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The Pharmacokinetics of Vitamin A in Relation to Its Teratogenicity in Healthy Women

By

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ABSTRACT

FACULTY OF MEDICINE
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Doctor of Philosophy

THE PHARMACOKINETICS OF VITAMIN A IN RELATION TO ITS TERATOGENICITY IN HEALTHY WOMEN

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Introduction and Aims : Vitamin A is a micro-nutrient essential for normal growth and physiology. It is found in many food sources, pharmaceutical preparations as well as cosmetic products. However, it has been found that high concentrations of vitamin A metabolites can cause teratogenic malformations. The aim of this project was to investigate the pharmacokinetics of vitamin A and the formation of its teratogenic metabolites under different conditions.

Methods : The project involved four clinical studies, investigating the various aspects of vitamin A pharmacokinetics following its administration in the three sources most commonly available to the general public i.e. animal liver, vitamin A supplements and transdermal creams. Investigated were the influence of (1) posture and previous dosing, (2) food and dosage, and (3) multiple dosing on the absorption of vitamin A and the formation of its teratogenic metabolites. A fourth study investigated the effect of transdermal vitamin A absorption in healthy women of child-bearing age.

Results : Teratogenic concentrations were not found in any of the four studies performed. Posture, previous dosing and multiple dosing did not alter the absorption of vitamin A and the formation of its metabolites. However, the source of vitamin A ingestion and the effect of dosing in conjunction with food were found to be of significance. Vitamin A and its metabolites levels were found to be many folds higher after vitamin A supplement dosing compared to dosing as a liver meal. Also, vitamin A supplement dosing in conjunction with a meal gave significantly faster absorption compared to dosing on an empty stomach. Long-term, high dose transdermal application of vitamin A creams resulted in negligible systemic amounts of vitamin A and its metabolites. In all four studies, it was observed that there were large inter- and intra-individual variations.

Discussion and Conclusions : Posture, previous dosing and multiple dosing did not affect vitamin A pharmacokinetics and therefore could not account for the large inter- and intra-individual variations seen in this study and in literature. Liver consumption does not increase the risk of teratogenicity and can be a valuable source of essential nutrients for pregnant women. Application of transdermal vitamin A cosmetic or medical creams does not represent a risk of teratogenicity. Vitamin supplements can be beneficial to the general public when used at the Recommended Daily Allowances. However, most sources of vitamin A are freely available to the general public, either as food sources or as over-the-counter products. Hence, abuse is a possibility, especially in the case of vitamin supplements. Therefore, it is of great importance to inform the general public and to increase its awareness about the risks and benefits of vitamin A.

Table of Contents

FOREWORD

To Richard,

Here it is ..... finally, after so many years of hard work. It looks really wonderful and I am very proud of you.
I can remember the many days that you have worked throughout the nights, sometimes with the excuse that you wanted to see the FA Cup Final the next day... Well, I have watched and worried, but most of all I have admired your courage for walking such a long way and climbing such high mountains. I am also privileged to be able to share this road with you.

When I first met you, you were working very hard on your PhD, on yourself, on your life ... Now I think the search is nearly over. It has taken a long time and many sacrifices on the way, but nothing is lost, nothing was in vain .... , because :

All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost.

From the ashes a fire shall be woken,
A light from the shadows shall spring;
Renewed shall be blade that was broken,
The crownless again shall be king.

J.R.R. Tolkien 1966

I know the last bit is always the hardest, but I hope to be able to hold your hand and enjoy the best view on the top of the mountain !

Your loving wife

ACKNOWLEDGEMENTS

I would to express my thanks to the following individuals and organizations for all the support and assistance given over the years towards the completion of this thesis.

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Hoffman La Roche

This thesis is dedicated to the one I love

Loan

You have been there at the end,

giving freely everything you have.

