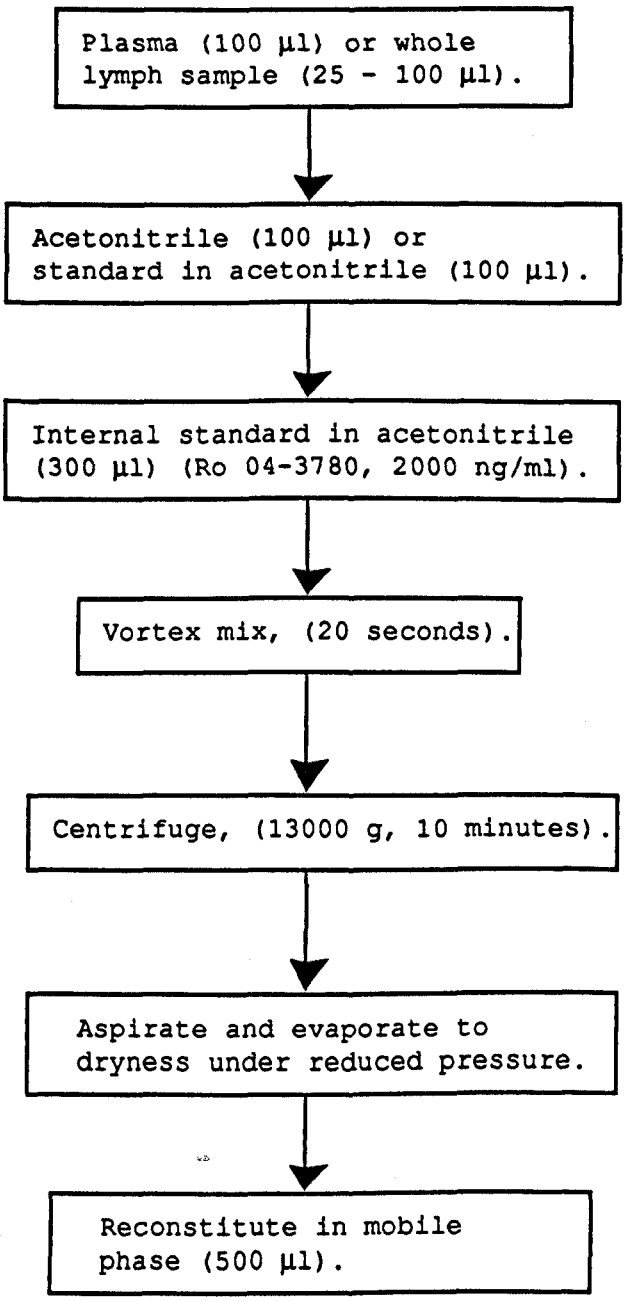


Table 11. Recovery and linearity studies on Ro 10-9359 after extraction of spiked plasma samples.

a) Recovery of Ro 10-9359.

Added ng/ml	Recovered ng/ml	Recovered %
25	22.5	90%
50	45.0	90%
100	93.9	94%
200	195.1	98%
400	371.7	93%
800	776.9	97%
1000	953.1	95%
	Mean recovery =	93.8% (± 4.93%)

(Recovery of the internal standard (Ro 04-3780, 2000 ng/ml) from extracted spiked plasma samples was 93.2% (± 4.66)).

b) Coefficients of the linear regression line of the form:

Peak area ratio = (B x Concentration (ng/ml)) + A,

obtained from extracted plasma standards were calculated using the method of least squares;

Intercept (A) = -6.9724×10^{-3}

Slope (B) = 2.2466×10^{-3}

Correlation coefficient (r^2) = 1.000

d) A standard curve plotted over the range 25 - 1000 ng/ml of Ro 10-9359 (Figure 8) showed a correlation coefficient of 0.999 with an intercept equivalent to 3.1 ng/ml.

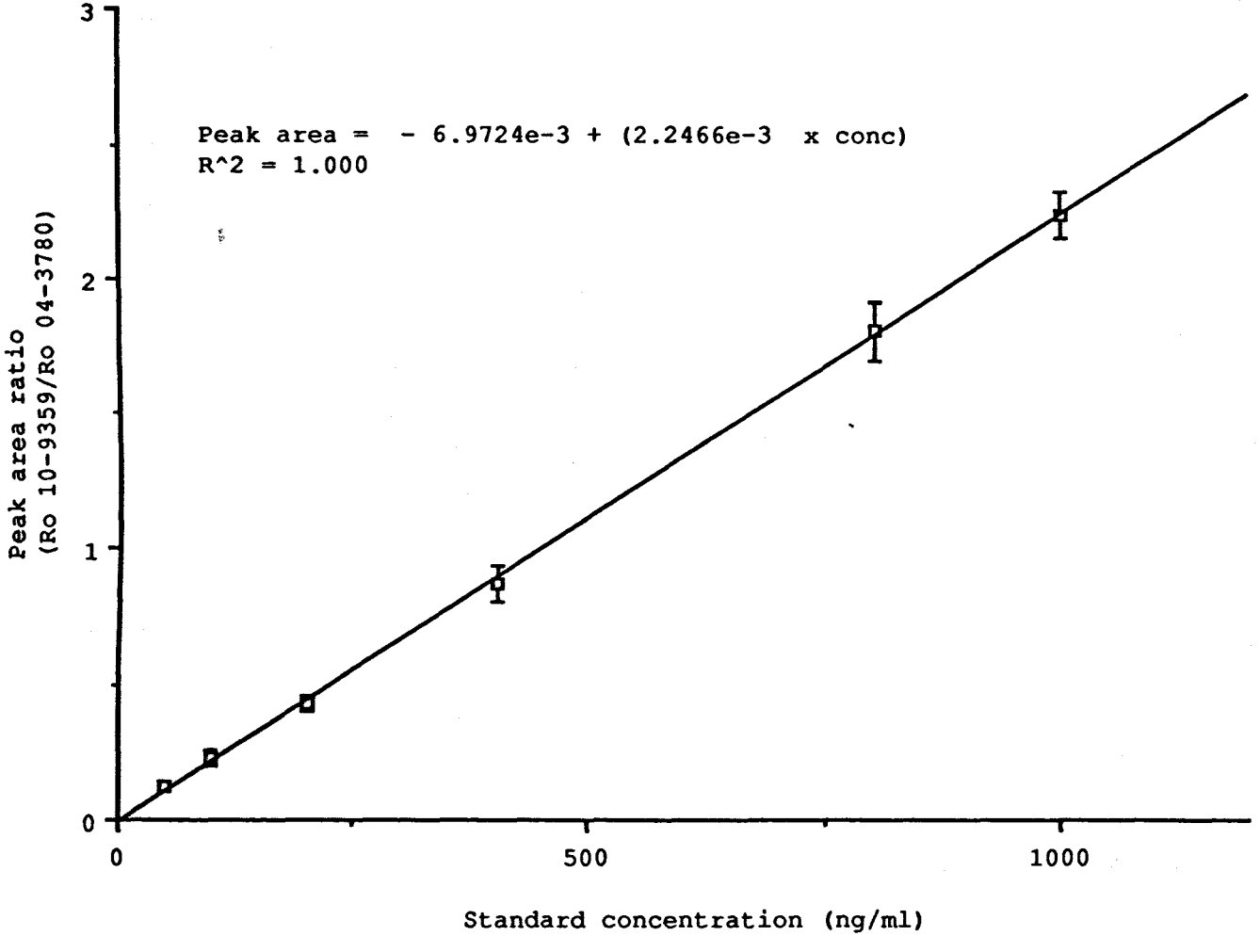
2.2.2.2. Conclusion.

A sensitive and specific normal phase hplc system has been developed and validated for the analysis of Ro 10-9359 in rat plasma and lymph samples.
The method allows the extraction of small concentrations of retinoid from small volumes of samples with a limit of detection of approximately 10 ng/ml from a 100 µl sample.
The extraction method allows the rapid analysis of numbers of samples on the hplc system with an analysis time of 12 minutes per sample.

2.2.3. Hplc method for Ro 15-0778.

Initially the hplc method which had been developed for the analysis of Ro 04-3780 was studied (section 2.1.1.). However, these indicated that a mobile phase containing 70% acetonitrile : 30% 0.01 M ammonium acetate buffer (pH 6.0) gave a retention time of 23.7 minutes (± 0.01) for Ro 15-0778. It was therefore necessary to increase the proportion of acetonitrile in the mobile phase in order to elute the retinoid with a shorter retention time.
The absorption maximum for Ro 15-0778 was found to

Figure 8. Calibration curve for the analysis of Ro 10-9359 by hplc.



be 279 nanometres (section 1.3.) and in consequence an internal standard with an absorbance maximum near to this value would also be required.

2.2.3.1. Mobile phase selection.

A standard containing Ro 15-0778 (2000 ng/ml) was prepared in acetonitrile. This solution was injected onto the 5 μm Spherisorb S5ODS column (100 x 4.6 mm) using a fixed volume loop injection of 100 μl. The mobile phase circulating through the column was adjusted to contain either 70%, 75%, 80%, 85% or 90% acetonitrile, the remaining volume was 0.01 M ammonium acetate buffer (pH 6.0). The retention times for the solvent front and the Ro 15-0778 peak were recorded and capacity factors (k') calculated.

2.2.3.2. Internal standard selection.

Ro 15-0778 is an arotinoid and as such differs in structure from the majority of the retinoids. The arotinoids do not possess the tetraene side group and terminal polar group traditionally found in the retinoids since these groups have incorporated into an aromatic terminal group (Figure 3). These conjugated aromatic molecules in consequence have a λ_{max} further into the ultraviolet than other retinoids. Temarotene (Ro 15-0778) has been shown to absorb at a maximum of 279 nanometres (section 1.3.) and it was therefore desirable to analyse the

compound at this wavelength in the hplc analytical method. An internal standard with similar absorption wavelength and physical properties to Ro 15-0778 was therefore sought for use as an internal standard. The absorbance maximum of the sulphone derivative (Ro 15-1570, Figure 3) of Ro 15-0778 was studied together with its retention properties on the proposed hplc system.

i) A standard solution of Ro 15-1570 (2000 ng/ml) was prepared in acetonitrile and diluted to allow a UV absorption spectrum to be measured over the range 200 - 400 nanometres.

ii) Samples (100 μl) of the Ro 15-1570 standard were injected onto the hplc system using a fixed volume loop injection. The retention time and capacity factor were recorded and evaluated.

2.2.3.3. <u>Extraction procedure for Ro 15-0778.</u>

A direct protein precipitation extraction procedure was used for the extraction of Ro 15-0778 and Ro 15-1570 from plasma and lymph. Essentially the method was identical to that described for Ro 04-3780. Plasma samples (100 μl) were transferred into polypropylene microcentrifuge tubes (1.5 ml) using a positive displacement pipette. The whole of a lymph sample (25 - 100 mg) was analysed in the tube into which it had been collected.

Acetonitrile (300 μl) was added to each sample.

Standard extracts were prepared by transferring blank rat (or human) plasma 100 µl into microcentrifuge tubes (1.5 ml) followed by acetonitrile (100 µl) containing the appropriate concentration of standard material (0, 50, 100, 200, 400, 800 or 1000 ng/ml). Blank acetonitrile (200 µl) was added to each tube.
To all lymph samples, plasma samples and standard tubes, acetonitrile (100 µl) containing the internal standard (Ro 15-1570, 2000 ng/ml) was added.
The tubes were mixed (20 seconds) using a vortex mixer and centrifuged in a microcentrifuge (13,000 x g, 10 minutes). The supernatant was transferred into an hplc autosampling vial.

2.2.3.3.1. <u>Validation of the extraction procedure for Ro 15-0778.</u>

A series of experiments was performed to validate the direct protein precipitation extraction procedure.
a) Blank rat and human plasma samples were analysed using the method described in section 2.2.2.3.3. and using the hplc system summarised in Table 6. The hplc traces were examined for the presence of interfering peaks at the elution times for Ro 15-0778 or Ro 15-1570.
Acetonitrile (100 µl) containing Ro 15-0778 at a range of concentrations from 25 ng/ml to 1000 ng/ml

was transferred to a series of rat (or human) plasma samples (100 µl) using a positive displacement pipette. A parallel set of standards samples was prepared in which acetonitrile (100 µl) replaced the plasma volume. Each set of samples was diluted using acetonitrile (300 µl) and acetonitrile (100 µl) containing the internal standard (Ro 15-1570, 2000 ng/ml).

The samples were mixed using a vortex mixer (20 seconds) and centrifuged (13,000 x g, 10 minutes). Aliquots of the supernatant from each sample was injected onto the hplc system (Table 6) using a fixed volume loop injection (100 µl). The peak areas obtained for the plasma extracts were compared with the parallel set of extracted control samples from which plasma was omitted. Recovery of the added Ro 15-0778 was calculated (Equation 10).

$$\text{Recovery (\%)} = \frac{\text{Peak area (plasma extract) x 100}}{\text{Peak area (acetonitrile extract)}}$$

Equation.....10

2.2.3.4. Results and Discussion.

A mobile phase system containing 80% acetonitrile and 20% 0.01 M ammonium acetate buffer (pH 6.0) was selected from the systems tested (Table 12) as the system of choice for the analysis of Ro 15-0778 using the reversed phase hplc, which is summarised in Table 6. This system gave retention times of

Table 12. Effect of acetonitrile content on the retention properties of Ro 15-0778 and Ro 15-1570.

a) **70% acetonitrile : 30% 0.01 M Ammonium acetate (pH 6.0)**

	k′	Retention time (minutes)
Solvent	-	1.0
Ro 15-0778	22.3	23.7
Ro 15-1570	8.2	9.3

b) **75% acetonitrile : 25% 0.01 M Ammonium acetate (pH 6.0)**

	k′	Retention time (minutes)
Solvent	-	1.2
Ro 15-0778	12.2	15.7
Ro 15-1570	4.5	6.5

c) **80% acetonitrile : 20% 0.01 M Ammonium acetate (pH 6.0)**

	k′	Retention time (minutes)
Solvent	-	1.4
Ro 15-0778	5.5	9.4
Ro 15-1570	2.2	4.6

d) **85% acetonitrile : 15% 0.01 M Ammonium acetate (pH 6.0)**

	k′	Retention time (minutes)
Solvent	-	1.4
Ro 15-0778	4.6	7.9
Ro 15-1570	1.3	3.2

Note. All systems were run at a flow rate of 1 ml/minute on a spherisorb S5ODS reversed phase column.

9.4 minutes for Ro 15-0778 (k′ = 5.5) and 4.6 minutes (k′ = 2.2) for Ro 15-1570.

A solution of Ro 15-1570 in acetonitrile was found to have a λ max of 295 nanometres, the compound showed a broad absorbance peak, similar to those found for other retinoids. Absorption for a solution of Ro 15-1570 at 279 nanometres was approximately 97% of the absorption at λ_{max} (295 nanometres) and this loss in sensitivity was considered to be acceptable.

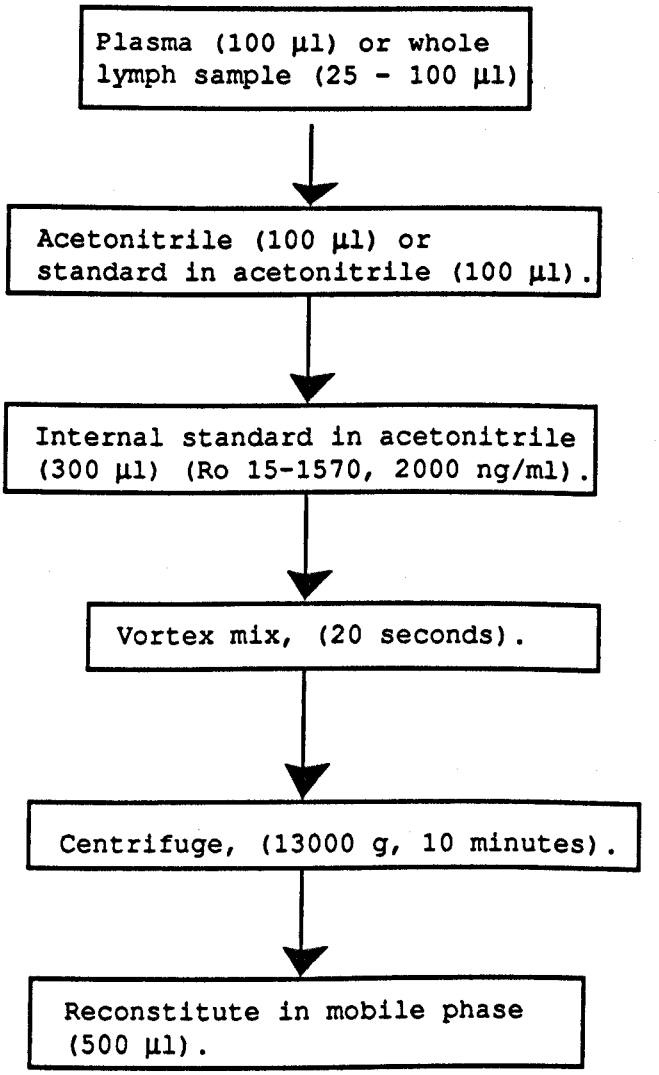
Ro 15-1570 has structural similarities to Ro 15-0778, and showed a retention time of 4.6 minutes on the proposed hplc system for Ro 15-0778, and was efficiently extracted (recovery 99% ± 3.48 at 1000 ng/ml) using the proposed direct protein precipitation extraction method.

These features suggested that the compound was therefore suitable for use as an internal standard in the analysis of Ro 15-0778.

The direct protein precipitation extraction method described in section 2.2.3.3. and summarised in Figure 9, proved successful in extracting both Ro 15-0778 and the internal standard, Ro 15-1570. The method was sensitive, gave a good recovery of the two retinoids with minimal interference from other extracted compounds.

No interfering substances were extracted which eluted near the elution times of either Ro 15-0778

Figure 9. Method for the extraction of Ro 15-0778 from plasma and lymph.



or Ro 15-1570.

Overall recovery of Ro 15-0778 in the concentration range 2.5 - 1000 ng/ml in spiked plasma was 92.6% (± 6.93) (Table 13). A standard calibration curve for Ro 15-0778 is shown in Figure 10.

The limit of detection for the assay of Ro 15-0778 was estimated to be approximately 2 ng/ml from a 100 µl sample, based on the concentration equivalent to three times the level of baseline noise on the hplc integrator trace.

A standard curve plotted over the range 2.5 - 1000 ng/ml of Ro 15-0778 showed a correlation coefficient of 0.983 with an intercept equivalent to 0.0015 ng/ml.

The extraction method allowed a rapid throughput of large sample batches with a mean analysis time of less than 12 minutes per sample.

Table 13. Recovery and linearity studies on Ro 15-0778 afte extraction of spiked plasma samples.

a) Recovery of Ro 15-0778.

Added ng/ml	Recovered ng/ml	Recovered %
2.5	1.9	76%
5	4.5	90%
10	9.5	95%
25	22.8	91%
50	49.0	98%
100	98.9	99%
250	229	92%
500	478	96%
1000	962	96%
	Mean recovery =	92.6% (± 6.93%)

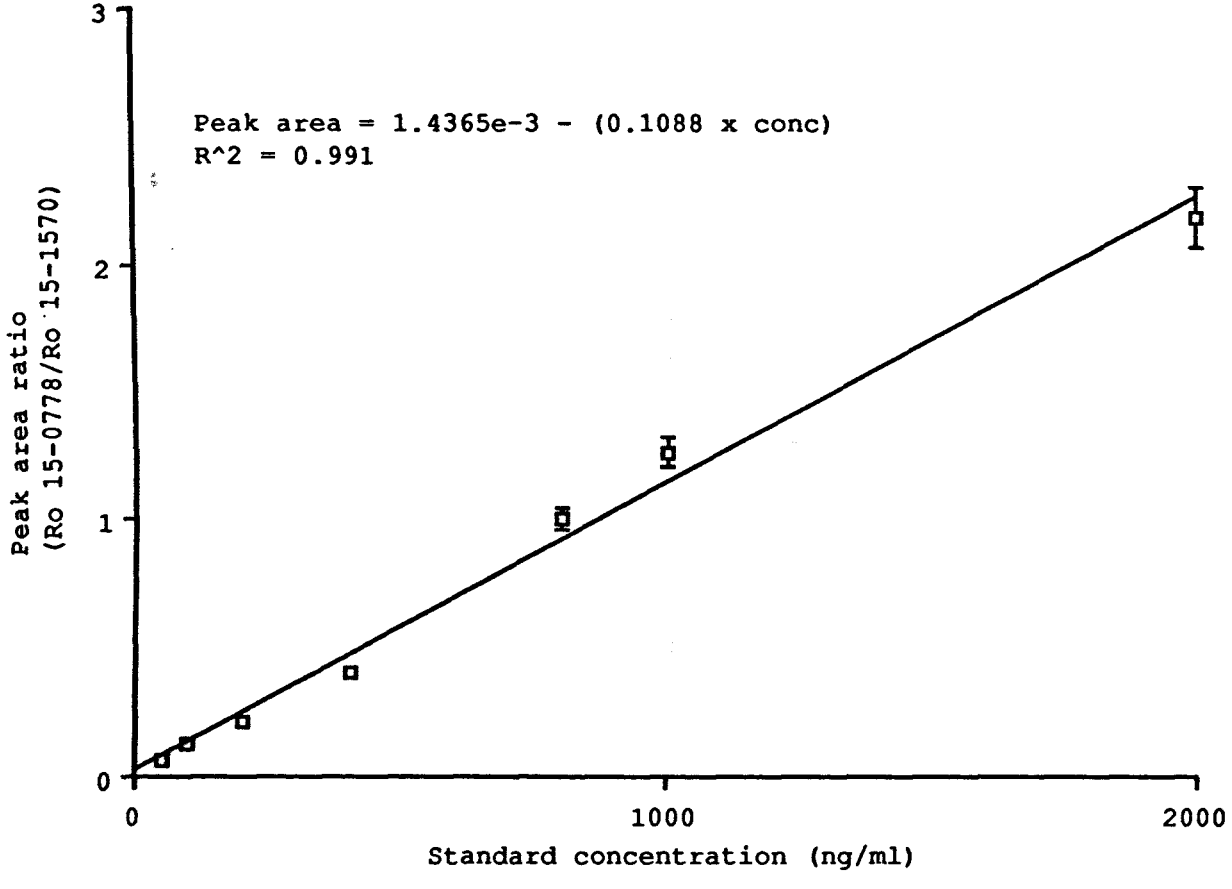
b) Coefficients of the linear regression line of the form: **Peak area ratio = (B x Concentration (ng/ml)) + A,** obtained from extracted plasma standards were calculated using the method of least squares;

Intercept (A) = 1.4365×10^{-3}

Slope (B) = 1.088×10^{-1}

Correlation coefficient (r^2) = 0.991

Figure 10. Calibration curve for the analysis of Ro 15-0778 by hplc.



CHAPTER 3

INVESTIGATION OF SELF-EMULSIFYING OIL SYSTEMS FOR THE ORAL DELIVERY OF RETINOIDS.

3. Introduction.

Dietary fat consists of triglyceride (tri-acyl glycerols), mainly as esters of glycerol and long chain fatty acids such as palmitic acid, stearic acid, oleic acid and linoleic acid. In addition, cholesterol, cholesterol esters and phospholipids (lecithin) are normally present in the diet. These lipid materials are emulsified in the stomach and the lipid emulsion enters the small intestine as tiny droplets of less than 5 μm in diameter (Carey et al., 1983). As a result of enzyme action both in the stomach and the small intestine and of the effects of bile salts, there is a marked change in the chemical and physical form of the ingested lipid. The lipid exists in the form of mixed micelles together with larger uni-lamellar vesicles (liposomes). It has been suggested that the mixed micelles are dissociated in the mucous layer which is present at the surface of the absorptive cells as a result of a pH gradient in this layer (Lucas et al., 1975). After dissociation of the mixed micelles the free fatty acids are subsequently incorporated into the membrane of the absorptive cells by passive diffusion. When the fatty acids have entered the cytoplasm of the enterocyte, an active transport process is necessary to carry them from the membrane into the cytoplasm of the cell (Ockner & Manning, 1974).

The incorporation of drug molecules into bile salt stabilized emulsions has been shown to promote absorption via the lymphatic route. For example Yoshikawa (Yoshikawa et al., 1985) found that mixed micelles of linoleic acid and the surfactant HCO 60 (poly-ethoxylated hydrogenated castor oil) enhanced enteral absorption of human interferon whereas neither linoleic acid alone or the surfactant alone was effective.

It was decided to investigate the formation of simple self-emulsifying oil systems (SES) which might be used to solubilise the retinoids and present them to an animal in a form which might enhance the absorptive process.

It was believed that this type of emulsifying system may present the retinoids in a form which would increase both chylomicron formation and lymph flow and hence improve lymphatic absorption.

3.1. Preparation of self-emulsifying oil emulsion systems.

In preparing SES it was desirable to achieve a system which would readily self-emulsify when mixed with the small volume of gastric fluid present in the rat stomach and remain stable in this form.

In order that emulsified systems resemble bile salt emulsions (Barrowman, 1984) in terms of particle

size it was necessary that the particle dimensions of the system be small, typically less than 500 nanometres. It is likely that fine droplets will empty rapidly from the stomach and thereby promote wide distribution of the drug throughout the intestinal tract. This effect will minimize the irritation frequently observed with extended contact between bulk drug substances and the gut wall (Charman et al., 1992).

A series of self-emulsifying oil-water systems was prepared, based on the oils selected for use in the oral dosing studies in the rat (Section 4.2.3.1). The oils included; Miglyol 812, peanut oil, cottonseed oil and linoleic acid. The oils were mixed with a range of surfactants, selected from the list in Table 14.

The suitability of a SES for oral administration was assessed after the determination of the particle size and the stability of the diluted SES.

Emulsions were to be visually examined for signs of instability and classified using the following terms:

i) **Stable.** System remains emulsified and of constant particle size over several days.

ii) **Biphasic (creaming).** System rapidly separates into two distinct layers.

iii) **Oily floaters.** Oily droplets seen floating on the surface of the emulsion system.

Table 14. Surfactant and co-surfactant systems used in self emulsifying oil systems.

Surfactant	Composition
Labrafil M1944CS	Unsaturated polyglycolised glycerol, (Apricot kernel oil PEG-6 complex).
Labrosol	Glyceryl caprylate/caprate and PEG-8 caprylate/caprate.
Softigen 767	PEG-6 caprylic/capric glycerides.
Span 20	Sorbitan monolaurate
Span 80	Sorbitan monooleate
Span 85	Sorbitan trioleate
Tween 20	Polyethylene sorbitan monolaurate
Tween 80	Polyethylene sorbitan monooleate
Tween 85	Polyethylene sorbitan trioleate

iv) **Unstable.** System breaks down irreversibly into individual components within 24 h.

v) **Overrange.** "Nano-Sizer" reading, out of range due to excessive turbidity. Sample requires further dilution to allow particle size measurement.

3.1.1. Preparation of artificial gastric contents.

A solution was prepared in which to dilute the SES systems. This solution contained both sodium chloride and hydrochloric acid in order to simulate the ionic content and pH of gastric juice.

Sodium chloride (2 g) was dissolved in distilled water (approximately 500 ml). To this solution was added hydrochloric acid (40 ml, 2 M) and the volume was adjusted to 1 litre with distilled water. The final pH of this solution was approximately 1.2 pH units.

3.1.2. Use of the Coulter "Nano-Sizer".

The particle size of the diluted emulsion systems was measured using the Coulter "Nano-Sizer". This instrument detects dynamic changes in laser light scattering intensity, which occurs when particles oscillate due to Brownian motion (Pouton, 1985). A mean particle size is computed for those particles between 40 and 3000 nm. The polydispersity index indicates the size range, as a score between 0 and 9, (narrow to wide size range).

The Coulter "Nano-Sizer" was allowed to equilibrate for at least 30 minutes before use.

The components for oil system of the SES were weighed into a tared 25 ml beaker. Artificial gastric contents were measured into the beaker and the emulsion was stirred gently on a magnetic stirrer.

The cuvette of the "Nano-Sizer" was filled with the diluted emulsified system and placed in the chamber of the instrument. The instrument controls were set to measure on automatic size ranging over a period of 2 minutes and each sample was assayed in triplicate to obtain a mean particle size measurement. A figure for the polydispersity index of the emulsion was also recorded.

The operation of the Coulter "Nano-Sizer" was validated on a daily basis by measuring the particle size in suspensions of polystyrene latex beads covering the range, 100 - 2000 nm

3.2.1. Effect of SES constituents on stability and particle size.

In order to establish the optimum proportions of components in a SES, a series of experiments was performed. For each SES the stability of the emulsion and its particle size were determined.

The main variables to be examined were:

The effect of different surfactants and co-

surfactants, changes in the surfactant : co-surfactant ratio, changes in the oil:surfactant/co-surfactant ratio, effect of diluent volume and effect of oil type.