Chapter 1

Introduction

Table of Contents

List of Tables

List of Figures

1.1 GENERAL INFORMATION

In recent years there has been a concerted focus of attention on the physiological and pharmacological properties of the micro nutrient vitamin A. Medically, vitamin A has been used unknownly since around 1500BC. It is recorded in an ancient Egyptian medical treatise (the Papyrus Ebers) that the condition of night blindness could be successfully treated with raw liver or liver extracts, which are prime sources of vitamin A (1). The condition of night blindness is called Xerophthalmia and most commonly affects young children, although during the recorded periods between the 18^{th} to 19^{th} centuries soldiers on military campaigns frequently suffered from this disease. During the American Civil war a prominent confederate surgeon is reported to have expressed his frustration at " this curious and obscure disease" affecting the confederate army (2). Xerophthalmia causes dryness and keratinization of the conjunctiva and cornea. The cornea can then soften, ulcerate, may perforate and becomes infected. This can lead to the destruction of the complete eye (3). Vitamin A was first identified in 1914 as the essential fat-soluble factor in certain foods essential for the growth of test animals (4;5). It was called "fat-soluble A" to differentiate it from the essential water soluble factors which were called "water soluble B". Research rapidly demonstrated a link between fat-soluble A and night blindness. In 1914 vitamin A was used successfully to treat xerophthalmia in soldiers of the first world war trenches (6). In 1925 vitamin A was linked to epithelial changes in relation to dyskeratotic skin conditions (7). It has also been linked to disorders such as Darier's diseases and pityriasis (8). It was suggested that the theoretical link

between these complaints was a deficiency of vitamin A. This resulted in a major change in the understanding of the actions of vitamin A (4;9). By the early 1930's research into vitamin A had progressed to a stage where a structure was determined for ß-carotene and for retinol (Figure 1.1) (10-12). As research progressed Vitamin A activity was found to be the result of several structurally similar compounds and ß-carotene was recognized as a natural precursor for vitamin A activity. By the 1960's vitamin A had become a generic term used to describe the series of biologically active compounds all structurally related to the originally elucidated molecule, now known as all-trans-retinol. In mammals, the biological activity is related to three major compounds as well as their metabolites. These compounds were identified as being all-trans-retinoic acid, all-trans- retinal and all-trans- retinol (Figure 1.1).

The wide range of biological activity expressed by vitamin A led to the investigation of these compounds as pharmacological agents. In 1969 all-trans-retinoic acid given orally was demonstrated to be an effective cure for acne vulgaris. However, it proved to have significant side effects when used orally and eventually this form of treatment was suspended in favour of other regimens(13;14). Clinical effectiveness of topical applications were investigated and were shown as effective treatments of such medical conditions as acneiform eruptions, disorders of keratinization and neoplasias. Vitamin A has also shown to have beneficial effects cosmetically in the reversal of skin ageing and the revitalization of hair follicles (15;16).

In the second half of the twentieth century the World Health Organization released data indicating that the deficiency of vitamin A was a wide-spread problem in developing countries (17). The World Health Organization took long term

measurements to combat this problem by means of dietary supplementation and education on healthy eating (18). The success of this project has lead to a world commitment to eliminate vitamin A deficiency related illnesses as a major public health problem by the year 2000 (18).

Changes in public awareness in regards to health occurred in the developed countries at a similar time. Over the past twenty years health foods and micronutrient supplementation have become a major business in both the human and animal markets. This has lead to instances of hypervitaminosis A in women taking oral medication or supplementation. Teratogenic side effects of vitamin A are well proven and covered in more detail in section 1.6.2. Therefore, excessive intake of vitamin A during pregnancy was discouraged by the medical profession (19). Research has demonstrated repeatedly, however, that a certain amount of vitamin A is required for normal growth patterns (20). Hence, animal supplementation with vitamin A increased the growth of meat animals and improved quality of the end product. However, animal livers were found to have much greater levels of retinol activity than previously found in the early half of this century (21). This led to concerns being raised of possible teratogenic effects being found after consumption of liver and liver products on a routine basis during pregnancy.

Across the developed world focus has been placed on vitamin A in terms of its effect on many forms of cancer and leukemias (22). Synthetic vitamin A analogues have been investigated to give improved medicinal properties with less severe side effects. Research has also focused on the teratogenic side effects seen in animal studies and in certain human cases where malformations linked to a vitamin A source have

occurred. Vitamin A undergoes a complex metabolic fate within the mammalian body. Research into the transport, metabolism, receptor signaling and genetic differentiation of cells are currently giving much greater understanding of vitamin A's importance. Research has also found the cosmetic benefits of retinol and its analogues, making vitamin A products available in much greater quantities to the general population of the developed world.

1.2 NOMENCLATURE, CHEMICAL PROPERTIES AND NATURAL SOURCES

Whereas vitamin A is a term used to describe natural sources of retinol activity, the term retinoid is used to describe the class of compounds based on the structure of retinol. These compounds can be with or without biological activity and may be either natural or synthetic. In the structure of retinol (figure 1.1) the aliphatic side chain contains a series of conjugated double bonds. The IUPAC name for retinol is 3,7, Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl) 2,4,6,8- nonantetraen-1-ol. The configuration gives rise to geometric isomerism and several isomers other than the "all-trans" form can exist. It is also known that certain changes in the structure of retinol yield new retinoids without removing the biological activity. In figure 1.2 structures for 9-cis-retinoic acid, 11-cis-retinal and 13-cis-retinoic acid are shown. These are geometric isomers that are found in mammalian systems. Also shown are some second and third generation synthetic retinoids: etretinate, etretin, acritin and artinoid ethyl ester.

Retinol and its derivatives are all hydrophobic compounds which are unstable in the presence of oxygen and particularly in the presence of strong sources of light. Retinol and its derivatives are stable if kept under dry anaerobic conditions, at low temperatures and in the absence of light. The physical properties of retinol, retinoic acid and 13-cis-retinoic acid are listed in table 1.1.

Table 1.1: - Common physical properties of vitamin A and its major metabolites. Adapted from (23).

Retinoid	MW	Solubility in alcohol	Solubility in water	physical state
retinol	286.5	soluble	practically insoluble	yellow crystals
all-trans-retinoic acid	300.4	slightly soluble	insoluble	yellow to light orange
13-cis-retinoic acid	300.4	slightly soluble	insoluble	yellow to orange

Natural sources of vitamin A occur as either the ester of retinol or as a carotenoid. The predominant ester is usually retinyl palmitate although the oleate and the stearate forms are also found. The retinoid esters found in animals are derived originally from carotenoid sources . Vitamin A moves up the food chain in animal products such as milk, liver and liver products and in fish oils of various types. Retinol is stored in the liver of mammals as retinyl palmitate . Carotenoids occur widely in nature and consist of eight five carbon isoprenoid units joined in a head to tail manner: an example of a carotenoid is given in figure 1 (ß-carotene). Over 500 carotenoids have been identified in nature, but of these only fifty show evidence of vitamin A activity. To exhibit vitamin A activity a carotenoid has to undergo a cleavage reaction at the central carbon of the isoprenoid chain. One or both of the retinoid fragments must

have vitamin A activity. Carotenoids with this potential are called vitamin A precursors or pro-vitamins. The carotenoid with the greatest vitamin A activity is ß-carotene (figure 1.1) which is cleaved to give two molecules of retinol. The conversion of ß-carotene to retinaldehyde is carried out in the intestinal mucosa and in the liver. The enzyme responsible for this cleavage is a dioxygenase where molecular oxygen reacts with the central two carbons. Retinaldehyde is then either reduced to retinol by enzymatic action or oxidized to all-trans retinoic acid (figure 1.3).

Other carotenoids found in nature are fucoxcenthin (a characteristic pigment of many algae), lutein, violaxanthin and neoxanthin (the three main carotenoids in green leaves). ß-Carotene is produced in relatively small amounts in plant life. However, it can be found over a wide range of plants and is the main source of vitamin A in nature.

With so many potential sources of vitamin A activity, a nutritional measure was devised to express the variety of forms as a single measurement of activity. This unit is called the retinol equivalent (RE) where 1µg of all-trans-retinol is equal to 1 RE. Generally, sources of vitamin A activity can be summarized as follows:

1µg all-trans-retinol = 6µg of ß-carotene = 12µg of mixed dietary carotenoids

Another unit that is in general use is an international unit (IU). One international unit is defined as 0.3µg equivalents of all-trans retinol.