Miglyol 812 was the oil used in the initial studies. This oil, when mixed with Tween 85 has been shown to produce systems which emulsify rapidly and produce emulsions which have small mean particle sizes (278 - 562 nm) (Pouton, 1985).

The most promising variables selected at each stage were used in subsequent investigations.

i) **Surfactant and co-surfactant.**

Surfactants and co-surfactants were selected from those known to be pharmaceutically acceptable.

A series of Miglyol 812 SES's were prepared based on the following basic formula;

0.08 ml Miglyol 812

0.02 ml Surfactant:co-surfactant (4:1)

and were emulsified in 25.00 ml of artificial gastric contents.

Surfactants were selected from Tween 85, Tween 80, Tween 20 and the co-surfactant selected from Span 85, Span 80, Span 20, Labrafil or Softigen.

ii) **Surfactant : co-surfactant ratio.**

Tween 85 and Span 85 were mixed in varying proportions for these SES's to obtain the optimum ratio for these components which produced a diluted emulsions with a small particle size.

A series of Miglyol 812 SES's was prepared based on the following basic formula;

0.08 ml Miglyol 812

0.02 ml variable ratios of Tween 85:Span 85

and were emulsified with 25.00 ml of artificial gastric contents.

iii) **Surfactant/co-surfactant to Miglyol 812 ratio.**

The ratio of Miglyol 812 was varied with respect to a fixed 4:1 ratio of the surfactant, Tween 85 and co-surfactant Span 85.

The composition of the SES was based on the following basic formula;

(0.1 - x) ml Miglyol 812

x ml Surfactant/co-surfactant,

and were emulsified in 25.00 ml of artificial gastric contents.

The volume of surfactant/co-surfactant (x) was varied between 0.01 ml and 0.04 ml.

iv) **Diluent volume.**

It was essential to measure the effect of dilution of a SES on the particle size in the emulsion since this variable may influence the volume of SES which could be used in-vivo to produce a successful absorption enhancing effect. The dilution of any SES produced a highly turbid emulsion and this required sufficient dilution to allow the Coulter "Nano-Sizer" to measure the particle size without being rejected by the instrument as "overrange". Therefore

only a limited series of dilutions were possible. The following basic system;

0.08 ml Miglyol 812

0.02 ml Tween 85 : Span 85 (4:1)

was emulsified with artificial gastric contents ranging from 10.0 ml to 28.75 ml and the emulsions formed were analysed for particle size using the "Nano-Sizer" and examined for stability.

v) **SES containing peanut oil.**

Some of the most successful SES's developed for Miglyol 812, based on their small particle sizes and emulsion stability, were repeated using peanut oil, using the following basic formula;

0.08 ml peanut oil

0.02 ml Surfactant:co-surfactant (4:1)

and was emulsified with 25.00 ml of artificial gastric contents.

Tween 85 was used as the surfactant and the co-surfactant was selected from Span 85, Labrafil and Labrasol.

vi) **SES containing cottonseed oil and linoleic acid.**

It was decided to test various mixtures of cottonseed oil and linoleic acid in a series of self-emulsifying oil systems. SES were investigated using mixtures in ratios of 3:1, 1:1 and 1:3, and using varying amounts of co-surfactant with Tween 85 as the surfactant.

The following basic system was used;

(0.1 - x) ml cottonseed:linoleic acid mixture

x ml Tween 85/co-surfactant (4:1)

and this was emulsified in 25.0 ml artificial gastric contents.

3.3. Results.

After measuring the particle size and visually assessing the stability of a number of the diluted SES's containing Miglyol 812 a number of observations were made.

The most successful Miglyol 812 SES system was prepared with combinations of surfactant and co-surfactant contained Tween 85 together with either Span 85 or Labrafil (Table 15). The systems containing these components readily emulsified at a dilution of 1 part SES to 250 parts aqueous phase and produced emulsions which remained stable over several days and with mean particle sizes of 509 and 584 nanometres respectively. The polydispersity index of these emulsion systems was however, generally high (7 - 9)). These particle sizes were similar to the diameter of chylomicrons (Barrowman, 1984). Miglyol 812 SES's prepared using mixtures of Tween 85 and either Softigen or Span 20 were unstable and visible oily droplets could be seen floating in the diluted SES. Miglyol 812 SES's prepared using Tweens 80 or 20 in combination with

Table 15. **Composition, particle size and stability of self emulsifying oil systems containing Miglyol 812: Effect of various surfactant/co-surfactants.**

Systems were based on the following composition:

0.08 ml Miglyol 812

0.02 ml Surfactant:Co-surfactant (4 : 1 by weight)

and were diluted with 25 ml artificial gastric contents

Surfactant	Co-surfactant	Size (nm ± sd)	Comments (PD)
Tween 85	Span 85	509 ± 20	Stable (7)
Tween 85	Labrafil	584 ± 21	Stable (8)
Tween 85	Softigen	499 ± 6	Oily floaters
Tween 85	Span 20	823 ± 72	Oily floaters
Tween 80	Span 80	---	Biphasic, unstable
Tween 20	Span 20	---	Biphasic, unstable

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

either Span 80 or 20 did not emulsify and were seen to be biphasic after dilution in the artificial gastric contents.

A mixture of Tween 85 and Span 85 was selected for further studies.

Variations in the ratio of Tween 85 and Span 85 in a series of Miglyol 812 SES's, showed the most stable system to be that which contained a ratio of 4:1 of Tween 85:Span 85. At ratios of Tween 85:Span 85 less than 4:1, the emulsions appeared to be stable but their mean particle size progressively increased to values of over 1000 nanometres. At higher Tween 85:Span 85 ratios and with Tween 85 alone, the emulsion formed was unstable and rapidly became biphasic. A 4:1 ratio of Tween 85 to Span 85 was used in all subsequent investigations. These data are shown in Table 16.

In order to optimise the incorporation of a lipophilic drug in the emulsion system, the proportion of oil in the SES should be high. The ratio of oil to surfactant : co-surfactant was varied to determine the optimum oil content in the SES. Increasing the proportion of oil:surfactant/co-surfactant from 8:2 to 9:1 produced a stable emulsion but the particle size increased twofold. Decreasing the oil : surfactant/co-surfactant ratio slightly increased the particle size of the emulsion but also resulted in an unstable system with the

Table 16. Composition, particle size and stability of self emulsifying oil systems containing Miglyol 812: Effect of surfactant:co-surfactant ratio.

Systems were based on the following composition:

0.08ml Miglyol 812

0.02ml Variable mixtures by weight of Tween 85:Span 85 and were diluted with 25 ml artificial gastric contents

Tween 85:Span 85 ratio	Size (nm ± sd)	Comments (PD)
Tween only	550 ± 18	Biphasic, unstable
6 : 1	545 ± 28	Biphasic, unstable
4 : 1	509 ± 20	Stable (7)
2 : 1	592 ± 29	Stable (8)
1 : 1	1940 ± 118	Stable (0)

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

presence of floating oily droplets. A ratio of 8 parts oil to 2 parts surfactant/co-surfactant was selected as the optimum for future studies. These data are shown in Table 17.

Dilution of an optimised Miglyol 812 SES containing 8 parts oil to 2 parts of a 4:1 mixture of Tween 85:Span 85 with artificial gastric contents in varying proportions showed the following results. At dilutions of 1:200 or less the emulsion formed was extremely turbid and the particle size of the emulsion could not be measured using the "Nano-Sizer". However, the diluted emulsions appeared stable at a dilution of 1:100. At higher dilutions from 1:240 to 1:287.5 the emulsion appeared stable but the results showed an increase in particle size with increasing dilution. These data are shown in Table 18.

It is difficult to extrapolate from these results to the anticipated low dilution volume in the rat when a 0.5 ml oral dose of SES is given. It may be postulated, however, that a bolus dose of oil might emulsify progressively in the stomach as gastric fluid is secreted and mixing proceeds.

Following an investigation of the variables which affect the production of SES's containing the mixed medium chain triglyceride oil, Miglyol 812. It was decided to repeat some of these studies using the

Table 17. Composition, particle size and stability of self emulsifying oil systems containing Miglyol 812: Effect of oil : surfactant/co-surfactant ratio.

Systems were based on the following composition:

(0.1 - x) ml Miglyol 812

x ml Tween 85:Span 85 (4 : 1 by weight)

and were diluted with 25 ml artificial gastric contents

Oil	Tween 85/ Span 85	Size (nm ± sd)	Comments (PD)
0.09 ml	0.01 ml	942 ± 143	Stable (7)
0.085 ml	0.015 ml	989 ± 96	Stable (7)
0.08 ml	0.02 ml	509 ± 20	Stable (7)
0.07 ml	0.03 ml	667 ± 32	Oily floater
0.06 ml	0.04 ml	694 ± 50	Oily floaters

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

Table 18. Composition, particle size and stability of self emulsifying oil systems containing Miglyol 812: Effect of diluent volume.

Systems were based on the following composition:

0.08 ml Miglyol 812

0.02 ml Surfactant:Co-surfactant (4 : 1 by weight)

Variable volumes of artificial gastric contents

Diluent volume (ml)	Size (nm ± sd)	Comments (PD)
10.0	---	Overrange
11.0	---	Overrange
12.0	---	Overrange
14.0	---	Overrange
16.0	---	Overrange
18.0	---	Overrange
20.0	---	Overrange
22.0	---	Overrange
24.0	548 ± 20	Stable (8)
25.0	575 ± 21	Stable (8)
26.25	580 ± 14	Stable (8)
27.5	565 ± 31	Stable (8)
28.75	626 ± 20	Stable (8)

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

mixed long chain fatty acid triglyceride oil, peanut oil, together with the more successful combinations of surfactant and co-surfactant.

The SES systems containing peanut oil with Tween 85 and Span 85, Labrasol or Labrafil in the ratio of 4:1 produced emulsions with particle sizes of 1903, 2656 and 2403 nanometres respectively. These systems were generally stable except for those which contained Labrasol. The polydispersity index of these emulsion systems was, however, much smaller than equivalent Miglyol 812 systems (0 - 2). These data are shown in Table 19. The systems containing peanut oil were not further investigated because the particle sizes in these emulsions were at least four times greater than the size in corresponding Miglyol 812 emulsions.

SES systems containing cottonseed oil alone or linoleic acid alone were biphasic when prepared with Tween 85 and Span 85 at a ratio of 4:1.

Mixtures of cottonseed oil and linoleic acid in ratios from 3:1 to 1:3 were generally unstable when prepared with Tween 85 and either Span 85 or Labrosol at a ratio of 4:1.

The proportion of surfactant/co-surfactant to oil phase was increased from 20% to 45%. Under these conditions the Tween 85:Labrosol (4:1) produced some stable systems. The particle sizes of these systems was, however, in excess of 1300 nanometres

Table 19. Composition, particle size and stability of self emulsifying oil systems containing Peanut oil: Effect of various surfactant/co-surfactants.

Systems were based on the following composition:

0.08 ml Peanut oil

0.02 ml Surfactant:Co-surfactant (4 : 1 by weight)

and were diluted with 25 ml artificial gastric contents

Surfactant	Co-surfactant	Size (nm ± sd)	Comments (PD)
Tween 85	Span 85	1903 ± 69	Stable (2)
Tween 85	Labrafil	2403 ± 191	Stable (0)
Tween 85	Labrasol	2656 ± 56	Unstable

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

(polydispersity index was generally low (0 - 2)). These systems were considered unacceptable because of the combination of low oil content and increased particle size, compared with the Miglyol 812 emulsion systems. These data are shown in Table 20.

3.4. Conclusions.

An investigation into the effects of varying oil, co-surfactant and surfactant systems on the particle size and stability of self-emulsifying oil systems was carried out. The results showed that the mixed medium chain fatty acid triglyceride, Miglyol 812, was the oil which could be most easily incorporated into a self-emulsifying oil system. An oil content of 80% and a surfactant : co-surfactant ratio of four parts Tween 85 to one part Span 85, produced emulsions which were stable and of the desired size (500 nanometres or less (Section 3.1.)), despite showing high polydispersity indices. Stable emulsions were produced using Labrafil to replace Span 85, these emulsions were of a slightly larger particle size (580 nm).

Self-emulsifying oil systems produced using the mixed long chain fatty acid triglyceride oil, peanut oil, were stable but with a mean particle diameter at least four times greater (1900 - 2660 nm) than with the equivalent Miglyol 812 systems.

These SES data compare favourably with data reported

Table 20. Composition, particle size and stability of self emulsifying oil systems containing varying proportions of cottonseed oil and linoleic acid: Effect of surfactant/co-surfactant types.

Systems were based on the following composition:

(0.1 - x)ml Cottonseed:Linoleic acid (Variable ratios)

x ml Tween 85:Co-surfactant (4 : 1 by weight)

and were diluted with 25 ml artificial gastric contents

Cottonseed/ Linoleic	Surfactant volume (x ml)	Co-surfactant	Size (nm ± sd)	(PD)	Comment
Cottonseed	0.02	Span 85	---		Biphasic
Linoleic	0.02	Span 85	---		Biphasic
1 : 3	0.02	Span 85	2590 ± 563		Unstable
1 : 1	0.02	Span 85	2370 ± 333		Stable(0)
3 : 1	0.02	Span 85	1863 ± 266		Unstable
1 : 3	0.02	Labrosol	1940 ± 730		Unstable
1 : 1	0.02	Labrosol	2760 ± 809		Unstable
3 : 1	0.02	Labrosol	1277 ± 93		Unstable
1 : 3	0.03	Labrosol	362 ± 59		Unstable
1 : 1	0.03	Labrosol	1893 ± 261		Stable(0)
3 : 1	0.03	Labrosol	1433 ± 491		Stable(0)
1 : 3	0.045	Labrosol	1377 ± 38		Stable(0)
1 : 1	0.045	Labrosol	1313 ± 65		Stable(0)
3 : 1	0.045	Labrosol	822 ± 21		Stable(0)

Notes:
i) Particle size is the mean (± sd) of three readings on an emulsion system taken at 2 minute intervals.
ii) (PD) is the polydispersity index. (Stable systems only)

by Charman (Charman et al., 1992), who investigated a self-emulsifying drug delivery system for the formulation of a lipophilic drug molecule, WIN 54954 (log. P = 5.3). They reported the smallest mean emulsion droplet diameter (MEDD) of 0.7 - 0.8 μm on a system which consisted of 40 % Neobee M5 (a mixed medium chain monoglyceride oil similar in fatty acid composition to the mixed medium chain tri-acyl glycerol oil, Miglyol 812), 30 % Tagat TO (ethoxylated glyceryl trioleate, nonionic surfactant) and 30 % WIN 54954.

In our studies, a mixed oil system containing the mixed long chain triglyceride oil, cottonseed oil, and the long chain fatty acid oil, linoleic acid, produced stable emulsion systems only if the amount of surfactant/ co-surfactant in the SES was at least 45% by weight, the limited quantity of oil in the SES may have limited the quantity of retinoid incorporated in a system of this type. The emulsions produced were also of a larger mean diameter (> 1000 nm).

It is difficult to explain the differences in particle size and stability of the SES's produced in these studies since no clear patterns were observed. The aim of these studies was to investigate the effect of various oral delivery systems on the absorption of the retinoids, therefore, the in-vivo studies on the absorption of the retinoids

(Chapter 4.2) are the most important indicator of the success of any oral SES's in retinoid absorption.

CHAPTER 4

IN-VIVO STUDIES ON THE ABSORPTION AND DISTRIBUTION OF THE RETINOIDS.

4.1. INTRAVENOUS PHARMACOKINETICS IN THE RAT.

4.1.1. Pharmacokinetic studies in the rat.

Intravenous dosing of the retinoids was essential to provide information on the pharmacokinetics of these compounds in the rat. Parameters which were considered essential for a complete understanding of the oral absorption of the retinoids included the total body clearance (Cl), volume of distribution (V_d) and half-life ($t_{½}$).

V_d was to be used in the estimation of the total body content of a retinoid in the body at a given time, using the plasma concentration data, after oral dosing (Section 4.2.). The magnitude of V_d was anticipated to be high, since it has been shown in Chapter 1 that the retinoids are highly lipophilic in nature, and are therefore expected to distribute extensively in the fatty tissues within the body of the rat. The elimination half-life ($t_{½}$) would be used to correct the estimates of total body retinoid content to allow for any elimination of drug which had occurred since absorption. The elimination half-life values for the retinoids were expected to be long, due to the extensive distribution and relatively low clearance. These three parameters, together with the area under the plasma concentration time curve (AUC), were to be used in the evaluation of absolute bioavailability of the retinoids after oral administration.

4.1.2. In-vivo techniques used to study the pharmacokinetics of the retinoids.

Two in-vivo techniques were used in these studies of the pharmacokinetics of the retinoids in the male Wistar rat. Cannulation of the jugular vein was required to permit the intravenous administration of oily emulsions of the retinoids used in the pharmacokinetic and bioavailability studies.
The carotid artery was cannulated in order to allow the collection of timed blood samples for analysis of retinoid concentrations after intravenous dosing.
The rat was chosen as a model animal species since the techniques for cannulating blood vessels and the mesenteric lymph duct (Section 4.2.2.4.1.) were already established using this animal and a breeding colony was available for use in the Animal Unit of the Queen's Medical Centre, Nottingham.

4.1.2.1. Cannulation of the carotid artery of the rat.

Animals were anaesthetised by an intra-peritoneal (i.p.) injection of sodium pentobarbitone (0.3 ml Sagatal 60 mg/ml in saline (72 mg/kg)). Anaesthesia was maintained throughout the procedure by the administration of further doses of Sagatal i.p. (0.1 ml) as necessary.
The animal was placed, ventral side uppermost, on a heated mat. A small section of skin at the front of

the neck was removed to expose the muscle which covered the trachea. The muscle was separated by blunt dissection, using curved-end forceps, to expose the carotid arteries, which lie perpendicular to the trachea on each side of the neck.
The left artery was dissected clear of surrounding tissues and a micro-spatula was slid under the vessel. Cotton sutures were placed at either end of the artery and the anterior end was tied. The posterior end of the vessel was clamped using an artery clip. The vessel was cut transversely using spring scissors and a saline filled, polythene cannula, (approximately 20 cm in length) having a sharply tapering bevel (75°) and clamped at the distal end with "Spencer Wells" forceps, was inserted into the vessel and tied in position using a double suture. The artery clip was removed and blood was seen to flow along the cannula. Timed blood samples (0.2 ml) were collected from this vessel into heparinised micro-centrifuge tubes. Plasma was separated by centrifugation (13,000 x g, 5 minutes) and transferred into micro-centrifuge tubes. Plasma samples were labelled and stored at -20°C.

4.1.2.2. <u>Cannulation of the jugular vein.</u>

Cannulation of the jugular vein was performed using a method similar to that used for cannulating the

carotid artery (Section 4.1.2.1.). The vessel was exposed and detached from the surrounding tissues using great care to prevent the vessel collapsing. The vessel was kept patent by the infusion of isotonic saline and was used for the administration of intravenous fluids.

4.1.2.3. Preparation of oily emulsions for intravenous administration.

Three soyabean oil-in-water emulsions were prepared. Each emulsion contained one of the three retinoids, Ro 04-3780, Ro 10-9359 or Ro 15-0778, for intravenous administration to fasting rats.
The retinoid was dissolved in soyabean oil at a concentration of approximately 15 mg/ml. Previous solubility studies on the retinoids had showed that this concentration exceeded the solubility of Ro 04-3780 in soyabean oil (Table 4). The following procedure was used to ensure maximum solubility of the retinoid in the oil. The retinoid (150 mg) was weighed into a borosilicate glass test tube (15 ml), which had been previously wrapped in aluminium foil to protect the retinoid from exposure to light. The retinoid was dissolved in the minimum volume of diethyl ether (0.2 - 0.5 ml) and soyabean oil (10 ml) was transferred to the tube using a glass pipette (10 ml). The contents of the tube were mixed by gentle rotation on a rotary mixer (30 rpm,

10 minutes). The diethyl ether was removed from the solution using a stream of helium. The retinoid solution was centrifuged (2200 x g, 20 minutes) to remove any precipitate which formed during this procedure.

An aliquot of the retinoid solution in soyabean oil (5 ml) was added to a solution of egg lecithin (1.2% Lipoid E80) in double distilled water (45 ml) and these solutions were homogenised in a micro-fluidiser (approximately 5 minutes). The resulting emulsion was transferred into a multi-dosing vial (50 ml), fitted with a rubber septum. The prepared emulsions were stored at room temperature (20°C) and appeared to remain stable for several weeks. The emulsions were normally, used in the iv. studies within a period of 7 days after preparation. The exact concentrations of each retinoid in the prepared emulsions were determined by dispensing an accurately weighed sample of each emulsion into a volumetric flask and diluting to volume using hplc grade THF. Samples of this mixture were analysed to determine the retinoid concentration following the extraction procedure for retinoids in oil (Section 1.2.1.2.) and using the appropriate hplc system (Section 2.1.1.).

4.1.3. Intravenous dosing of rats with an emulsion containing retinoid.

Two groups of six male Wistar rats (200 - 250 g) were used in each pharmacokinetic study and were fasted overnight on wire grids to prevent coprophagia. Each animal was anaesthetised as previously described (Section 4.1.2.1.). The animal was placed on its back and the carotid artery and jugular vein were cannulated as previously described (4.1.2.1. and 4.1.2.2.).

A bolus intravenous dose of the soyabean oil emulsion (group a; 0.2 ml and group b; 0.6 ml) was administered to each animal *via* the jugular vein and the dose was washed into the animal using isotonic saline (approximately 0.3 ml). Intravenous administration of the emulsion (including the saline wash) was completed in less than 30 seconds.

Blood samples (0.2 ml) were collected from the carotid artery of each animal at timed intervals after intravenous dosing. In studies using Ro 04-3780, samples were collected at 5, 10, 20, 30, 45, 60, 90, 120, 150, 180 and 210 minutes and in studies using Ro 10-9359 and Ro 15-0778, samples were also collected at 240, 270 and 300 minutes. After centrifugation (13,000 x g, 10 minutes), plasma was separated and stored at -20°C.

Samples of plasma were analysed to determine the retinoid concentration of retinoid using the

appropriate hplc system (Chapter 2). Pharmacokinetic parameters were calculated from the plasma concentration data.

4.1.4. Calculation of pharmacokinetic parameters on the retinoids.

The concentration of retinoid in each of the soyabean oil emulsions were determined by hplc. The administered doses of each retinoid were found to contain;

	Group a, low dose (0.2ml)	**Group b, high dose (0.6ml)**
Ro 04-3780	0.264 mg/kg	0.792 mg/kg
Ro 10-9359	0.655 mg/kg	1.964 mg/kg
Ro 15-0778	0.732 mg/kg	2.200 mg/kg

The area under the logarithmic plasma concentration time curve (AUC) was calculated using the trapezoid approximation method. An estimate of AUC from the last data time point to infinite time ($AUC_{0-\infty}$) was obtained by dividing the final concentration data point by the terminal elimination rate constant (β). The terminal elimination rate constant was determined by measuring the gradient of the terminal linear section of the logarithmic concentration time curve. The terminal phase elimination rate constant was used in Equation 11 to determine the terminal half-life ($t_{½}$) of the respective retinoid.

Elimination half life, $t_{½} = \frac{0.693}{\beta}$ Equation.....11

β = terminal phase elimination rate constant.

The mean residence time (MRT) was calculated from the ratio of the area under the first moment curve (AUMC) and the area under the plasma concentration time curve (AUC) using Equation 12.

Mean residence time, $MRT = \frac{AUMC}{AUC}$ Equation.....12

AUMC = Area under the first moment curve, extrapolated to infinity.

AUC = Area under the plasma concentration time curve, extrapolated to infinity.

Clearance (Cl) was calculated from the intravenous dose and AUC using Equation 13.

Clearance, $Cl = \frac{Dose}{AUC_{0-\infty}}$ Equation.....13

AUC = Area under the plasma concentration time curve, extrapolated to infinity.

The volume of distribution (V_d) was calculated from clearance (Cl) and the terminal elimination rate constant using Equation 14.

Volume of distribution, $V_d = \frac{Cl}{\beta}$ Equation.....14

The volume of distribution at steady state (V_{ss}) was calculated as the product of the MRT and the clearance using Equation 15.

Volume of distribution = MRT x Cl Equation.....15
at steady state, V_{ss}.

These pharmacokinetic data are displayed in Table 21, Table 22, and Table 23.

4.1.5. Results and discussion.

Pharmacokinetic data were obtained from fasted male Wistar rats after intravenous dosing of retinoids in soyabean oil emulsions. The data obtained from these studies and especially data on the clearance (Cl), the volume of distribution (V_d) and the elimination half life ($t_{½}$), were necessary for the calculation of plasma uptake rate after oral dosing studies (Section 4.2.).

All retinoids have been shown to be highly lipophilic in nature and were expected to distribute widely into the fatty tissues of the body. The volumes of distribution were expected to be large and the elimination half-life was consequently expected to be relatively long.

From the pharmacokinetic data for Ro 04-3780 (Table 21 and Figure 11), a non-proportional increase in AUC was observed with increasing dose,

Table 21. **Pharmacokinetic data for Ro 04-3780 (Isotretinoin in the rat after intravenous administration of a soya oil emulsion.**

Dose (mg/kg)	0.264	0.792
A.U.C. (ug/ml.min)	55 ± 31	317 ± 34 *
Elimination half-life, $t_{\frac{1}{2}}$ (min).	48 ± 5	61 ± 7 *
MRT (min)	73 ± 16	79 ± 3
Clearance (ml/min/kg)	6.0 ± 3.4	2.5 ± 0.2 *
Volume of Distribution (ml)	421 ± 227	222 ± 19
Volume of Distribution at steady state (V_{ss}, ml).	438 ± 199	197 ± 48

(* $p < 0.05$)

Table 22. Pharmacokinetic data for Ro 15-0778 (Temarotene) in the rat after intravenous administration of a soya oil emulsion.

Dose (mg/kg)	0.732	2.200
A.U.C. (ug/ml.min)	195 ± 37	852 ± 100 *
Elimination half-life, $t_{\frac{1}{2}}$ (min).	111 ± 38	114 ± 45
MRT (min)	111 ± 68	62 ± 14
Clearance (ml/min/kg)	3.9 ± 0.6	2.6 ± 0.4 *
Volume of Distribution (ml)	983 ± 746	1221 ± 638
Volume of distribution at steady state (V_{ss}, ml)	432 ± 269	325 ± 349

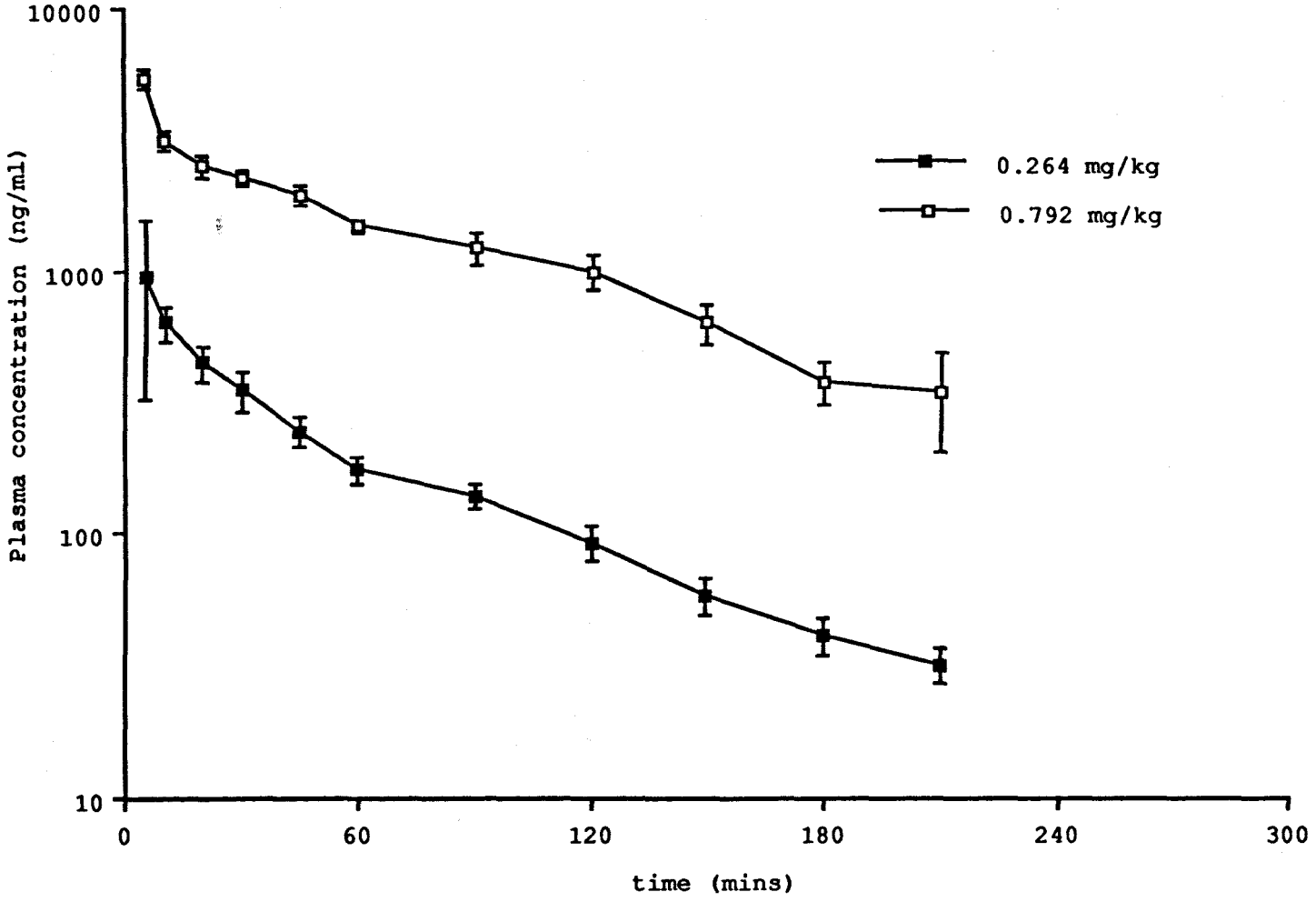
(* $p < 0.05$)

Table 23. Pharmacokinetic data for Ro 10-9359 (Etretinate) in the rat after intravenous administration of a soya oil emulsion.

Dose (mg/kg)	0.655	1.964
A.U.C. (ug/ml.min)	114.6 **	579.6 ± 130.3
Elimination half-life, $t_{\frac{1}{2}}$ (min).	3.94 **	4.95 ± 1.47
MRT	10 ± 7	38 ± 20
Clearance (ml/min/kg)	5.72 **	3.53 ± 0.64
Volume of Distribution (ml)	32.4 **	25.99 ± 10.7
Volume of distribution at steady state (Vss, ml).	57 ± 15 **	134 ± 133

(** Insufficient data for statistical analysis)

Figure 11.Plasma concentration-time profile for Ro 04-3780 in the rat after intravenous dosing in a soyabean emulsion.



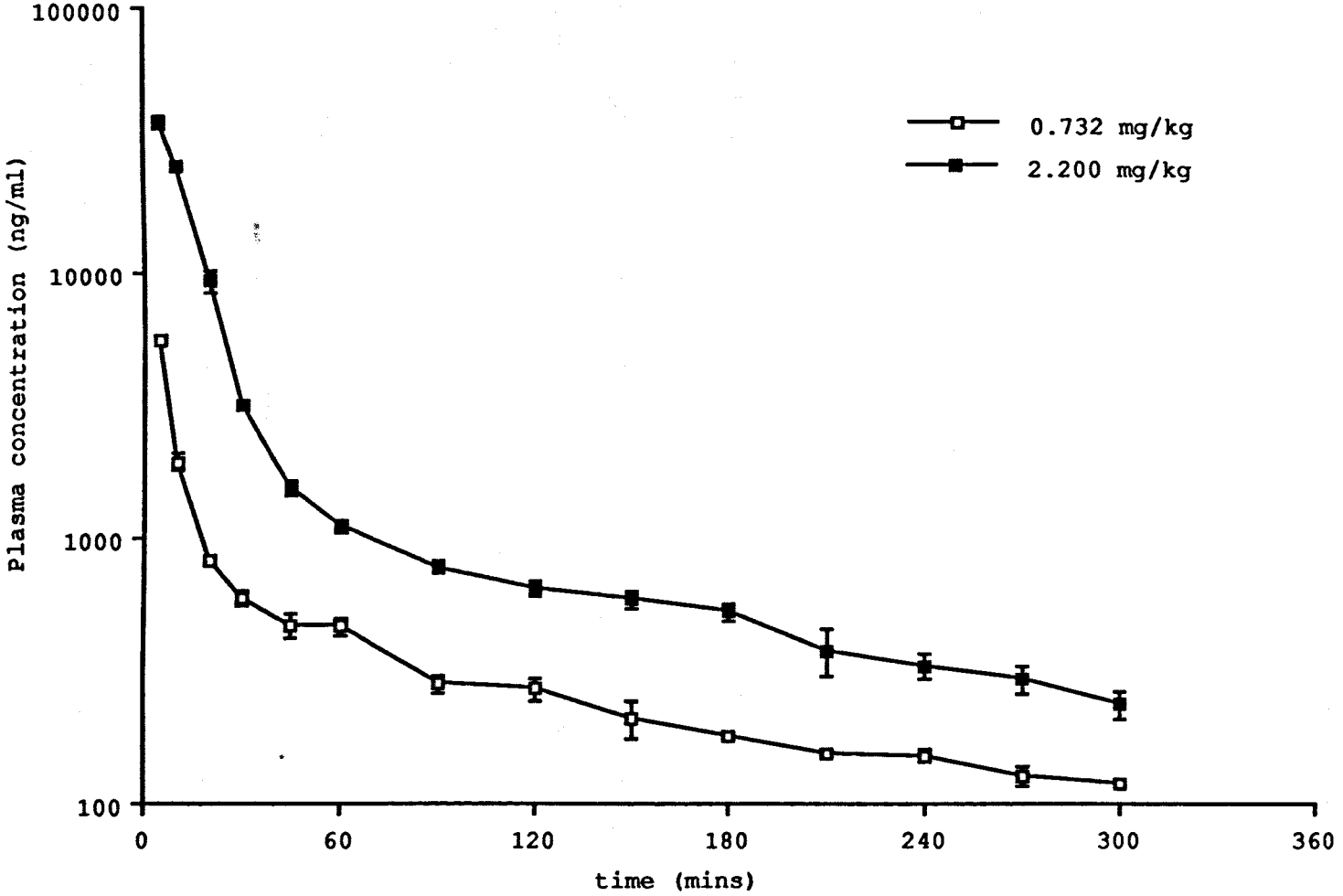
resulting from a decrease in both the total body clearance and the volume of distribution. The elimination half-life also increased with dose. The mean half-life for Ro 04-3780 was found to be 55 ± 5.6 minutes, slightly shorter than data reported in the literature ($t_{½}$ = 76 minutes) (Liu et al., 1990), however the reported data were obtained from studies in Sprague-Dawley rats of much larger body weight (383 ± 33 g).

The mean residence time, MRT, an estimate of the time taken for 63.2% of the dose to be eliminated from the body (Gibaldi, 1990), was 76 minutes and was consistent with the observed elimination half-life ($t_{½}$) of 55 minutes.

At the lower dose of 0.264 mg/kg, a volume of distribution (V_d) of 220 ml, a volume of distribution at steady state (V_{ss}) of 200 ml and total body clearance of 2.5 ml/min were obtained, which were consistent with data reported in rats (V_{ss} = 230 ml and Cl = 2.1 ml/min, (Liu et al., 1990)) and dogs (V_d = 2020 ml and Cl = 5.2 ml/min, (Cotler et al., 1983)).

From the pharmacokinetic data for the more lipophilic retinoid, Ro 15-0778 (Table 22 and Figure 12), a non-proportional increase in AUC was observed with increasing dose, due to a increased $t_{½}$ at the high dose, this resulted from a decrease in total body clearance. There were considerable inter-

Figure 12.Plasma concentration-time profile for Ro 15-0778 in the rat after intravenous dosing in a soyabean emulsion.



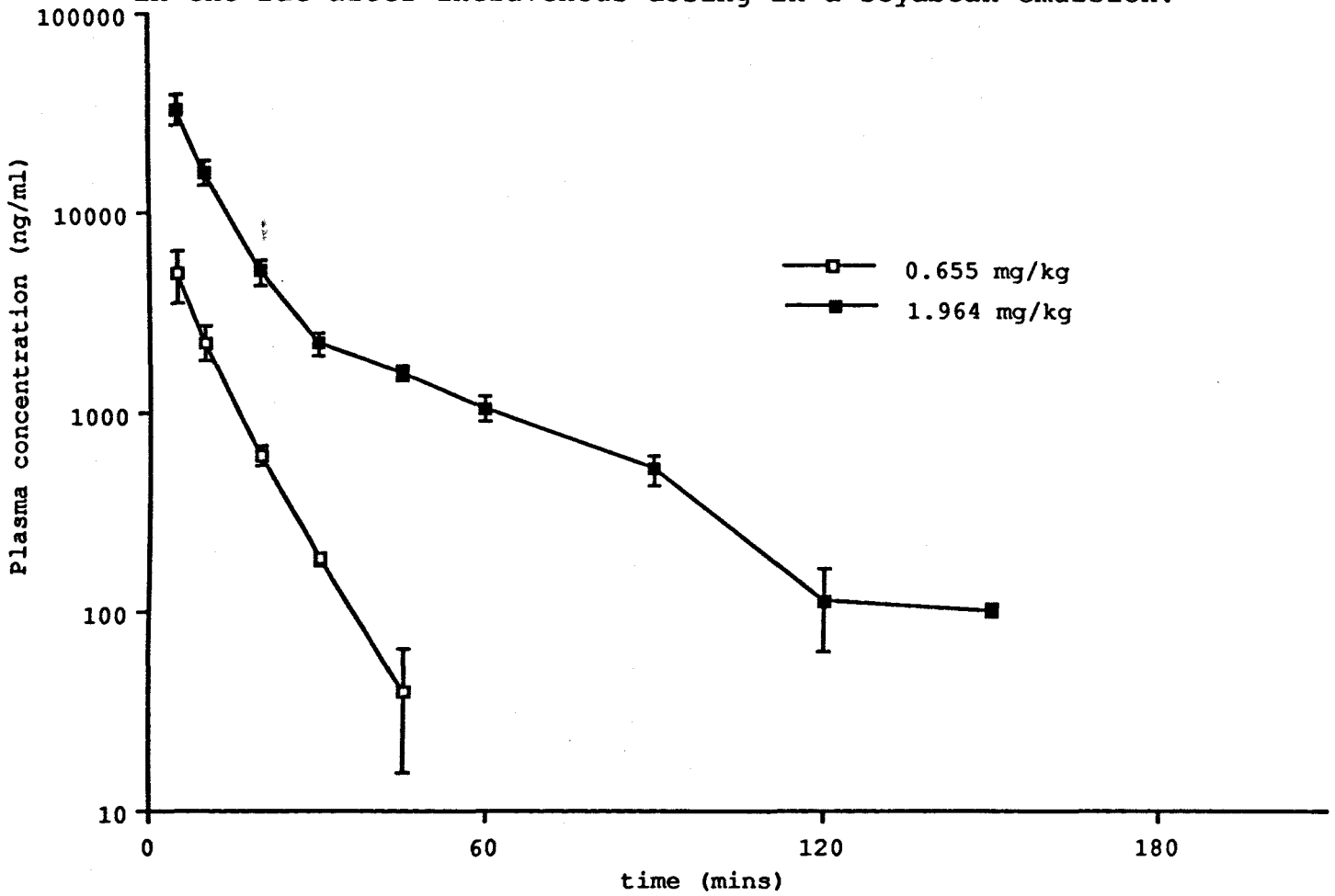
subject variability in the mean data for V_d (1100 ± 690 ml) and V_{ss} (380 ± 340 ml). The volume of distribution (V_d) at the low dose (980 ml) and at the high dose (1220 ml) and the volume of distribution at steady state (V_{ss}) calculated at the low dose (430 ml) and at the high dose (325 ml), were not significantly different ($p > 0.05$).

The MRT at the lower dose (MRT = 111 ± 68 min) was consistent with the elimination half-life observed at the lower dose ($t_{½}$ = 111 ± 38 min), however, the MRT at the higher dose was much lower (MRT = 62 ± 14 min) than either the MRT at the lower dose or the elimination half-life at either dose. These differences, however, were not significant ($p > 0.05$) because of the considerable inter-subject variation in the data.

There appear to be no reported pharmacokinetic data relating to this compound in the literature. However, the data were consistent with those expected for a highly lipophilic retinoid.

After intravenous dosing of Ro 10-9359 at the higher dose (1.964 mg/kg) (Figure 13), plasma concentrations fell rapidly from 33000 ng/ml at 5 minutes post dose to less than 100 ng/ml after 150 minutes and below the limit of detection at 180 minutes. At the low dose (0.655 mg/kg), a similar rapid fall in plasma concentrations from

Figure 13. Plasma concentration-time profile for Ro 10-9359 in the rat after intravenous dosing in a soyabean emulsion.



approximately 5000 ng/ml at 5 minutes post dose to less than 40 ng/ml after 45 minutes post dose and below the limit of detection at 60 minutes (Figure 13). The pharmacokinetic data showed a mean elimination half-life of 3.5 minutes and a mean MRT of 24 min. The volume of distribution was estimated to be 32 ml at the lower dose and 26 ml at the higher dose. At steady state, the volume of distribution was slightly higher, being 57 ml at the low dose and 134 ml at the higher dose.

These data (Table 23) were not consistent with those expected after studying values reported in humans (Paravicini et al., 1981) nor with the lipophilic nature of the compound nor with the pharmacokinetic data obtained for other retinoids in this study (Table 21 and Table 22). The data suggest that a prolonged distribution phase was present and the terminal elimination phase had not been observed. This prolonged distribution phase presented problems in accurately measuring the resulting low retinoid concentrations in plasma samples after 180 minutes post dose.

Paravicini (Paravicini et al., 1981) has suggested that there are at least four elimination phases for Ro 10-9359 after intravenous dosing studies in human volunteers. These phases had reported half-lives of, 5 - 10 minutes, 30 - 60 minutes and 6 - 12 hours after a single intravenous dose. Although there was

evidence in these studies of at least one longer phase, it was not possible to quantitate it fully. This very long elimination half-life has been estimated to be about 100 days. These data were obtained from a trial study in psoriatic patients undergoing chronic therapy with 10 - 25 mg/day etretinate for more than a year (Paravicini et al., 1981).

In order to determine the plasma concentrations of this retinoid over a prolonged time period in the rat, it would have been necessary to increase greatly the plasma concentrations of the drug. It was possible to achieve this by administering a much larger dose of retinoid to the animal (a dose of 20 mg/kg may have been required). However, it was thought inappropriate to exceed the volume of intravenous soyabean emulsion beyond the 0.6 ml dose size (approximately 5.5 % of the total blood volume in a 250 g rat (Griffith & Campbell, 1934). It was also impractical to administer a higher dose of retinoid in other physiologically acceptable vehicles because of the limited solubility of Ro 10-9359 in these solvents. The proposed high dose would have also greatly increased the potential toxicity of the drug to the animal and would have far exceeded the upper therapeutic limit for a human (2 mg/kg). Alternative procedures would have been to use much greater blood sample volumes at each data

point, extracting the whole animal blood volume (10 - 12 ml) for each data point, however, this procedure was considered an unacceptable use of animals. Alternatively, the dose could have been administered over an extended time period by intravenous infusion or a more sensitive analytical procedure could have been investigated.

In subsequent estimations of the venous uptake rate for Ro 10-9359 from plasma concentrations after oral dosing, a volume of distribution value of 1175 ml (Liu et al., 1990) was used.

CHAPTER 4.

IN-VIVO STUDIES ON THE ABSORPTION AND DISTRIBUTION OF THE RETINOIDS.

4.2. ORAL DOSING STUDIES IN THE RAT.

4.2.1. Introduction.

It is now well established that many lipophilic compounds are absorbed via the lymphatic route following oral administration (Table 1).

The mechanisms whereby lipophilic molecules are absorbed from the gastrointestinal tract and transported to the general circulation are complex and poorly understood (Patton, 1981). However, certain factors, thought to be important in lymphatic absorption, have been identified.

Absorption is considered to involve liberation of a drug from the vehicle in which it is dosed, into the aqueous luminal fluid, followed by passage through the gastrointestinal wall. It has been reported that the rate of release of drug from a solution is an inverse function of its solubility in a solvent (Armstrong & James, 1980). These data therefore imply that a vehicle in which a drug has a lower solubility will promote greater partitioning of the drug from the vehicle into the luminal fluid, thereby enhancing absorption. This advantage of poorly solvating vehicles for the absorption of drugs is limited, however, by the total amount of drug which the vehicle can dissolve since this limiting factor will control drug availability at the site of absorption.

Armstrong et al. (1979) have determined the in-vitro rates of release and distribution coefficients of a

range of benzoic and phenylacetic acid derivatives between either isopropyl myristate and water or octanol and water, and compared these with the *in-vivo* bioavailability of these compounds in the rat. Absorption *in-vivo* followed an inverse rank order of drug-lipid solubility, but was related to the *in vitro* solvent-water transfer rate constant, rather than the distribution coefficient. The inference from these studies was that bioavailability was dependent on the concentration of drug in solution in the gastrointestinal fluid which in turn was dependent on the rate of supply from the oily phase.

4.2.1.1. Factors which are thought to affect the lymphatic absorption of drugs.

There is evidence to suggest that fatty acids of all chain lengths can alter the permeability of the intestinal mucosa to drugs. The short chain fatty acids, propionic, butyric and caproic have been found to inhibit the absorption of anionic drugs but enhance the absorption of neutral and cationic drugs at pH 6.5 in the rat, these effects having been related to water-flux and changes in the microclimate pH (Inui *et al.*, 1974).

Diet has been shown to increase the overall bio-availability of retinoids in humans (Gibson *et al.*, 1984., Holazo *et al.*, 1990., Colburn *et al.*, 1983a). This effect has been explained (Colburn *et al.*,

1983a) by an increase in splanchnic-hepatic blood flow and an increase in the rate of dissolution of the drug, possibly by an increase in the flow of bile. Colburn et al. (1983a) have also suggested that food will increase the total absorption of drugs by delaying gastric emptying.

Structurally similar drugs may vary in their lymphatic absorption when presented in the same vehicle. For example, Fidge et al. (1968) have showed that after presenting radio-labelled vitamin-A compounds to rats, in bile salt-lipid emulsions resembling normal intestinal contents, 70% - 80% of the radioactivity associated with the vitamin-A or vitamin-A aldehyde was found in lymph with only 15% - 20% being found in the bile, suggesting predominantly lymphatic absorption. In contrast, only 5% - 7% of labelled vitamin-A acid was found in the lymph, much of the remainder (92% - 95%) being located in the bile, suggesting portal absorption. This difference may relate to differences in lipophilicity, however, the predicted difference in log P is only slight (0.031 units from Rekker fragmental data (Table 2)). It should be noted that log P data are calculated on the unionised forms of the retinoids and values will be significantly lower for a molecule with an dissociated acidic proton (Lyman, 1982).

In order to assess the importance of the oil

solubility of a retinoid, retinoid lipophilicity and the effect of co-administering a retinoid with oil, on the lymphatic uptake of the retinoid, a series of in-vivo experiments were performed.

4.2.2. In-vivo techniques used to study the lymphatic absorption of the retinoids.

A number of in-vivo techniques using male Wistar rats were used in the study of the lymphatic absorption of the retinoids.

Following some initial investigations, rats were selected with a body weight between 200 g and 250 g. Animals of greater body weight had been found to contain increasing quantities of body fat which made cannulation of the mesenteric lymph duct technically more difficult. Animals of body weight less than 200 g had mesenteric lymph vessels which were difficult to cannulate because of their small size and which gave very low lymph flow rates.

Several in-vivo techniques were used in the oral dosing studies, retinoids were delivered into the stomach of the rat in a series of oily vehicles, in order to study the effect of various formulations on the absorption of the retinoids. The carotid artery was cannulated in order to collect blood samples for analysis of retinoid concentrations after oral dosing and the mesenteric lymph duct of the rat was cannulated in order to collect intestinal lymph

samples for analysis of retinoid concentrations after oral dosing.

Lymph flow rates were assessed from individual lymph sample volumes and the times to collect the samples. Lymph turbidity was determined by measuring the spectrophotometric absorbance of the lymph samples.

4.2.2.1. Oral dosing technique in the rat.

Oily solutions of the retinoids (0.5 ml) or liquid food material (2.0 ml) were administered into the stomach of the rat from a disposable syringe (1.0 or 2.5 ml) through a metal oral dosing needle (16 g x 3").

The animal was restrained by the skin on the back, the dosing needle was inserted into the stomach and the contents of the syringe were dosed into the stomach. Care was taken to prevent accidental dosing into the trachea or lungs. This procedure was performed without anaesthesia and was completed in less than 30 seconds.

4.2.2.2. Cannulation of the cartotid artery.

Cannulation of the carotid artery was performed as previously described in Section 4.1.2.1.

4.2.2.3. Cannulation of the mesenteric lymph duct.

A search of the literature has revealed that thoracic lymph duct cannulations appear to have been

performed more frequently than mesenteric lymph duct cannulations in studies of lymphatic absorption (Forsgren, 1969., Kamp & Neumann, 1975., Saldeen & Linder, 1960., Sieber et al., 1974., Tucker, 1983., Yanagawa et al., 1989., Gowan & Stavchansky, 1986., Hashida et al., 1977., Hirate & Ueda, 1989).

The thoracic lymph duct in the rat, is large thick walled vessel, approximately 1 - 2 mm in diameter, which runs posterior to the aorta (Bollman et al., 1948). Cannulation of this vessel is relatively easy and large volumes of lymph can be collected from the vessel. The high lymph flow through the thoracic duct is thought to be due to extensive drainage of fluid from tissues around the body including the small intestine (Olszewski, 1985). In contrast, mesenteric lymph flows directly from the intestine and is more likely to give a good estimate of direct lymphatic absorption (de Nijs, 1987). This factor would tend to dilute any mesenterically absorbed compounds and thus cause problems in the analysis of minute quantities of lymphatically absorbed drugs due to the effect of dilution.

The importance of cannulating the mesenteric duct in studies of lymphatic absorption was demonstrated by Noguchi et al. (1985) who studied the lymphatic absorption of the highly lipophilic compound DDT (1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane) in the rat. After intravenous dosing of DDT in oleic

acid, high levels of the compound were detected in the thoracic lymph (3.6% of the administered dose / 24 hours). Small quantities of DDT were also detected in mesenteric lymph (0.6% of the administered dose / 24 hours) but these were explained as being due to extrahepatic recirculation of DDT which has been excreted into the duodenum through the bile (Noguchi et al, 1985., Levine et al., 1969).

DDT has also been detected in thoracic lymph after occlusion of the mesenteric lymph duct. These data suggest that DDT is re-circulated from plasma into thoracic lymph after intravenous dosing.

These data indicate that the collection of thoracic lymph may tend to overestimate the extent of direct intestinal lymphatic absorption. It was therefore decided to collect lymph from the mesenteric lymph duct in order to study the lymphatic absorption of the retinoids from the gastrointestinal tract.

4.2.2.3.1 Method for cannulation of the mesenteric lymph duct.

Cannulation of the mesenteric lymph duct was carried out based on the method of Bollman (Bollman et al., 1948) and modified by Warshaw (Warshaw, 1972).

Male rats were fasted overnight on wire grids to prevent coprophagia. Animals were anaesthetised as previously described (Section 4.1.2.1.).

The anaesthetised animal was placed, ventral side uppermost, on a heated mat and was shaved on the right side from the rib margin to the pelvic area. A diagonal incision was made through the body wall, using a scalpel, extending from the xiphisternum to the right pelvic margin. Scissors were used to cut through the peritoneum and the intestines were displaced to the left to observe the right kidney. The mesenteric lymph duct can be seen as a vessel of slightly less than 1 mm in diameter, running parallel to the mesenteric artery and perpendicular to the vena cava.The vessel can be observed to contain a milky white fluid after orally dosing the animal with a fatty meal.

A saline filled cannula was prepared using a length of polythene tubing (internal diameter; 0.5 mm, external diameter; 1.0 mm), cut with a 45° bevel, and this was fed under the right kidney and vena cava. The area around the mesenteric lymph duct was examined using a binocular dissecting microscope and was cleared using cotton buds. The main mesenteric lymph duct was cut transversely using a small pair of spring scissors. The cannula was inserted into the leaking vessel and glued into position using a small drop of cyanoacrylate cement. Lymph was seen to flow into the cannula. The cannula was exteriorised, cut short (approximately 3 cm outside the body of the animal) and immobilised using 'blu-

tack'. A further length of saline filled cannula (approximately 50 cm in length) and containing a small air space (5 μl), was attached to the lymphatic cannula using a broken off hypodermic syringe needle. The second length of cannula was allowed to hang vertically over the edge of the bench to provide a slight negative pressure on the mesenteric lymph duct and thereby assist the flow of lymph. The small air space in the cannula was used to monitor the flow of lymph (a 40 cm length of cannula gives an internal volume of approximately 100 μl). The second cannula was replaced periodically and lymph was drained from this into pre-weighed micro-centrifuge tubes (1.5 ml) and stored at -20°C. The time of collection of each lymph sample and its volume (mass) was recorded.

4.2.3. Oral dosing studies.

It has been suggested that most lymphatically transported lipophilic drugs are present in the chylomicron fraction of the intestinal lymph and two factors may influence the quantity of drug carried via this intestinal transportation pathway. Firstly, the quantity of lipid transported in the lymph in the form of chylomicron and secondly, the amount of drug per chylomicron (Charman & Stella, 1986b). Both of these factors may be manipulated in order to optimize or promote the lymphatic absorption of a

particular drug.

Chylomicron flow may be influenced by factors such as diet and the type of lipid vehicle which is co-administered with the drug.

In the present studies it was decided therefore, to select a series of oils and oil systems with which to investigate the relationships between the lymphatic absorption of the retinoids and the nature of the oral formulation dosed.

4.2.3.1. Selection of oil systems for oral dosing studies.

A series of oils was selected from the list in Table 3. The oils were chosen to test several factors which were believed to be important in the promotion of lymphatic absorption.

The effect of the fatty acyl-ester content of mixed long chain fatty acyl glycerol oils was to be assessed. The mixed long chain fatty acyl triglycerol oils (peanut oil, soyabean oil and cottonseed oil) were chosen in order to compare the effect of different proportions of three component long chain fatty acids; palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) on the lymphatic absorption of the retinoids. These three triglyceride oils contain between 87% and 96% of these fatty acyl esters, although in differing proportions (Mead et al., 1986).

It may be seen from the data in Table 3 that peanut oil and soyabean oil contain similar compositions of palmitate esters but approximately inversed proportions of oleate and linoleate esters.
Soyabean oil and cottonseed oil contain similar amounts of oleate and linoleate esters but cottonseed oil contains a greater proportion of the palmitate ester.
Mixed long chain fatty acyl glycerol oils were also selected to enable comparison of these with the effects of the free long chain fatty acid oils (oleic acid and linoleic acid).
Mixed long chain fatty acyl glycerol oils and mixed medium chain fatty acyl glycerol oil (Miglyol 812) were chosen in order to test the effect of fatty acid chain length in triacyl glycerol oils on the lymphatic absorption of the retinoids.
Initially, Ro 04-3780 was orally administered in each of six oils; cottonseed, soyabean, peanut, oleic acid, linoleic acid and Miglyol 812. Oral dosing studies were also carried out on Ro 10-9359 and Ro 15-0778 using three of these oils, cottonseed oil, linoleic acid and Miglyol 812.
In addition, the lymph flow rate and chylomicron concentration (lymph turbidity) were measured after oral dosing with each of the oil systems in order to examine any relationship between these factors and lymphatic absorption.

Several other factors which were thought may be important in influencing the rate and extent of lymphatic absorption, including the effect of food and the effect of oral oil emulsion systems on lymphatic absorption, were also studied (Sections 4.2.3.3. and 4.2.3.4.).

4.2.3.2. Method for studying absorption of retinoids after oral administration in oil systems.

Reports in the literature have suggested that some lipophilic compounds are extensively absorbed via the lymphatic route, including the parent compound of the retinoids, vitamin-A (Blomhoff et al., 1984., Palin et al., 1982., Drummond et al., 1935) and the highly lipophilic compound, DDT (Sieber et al., 1974., Palin et al., 1982., Noguchi et al., 1985., Charman et al., 1986a., Charman et al., 1986b).

In a study of the blood and lymph transport of DDT, O'Driscoll (O'Driscoll et al., 1991) reported low blood levels of DDT in mesenteric lymph cannulated rats (AUC = 18.2 $\mu g.h.ml^{-1}$), following intraduodenal administration. When blood alone was sampled, much higher levels of DDT were observed (AUC = 28.1 $\pm$ 5.8 $\mu g.h.ml^{-1}$). The higher mean blood values reported,when blood alone was sampled, were explained by a redistribution of DDT from lymph to blood.

In the present study of the lymphatic absorption of

the highly lipophilic retinoids, it was considered necessary to collect lymph and blood from separate groups of animals since cannulation of the mesenteric lymphatic duct and the subsequent sampling of mesenteric lymph may remove a major route of absorption for retinoids into the systemic circulation.