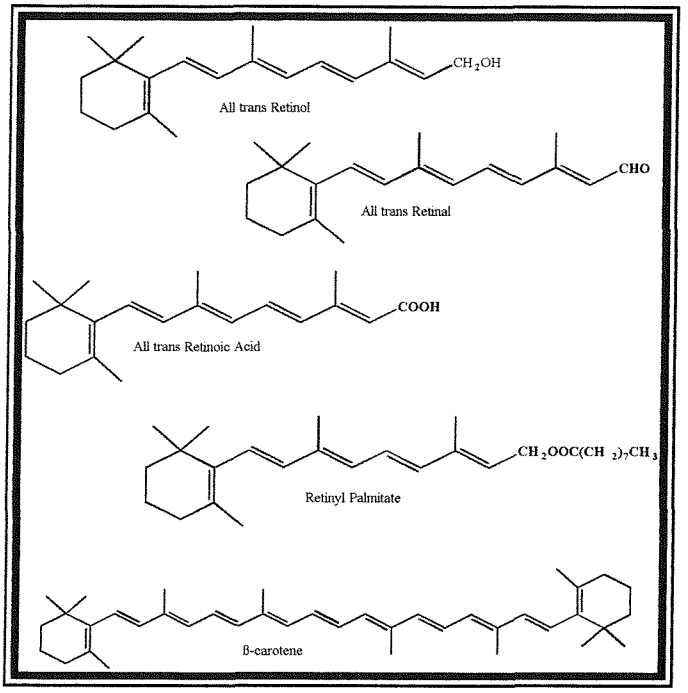

Figure 1.1 Structures of retinoids and carotenes found in nature in relation to vitamin A activity.

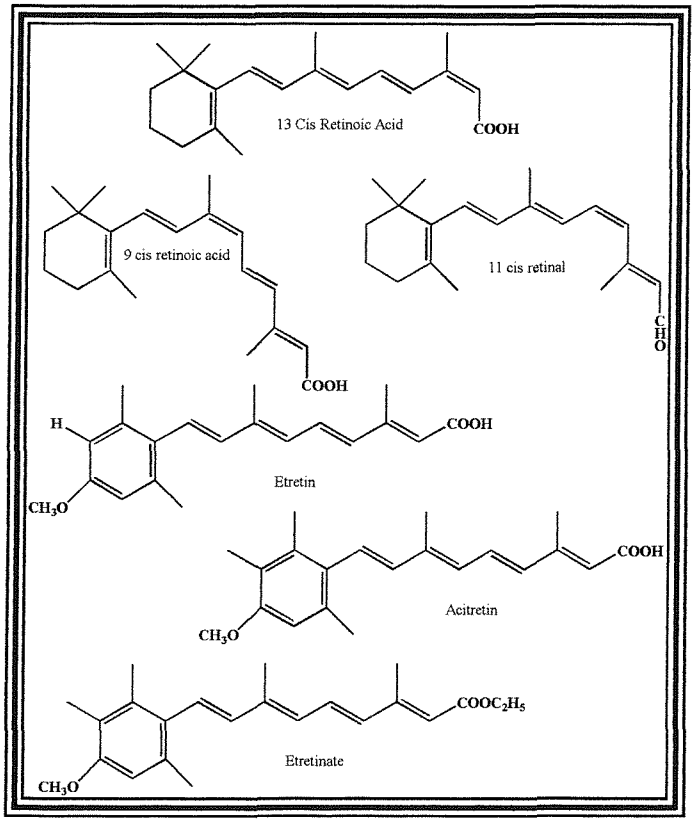

Figure 1.2: Some of the common metabolites of retinoic acid and 2nd - 3rd generation synthetic retinoids.

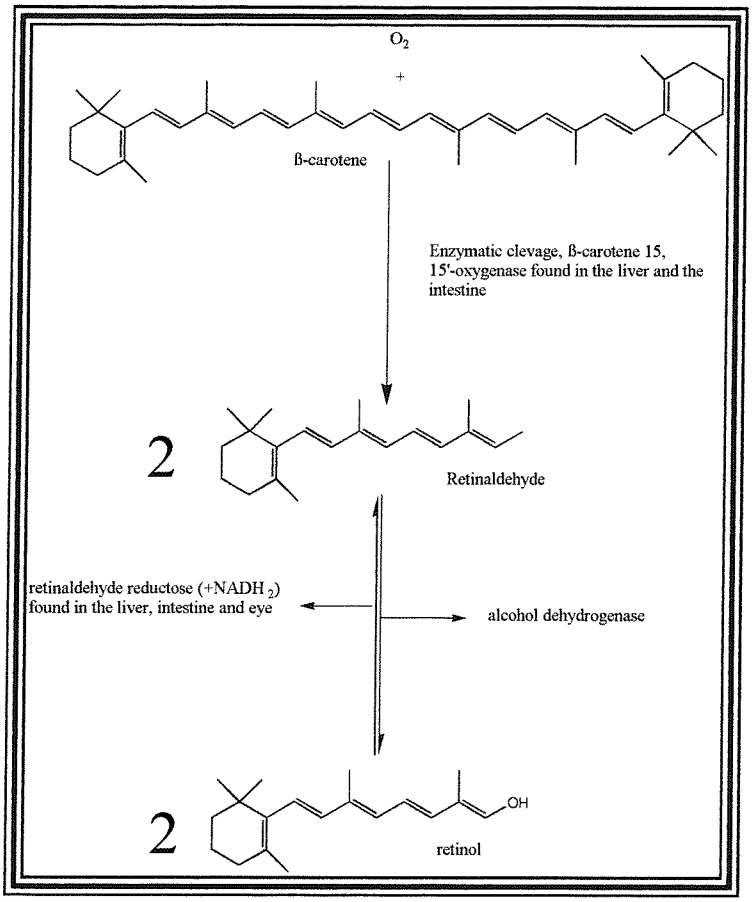

Figure 1.3 Cleavage reaction of ß-carotene. Adapted from (23).

1.3 ABSORPTION, METABOLISM AND TRANSPORT

1.3.1 Absorption

Animals must obtain all their vitamin A from dietary sources, either from other animals or from plant sources (24). Absorption of vitamin A is an important role in the understanding of vitamin A's effect on the physiological properties of the mammalian system. For dietary purposes retinol is obtained as either a carotene or

as long chain fatty acid esters (most notable retinyl palmitate) (25). Carotene comes in several different structural forms as mentioned above, but the most active is ß-carotene. Intestinal enzyme activity targets this molecule for absorption and cleavage (26).

Once the dietary forms of vitamin A are consumed with other fats, proteins and micro nutrients, the chemical and physical properties of the molecules dictate how they are absorbed into the body. Although absorption of retinol and ß-carotene takes place in the intestinal tract, the overall process is not efficient and much can be eliminated unprocessed in the feces. Once in the stomach, mechanical action and acidic conditions combine to cause the lipid soluble molecules to form a crude emulsification. This crude emulsification is made up of a triglyceride bilayer surrounding an oil droplet containing the lipid soluble fraction (27). This structure is very unstable and upon entry to the small intestine, environmental conditions change significantly, for example there is a large pH change (from 1.5 to 5.3). Also, the physical transfer through the pyloric channel causes strong shear forces which tear the liquid interfaces apart. During this action the fatty acid molecules diffuse to the surface of the triglyceride droplets and become partly ionized. In this state the fatty acids will mix with pancreatic secretions, bile salts, dietary phospholipids and amphiphilic peptic digests of dietary proteins. The end result is an increase of ionization at the surface of the lipid droplets and an increase in the stability of finer emulsified droplets. During all this action vitamin A is contained within these oil droplets as either a carotenoid or as an ester of retinol (25).

The carotenoids are thought to be absorbed directly from the fine oil droplets into the enterocytes via the brush border membranes (28). Evidence suggests that this is a passive diffusion process, although research has not been conducted at very low levels to investigate the possibility of a carrier mediated transfer (28). The retinyl esters have been shown to require hydrolysis before absorption can take place (29;30). Traditionally, it was believed that pancreatic enzymes and bile salts were responsible (31). Deoxycholate, a dihydroxy bile salt, has been shown to stimulate the hydrolysis of long chain fatty acids while taurocholate stimulated the hydrolysis of shorter chain fatty acyl esters and the inhibition of the hydrolysis of retinyl palmitate (31). Activity observed can not be explained solely by the hydrolysis reactions of pancreatic lipases or bile salts. Animal investigations have indicated an enzyme activity in the brush border membranes capable of hydrolysing retinyl esters (32). To determine the sources of the enzymatic activity, rat ligation of the common ducts was performed and activity was measured in the brush border membrane in comparison to sham operated animals. Such ligations demonstrated little decrease in brush border membrane activity for the hydrolysis of long chain retinyl esters, but almost complete inhibition of the shorter chain retinyl esters. Conclusions were drawn indicating that the enzyme hydrolysis of dietary retinyl esters occurs primarily in the brush border membrane (32). Activity rates demonstrate that the brush border membrane activity is capable of hydrolysing 25nmol of retinyl palmitate per minute in the rat intestine. Since the growing rat generally requires 20IU of vitamin A /day (~20nmol) the intrinsic activity of the brush border membrane is potentially capable of hydrolysing retinyl esters to retinol to supply the rats total daily requirements in under 2 mins with ideal

conditions. In summary, two sources of retinyl esterase activity are known, pancreatic esterases/bile salts and the activity discovered within the brush border membrane. The level of activity within the brush border membrane suggests a significant role within the absorption processes of dietary vitamin A (33).