Male Wistar rats were fasted overnight (16 hours) on wire grids, to prevent coprophagia, water was available ad-libitum.

Animals were orally dosed with 0.5 ml of an oily solution containing the retinoid at a concentration of 3 mg/ml (6 mg/kg in a 250 g rat), using the method described in Section 4.2.2.1. The animals were returned to their cage for 30 minutes to allow absorption of the oil to begin.

The mesenteric lymph duct or the carotid artery was cannulated using the methods described above (Section 4.2.2.3.1 or Section 4.2.2.2.). Samples of lymph or plasma were collected at timed intervals. At least twelve animals were used for each oily dosing formulation studied. The animals were divided into groups of equal size, the first group of six animals had a carotid artery cannulation performed on them (Section 4.2.2.2.) and were used only for the collection of timed blood samples. The second group of six animals had a mesenteric lymph duct cannulation performed on them (Section 4.2.2.3.1)

and were used only for collection of timed lymph samples.

A maximum of 10 - 12 samples of lymph or plasma were collected from each animal. Blood samples (approx. 200 µl) were collected at 15 - 30 minute intervals and lymph samples were collected when a sufficient volume had been collected in the cannula to permit analysis (25 - 100 µl). The number of lymph sample collections which could be made was often limited by the appearance of "clots" within the cannula which eventually prevented the flow of lymph.

The retinoid concentration in each sample of lymph or plasma was determined using the appropriate analytical procedure (Section 2.1.1.). The concentration of retinoid determined in each sample was corrected for sample volume and expressed as nanograms retinoid per millilitre of sample per milligram dose per kilogram body weight (Dose adjusted concentration, (ng/ml)).

This series of procedures was repeated for each oil system and retinoid combination.

4.2.3.3. <u>The effect of oral dosing in a self-emulsifying oil system.</u>

In an attempt to improve the overall absorption of retinoids it was decided to study the effect of a self-emulsifying oil system (Chapter 3) on the absorption of Ro 15-0778. The self-emulsifying oil

system chosen for this study was MTS, a system containing 80% Miglyol 812, 16% Tween 85 and 4% Span 85. This self-emulsifying oil system produced an emulsion which was stable for several days and showed a small particle size (509 ± 20 nm).
Ro 15-0778 was selected as the retinoid for use in these studies because it showed similar solubility in MTS to that measured in Miglyol 812. It was also expected to demonstrate a high extent of absorption because of its extremely lipophilic nature, thus allowing precise analytical data to be obtained.
Ro 15-0778 (6 mg/kg) was dissolved in MTS and orally administered to animals as a bolus dose (0.5 ml) as described in Section 4.2.4.1. and the absorption of Ro 15-0778 was studied using the procedures described in Section 4.2.3.2.

4.2.3.4. The effect of food on the lymphatic absorption of the retinoids.

A series of experiments was carried out using a selected oil (linoleic acid) to test the effect of food on the lymphatic absorption of a retinoid (Ro 10-9359).
Two groups of rats (6 animals per group) were fasted overnight (16 hours). The animals were orally dosed with a liquid rat diet (2 ml of SDS water soluble diet) and returned to their cage for 30 minutes. The animals were then orally dosed with linoleic acid

(0.5 ml) containing the retinoid Ro 10-9359 (6 mg/kg). Water was available ad-libitum at all times. After a further 30 minutes the first group of animals had a mesenteric lymph duct cannulation performed on them (Section 4.2.2.3.1) whilst the second group had a carotid artery cannulation procedure (Section 4.2.2.2.) performed on them. Samples of lymph or blood were collected and the absorption of Ro 10-9359 was studied using the procedure described in Section 4.2.3.2. These data were compared with data obtained on a control group of animals which had been fasted overnight but had not received any food prior to oral dosing with Ro 10-9359 (6 mg/kg) in linoleic acid.

4.2.3.5. Studies of lymph turbidity.

Charman & Stella (1986b) have reported that chylomicron flux is important for the transport of lipophilic drugs and have studied the lymph optical density at 560 nm as a relative index of chylomicron lipid content (Charman et al., 1986b). In this study it was decided to study the turbidity of lymph (as an index of chylomicron transport) as a function of orally dosed lipid vehicle.

A group (18 animals) of fasted male Wistar rats was fasted overnight (16 hours) and pairs of animals were orally dosed with either isotonic saline (0.5 ml), one of a series of oils (0.5 ml) or the

SDS water soluble diet (2 ml). Mesenteric lymph duct cannulations were performed on each animal (Section 4.2.2.3.1) and lymph samples were collected for turbidity estimation.

Aliquots (20 - 50 mg) in duplicate from each lymph sample were diluted in isotonic saline (2 ml) in order to give an absorbance (λ = 400 nm) value within the range 0.20 - 1.00 when measured on a spectrophotometer. The wavelength of 400 nm was chosen for these absorbance measurements since at lower wavelengths non-specific absorbance was observed, possibly due to other chromophores in the lymph samples (proteins, vitamins etc.), whilst at higher wavelengths the absorbance decreased rapidly and may have presented difficulties in obtaining sufficient volumes of lymph for analysis.

The product of the absorbance for each diluted lymph sample and its dilution factor was used to determine the absorbance of undiluted lymph.

4.2.4. <u>Results.</u>

a) Ro 04-3780.

The mean mesenteric lymph flow rate was calculated from the volume of lymph collected in each sample (mg) and the time of collection (min), after dosing rats with Ro 04-3780 in each of the six oily vehicles. These data are shown in Table 24. The greatest lymph flow rate was measured after dosing

Table 24. Lymphatic absorption data for Ro 04-3780 after oral administration in a series of oil systems to fasting rats.

a) Lymphatic absorption.

Oil	Lymph flow rate. mg/h/kg	Dose adjusted lymphatic concentration. ng/ml	Total lymphatic uptake. ng/h
Cottonseed	3.14 ± 0.82 *	48.0	151
Soyabean	3.31 ± 1.76 *	37.5	124
Peanut	1.73 ± 0.37	41.5	72
Miglyol 812	2.05 ± 0.70	26.6	55
Oleic acid	1.94 ± 0.87	13.5	26
Linoleic acid	0.80 ± 0.23 *	1.4	1.1

b) Plasma absorption.

Oil	Total body retinoid. ng	Estimated plasma uptake. ng/h	Lymph:plasma uptake ratio (x 100)
Cottonseed	11520	7530	2.00
Soyabean	14950	9840	1.26
Peanut	8630	5680	1.27
Miglyol 812	16050	10650	0.52
Oleic acid	4370	2890	0.90
Linoleic acid	230	154	0.72

* Indicates result significantly different ($P < 0.01$) from basal flow rate after animals were orally dosed with saline (0.5 ml).

Notes:

i) Total lymphatic uptake; product of measured lymph flow rate and mean 90 minutes dose adjusted lymph concentration.

ii) Total body retinoid; product of volume of distribution and mean 90 minute dose adjusted plasma concentration.

iii) Plasma uptake; difference between total uptake and lymphatic uptake rates.

Ro 04-3780 in soyabean oil (3.31 mg/min). The remaining oils gave lymph flow rates of 3.14 mg/min (cottonseed oil), 2.05 mg/min (Miglyol 812), 1.93 mg/min (oleic acid), 1.73 mg/min (peanut oil) and 0.80 mg/min (linoleic acid). The least significance ($p < 0.01$) on these results was approximately 0.8 ml/h.

The dose adjusted concentration data for Ro 04-3780 in lymph and plasma have been plotted as a function of time after oral dosing with each of six oils. The results show that the mixed long chain tri-acyl glycerol oils, cottonseed oil (Figure 14), soyabean oil (Figure 15) and peanut oil (Figure 16), gave the greatest dose adjusted lymphatic concentrations of Ro 04-3780 (48.0 ng/ml, 37.5 ng/ml and 41.5 ng/ml respectively). The mixed medium chain tri-acyl glycerol oil, Miglyol 812 (Figure 17) and the long chain fatty acid oil, oleic acid (Figure 18) gave slightly lower dose adjusted lymphatic concentrations (26.6 ng/ml and 13.5 ng/ml). Linoleic acid (Figure 19) gave the lowest dose adjusted lymphatic concentration (1.4 ng/ml).

The lymphatic uptake rates for Ro 04-3780, were calculated from the lymphatic concentration data and the lymph flow rate (Table 24). The results show that the mixed long chain tri-acyl glycerol oils,

Figure 14. Dose adjusted plasma and lymph concentrations of Ro 04-3780 in the rat after oral dosing in cottonseed oil (6 mg/kg).

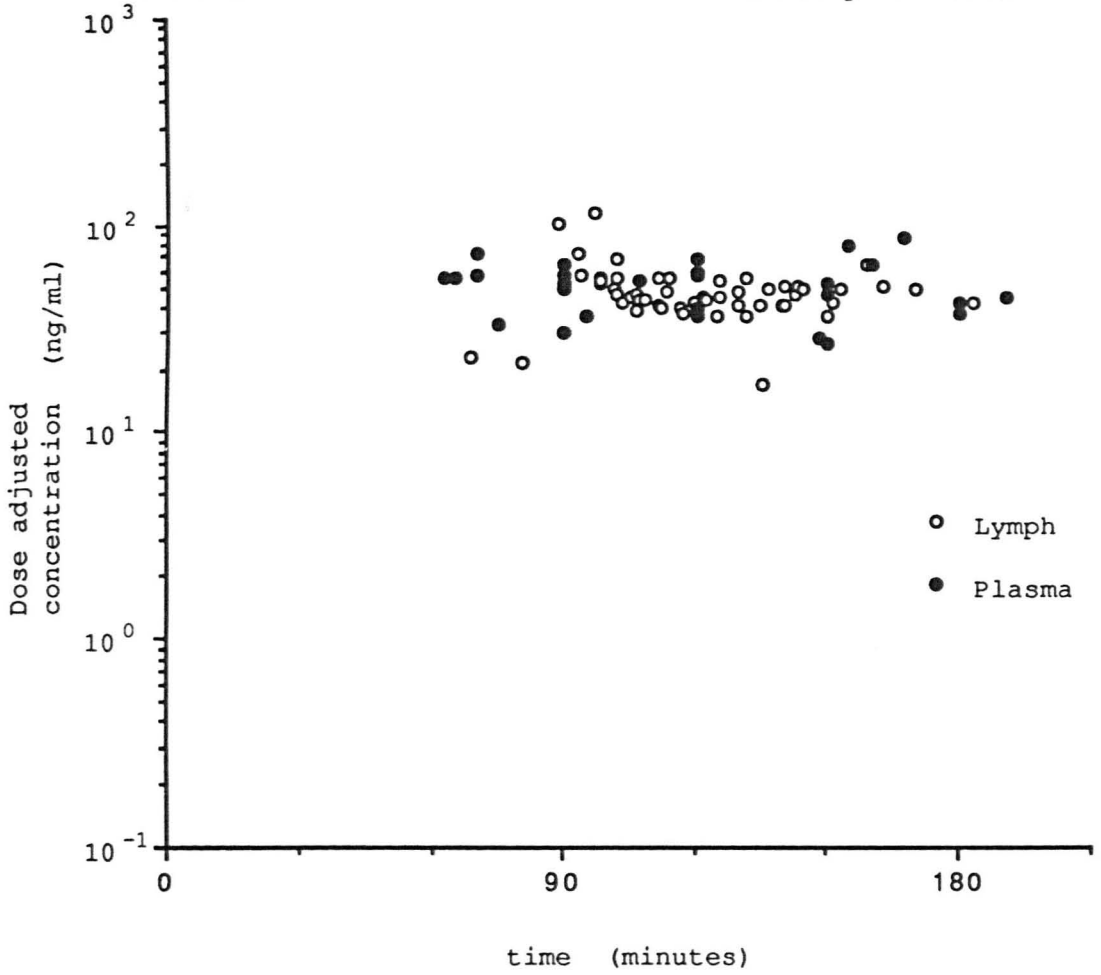


Figure 15. Dose adjusted plasma and lymph concentrations of Ro 04-3780 in the rat after oral dosing in soyabean oil (6 mg/kg).

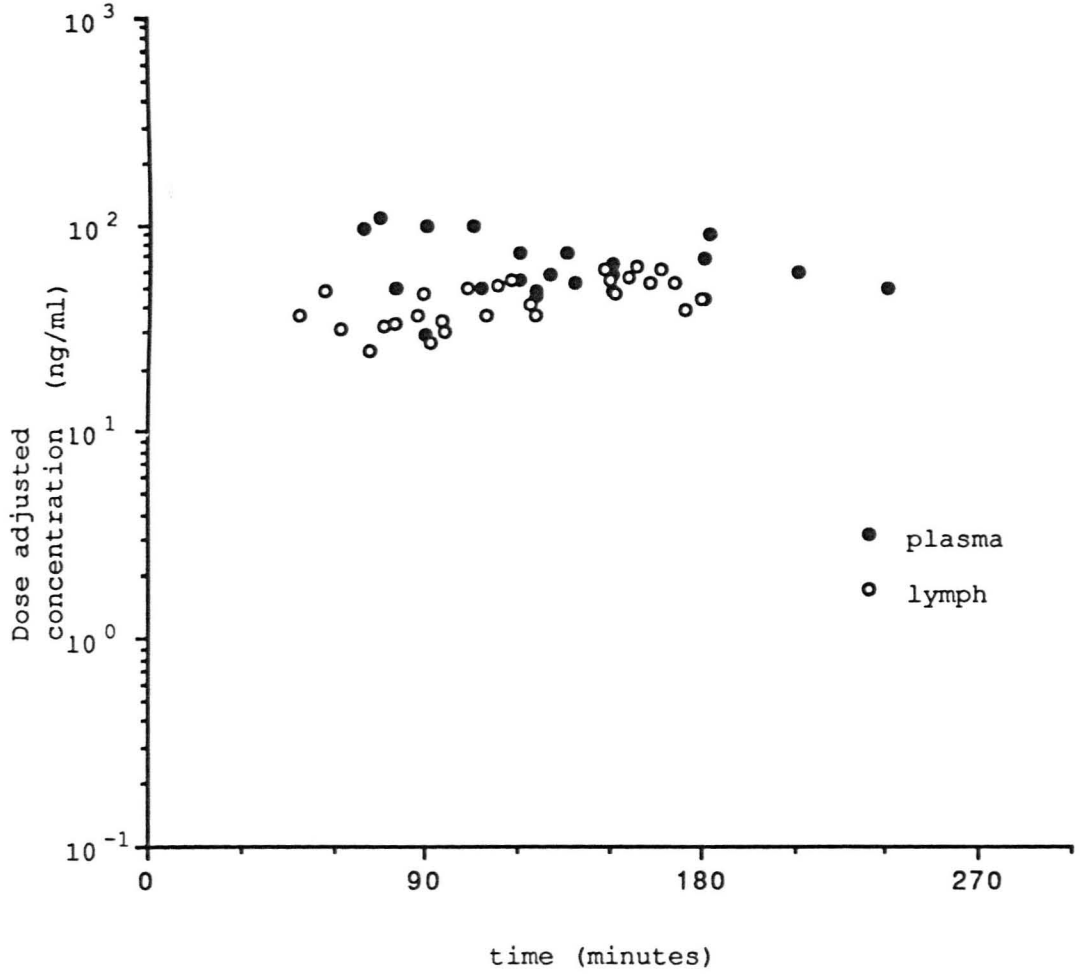


Figure 16. Dose adjusted plasma and lymph concentrations of Ro 04-3780 in the rat after oral dosing in peanut oil (6 mg/kg).

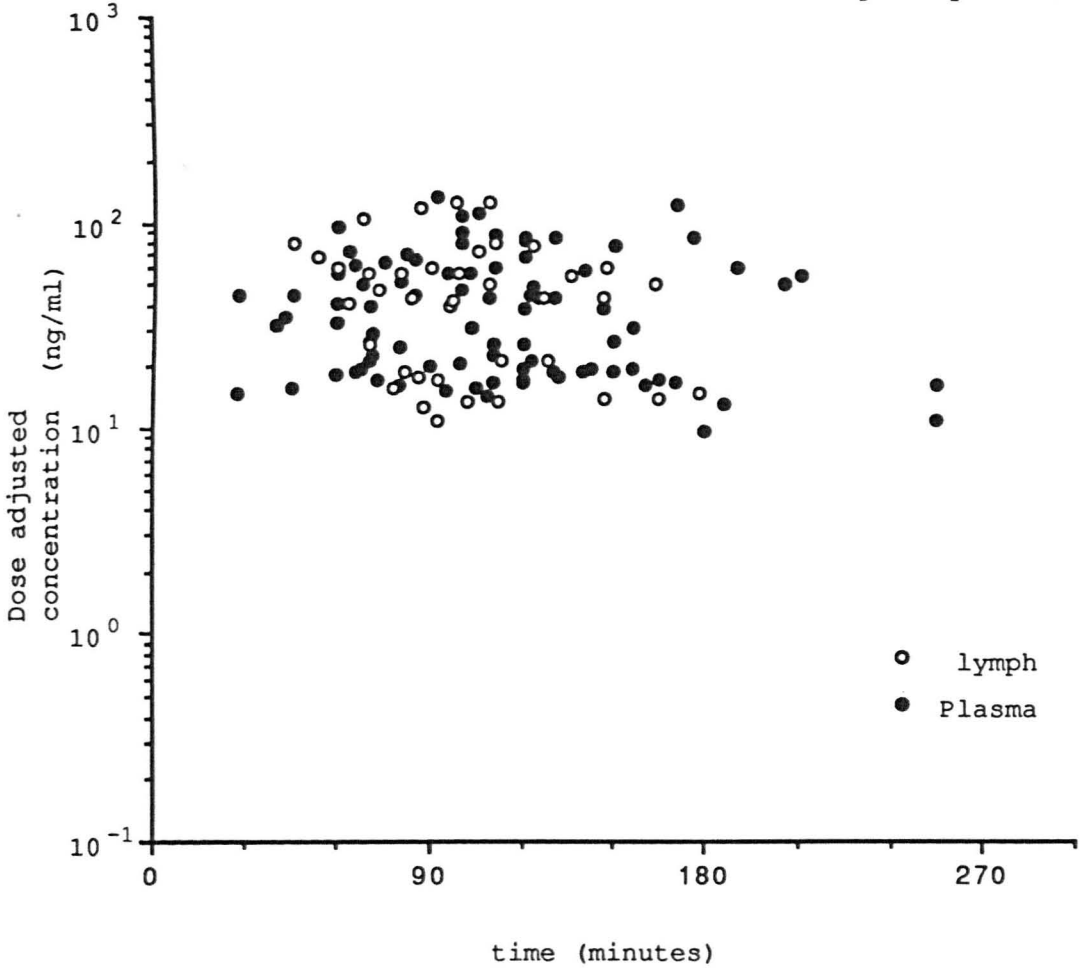


Figure 17. Dose adjusted lymph and plasma concentrations of Ro 04-3780 in the rat after oral dosing in Miglyol 812 (6 mg/kg).

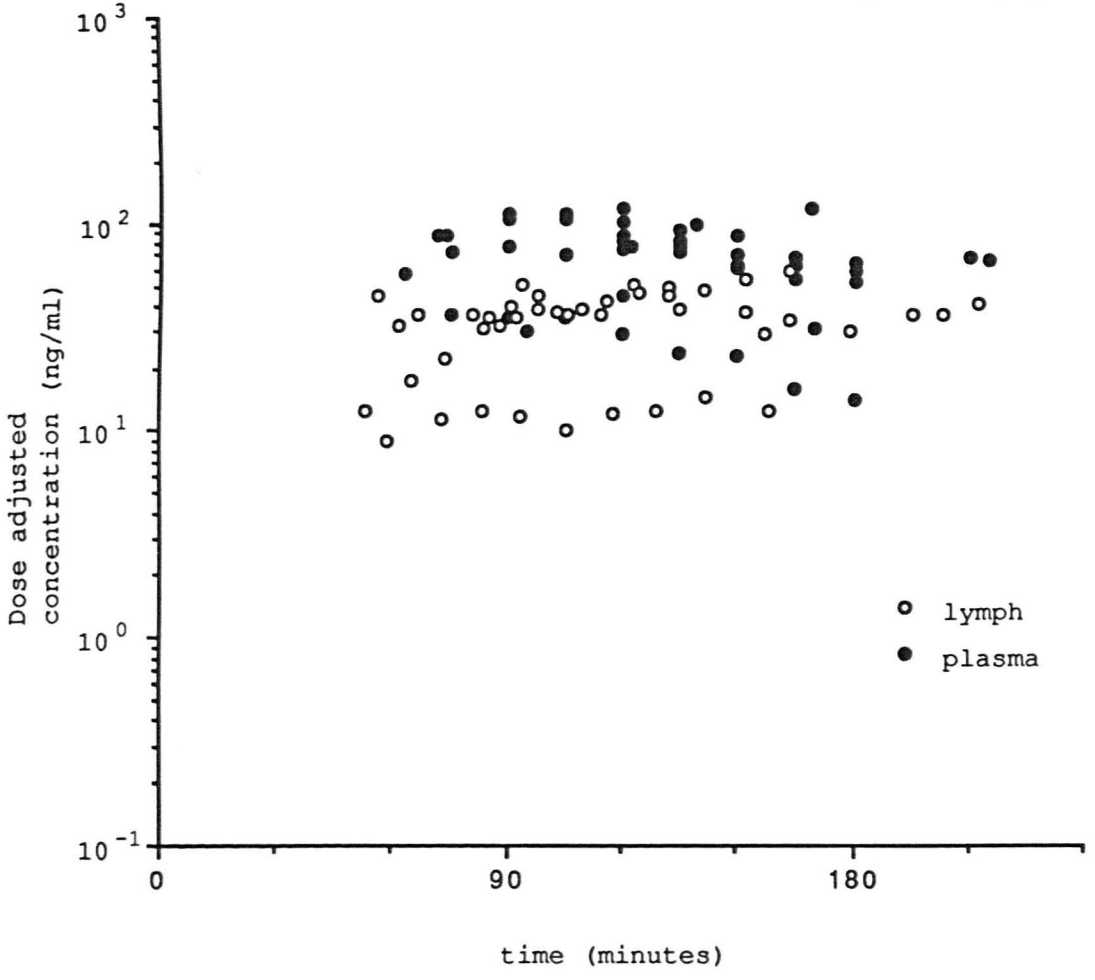


Figure 18. Dose adjusted plasma and lymph concentrations of Ro 04-3780 in the rat after oral dosing in oleic acid (6 mg/kg)

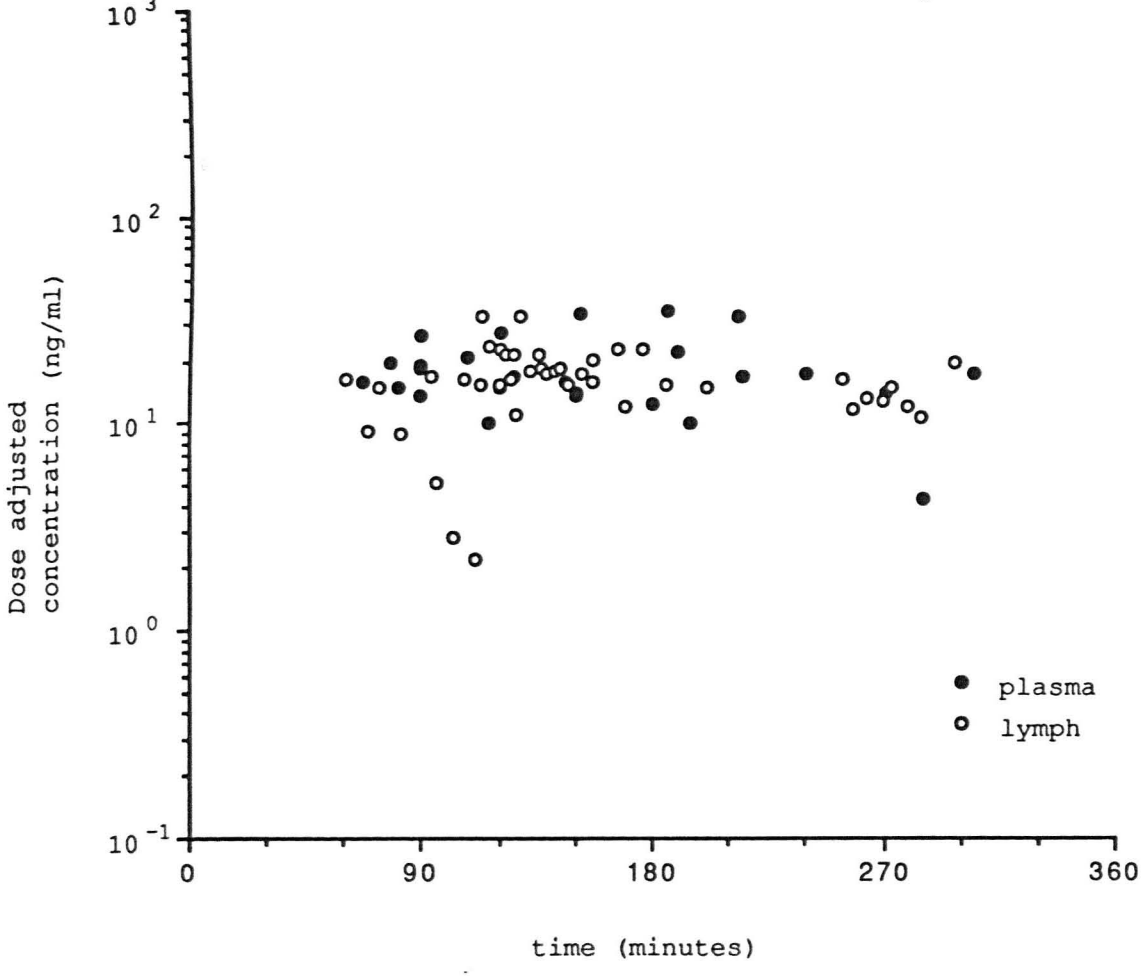
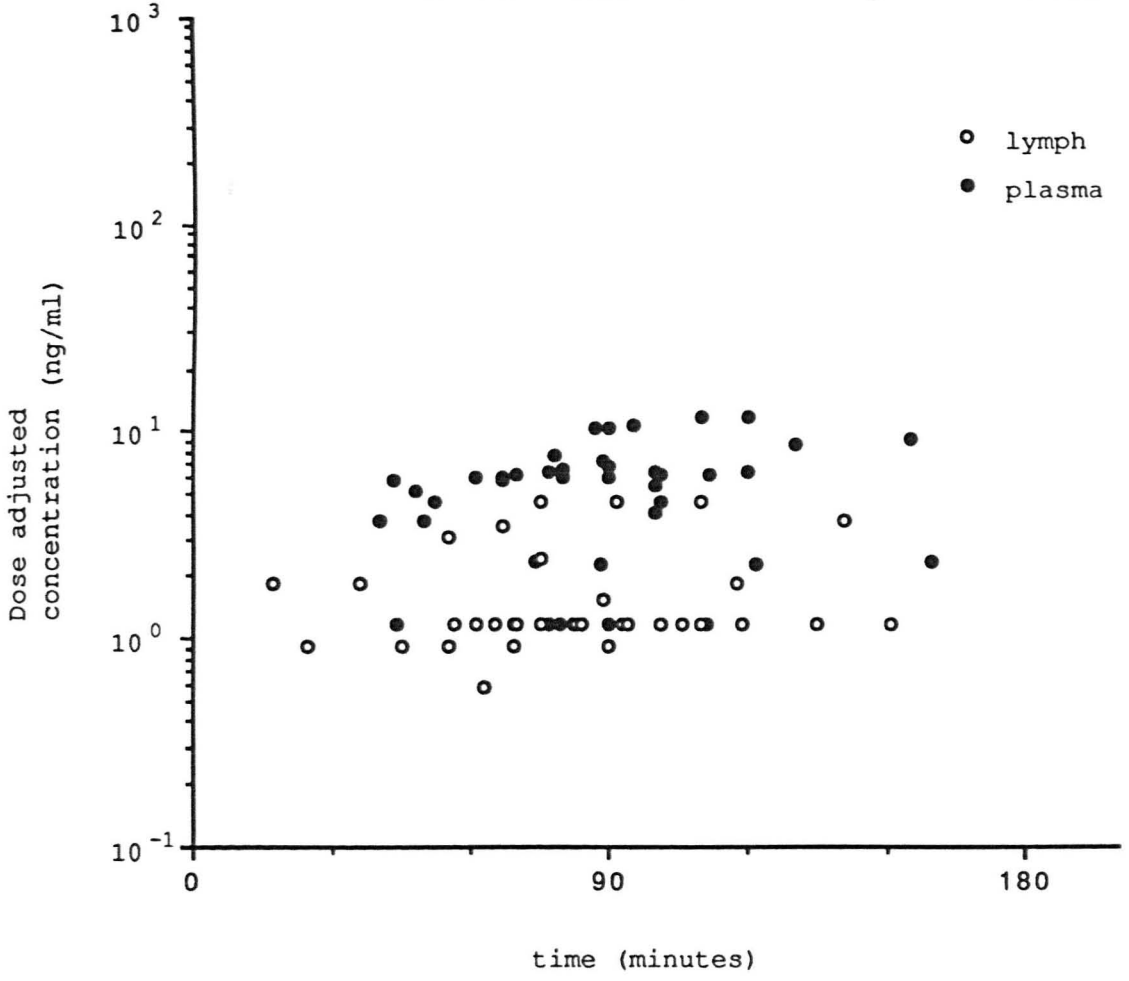


Figure 19. Dose adjusted plasma and lymph concentrations of Ro 04-3780 in the rat after oral dosing in linoleic acid (75 mg/kg).



cottonseed oil, soyabean oil and peanut oil, gave the greatest lymphatic uptake rate for Ro 04-3780 (151 ng/h, 124 ng/h and 72 ng/h respectively. The mixed medium chain tri-acyl glycerol oil, Miglyol 812 and the long chain fatty acid, oleic acid gave slightly lower lymphatic uptake rates (55 ng/h and 26 ng/h) and linoleic acid, gave the lowest lymphatic uptake rate (1.1 ng/h).