Research showed that the fine emulsification formed in the small intestine deposits liquid vesicles and bile salt micelles in the lumen which transport the hydrolysis products to the enterocytes for absorption. Retinol has been determined to be absorbed from the intestinal lumen into the enterocytes by means of a specific carrier protein called Cellular Retinoid Binding Protein II (CRBP II) (34;35). However, at concentrations of retinol greater than 5μM a simple passive diffusion system has been observed to dominate. This indicates that the carrier mediated absorption is possibly a saturable process (33). Research has also demonstrated that retinol bound to a retinol binding protein in the gut has enhanced absorption rates. There is some suggestion that protein factors in the gut may enhance retinol absorption. However, more research is still required to investigate this area.

1.3.2 Transport From Gut To Systemic Circulation

Absorption of retinol and the carotenoids from the gut is but the first step in the fate of retinol within the human body. From the enterocytes the retinol molecules are transported to enzymatic sites where they are re-esterified (36). The enzyme system that accomplishes this is the lecithin:retinol acyl transferase (LRAT) (36). The transport mechanism is thought to consist of CRBP II, which contains an active

binding site with a high degree of specificity for retinol. The CRBP II transports the lipid soluble retinol through the aqueous intestinal fluid to the enzymatic system. It has been demonstrated *in vitro* that retinol bound to CRBP II is not enzymatically hydrolysed by enzymes within the intestinal system with the exception of LRAT (33). The mRNA activity for CRBP II has been found to exist at high levels within the specific tissue of the gut. However, an analogue of CRBP II has a mRNA activity in nearly all of the other major organs and tissues of the human body and is known as CRBP I, although mRNA levels are less than that found for CRBP II in the intestine (37).

Upon regeneration of the retinyl esters within the enterocyte (mainly the palmitate but also some stearate and some oleate) the molecule is incorporated within the forming chylomicrons (25). Chylomicrons are the structures formed out of the aggregation of triacylglycerol and phospholipids, packed together in a circular form. Incorporated within these lipid bilayers are the carotenoids, the retinyl esters and all the other fat soluble vitamins, cholesteryl esters and certain specific apolipoproteins. Chylomicron aggregates are many thousands of daltons in size. Hence, they leave the intestinal cells where they are formed by exocytosis. Exocytosis is the process by which a vesicle is formed within the cell on the membrane surface, contained within this vesicle are the large particles created within the cell. The vesicles then is sealed and detached from the cell membrane into the interstitial fluid. The vesicle membrane is dissolved and/or broken down to release the vesicle contents including the chylomicrons into the interstitial fluid. Chylomicrons cannot then be absorbed into the blood capillaries since they are too large. However, there is another transport

system and the chylomicrons are drained from the interstitial fluid into the lymphatic system (38).

The lymphatic system is a network of tubes much like the blood vessels within the body. The walls of this system have a cellular structure which permits the passage of very large particles between the cells within the tube wall. Transport in this system is essentially mono-directional. A series of valve structures and reflex contraction of the tubular walls cause the fluid to be circulated slowly around the body. Within the lymphatic system are strategic areas of the body (such as limb junctions) where filtration and immune response actions to toxic antigens take place. These areas are called lymph nodes. The chylomicrons are transported via the lymphatic system to the largest of the nodes at the junction of the thorax where the lymph is emptied into the blood system. Within the blood the chylomicrons have a half-life of approximately one hour (39). The fat content of each chylomicron is removed by either hydrolysis of the triglyceride components or through uptake and storage of fatty components by the fat cells around the body. The remains of the chylomicrons retain their structure but are smaller and are referred to as chylomicron remnants. At this stage the absorbed vitamins and cholesterol esters are still contained within the chylomicrons remnants. The remnants are mainly cleared by the liver (figure 1.4).

Another suggested role for the chylomicrons remnants is the transport of essential components (e.g retinol and carotenoids) to areas of intense cellular differentiation and/or proliferation (40). Tissues under investigation utilizing this delivery method are adipose tissue, skeletal muscle, kidney and bone marrow (28). Clearance within the liver is the main mechanism for removal of chylomicron remnants. Chylomicron

remnants are thought to complex with either LDL's (low density lipoproteins) or LDL receptors (figure 1.4). The relative role of either in the uptake of chylomicrons remnants by the liver is debatable and intense research is currently being undertaken in this field. However, it is known that retinyl esters are taken up by the liver, and stored within its cellular structure (24,28).