The dose adjusted plasma concentration data, together with the volume of distribution (Vd) for Ro 04-3780 (Table 21), allowed an estimate of the total body Ro 04-3780 content to be calculated. These data (Table 24) show a maximum total body retinoid content for Ro 04-3780 of 1.60 % of the administered dose (16,000 ng from a 1 mg dose) after 90 mins post dosing in Miglyol 812, (Table 24). The other oils gave a total body retinoid content of 15,000 ng (1.50% of the dose) after dosing in soyabean oil, 11,500 ng (1.15%) after dosing in cottonseed oil, 8,500 ng (0.85%) after dosing in peanut oil, 4,500 ng (0.45%) after dosing in oleic acid and 200 ng (0.02%) after dosing in linoleic acid.

An estimate of the plasma uptake rate of Ro 04-3780 was made by subtracting the calculated lymphatic uptake rate from the total body retinoid uptake

rate. Miglyol 812 and soyabean oil gave the greatest plasma uptake rate (10,500 ng/h and 10,000 ng/h respectively). The plasma uptake rate was 7,500 ng/h after dosing Ro 04-3780 in cottonseed oil, 5,500 ng/h after dosing in peanut oil, 3,000 ng/h after dosing in oleic acid, and 150 ng/h after dosing in linoleic acid.

The lymph : plasma uptake ratios of Ro 04-3780 after dosing in the six oils ranged from 0.5% (Miglyol 812) to 2.0% (cottonseed oil).

b) Ro 10-9359.

The mean mesenteric lymph flow rate was calculated, after dosing fasted rats with Ro 10-9359 in each of three oily vehicles. These data are shown in Table 25. The greatest lymph flow rate was measured after dosing Ro 10-9359 in cottonseed oil (2.57 mg/min). Miglyol 812 gave a flow rate of 1.47 mg/min and linoleic acid gave a flow rate of 1.48 mg/min. The lymph flow rate after dosing Ro 10-9359 in linoleic acid after pre-feeding the rats with liquid food was 1.38 mg/min.

The dose adjusted concentration data for Ro 10-9359 in lymph and plasma have been plotted as a function of time after oral dosing with each of three oils. The results show that the mixed long chain tri-acyl glycerol oil, cottonseed oil (Figure 20), gave the

Table 25. Lymphatic absorption data for Ro 10-9359 after oral administration in a series of oil systems to fasting and fed rats.

a) Lymphatic absorption.

Oil	Lymph flow rate. mg/h/kg	Dose adjusted lymphatic concentration. ng/ml	Total lymphatic uptake. ng/h
Cottonseed	2.57 ± 0.85 *	1550	3980
Miglyol 812	1.47 ± 0.26	350	514
Food/Linoleic	1.38 ± 0.54	570	790
Fast/Linoleic	1.48 ± 0.70	690	1030

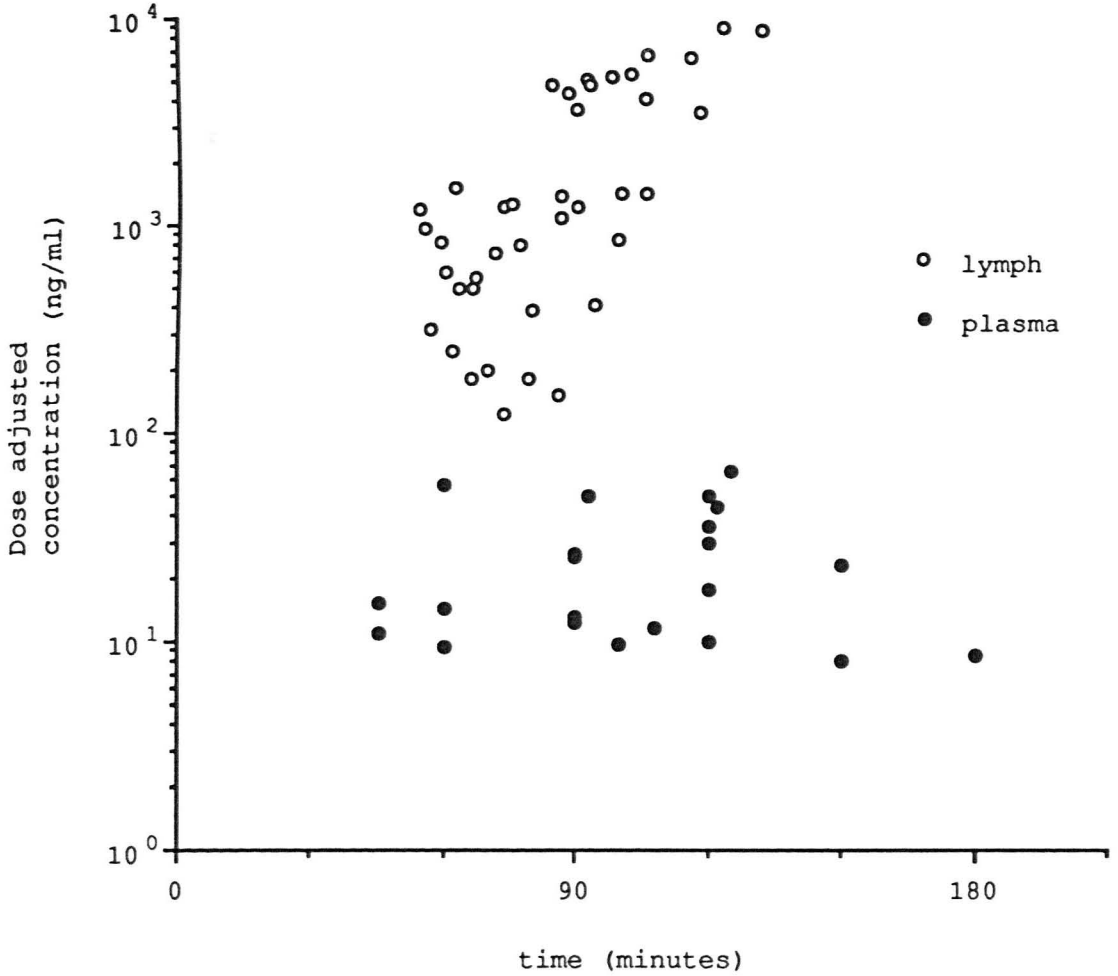
b) Plasma absorption.

Oil	Total body retinoid. ng	Estimated plasma uptake. ng/h	Lymph:plasma uptake ratio (x 100)
Cottonseed	23150	11454	34.7
Miglyol 812	38660	25260	2.03
Food/Linoleic	1120	< 25	> 3000
Fast/Linoleic	2000	310	332

* Indicates result significantly different ($P < 0.01$) from basal flow rate after animals were orally dosed with saline (0.5 ml).

Note:
Total body retinoid was calculated as the product of volume of distribution and mean 90 minute dose adjusted plasma concentration. Volume of distribution (1175 ml) was taken from Liu (Liu et al., 1990).

Figure 20. Dose adjusted plasma and lymph concentrations of Ro 10-9359 in the rat after oral dosing in cottonseed oil (6 mg/kg).



greatest dose adjusted lymphatic concentrations of Ro 10-9359 (1550 ng/ml). Linoleic acid (Figure 21), gave a dose adjusted lymphatic concentration of 690 ng/ml and Miglyol 812 (Figure 22) gave the lowest dose adjusted lymphatic concentration of 350 ng/ml. The lymph and plasma data, obtained effect of feeding rats prior to dosing with Ro 10-9359 in linoleic acid, were compared with the data obtained from fasted rats dosed with Ro 10-9359 in linoleic acid and are plotted as a function of time in Figure 23. Fed rats, dosed with linoleic acid, gave a dose adjusted lymphatic concentration of 570 ng/ml compared with 690 ng/ml in fasted rats (Table 25).

The lymphatic uptake rate data for Ro 10-9359 were calculated from the lymphatic concentration data using the lymph flow rate (Table 25). The results show that the mixed long chain tri-acyl glycerol oil, cottonseed oil, gave the greatest lymphatic uptake rate for Ro 10-9359 of 3980 ng/h. The long chain fatty acid, linoleic acid, gave a lymphatic uptake rate of 1030 ng/h and the mixed medium chain tri-acyl glycerol oil, Miglyol 812, gave the lowest lymphatic uptake rate (514 ng/h). Fed rats, dosed with linoleic acid, gave a lymphatic uptake rate of 790 ng/h (Table 25).

Figure 21. Dose adjusted lymph and plasma concentrations of Ro 10-9359 in the rat after oral dosing in linoleic acid (6 mg/kg).

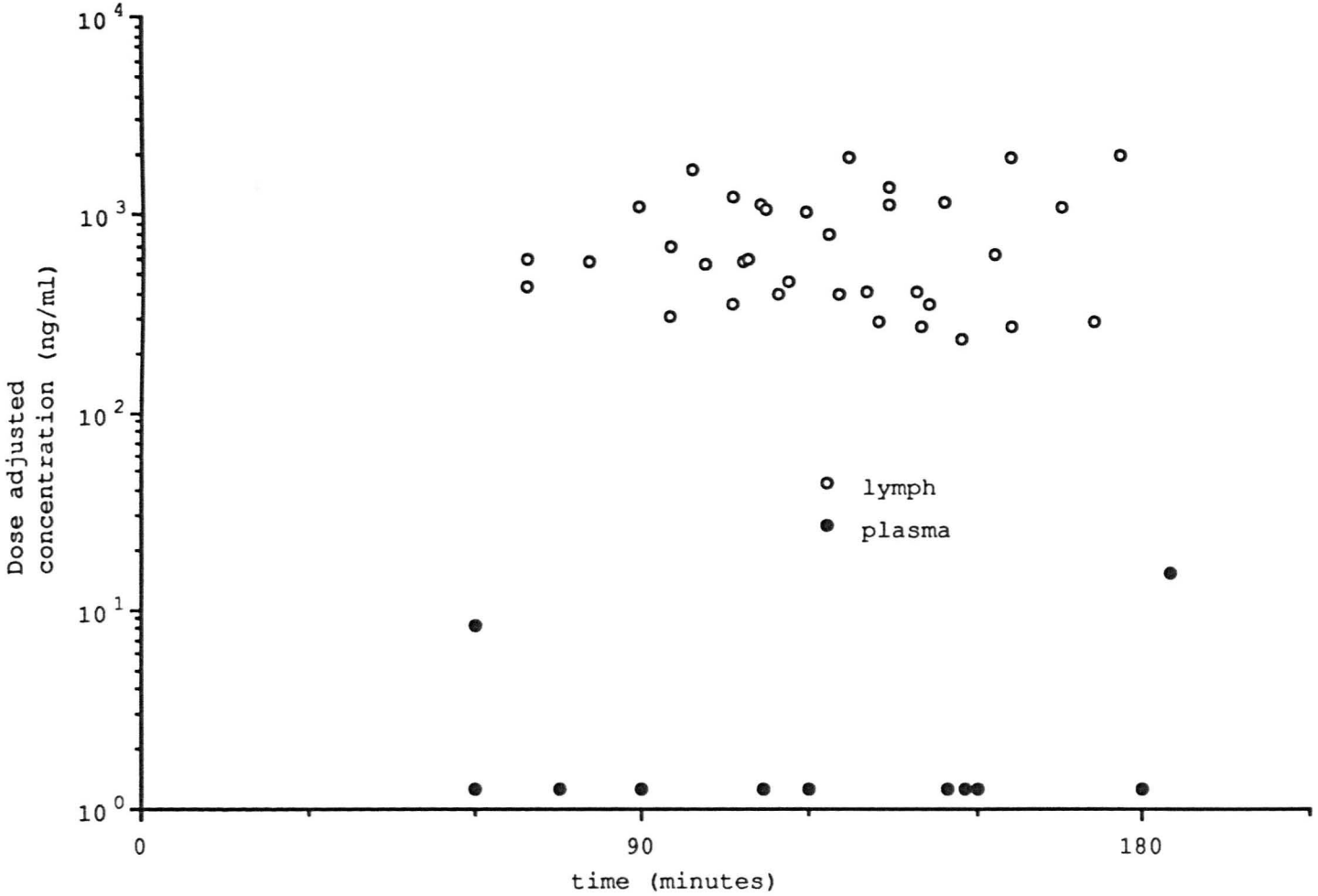


Figure 22. Dose adjusted plasma and lymph concentrations of Ro 10-9359 in the rat after ral dosing in Miglyol 812 (6mg/kg).

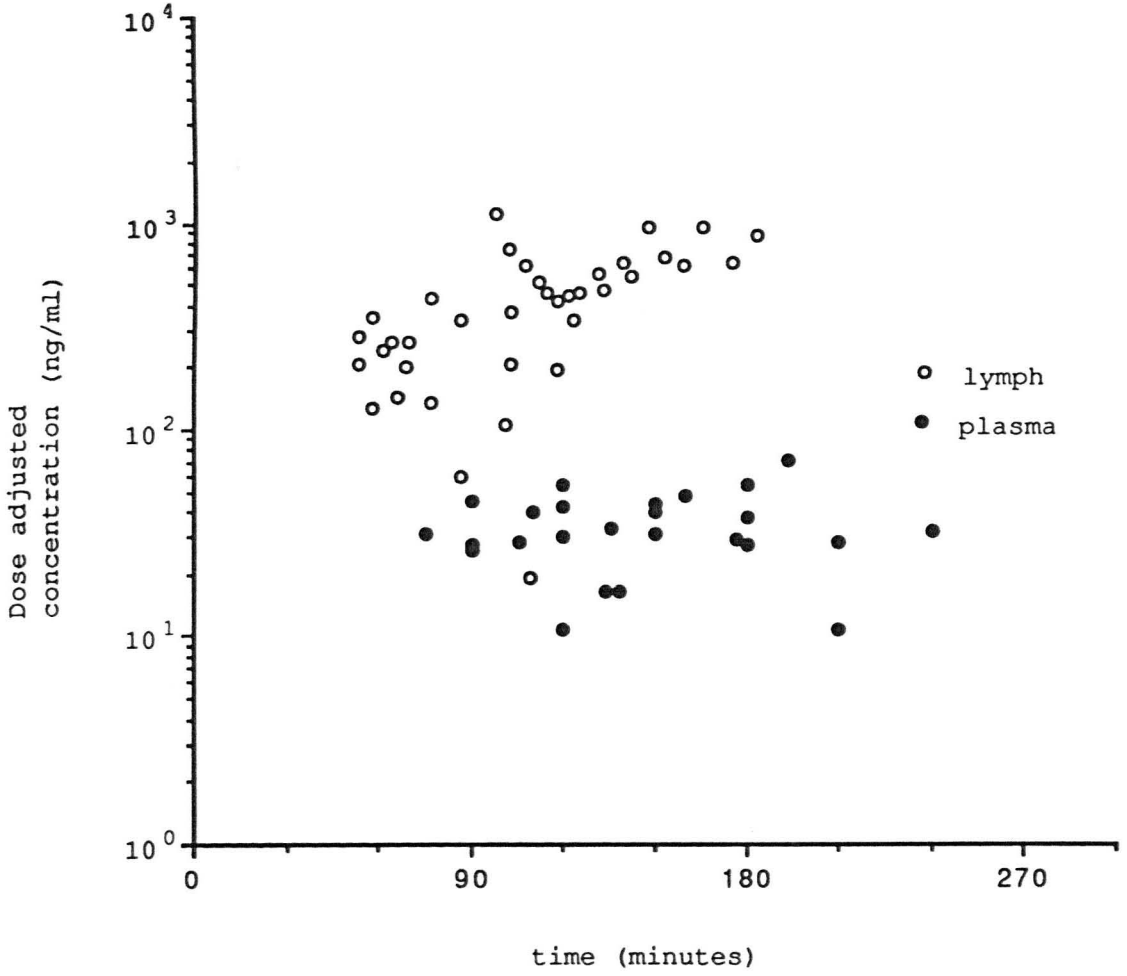
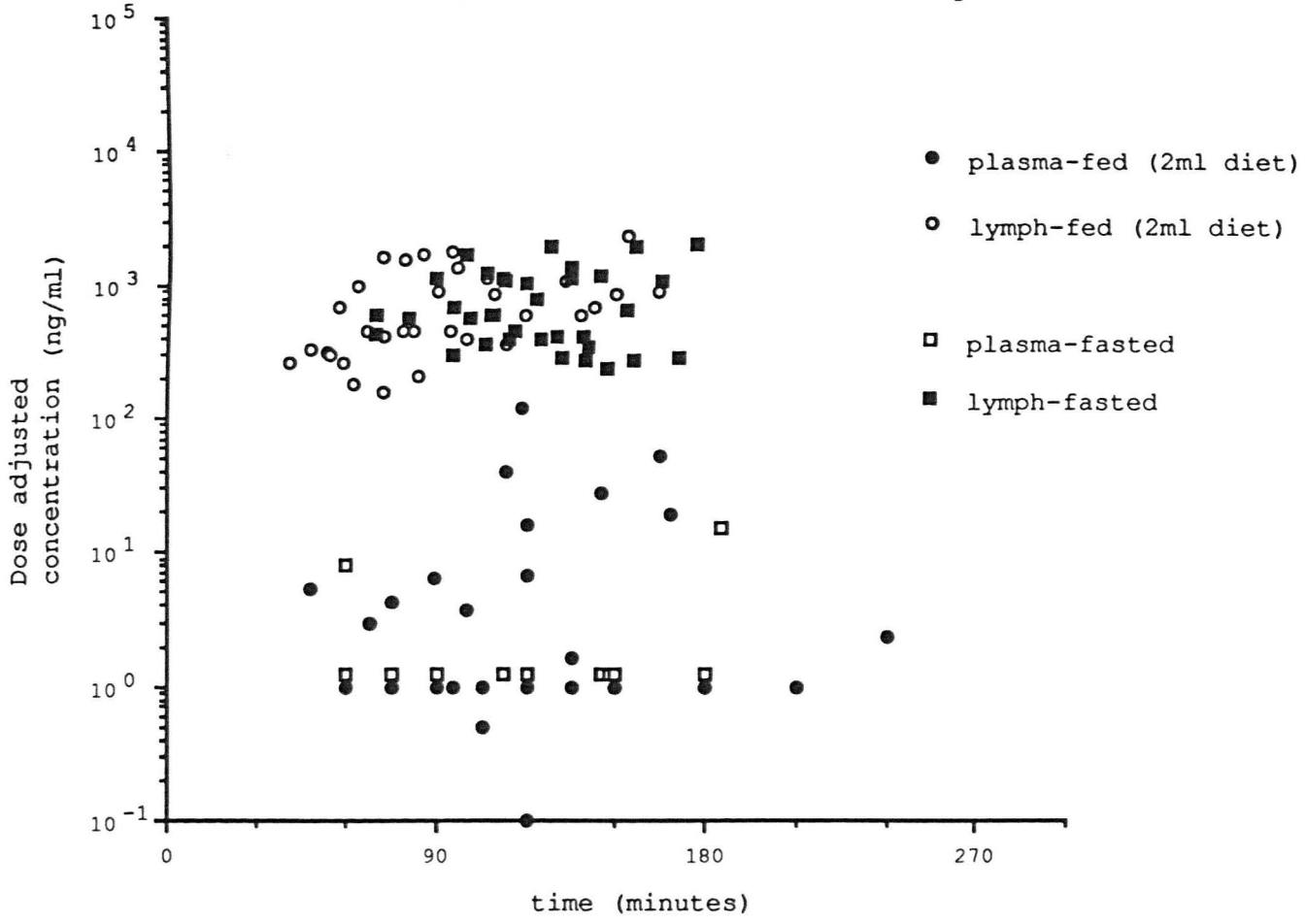


Figure 23. Effect of pre-feeding on the lymph and plasma concentrations of Ro 10-9359 in the rat after oral dosing in linoleic acid (6 mg/kg).



The dose adjusted plasma concentration data, together with the volume of distribution (Vd) for Ro 10-9359 (Liu et al., 1990), were used to estimate the total body Ro 10-9359 content. These data (Table 25) show a maximum total body retinoid content of Ro 10-9359, of 3.87% of the administered dose (38,660 ng from a 1 mg dose) after 90 mins post dosing in Miglyol 812, (Table 25). Cottonseed oil gave a total body retinoid content of 23,150 ng (2.32% of the dose) and linoleic acid gave a total body retinoid content of 2,000 ng (0.20%). The total body retinoid content of Ro 10-9359 in fed rats, after dosing in linoleic acid, was 1,120 ng (0.11%).

Miglyol 812 gave the greatest plasma uptake rate (25,000 ng/h). The plasma uptake rates from cottonseed oil and linoleic acid were 11,500 ng/h and 300 ng/h. The plasma uptake rate in fed rats, after dosing in linoleic acid, was less than 25 ng/h.

The lymph : plasma uptake ratios for the oils ranged from 0.02:1 (Miglyol 812) to 3.3:1 (linoleic acid), however, in fed rats, the lymph : plasma uptake ratio was greater than 30:1.

c) Ro 15-0778.

The three oils which had been chosen for use in studies of the lymphatic absorption of Ro 10-9359

were also studied using Ro 15-0778.
The mean lymph flow rate was calculated, after dosing fasted rats with Ro 15-0778 in each of the three oily vehicles and in a self-emulsifying oil system (MTS). These data are shown in Table 26. The greatest lymph flow rate was measured after dosing Ro 15-0778 in cottonseed oil (1.99 mg/min). Linoleic acid gave a flow rate of 1.14 mg/min and Miglyol 812 gave a flow rate of 0.97 mg/min. The lymph flow rate after dosing Ro 15-0778 in the self-emulsifying oil system (MTS) was 1.38 mg/min.

The dose adjusted concentration data for Ro 15-0778 in lymph and plasma have been plotted as a function of time after oral dosing with each of the three oils. The results show the greatest dose adjusted lymphatic concentration after dosing Ro 15-0778 in the long chain fatty acid, linoleic acid (Figure 24), (3590 ng/ml). The mixed long chain tri-acyl glycerol oil, cottonseed oil (Figure 25), gave a dose adjusted lymphatic concentrations of 2040 ng/ml and Miglyol 812 (Figure 26) gave the lowest lymphatic concentration (1270 ng/ml) (Table 26).

After dosing Ro 15-0778 in MTS, which contains 80% Miglyol 812, a dose adjusted lymphatic concentration of Ro 15-0778 of 3670 ng/ml was obtained (Table 26).

Figure 24. Dose adjusted plasma and lymph concentrations of Ro 15-0778 in the rat after oral dosing in linoleic acid (6 mg/kg).

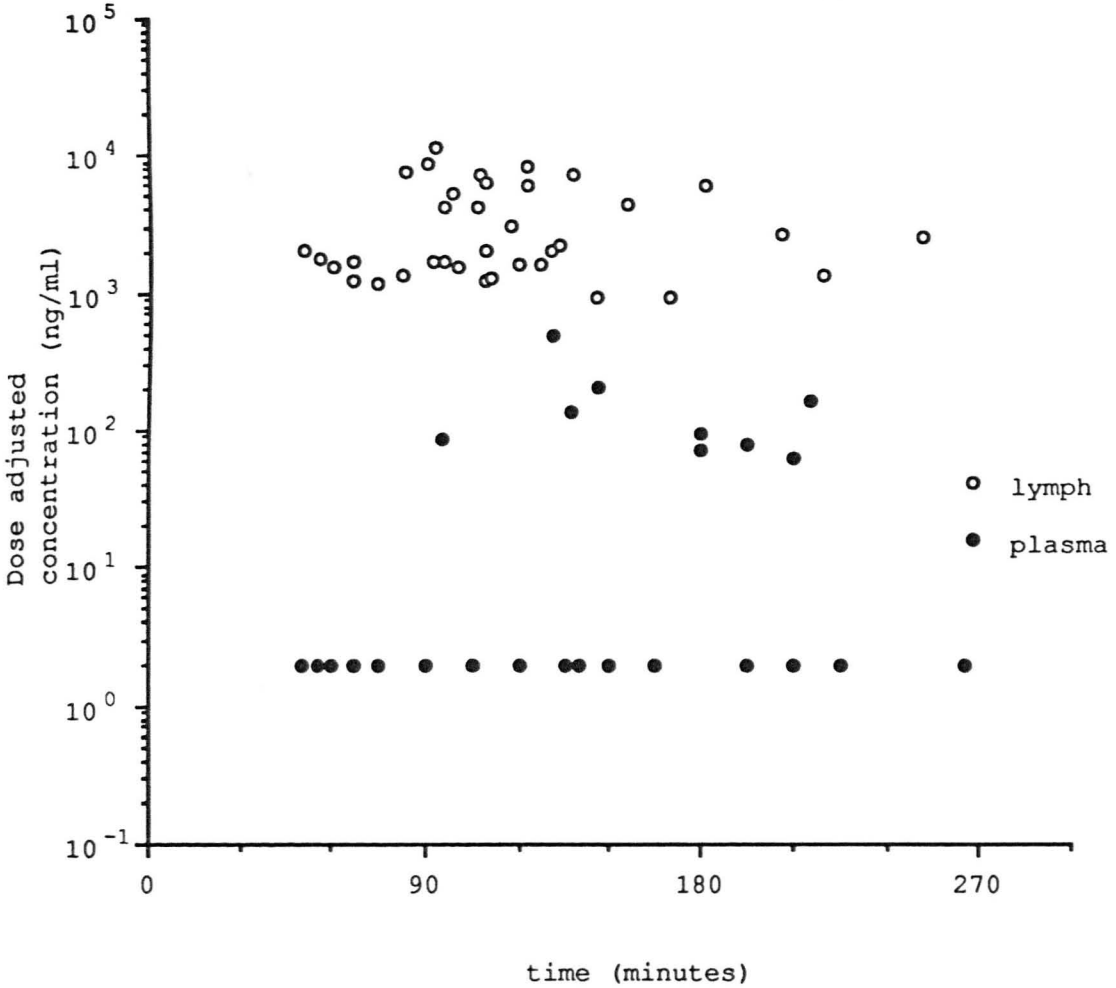


Figure 25. Dose adjusted plasma and lymph concentrations of Ro 15-0778 in the rat after oral dosing in cottonseed oil (6 mg/kg).

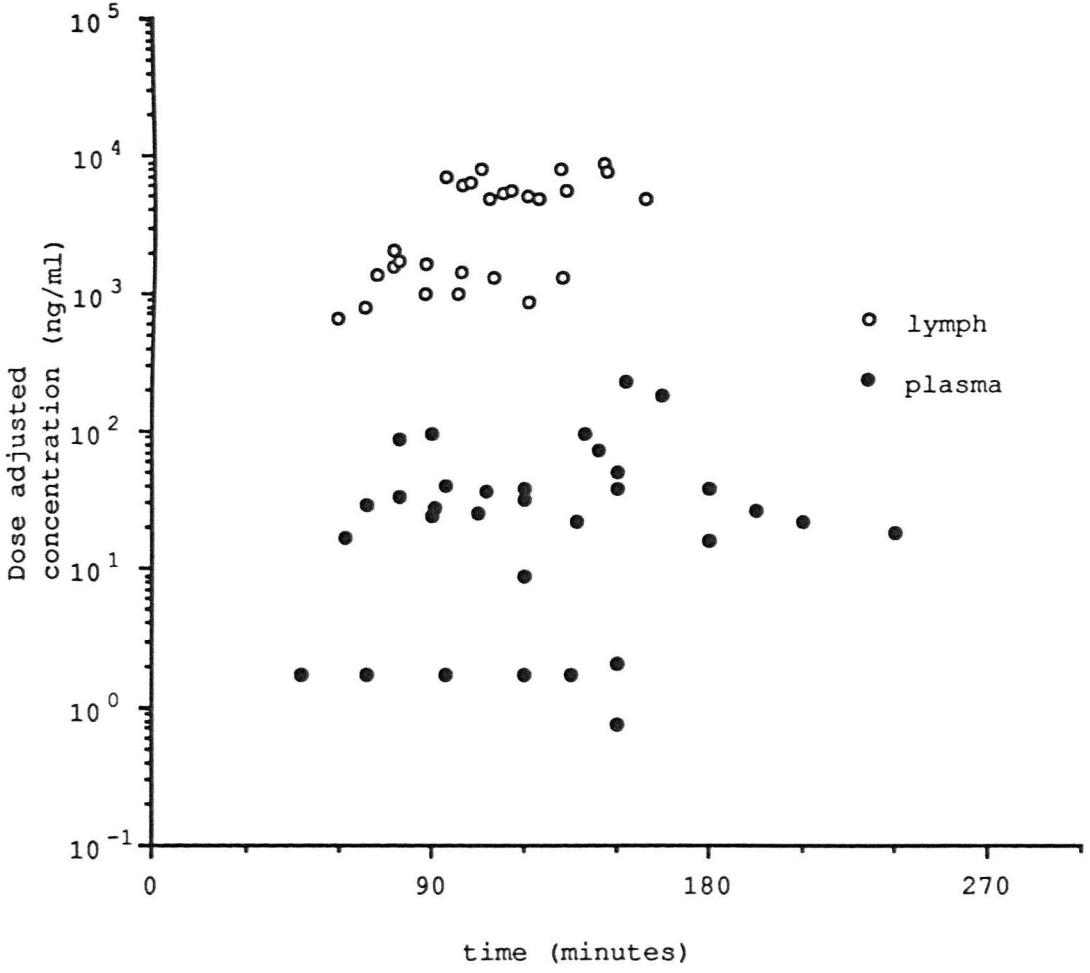


Figure 26. Dose adjusted lymph and plasma concentrations of Ro 15-0778 in the rat after oral dosing in Miglyol 812 (6 mg/kg).

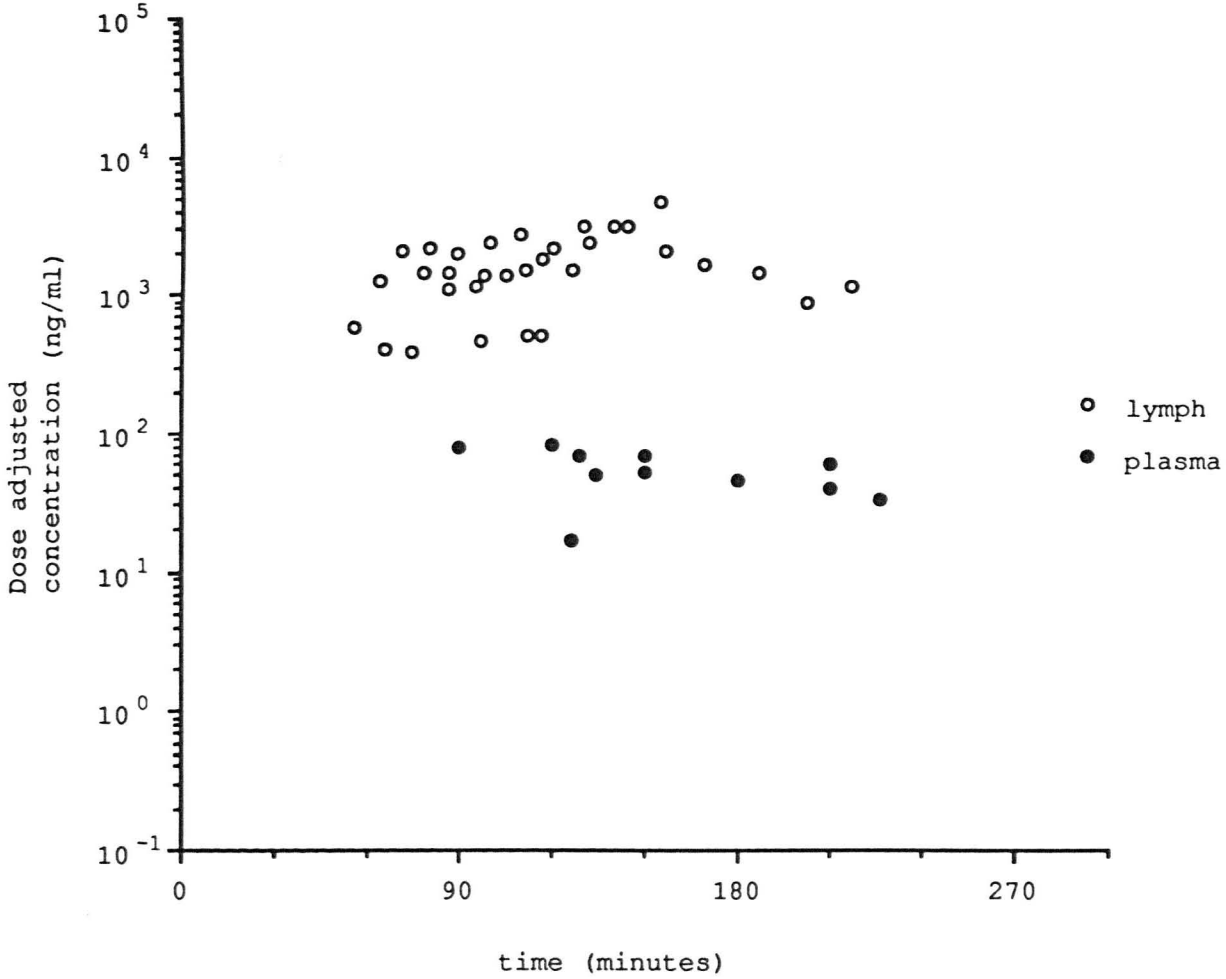


Table 26. Lymphatic absorption data for Ro 15-0778 after oral administration in a series of oil systems to fasting rats.

a) Lymphatic absorption.

Oil	Lymph flow rate. mg/h/kg	Dose adjusted lymphatic concentration. ng/ml	Total lymphatic uptake. ng/h
Cottonseed	1.99 ± 0.64	2040	4060
Miglyol 812	0.97 ± 0.33	1270	1230
MTS	0.95 ± 0.26	3670	3480
Linoleic acid	1.14 ± 0.39	3594	4110

b) Plasma absorption.

Oil	Total body retinoid. ng	Estimated plasma uptake. ng/h	Lymph:plasma uptake ratio. (x 100)
Cottonseed	14220	4830	84.0
Miglyol 812	14280	8290	14.9
MTS	59750	36170	9.6
Linoleic acid	26400	13493	30.5

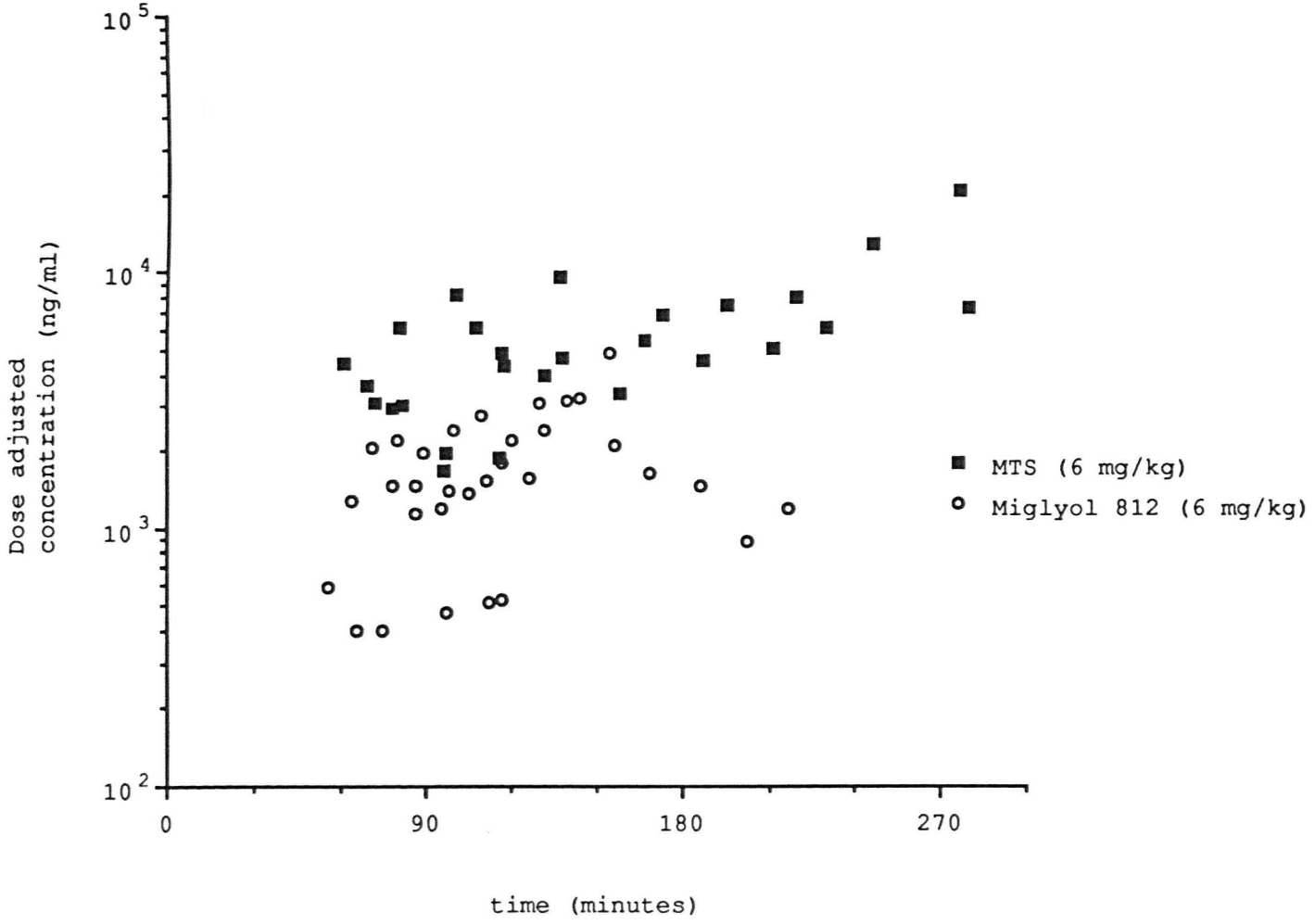
Note:
MTS is an self emulsifying oil system containing Miglyol 81 (80 parts), Tween 85 (16 parts) and Span 85 (4 parts).

This result being higher than that obtained with Miglyol 812 alone (1279 ng/ml). These dose adjusted lymphatic concentration data are plotted as a function of time in Figure 27.

The lymphatic uptake rates were calculated from the lymphatic concentration data using the lymph flow rate (Table 26). The results show that the long chain fatty acid, linoleic acid, gave the greatest lymphatic uptake rate for Ro 15-0778 (4110 ng/h). The mixed long chain tri-acyl glycerol oil, cottonseed oil, gave a slightly lower lymphatic uptake rate (4,060 ng/h) and the mixed medium chain tri-acyl glycerol oil, Miglyol 812, gave the lowest lymphatic uptake rates (1,230 ng/h). Rats which were dosed with Ro 15-0778 in MTS, gave a lymphatic uptake rate of 3,480 ng/h (Table 26).

The total body content of Ro 15-0778 after oral dosing in each of the three oils (Table 26) reached a maximum of 2.64% of the administered dose (24,600 ng from a 1 mg dose) after 90 mins after dosing in linoleic acid. The other oils show a total body retinoid content of 14,280 ng (1.43% of the dose) after dosing in Miglyol 812 and 14,220 ng (1.42%) after dosing in cottonseed oil. However, after dosing Ro 15-0778 in MTS, the total body retinoid

Figure 27. A comparison of lymph concentrations of Ro 15-0778 in the rat after oral dosing in MTS or Miglyol 812 (6 mg/kg).



content reached a still higher value of 59,770 ng (5.98%).

Of the three oils, linoleic acid gave the greatest plasma uptake rate (13,500 ng/h). The plasma uptake rates from Miglyol 812 and cottonseed oil were 8,300 ng/h and 4,800 ng/h. The plasma uptake rate in rats which had been dosed with Ro 15-0778 in MTS (36,200 ng/h) was much greater than those found after dosing in oils.

The lymph : plasma uptake ratios for the oils ranged from 15% (Miglyol 812) to 84% (cottonseed oil). In rats dosed with MTS, the lymph : plasma uptake ratio was greater than 10%.

4.2.5. Discussion.

A method for the cannulation of the mesenteric lymphatic duct in the male Wistar rat has been developed. Lymph was allowed to flow into a polythene cannula under a slight negative pressure and the method permitted the collection of small amounts of lymph (20 - 100 mg) over a number of hours.

Lymph collections were seen to become turbid and creamy in appearance several minutes after dosing the animals with an oily meal. This was believed to be caused by an increase in chylomicron formation in the enterocytes of the gut and the subsequent

transport of these chylomicrons, *via* the lymphatic network, into the venous circulation.
Lymph flow rate was determined after each sample of lymph was collected. The lymph flow rate was also seen to vary after dosing the animals with an oily meal.

4.2.5.1. Lymph turbidity after oral dosing.

After performing numerous mesenteric lymph duct cannulations on animals dosed with oily vehicles containing retinoid, it was observed that certain oils produced a very creamy lymph whilst other oils gave rise to a very pale lymph. Charman *et al.* (1986b) have confirmed a strong correlation (r^2 = 0.9561) between lymph turbidity and lymph chylomicron lipid concentration. Since lymph chylomicrons are almost certainly the carrier vehicle for lipophilic compounds (Sieber, 1976., Vost & Maclean, 1984), it was decided to investigate the turbidity of lymph after various dosing regimens and examine these data in relation to lymphatic uptake rates from the same oily vehicles. Turbidity estimations were made by measuring the visible absorption of lymph samples diluted in saline as described in Section 4.2.4.4.
The lymph turbidity data obtained from animals dosed with oils and food were compared with those obtained from animals which were dosed with an equivalent

volume of saline (Table 27).

In general, the turbidity of lymph seemed to follow the same pattern which had been observed subjectively during mesenteric duct cannulation procedures. Three distinct groups of oily dosing vehicles could be categorised in terms of their ability to produce turbid lymph. The first group included the three long chain fatty acid triacyl glycerol oils: soyabean oil, cottonseed oil and peanut oil; these gave the greatest turbidity (absorbance greater than 25 x lymph from animals dosed with saline).

The second group contained long chain fatty acids, oleic acid and linoleic acid, the self-emulsifying oil system containing Miglyol 812, Tween 85 and Span 85 and the special liquid rat diet. These materials gave intermediate turbidity (absorbance greater than 10 x lymph from animals dosed with saline).

The third group contained the mixed medium chain fatty acid triacyl glycerol oil, Miglyol 812, which gave only a very slight increase in turbidity (absorbance less than 5 x lymph from animals dosed with saline).

These data are consistent with lymph turbidity data (0 - 50 absorbance units/h at 360 nanometres after infusion of a 0.2% Tween/saline solution containing DDT, into the duodenum) obtained from fasted Sprague-Dawley rats (Charman et al., 1986b). Cheema

Table 27. Lymph turbidity data after orally dosing fasted rats with oils (0.5 ml), SES (0.5 ml), liquid food (2.0 ml) or saline (0.5 ml).

Oral dose.	Lymph absorbance (400 nanometres)	Turbidity relative to saline dose.
Saline #	1.07 ± 0.346 (8)	---
Soyabean	51.3 ± 9.59 (6)	48.2
Cottonseed	38.0 ± ** (4)	35.7
Peanut	26.6 ± ** (3)	25.0
Oleic acid	12.9 ± 1.92 (10)	12.1
MTS	12.5 ± 2.83 (8)	11.0
Liquid food	10.5 ± 6.49 (12)	9.9
Linoleic acid	10.4 ± ** (3)	9.8
Miglyol 812	4.7 ± 0.91 (6)	4.4

Note:
i) Figures are the lymphatic absorbance (absorbance units/ml) for lymph after correcting for dilution ± standar deviation. Value in brackets indicates number of values measured.
ii) ** Insufficient data for analysis.
iii) MTS is an self emulsifying oil system containing Miglyol 812 (80 parts), Tween 85 (16 parts) and Span 85 (4 parts).
iv) # Lymph flow rate after dosing saline (0.5 ml) was 1.61 ± 0.69 ml/h/kg.

et al. (1987) have also reported the highest lymphatic concentration of chylomicrons after the intra-duodenal administration of the mixed long chain tri-acyl glycerol oil, arachis oil (peanut oil), in the rat. However, similar lymphatic chylomicron concentrations were also reported after administration of the free long chain fatty acid, linoleic acid.

The mixed long chain triacyl glycerol oils promoted the highest lymph turbidity and also the greatest lymphatic absorption rates suggesting a possible link between these two factors. The correlation coefficients between lymph turbidity and lymphatic transport of a retinoid were highly significant (Ro 04-3780; $r^2 = 0.495$, $p < 0.001$ ($n = 292$) (Figure 28): Ro 10-9359; $r^2 = 0.998$, $p < 0.001$ ($n = 136$) (Figure 29): Ro 15-0778; $r^2 = 0.147$, $p < 0.001$ ($n = 97$) (Figure 30)).

4.2.5.2. Lymph flow rates after oral dosing.

Lymph flow rates measured in mesenteric lymph duct cannulae varied between 0.8 ml/h and 3.3 ml/h (the least significant difference using $p < 0.01$ (1% LSD = 0.8 ml/h). These lymph flow rates were consistent with data (5.7 ml/h/kg) reported in the literature, (Tso et al., 1985) after the infusion of an oleic acid/1-monoolein emulsion into the duodenum of the rat.

Figure 28. A comparison between the lymph turbidity and lymphatic uptake of Ro 04-3780 in the rat after dosing in a series of oils.

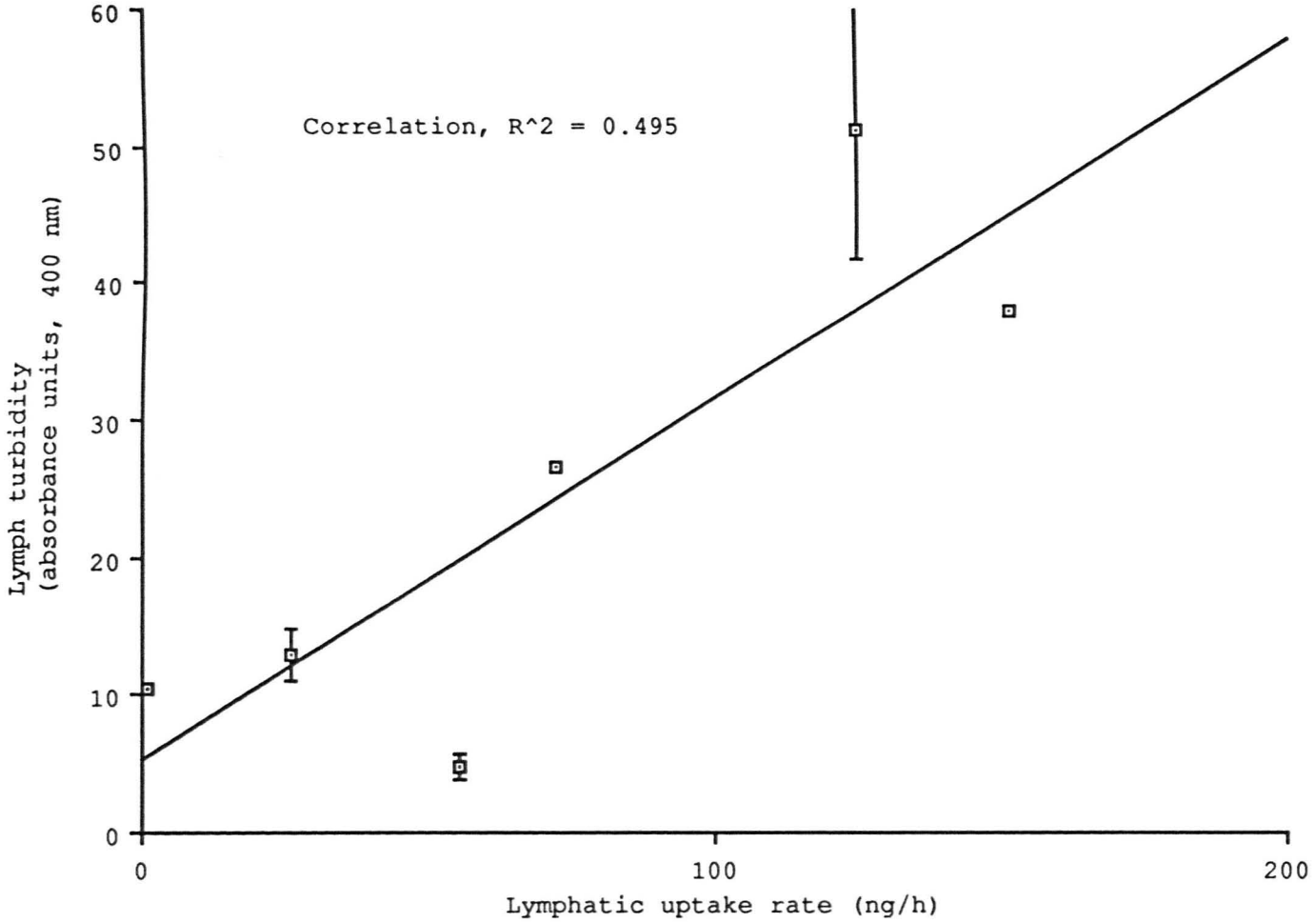


Figure 29. A comparison between the lymph turbidity and lymphatic uptake of Ro 10-9359 in the rat after dosing in a series of oils.

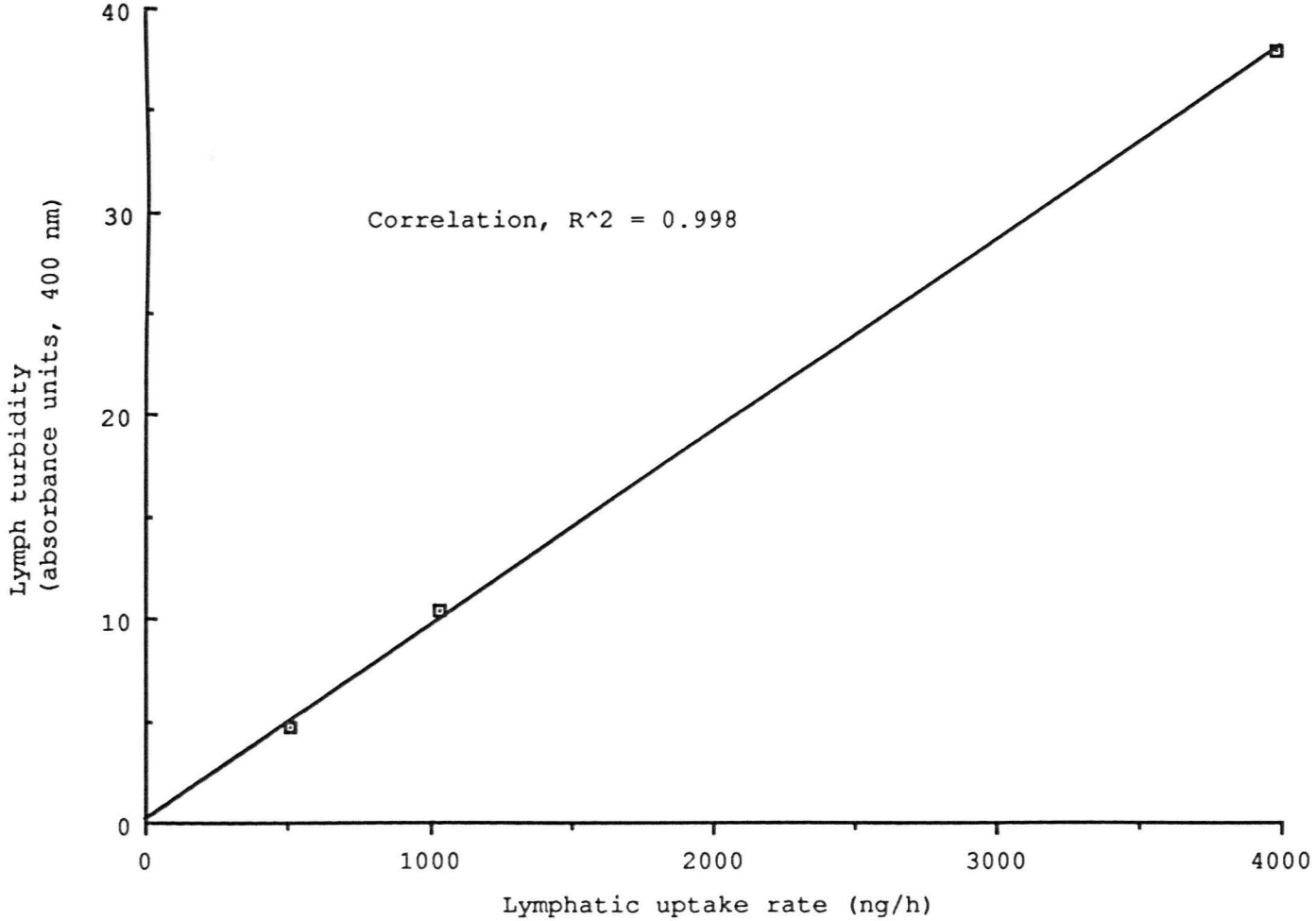
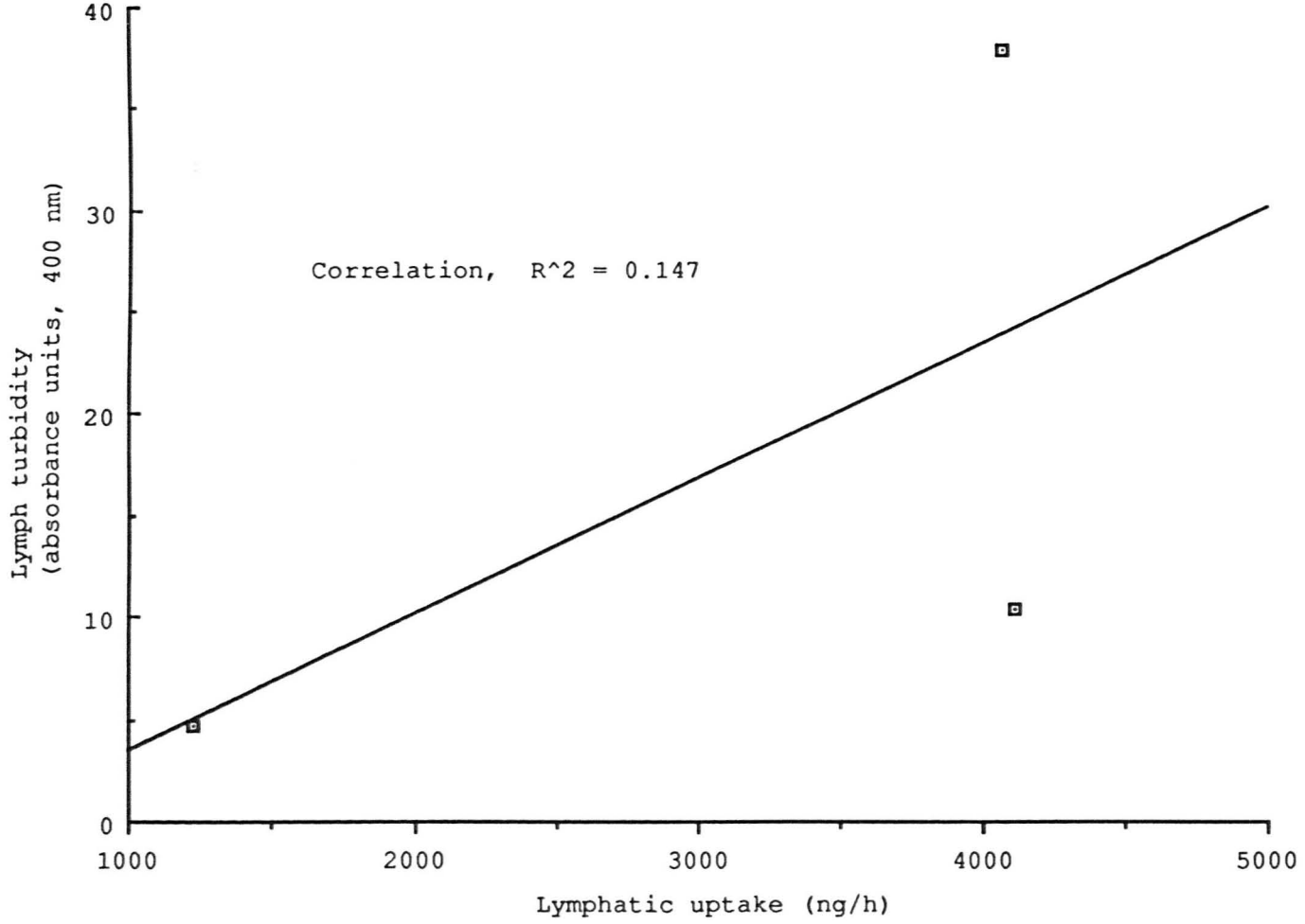


Figure 30. A comparison between the lymph turbidity and lymphatic uptake of Ro 15-0778 in the rat after oral dosing in a series of oils.



Lymph flow rate may be influenced by the type of oil used to formulate a particular retinoid for oral dosing and also by the retinoid itself.

When the data obtained from lymph turbidity studies (Table 27) were examined, it was observed that the mean intrinsic lymph flow rate, measured in fasted animals which had been orally dosed with isotonic saline (0.5 ml), was 1.61 $\pm$ 0.69 ml/h. These data demonstrate that not only do certain oils enhance lymph flow rate but that other oils would appear to suppress it. There was considerable inter-subject variation in the lymph flow rate data and therefore only flow rates measured after dosing Ro 04-3780 in cottonseed oil (3.1 ml/h) or soyabean oil (3.3 ml/h) and Ro 10-9359 in cottonseed oil (2.6 ml/h) were significantly elevated ($p < 0.01$) whilst the flow rate after dosing Ro 04-3780 in linoleic acid (0.8 ml/h) was significantly suppressed ($p < 0.01$).

The lymph flow rate data also show a variable effect of lymph flow depending upon the oil / retinoid combination studied. After orally dosing retinoids in cottonseed oil, lymphatic flow rate decreased from 3.1 ml/h with Ro 04-3780 to 2.6 ml/h with Ro 10-9359 and 2.0 ml/h with Ro 15-0778. Similarly, after orally dosing in Miglyol 812, lymph flow rates decreased from 2.1 ml/h with Ro 04-3780 to 1.5 ml/h with Ro 10-9359 and 1.0 ml/h with Ro 15-0778. These decreases in lymphatic flow rate with increasing

retinoid lipophilicity, although only marginally significant (5% and 1% LSD's approximately 0.79 and 0.56 ml/h), appeared to correlate with an overall increase in the lymphatic absorption of these retinoids and show an effect of the retinoid itself on the lymph flow rate. However, after the flow rate data obtained after dosing each of the three retinoids in linoleic acid were examined, the lowest flow rate was observed with Ro 04-3780 (0.8 ml/h) and the highest with Ro 10-9359 (1.5 ml/h), Ro 15-0778 gave a flow rate of 1.1 ml/h. These data suggest that lymph flow rate is not exclusively dependent upon retinoid type (lipophilicity) nor on the type of oil in which it is administered but upon some interaction between these factors. The mechanism by which the retinoid has an effect on lymph flow rate is not clear, but this may be a result of either a pharmacological action of the retinoid on the enterocytes to alter the lymph formation or a more direct effect on lymphatic vessels.

When the lymph flow rate and lymphatic uptake data were analysed for each retinoid, the correlation coefficients were found to be highly significant (Ro 04-3780; $r^2 = 0.709$, $p < 0.01$ ($n = 292$) (Figure 31): Ro 10-9359; $r^2 = 0.966$ $p < 0.001$ ($n = 136$) (Figure 32):Ro 15-0778 $r^2 = 0.145$, $p < 0.001$ ($n = 97$) (Figure 33)).

Figure 31. A comparison between the lymph flow rate and lymphatic uptake rate of Ro 04-3780 in the rat after dosing in a series of oils.

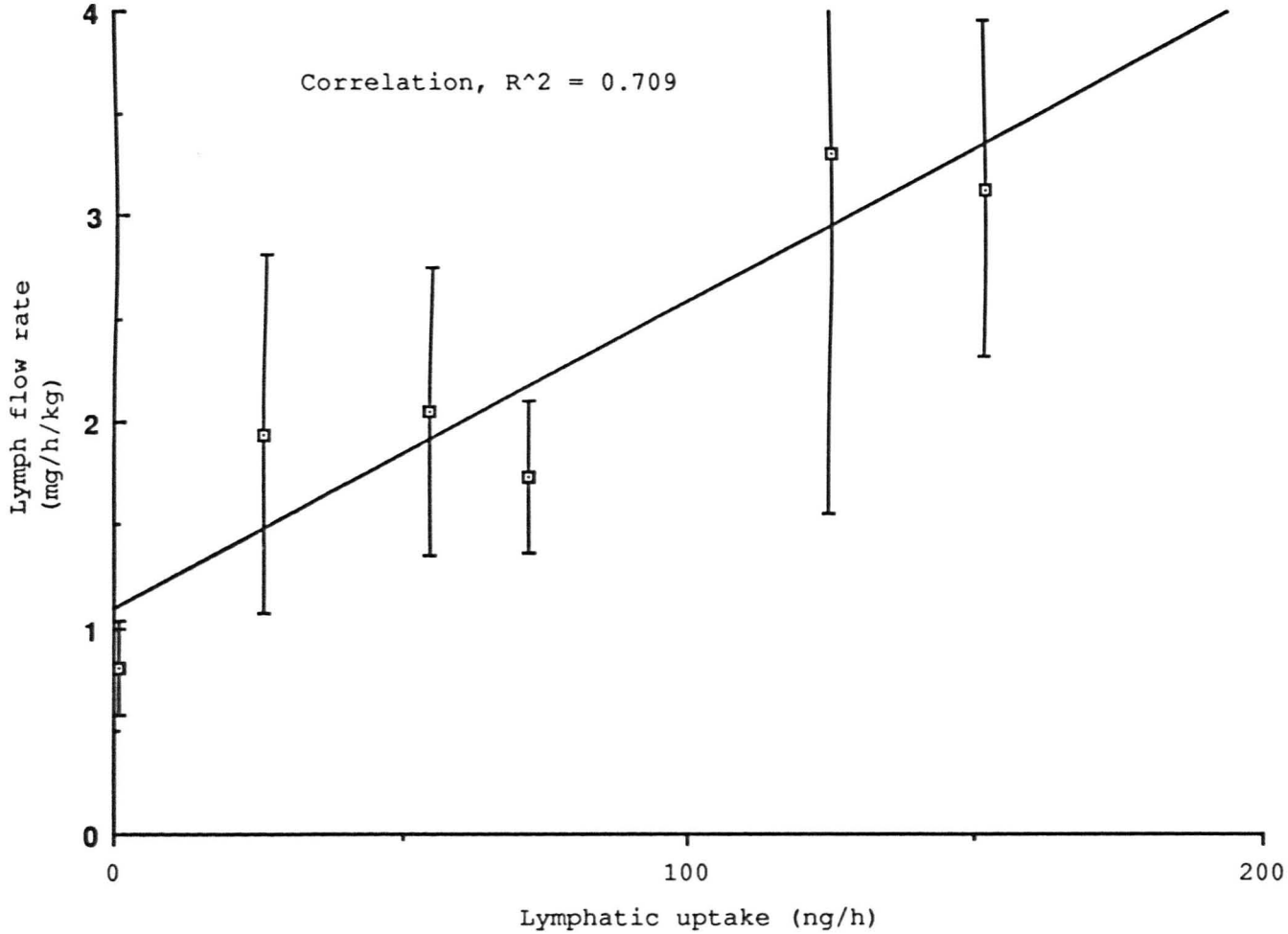


Figure 32. A comparison between the lymph flow rate and lymphatic uptake rate of Ro 10-9359 in the rat after dosing in a series of oils.

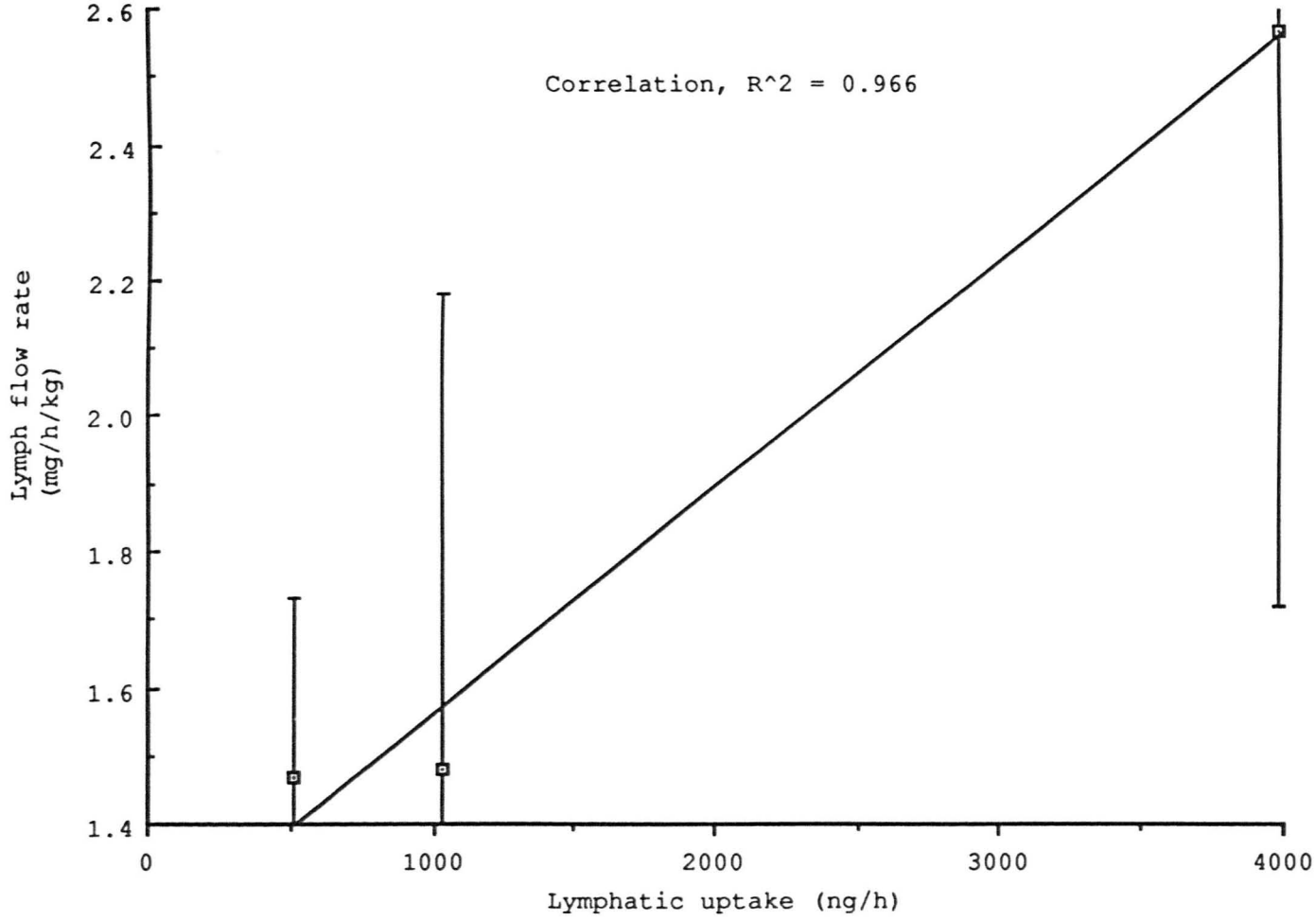
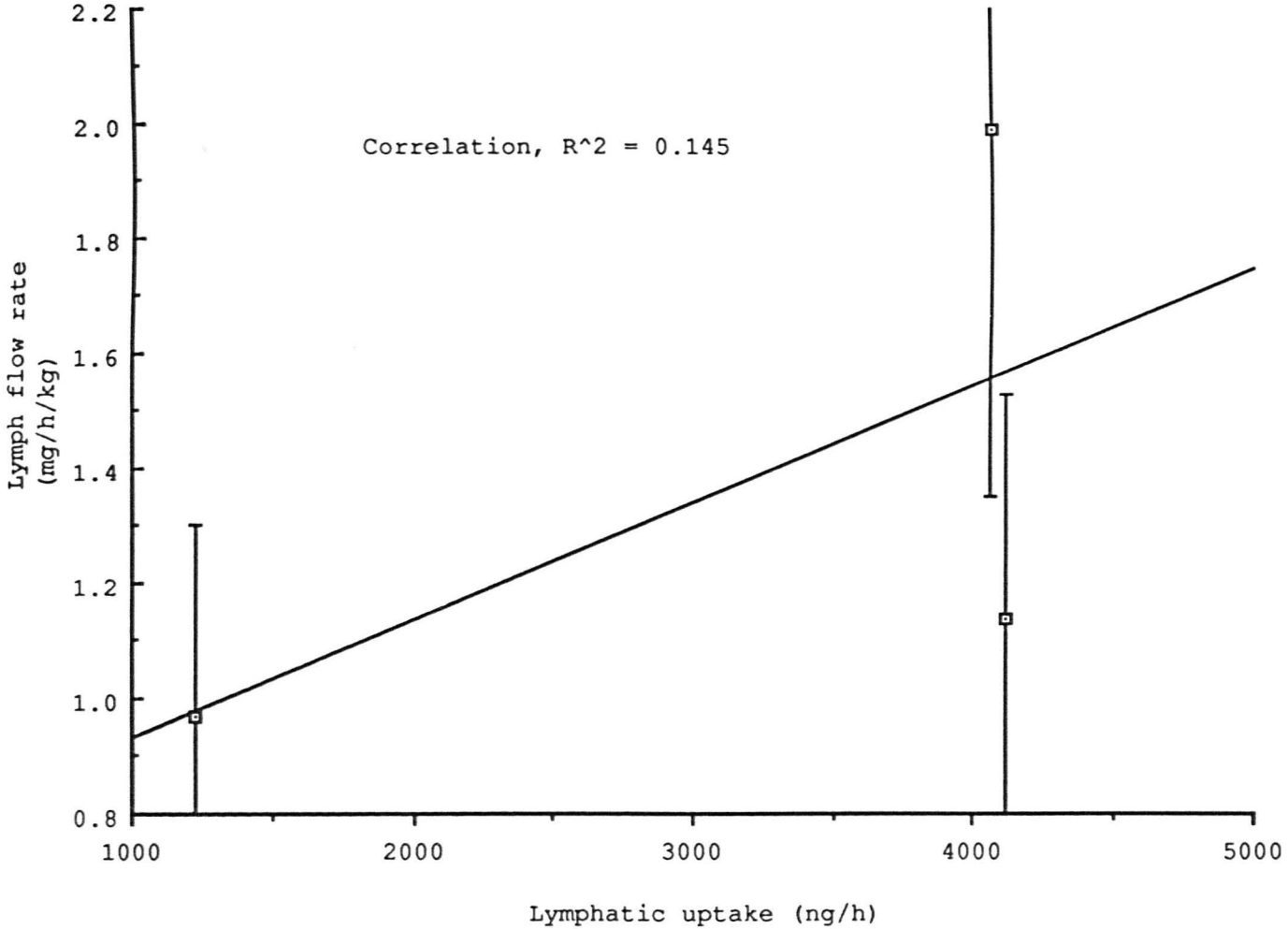


Figure 33. A comparison between the lymph flow rate and lymphatic uptake of Ro 15-0778 in the rat after dosing in a series of oils.



These data, together with the lymph turbidity data (Section 4.2.5.1) indicate that both the lymph flow rate and the lymph chylomicron transport rate are important determinants in the lymphatic absorption of the retinoids and confirm the findings of Charman & Stella (1986b).

4.2.5.3. Effect of oil type on lymphatic uptake of retinoids after oral dosing.

Several workers have studied the effects of various vehicle systems on the bioavailability of drugs. Palin et al. (1980) have studied the effects of three oily vehicles on the absorption of the highly lipophilic (log P = 6.2) model compound DDT in the rat. They showed that the area under the plasma concentration time curve (AUC) was greater after orally dosing DDT in peanut oil compared with dosing in Miglyol 812 or liquid paraffin. These workers also reported (Palin et al., 1982) that the absorption of DDT was almost totally via the lymphatic route and that lymph flow was not important in determining lymphatic uptake rate since both peanut oil and liquid paraffin stimulated lymph flow to the same extent.

Holmberg et al. (1990) studied the absorption of vitamin-D in humans after administration in gelatin capsules containing either peanut oil or Miglyol 812. In fasting subjects, the mean peak plasma

concentration was approximately three times greater when vitamin-D was administered in a mixed long chain fatty acid triacyl glycerol oil (peanut oil) than in a medium chain triacyl glycerol oil (Miglyol 812). These data supported the findings of Palin et al. (1980).

Similar data were obtained using the even more lipophilic compound, Probucol (estimated log P = 11),(Palin & Wilson, 1984., Palin et al., 1984), which confirmed peanut oil to be the oil which promoted the greatest increase in lymphatic absorption compared with Miglyol 812 or liquid paraffin.

Charman et al. (1986a) have studied the effects of peanut oil and the long chain fatty acid, oleic acid, on the oral absorption of DDT and showed that oleic acid gave even greater lymphatic absorption than peanut oil. These findings have been further supported by Gowan & Stavchansky (1986) who obtained the greatest bioavailability and also the highest lymph flow rate when radio-labelled phenytoin was administered in the salt of a long chain fatty acid, sodium oleate.

Charman et al. (1986b) went on to study the lymphatic transport of DDT in rats after oral (intra-duodenal) administration in oleic acid, showed a good linear relationship with chylomicron transport rather than lymph flow and demonstrated

the importance of chylomicron flow (determined from lymph turbidity measurements) in the lymphatic absorption of DDT.

A review of the literature has therefore suggested that long chain fatty acids and their salts promote greater lymphatic absorption than mixed long chain fatty tri-acyl glycerol oils, and these in turn promote greater lymphatic absorption than mixed medium chain fatty tri-acyl glycerol oils. However, Hollander (1980) has reported some conflicting data. When a vitamin-A : bile salt emulsion system was perfused into the duodenum of a rat, the addition of a medium chain fatty acid, caprylic acid, to the emulsion, increased the lymphatic absorption of vitamin-A. In contrast, the addition of one of the unsaturated long chain fatty acids, oleic, linoleic or arachidonic (C20:0) resulted in a decrease in lymphatic absorption. These data therefore suggest that medium chain fatty acids would promote lymphatic absorption whilst the longer chain fatty acids would decrease it.

Six oils were selected in order to study their effects on the lymphatic absorption of the retinoids (Section 4.2.3.1.). After studying the effect of these oil systems on the lymphatic absorption of Ro 04-3780, the results suggested a number of features of the oils which may be important in

influencing lymphatic absorption. The mixed long chain fatty acyl triglyceride oils, cottonseed, soyabean and peanut oil, promoted the lymphatic absorption of Ro 04-3780 to the greatest extent. The free long chain fatty acids, oleic acid and linoleic acid, gave very poor lymphatic uptake whilst the mixed medium chain fatty acid triacyl glycerol oil, Miglyol 812, gave lymphatic uptake intermediate in extent between the mixed long chain fatty acyl triglyceride oils and the unsaturated long chain free fatty acids. These data are consistent with reported data (Palin et al., 1980)., Palin & Wilson, 1984., Palin et al., 1984., Holmberg et al., 1990). Lymphatic uptake of Ro 04-3780 from any of the oil systems was extremely poor, with a maximum rate of 150 ng/h from a adjusted oral dose of 1 mg/kg (cottonseed oil). This figure represented a lymphatic absorption rate of only 2.00% of the absorbed dose per hour and 0.015% of administered oral dose per hour. Data obtained on the absorption of Ro 04-3780 from linoleic acid, which gave the poorest lymphatic uptake rate for this retinoid, showed a mean lymphatic uptake rate of 0.71% of the absorbed dose per hour and approximately 0.00010 % of administered oral dose per hour.

Oral dosing studies using the more lipophilic retinoid, Ro 10-9359 in the mixed long chain fatty acid triacyl glycerol oil, cottonseed oil, continued

to give a higher lymphatic uptake compared with either Miglyol 812 or linoleic acid. The lymphatic uptake values for Ro 10-9359 after dosing in each of the three oils were much higher than data obtained with Ro 04-3780. Linoleic acid in particular gave a much improved rate of lymphatic uptake (1030 ng/h), compared with that obtained for Ro 04-3780 in linoleic acid (150 ng/h). This lymphatic uptake for Ro 10-9359 in linoleic acid was almost twice the rate obtained from Miglyol 812 (514 ng/h). Cottonseed oil however, gave the highest lymphatic uptake for Ro 10-9359 (3980 ng/h).
Ro 15-0778 in cottonseed oil gave a mean lymphatic uptake value (4110 ng/h) which were slightly greater ($p > 0.05$) than linoleic acid (4060 ng/h) and more than three times greater than Miglyol 812 (1230 ng/h). All three oils exhibited greater lymphatic uptake of Ro 15-0778 compared with Ro 10-9359, cottonseed oil and Miglyol 812 giving approximately three fold greater lymphatic uptake.

The lymphatic uptake data for Ro 04-3780 shows a 150 fold range for the six oils used for oral dosing however, the corresponding estimates for plasma uptake show a similar trend and therefore the lymph : plasma uptake ratios for each oil were similar and between 0.5% and 2.0%. Therefore, although certain oils increase the lymphatic uptake rate of

Ro 04-3780, they also increased portal absorption to approximately the same extent and hence, preferential delivery of Ro 04-3780 to the lymphatics was not achieved.

When the data for Ro 10-9359 were examined, certain orally dosed oil systems were identified which greatly increased lymphatic uptake rate with only a marginal increase in portal absorption, compared with data obtained after dosing with Ro 04-3780. The lymph : plasma uptake ratio for Ro 10-9359 is greater than 332 % after dosing linoleic acid to the fasted rat compared with only 0.72 % when Ro 04-3780 is dosed in linoleic. These data suggest that certain oil / retinoid combinations selectively deliver retinoids into the lymphatics and suggests that the higher lipophilicity of Ro 10-9359, compared with that of Ro 04-3780, may be an important factor in this process. Data obtained after oral dosing of the more lipophilic, Ro 15-0778 in oils seemed to confirm the importance of high lipophilicity of a retinoid in promoting lymphatic absorption. The lymphatic uptake was 2% greater after dosing Ro 15-0778 in cottonseed oil compared with Ro 10-9359 (4060 ng/h and 3980 ng/h), 140% greater from Miglyol 812 (1230 ng/h and 514 ng/h) and 300% greater from linoleic acid (4110 ng/h and 1030 ng/h).

The lymph : plasma ratios obtained after dosing

Ro 15-0778 in oil were between 15% and 84%, generally lower than with Ro 10-9359. These data suggest that although high lipophilicity is an important factor in increasing the overall absorption of the retinoids, it does not increase the selective targeting of retinoids to the lymphatics.

4.2.5.4. A self-emulsifying oil system for the oral delivery of Ro 15-0778.

A number of self-emulsifying oil systems were developed (Chapter 3) which would spontaneously emulsify when gently agitated with an aqueous medium. One of the most promising systems was MTS, (a Miglyol 812 oil based self-emulsifying system containing Tween 85 and Span 85). This system produced an emulsion which was stable for several days and possessed a mean particle size of 509 $\pm$ 20 nm. In order to study the effect of this self-emulsifying oil system in the rat, a series of oral dosing studies was performed with the retinoid, Ro 15-0778.

The lymph flow rates after orally dosing Ro 15-0778 (6 mg/kg) in MTS (0.95 $\pm$ 0.26 ml/h/kg) or in Miglyol 812 (0.97 $\pm$ 0.33 ml/h/kg) were almost identical (Table 26) but lymph turbidity was almost 2.7 times greater ($p < 0.001$) after dosing Ro 15-0778 in MTS. Oral dosing data (Table 26) demonstrate that

administration of Ro 15-0778 in a self-emulsifying oil system (MTS) compared with Miglyol 812 augmented the overall absorption rate by approximately four fold and lymphatic absorption rate by three fold. Reports in the literature have demonstrated three to four fold increases in the bioavailability of penclomidine after administration in a long chain triacyl glycerol emulsion system (Myers & Stella, 1989). Myers (Myers & Stella, 1989) concluded that long chain triacyl glycerol oils increased lymphatic transport of penclomidine by an increase in chylomicron formation.

4.2.5.5. Effect of food on the oral bioavailability of Ro 10-9359.

The timing of a lymphatic cannulation with respect to the fasted state of the animal appears to affect the rate and extent of lymphatic absorption. Data from Colburn et al. (1983b) demonstrated that mesenteric lymphatic cannulations performed in rats, fed three hours before oral dosing of DDT, produced higher mesenteric lymphatic absorption of DDT over 8 hours post dosing compared with cannulations performed after a 24 hour fast, but three hours before oral dosing.

Cannulations prior to a 48 hour fast produced greater mesenteric lymph levels of DDT over an 8 hour period post dosing compared to levels found

following surgery after a 24 hour fast (Colburn et al., 1983b); overall lymph flow was also found to be greater after food.

These effects on absorption may have been due to the effects of anaesthesia or changes in lipid metabolism and gut absorption. Lee (1965) studied an isolated gut preparation with intact mesentery and found that levels of sodium pentobarbitone could abolish gut motility and reduce lymphatic pressure. Kalin (1984) also showed that phenobarbitone appeared to reduce levels of retinoids found in tissues after intra-gastric feeding.

Studies in humans (Holazo et al., 1990., Gibson et al., 1984., Colburn et al., 1983a) have shown increased bioavailability of the retinoids, Ro 15-0778, Ro 04-3780 and Ro 10-9359 after a fatty meal.

In view of these reports, all the oral dosing studies in the present work, have been carried out on animals which had been fasted for at least 16 hours to eliminate any effects due to feeding. However, it may be argued that this was not a physiologically normal state for the animals and the data would have been best obtained on fed animals. A series of dosing studies was therefore carried out in order to study the effects of diet on the overall bioavailability of the retinoids in the rat and to investigate any selective targeting of retinoids due

to the effects of food.

The level of anaesthesia within the animals could not be perfectly controlled, but was administered at a constant inter-peritoneal dose of 72 mg/kg sodium pentobarbitone (Sagatal).

Animals were dosed with a specially prepared liquid animal diet prior to dosing with Ro 10-9359 in linoleic acid.

Linoleic acid was the oil of choice for this study because the lymphatic uptake of Ro 10-9359 in the fasted rat, after dosing with linoleic acid (1030 ng/h), was sufficiently high to allow sensitive detection of small changes in retinoid concentrations. The lymphatic uptake of Ro 10-9359 with cottonseed oil (3980 ng/h), although higher than with linoleic acid, may have been limited by factors other than the oil type (a possible upper threshold limit of lymphatic uptake rate) and may therefore have been a poor indicator of further increases in lymphatic uptake.

Animals were pre-dosed with a 2 ml bolus of the liquid diet, 30 minutes prior dosing with linoleic acid containing Ro 10-9359. The lymph which was produced after food was almost identical in turbidity (10.4 absorbance units/ml) to the lymph produced when linoleic acid was dosed to fasting animals (10.5 absorbance units/ml),(Table 27). The lymph flow rate was marginally lower after food

(1.38 ml/h compared with 1.48 ml/h),(Table 25). The lymphatic concentration data for Ro 10-9359 in fasted and fed animals after dosing in linoleic acid were found to be almost identical, even after correcting the data for the slight difference in lymph flow rate. Feeding, prior to oral dosing with retinoid in an oil, was therefore ineffective in increasing the lymphatic uptake rate of Ro 10-9359 in the rat. In contrast, plasma concentrations of Ro 10-9359 in fed animals were approximately one tenth of those found in the fasted group and after correcting the data using the volume of distribution of Ro 10-9359, the estimated plasma uptake rates was at least twelve times greater in the fasted group. These data have important consequences on the effective therapeutic dose of Ro 10-9359 if these findings are extrapolated to man. Since the estimated plasma uptake rate has been shown to decrease after food, this may imply that a lower dose of Ro 10-9359 will need to be administered to give the desired therapeutic effect in a patient who was unable to consume a normal diet, since the dose will be more readily absorbed *via* the portal route. Conversely, in a subject on a normal diet, the effective therapeutic dose will need to be increased, because of the decreased uptake rate, which is occurring primarily *via* the lymphatic route.

The literature data show greater plasma concentrations of the retinoids after a fatty meal (Holazo et al., 1990., Gibson et al., 1984., Colburn et al., 1983a), but none of these authors discussed the route of delivery of the retinoids into the plasma. The data presented here allow a somewhat different interpretation of the findings, concerning the effect of food on the bioavailability of the retinoids, namely that food decreases the plasma uptake rate of the retinoids and increases the lymphatic uptake of the retinoid.

4.2.5.6. The relationship between lymphatic uptake rate and oil solubility.

Solubility is defined as the amount of dissolved solute in equilibrium with its pure state (Szets & Harosi, 1991). The solubility of a non-electrolyte in water may be determined from the relationship in equation 16, (Yalkowsky & Morozowich, 1980).

$$\log S_w = -\log P - 0.01\ M.P. + 0.5 + A$$

Equation.....16

S_w = Solubility in water, of a nonelectrolyte compound.

M.P. = Melting point of the compound.

A = A variable to allow for the presence (A = 1.0) or absence (A = 0) of an acidic proton in the compound.

Using this relationship, together with the

calculated log. P data for the retinoids (Table 2) and their melting points (Day, 1992) the solubility of the retinoids have been calculated. As expected, the most polar retinoid, Ro 04-3780 (calculated log. P = 6.76, M.P. = 176°C), shows the greatest calculated solubility (log. S_w = -6.99), dependent upon the degree of dissociation of the terminal carboxyl group (Figure 3)). The more lipophilic retinoid, Ro 10-9359 (log. P = 7.82, M.P. = 103°C), shows a lower solubility (log. S_w = -8.35), and the most lipophilic retinoid, Ro 15-0778 (calculated log. P = 8.66, M.P. = 90°C), shows a very low solubility (log. S_w = -9.06). These calculated solubility data would suggest that the least lipophilic retinoid, Ro 04-3780, would be more readily absorbed from the gastro-intestinal tract (de Blaey & Polderman, 1980), however this was not confirmed by the *in vivo* studies.

Using the oil solubility data (Table 4), it is possible to accurately predict a rank order for oily vehicles, which will promote lymphatic absorption of a retinoid. Since there is an inverse relationship between lymphatic absorption after oral administration in the oil and the solubility of the retinoid in the oil. These results were surprising, since it has been reported that lipophilic drugs are preferentially absorbed via the lymphatic route (Noguchi *et al.*, 1985., Sieber, 1976), therefore an

increase in solubility of compound in an oil may have been expected to increase the lymphatic absorption of that compound.

The absorption of a retinoid from the gastro-intestinal tract may involve the release of retinoid from the oily vehicle, into the fluid in the gut. Therefore, a higher solubility of retinoid in water, together with a lower solubility in the oily vehicle may be postulated to enhance absorption (de Blaey & Polderman, 1980).

A hypothesis which would explain the inverse oil solubility : lymphatic uptake is as follows. It is postulated that a retinoid will escape from the oil before passing across the un-stirred water layer barrier which may exist in the gut. This phenomenon will relate to the solubility (affinity) of the retinoid in an oil (Armstrong & James, 1980). The more easily the process occurs (the lower the solubility of the retinoid in the oil), the more thermo-dynamically favourable it will be for the retinoid partition out of the oil and subsequently be diffuse across the un-stirred water layer.

After this stage of the absorption process, the oil must then play a secondary role in facilitating the incorporation of drug into lymph and its subsequent lymphatic transport, potentially by stimulating the lymph flow and chylomicron formation.

The extent of lymphatic uptake has been demonstrated

here to be related to the type of oil. In the oral dosing studies, the retinoid concentration present in each oil system was kept constant (3 mg/ml), in order that the same volume of oil (0.5 ml), would deliver the same dose of retinoid (6 mg/kg) was given to each animal (250 g body weight).
The percentage solubility of Ro 04-3780 in each of the six oils, was determined using equation 17. The solubility data for the retinoids were determined in Section 1.2. and are shown in Table 4.

$$\text{Percentage saturation of retinoid in oil.} = \frac{\text{concentration of retinoid in oil}}{\text{saturation solubility of retinoid in oil}} \times 100$$

Equation.....17

The percentage saturation of Ro 04-3780 (3 mg/ml) is 58 % in cottonseed oil, 53 % in soyabean oil, 49 % in peanut oil, 25 % in Miglyol 812, 12 % in oleic acid and 10 % in linoleic acid. This decrease in percentage saturation parallels the downward trend in lymphatic uptake from 150 ng/h to 1 ng/h found in the oral dosing studies. It may be postulated therefore, that either an increase in the percentage retinoid saturation in the oil or a decrease in the solubility of the retinoid in the oil may be important in increasing lymphatic uptake. However, when Ro 04-3780 was presented in linoleic acid (0.5 ml) at an oral dose of 75 mg/kg (percentage saturation, 93%), the dose adjusted lymphatic uptake

was 150 times smaller than when Ro 04-3780 was administered in cottonseed oil (0.5 ml) at an oral dose of 6 mg/kg (59 % saturation) (Table 24). These data therefore support the hypothesis that a lower solubility of the retinoid in an oil is a key determinant in increasing lymphatic absorption of the retinoid.

CHAPTER 5

GENERAL CONCLUSIONS

5. General conclusions.

The retinoids are a series of vitamin-A analogues which have been extensively investigated in their role as dermatological, chemotherapeutic and chemopreventive agents.

The retinoids are extremely lipophilic in nature and as such are thought to be absorbed into the venous circulation *via* the lymphatic route after oral administration in a way analogous to vitamin-A.

It was proposed to study the physicochemical properties of the retinoids and various formulation factors which were thought to be important in the lymphatic absorption of the retinoids after oral administration.

Three retinoids were to be used in a series of oral dosing studies, one being selected from each of the three main groups (generations) of retinoids.

Isotretinoin (Ro 04-3780, 13-*cis* retinoic acid) was selected from the first generation of retinoids.

Etretinate (Ro 10-9359) was selected from the second generation of retinoids and Temarotene (Ro 15-0778) was selected from the third generation of retinoids.

Numerous factors have been examined which were believed may be important determinants in the lymphatic absorption of the retinoids. Many of these factors have been found to be interrelated in their effects.

5.1. Physicochemical properties of the retinoids.

Some of the physicochemical properties of the retinoids have been examined, these include their ultraviolet absorption, their lipophilicity and their solubility in a range of oil systems.

5.1.1. Ultraviolet absorption and photosensitivity of the retinoids.

The first generation retinoid, Ro 04-3780 (13-cis retinoic acid) and the second generation retinoids, Ro 10-9359 (Etretinate) and Ro 11-5036 have been shown to have ultraviolet absorption maxima of 349 and 360 nanometres respectively.

The third generation retinoids, Ro 15-1570 (Etarotene) and Ro 15-0778 (Temarotene), have been shown to have absorption maxima further into the ultraviolet at 295 and 279 nanometres, respectively.

All of the retinoids studied show a very broad band of absorption around their absorption maximum, for example Ro 04-3780 shows 90% peak absorption at $\pm$ 16 nanometres around its peak wavelength.

The molar absorptivity of the retinoids is typically high and was found to be 2.388×10^4 litres.mol^{-1}.cm^{-1} for Ro 15-0778, 4.358×10^4 litres.mol^{-1}.cm^{-1} for Ro 04-3780 and 5.192×10^4 litres.mol^{-1}.cm^{-1} for Ro 10-9359. These data confirm the strong ultraviolet absorption of the retinoids and favoured the use of this attribute as a method of choice in

the detection and quantitation of the retinoids. Although this strong ultraviolet absorption of the retinoids is of benefit in their detection and quantitation, it is a disadvantage in terms of the handling of these compounds. Extensive degradation of the retinoids has been demonstrated after brief exposure to light. This degradation was thought to be due to either photo-decomposition or to photo-isomerism of the retinoids.

Sunlight was found to have the most damaging effect on the retinoids, decreasing the quantity of parent compound to less than 50% after 60 seconds of exposure ($P < 0.001$). Using the hplc system developed for the analysis of Ro 04-3780, it was not possible to detect any isomerisation of 13-*cis*-retinoic acid to its all-*trans* isomer.

Laboratory fluorescent tubes caused a significant decrease ($P < 0.05$) in concentration after 30 minutes exposure. In contrast, exposure of the retinoids to light from yellow fluorescent tubes, which do not emit light below 500 nanometres, results in little loss or isomerisation of the retinoids ($P > 0.05$), even after periods of 180 minutes.

These observations require the use of great care when retinoids are being handled. Aluminium foil must be used to mask the sample tubes or the use of protective yellow lighting conditions is recommended

during handling of the retinoids to protect the samples from light.

5.1.2. Lipophilicity of the retinoids.

Experimentally determined values for the octanol-water partition coefficient (log. P) of extremely lipophilic compounds, such as the retinoids, have been shown to be difficult to measure using the traditional shake flask method or by the slow stir technique. This arises from the difficulties in accurately determining the very small quantities of compound which partitions into the aqueous (water) phase when the compound is allowed to distribute between octanol and water.

It is proposed that the relatively high solubility of octanol in water (1.36×10^{-4} mol/l, 17.7 parts per million), may lead to underestimated values of log. P for solutes with log. P values greater than 5. For example, in a slow stir flask containing a solute with a calculated log. P of 6 there will be 17.7 times more solute present in the octan-1-ol which is dissolved in the water phase than the amount of solute dissolved in the water itself. This predicted negative deviation from the theoretical log. P might explain some of the discrepancies between experimentally determined and computer predicted log. P values.

It was decided, to use a computer database of

fragmental constant data to calculate the log. P values for the retinoids. Two commonly used methods have been studied, the Rekker method, which is thought to be more appropriate for calculations based on the complete chemical structure of the molecule and the Hansch method which is more convenient when the log. P value of a parent compound is known and changes in various functional groups are been made.

In these studies, calculated log. P data obtained after using the Hansch π method were in close agreement with data provided for log. P values calculated using the Rekker fragmental (Van der Waterbeemd, 1990).

All the retinoids show log. P values in excess of 6 and could be therefore classed as highly lipophilic. The most lipophilic compound was Temarotene (mean log. P = 8.48) followed by Etretinate (mean log. P = 7.82) and Isotretinoin (mean log. P = 6.77).

Other physicochemical data have been used to assess the validity of these predicted log. P values. Hplc retention times and structural data have been examined and although the gross trends in log. P are consistent with the theoretically expected results, there are some inconsistencies. On a reversed phase chromatographic system, polar solutes (less lipophilic) would be expected to be elute from the

chromatographic system more rapidly than less polar solutes (more lipophilic). However, the order of elution for Ro 01-4955, Ro 01-5488 and Ro 04-3780 on the reversed phase hplc system does not follow this expected pattern. Ro 01-4955 elutes with a retention time almost twice that of Ro 01-5488 or Ro 04-3780 despite having a predicted log. P slightly less than either of these compounds. In addition, Ro 01-5488 and Ro 04-3780 have the same empirical chemical formula, differing only in the geometry of the terminal group but were predicted to show identical log. P values using the two methods. However, these two compounds were resolved using the hplc system, suggesting Ro 04-3780 to be slightly less polar. Possible explanations which might explain this anomaly include;

i) The Rekker or Hansch methods do not take into consideration any effect of ionisation of polar groups in the molecules and indeed assume the molecule to be completely unionised and undissociated. Compounds such as Ro 04-3780 or Ro 01-5488, both contain a ionisable acid group and would partially ionise in an aqueous environment, such as in the fluids within the gastrointestinal tract (approximately 2-8 pH units) or in the mobile phase (pH 6.0) of the hplc system. The degree of ionisation will obviously be dependent upon the pH of the solvent and the pK of the dissociable group.

In the mobile phase at pH 6.0 and with an expected pKa for the acidic retinoids of approximately 4 - 5 the acidic group in both Ro 01-5488 and Ro 04-3780 is likely to be 90% - 99% ionised. This would increase the polarity of these compounds and decrease their expected log. P values and help to explain their more rapid elution from the reversed phase hplc system compared with Ro 01-4955.

ii) When the structures of the two retinoids, Ro 01-5488 and Ro 04-3780 are examined, it may be postulated that hydrogen bonding between the carbonyl oxygen and the hydrogen atom on carbon-11 may occur in the 13-cis isomer (Ro 04-3780). This would decrease the charge density around the terminal group and result in a marginally higher lipophilicity and greater log. P value for this isomer.

5.1.3. Solubility of the retinoids in various oil systems.

It is well established that the retinoids are highly lipophilic in nature. However, lipophilicity is not easy to define. The ratio between the solubility of a compound in each of two immiscible solvents can be misleading. It was considered necessary to examine the relationship between solubility and lipophilicity for the retinoids using oil systems

which were to be used as oral delivery vehicles in these studies.

For each retinoid, a rank order of increasing solubility in a range of oil systems was constructed. This revealed many interesting features.

i) The more lipophilic the retinoid, as defined by the magnitude of its calculated log. P value, the greater is its solubility in a particular oil. In the oil systems studied, Ro 04-3780 shows solubilities between 2.2 and 73.3 mg/ml (median = 19.9 mg/ml), Ro 10-9359 from 6.6 to 68.7 mg/ml (median = 25.7 mg/ml) and Ro 15-0778 between 47.8 and 201.8 mg/ml (median = 149.5 mg/ml). These are consistent with predictions made after considering the physical interactions of lipophilic solutes in non-polar solvents.

ii) The mono-acyl glycerol oils, monocaprylin and monoolein, give low solubility for all three retinoids. This may be explained by the presence of free hydroxyl groups on the glycerol molecule which make these oils slightly more polar in nature and decreasing their affinity for the highly lipophilic retinoids.

iii) The monoglyceride oil, Neobee M5, contains a mixture of both monocaprylate and monocaprate esters of glycerol but, with the exception of Ro 04-3780, the solubility of the retinoids in this oil is far

greater than in the monoglyceride oil, monocaprylin (Neobee : Monocaprylin solubility ratio, for Ro 10-9359 is 2.63 and for Ro 15-0778 is 4.22). These findings may be explained by hydrophobic interactions between the slighter longer chain length capric ($C_{10:0}$) ester and the tetraene side chain of the retinoid molecule.

iv) The solubility of retinoids in the mixed long chain triglyceride oils was lower in all cases than in the mixed medium chain triglyceride oil, Miglyol 812. This may be due to the effect of the more electrophilic nature of the LCFA triglyceride oils, which contain varying quantities of esters of the unsaturated fatty acids, linoleic and linolenic acids. This theory may also explain the poor retinoid solubility in trilinolein (glyceryl trilinoleate) compared with Miglyol 812 and lower solubility of retinoids in the unsaturated long chain free fatty acids, oleic and linoleic acid, compared with the saturated medium chain fatty acid caprylic ($C_{8:0}$).

The relationship between solubility in pairs of oils varied with retinoid lipophilicity.

v) The solubility of retinoids in linoleic acid compared with that in cottonseed oil decreased as the lipophilicity of the retinoid increased. Ro 04-3780 shows a linoleic/ cottonseed solubility ratio

of 5.98, the ratio for Ro 10-9359 is 1.18 and for Ro 15-0778 is 0.66.

In a similar way the ratio of the solubility of retinoids in monocaprylin to that in monoolein decreased from 12.5 for Ro 04-3780 to 2.4 for Ro 10-9359 to 0.82 for Ro 15-0778.

These differences in relative solubility of the retinoids in pairs of oils may reflect differences in the polarity of oils and the effect these changes have on the solubility of a retinoid as it becomes more lipophilic. The differences may also be explained by differences in the physical associations of the retinoid with an oil, such as hydrophobic interactions (Van der Waals forces) and hydrogen bonding.

Many of the variations in solubility have been explained by interactions between the retinoid and the solvent, however, some trends cannot be explained in this way. For example;

vi) The solubility of Ro 04-3780 in a equivolume mixture of Miglyol 812 and oleic acid (36.7 mg/ml) is considerably higher than in either component alone (Miglyol 812 = 12.0 mg/ml, oleic acid = 24.4 mg/ml), whereas for the other retinoids it lies, as expected, between the two values.

vii) A discordant observation is seen between the solubility of Ro 10-9359 in an equivolume mixture of Miglyol 812 and caprylic acid (36.0 mg/ml) which is

less than that found in Miglyol 812 (44.5 mg/ml) or caprylic acid alone (68.7 mg/ml). These inconsistencies may be explained by a solvent:solvent interaction in which each oil component has associated with its partner and made it less polar in nature, however why this interaction is so inconsistent is uncertain.

viii) The solubility of retinoids in a self emulsifying oil system (SES) which contained 80% peanut oil, may have been expected to be approximately 20% lower than in peanut oil alone to reflect the lower oil content. However, Ro 15-0778 has a much lower solubility in this SES than predicted, whereas the more polar Ro 04-3780 shows a greater solubility than in peanut oil. These findings suggest that the ionic nature of the surfactant system has either a desolubilising effect on the non-polar compound or an ion pairing effect on the more polar retinoid.

Ro 10-9359 shows almost identical solubility in the SES and peanut oil.

5.2. Analytical and in-vivo studies on the retinoids.

Having established some of the physicochemical properties of the retinoids it was necessary to establish and validate a viable assay for the quantitation of low concentrations of retinoids in

small volumes of biological fluids and achieve a reliable animal model as an in-vivo screen for retinoid formulations.

5.2.1. Analytical methods.

Sensitive and specific hplc methods have been developed for the analysis of the retinoids in biological fluids. The hplc analytical methods had limits of detection of 10 ng/ml for Ro 04-3780, 10 ng/ml for Ro 10-9359 and 15 ng/ml of Ro 15-0778, all based on the extraction of 100 μl of plasma or lymph.

A reversed phase hplc system was used for both Ro 04-3780 and Ro 15-0778 using a mobile phase containing acetonitrile and ammonium acetate buffer (70:30 and 90:10 respectively). The minimum analysis times for these two compounds on their respective systems were approximately 8 and 12 minutes, using Ro 11-5036 and Ro 15-1570 as the internal standards in the respective analytical methods. These compounds show similar retention behaviour and ultraviolet absorption properties to the retinoid being analysed.

The reversed phase hplc system, used for the analysis of Ro 04-3780 and Ro 15-0778 was investigated for use in the analysis of Ro 10-9359. However, after testing several retinoids as a potential internal standard in this analytical

system, none was found which was sufficiently lipophilic to allow elution with a sufficiently long retention time which would allow it to be resolved from the solvent front when Ro 10-9359 was eluted with an acceptable retention time. A normal phase hplc system were therefore applied to the analysis of this retinoid. The system which was found to give good resolution of Ro 10-9359 from the internal standard, Ro 04-3780, used a mobile phase containing hexane, propionic acid and methyl benzoate and a cyano-bonded silica column (Spherisorb S3CN). The system allowed a minimum analysis time of approximately 12 minutes.

5.2.2. Extraction systems.

Extraction of the three retinoids from biological matrices including plasma and lymph, was achieved by direct protein precipitation using acetonitrile containing the appropriate internal standard. Direct injection of this acetonitrile showed excellent recovery of retinoid when the acetonitrile to plasma ratio was at least three parts to one (Ro 04-3780: 99.6% ± 3.42% from 25 - 1000 ng/ml, Ro 10-9359: 93.8% ± 4.93% from 25 - 1000 ng/ml, Ro 15-0778: 92.6% ± 6.93% from 2.5 - 1000 ng/ml).
In the analysis of Ro 10-9359, an additional step was introduced prior to sample injection to remove traces of aqueous solvents which may have

contaminated the normal phase column. The acetonitrile extract was removed completely under reduced pressure and the samples were reconstituted in mobile phase prior to analysis.
Recovery of retinoids was greater than 90% at concentrations > 25 ng/ml for Ro 04-3780 and Ro 10-9359 and greater than 5 ng/ml for Ro 15-0778. All recovery figures are based on the extraction of a 100 μl sample of plasma.

5.3. In-vivo studies on the retinoids.

5.3.1. Pharmacokinetic studies.

From the pharmacokinetic data for Ro 04-3780, a non-proportional increase in AUC was observed with increasing dose, resulting from a decrease in both the total body clearance and the volume of distribution. The elimination half-life also increased with dose.
The mean residence time (MRT), was 76 minutes and consistent with the observed elimination half-life ($t_{½}$) of 55 minutes.
At the lower dose of 0.264 mg/kg, a volume of distribution (V_d) of 220 ml, a volume of distribution at steady state (V_{ss}) of 200 ml and total body clearance of 2.5 ml/min were obtained. These data are consistent with reports in the literature.
From the pharmacokinetic data for the more lipophilic retinoid, Ro 15-0778, a non-proportional

increase in AUC was observed with increasing dose, resulting from a decrease in total body clearance. The elimination half-life also varied with dose. The MRT at the lower dose (MRT = 111 ± 68 min) was consistent with the elimination half-life observed at the lower dose ($t_{½}$ = 111 ± 38 min) however, the MRT at the higher dose was much lower (MRT = 62 ± 14 min) than either the MRT at the lower dose or the elimination half-life at either dose. These differences, however, were not significant ($p > 0.05$) due to considerable inter-subject variation in the data.

There was considerable inter-subject variation in the mean data for V_d (1100 ± 690 ml) and V_{ss} (380 ± 340 ml). The volume of distribution (V_d) at the low dose (980 ml) and at the high dose (1220 ml) and the volume of distribution at steady state (V_{ss}) calculated at the low dose (430 ml) and at the high dose (325 ml), were not significantly different ($p > 0.05$).

There appear to be no reported pharmacokinetic data on this compound in the literature, however, the data were consistent with those expected for a highly lipophilic retinoid.

After intravenous dosing of Ro 10-9359 at the higher dose (1.964 mg/kg), plasma concentrations fell exponentially to below the limit of detection at 180 minutes. At the low dose (0.655 mg/kg), a similar

rapid fall in plasma concentrations was observed. The pharmacokinetic data showed a mean elimination half-life of 3.5 minutes and a mean MRT of 24 min. The volume of distribution was estimated to be 32 ml at the lower dose and 26 ml at the higher dose. At steady state, the volume of distribution was slightly higher, being 57 ml at the low dose and 134 ml at the higher dose.

These data were not consistent with those expected after studying values reported in humans nor with the lipophilic nature of the compound nor with the pharmacokinetic data obtained for other retinoids in this study. These data suggest that a distribution phase was being observed and distribution equilibrium had not been reached.

This prolonged distribution phase presented problems in accurately measuring the low retinoid concentrations in plasma samples after 180 minutes post dose.

In order to determine the plasma concentrations of this retinoid over a prolonged time period in the rat, it would have been necessary to increase greatly the plasma concentrations. This could have been achieved by administering a much larger dose of retinoid to the animal (a dose of 20 mg/kg may have been required). However, it was thought to be inappropriate to exceed the volume of intravenous soyabean emulsion beyond the 0.6 ml dose size

(approximately 5.5 % of the total blood volume in a 250 g rat (Griffith & Campbell, 1934). It was found impossible to administer a higher dose of retinoid in other physiologically acceptable vehicles because of the limited solubility of Ro 10-9359 in these solvents. The proposed high dose would have also greatly increased the potential toxicity of the drug to the animal and would have far exceeded the upper therapeutic limit for a human (2 mg/kg). Alternative procedures would have been to use much greater blood sample volumes at each data point, extracting the whole animal blood volume (10 - 12 ml) for each data point, however, this procedure was considered an unacceptable use of animals. Alternatively, the dose could have been administered over an extended time period by intravenous infusion or a more sensitive analytical procedure could have been investigated.

5.3.2. Oral dosing studies in the rat.

The data presented here on the oral dosing of retinoids in the rat, suggest that the processes involved in the lymphatic absorption of a retinoid are very complex. There appear to be many interacting effects of oral dosing vehicle, retinoid solubility in the vehicle, retinoid lipophilicity, lipid content of the lymph and lymph flow rate. In summary;

i) The more lipophilic the retinoid as defined by

the logarithm of the octanol water partition coefficient (log. P), the higher the proportion of the dose which is absorbed via the lymphatic route, (Ro 15-0778 > Ro 10-9359 > Ro 04-3780).

ii) The solubility of a retinoid in an oil shows an inverse relationship with the lymphatic uptake from the oil. This advantage of poorly solvating vehicles for the absorption of drugs is limited, however, by the total amount of drug which the vehicle can incorporate since this will control drug availability at the site of absorption.

iii) Certain oils appear to enhance the lymphatic uptake of a retinoid. However, it is difficult to predict which oils which will promote the greatest lymphatic uptake of a retinoid, since this depends upon both the lipophilicity of the retinoid and its solubility in the oil.

iv) Oils may have a positive or a negative effect on the fasting lymph flow rate in the rat. For example the mean lymph flow rate after an oral dose of saline (0.5 ml) was 1.61 ml/h compared with a flow rate of 3.31 ml/h after dosing Ro 04-3780 in soyabean oil (0.5 ml) and only 0.80 ml/h after dosing Ro 04-3780 in linoleic acid.

The lymph flow rate correlate strongly with the lymphatic uptake of a retinoid ($p < 0.001$). Oils which gave the highest lymph flow rate also gave the highest lymphatic uptake, whereas those which give

the lowest flow rates gave the lowest lymphatic uptake.

v) Lymph turbidity (lymph chylomicron concentration) was increased after rats were orally dosed with oils compared with rats dosed with saline. The long chain tri-acyl glycerol oils, were most effective in this role, increasing turbidity by up to 48 fold, whereas oils such as linoleic acid and Miglyol 812 increased turbidity by less than 10 fold. This increase in turbidity was strongly correlated ($p < 0.001$) with an increase in lymphatic uptake after the dosing of retinoid in these oils.

vi) Food was not found to affect the lymph flow rate ($p > 0.05$), the lymph turbidity ($p > 0.05$) or the lymphatic uptake of Ro 10-9359. However, the estimated plasma uptake rate after food was decreased, resulting in an increase in the lymph : plasma uptake ratio. These effects result in selective targeting of the retinoid *via* the lymphatics after food.

vii) Administration of a retinoid (Ro 15-0778) in a self-emulsifying oil system (MTS), increased the lymph turbidity ($p < 0.01$) compared with dosing the retinoid in Miglyol 812 alone, however, the lymph flow rates, (0.95 ml/h after MTS, 0.97 ml/h after Miglyol) were not significantly affected ($p > 0.05$). The lymphatic uptake and estimated plasma uptake of Ro 15-0778 from the self-emulsifying oil system,

were almost three times greater than after dosing in Miglyol 812.

This increase in overall bioavailability of the retinoids after oral dosing in a self emulsifying oil system indicates a potential advantage of such systems in the absorption of lipophilic drugs.

viii) The fasted state of the animal plays a role in enhancing the relative amounts of retinoid which enters the body via both the lymphatic and portal venous routes of absorption.

The results show that feeding a rat, 30 minutes prior to dosing with the retinoid (Ro 10-9359), has little or no effect on the lymphatic concentrations of Ro 10-9359. However, there is a marked decrease in the plasma concentrations of retinoid observed compared with the fasted animal. These changes in plasma retinoid concentrations after feeding give rise to a ten fold increase in the lymph : plasma uptake ratio, compared with the fasted animal.

The data given here, although presenting a very complex picture, suggest many possible ways in which to promote or enhance the lymphatic absorption of the poorly absorbed retinoids. The most valuable results, which enable a prediction of the vehicles which will give the highest lymphatic uptake of a given compound, are the solubilities of the retinoids in the oily vehicles. There exists a definite inverse relationship between solubility of

a retinoid (Table 4) and its lymphatic uptake (Table 24, Table 25 and Table 26). The retinoids were orally dosed in a fixed volume of oil (0.5 ml) and it may be postulated therefore, that either an increase in the percentage retinoid saturation in the oil or a decrease in the solubility of the retinoid in the oil may be important in increasing lymphatic uptake. However, it has been demonstrated in these studies that when Ro 04-3780 was presented in linoleic acid (0.5 ml) at an oral dose of 75 mg/kg (percentage saturation, 93%), the dose adjusted lymphatic uptake was 150 times smaller than when Ro 04-3780 was administered in cottonseed oil (0.5 ml) at an oral dose of 6 mg/kg (59 % saturation).

Interpretation of these data from the rat in relation to its significance in man must be made with caution however, since the volume of the oral dose for the oily vehicle used in these studies in the rat (2 ml/kg) would extrapolate to approximately 150 ml of oil in man.

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ERRATA

Page 21. Delete the sentence, "The lymphatic absorption of retinoid and lymph flow rate were slightly increased ($p > 0.05$) after food."

Page 38. The line which reads, "divided over 60 and 7 days respectively." should be replaced by "divided over 5 and 7 days respectively."

Page 85. Insert the word, "is" after "the polarity of the mobile phase"

Page 86. Delete the word "be" from the line which reads, "analysis of the retinoids had to be fulfil the following criteria;"

Page 113. Insert the word "been" after the line which reads, "retinoids since these groups have".

Page 145. The line which reads, "At the lower dose of 0.264 mg/kg," should be replaced by, "At the higher dose of 0.792 mg/kg,"

Page 173. The figure "1.38 mg/min" should be replaced by the figure "0.95 mg/min".

Page 175. Replace "the lymph : plasma uptake ratio was greater than 10%." with "the lymph : plasma uptake ratio was approximately 10%."

Page 185. The figure "(150 ng/h)" should be replaced by the figure "(1.1 ng/h)".

Table 24. The estimated plasma uptake for linoleic acid of "154 ng/h" should be replaced by "152 ng/h".