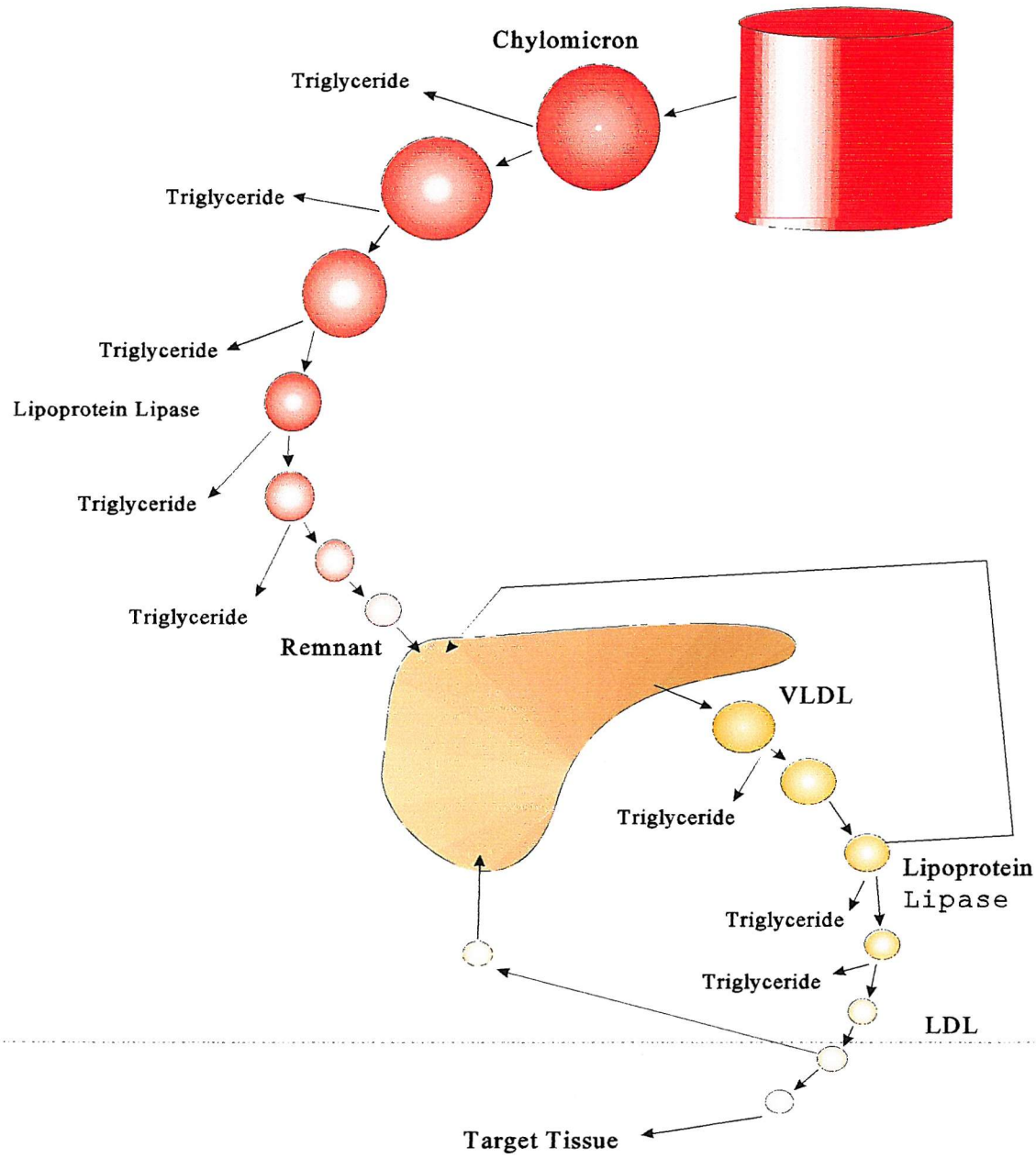

Figure 1.4 Metabolism of triglyceride-rich lipoproteins secreted by the gut and liver. Adapted from Weatherall 1999.

1.3.3 Storage And Homeostasis

Within the liver, retinyl esters are rapidly hydrolyzed within the hepatic cells (parenchymal cells specifically) (41). Several enzyme systems have been suggested to be involved in this hydrolysis. However, at this time it is unknown which are actually involved. Retinol once formed is rapidly transferred from the parenchymal

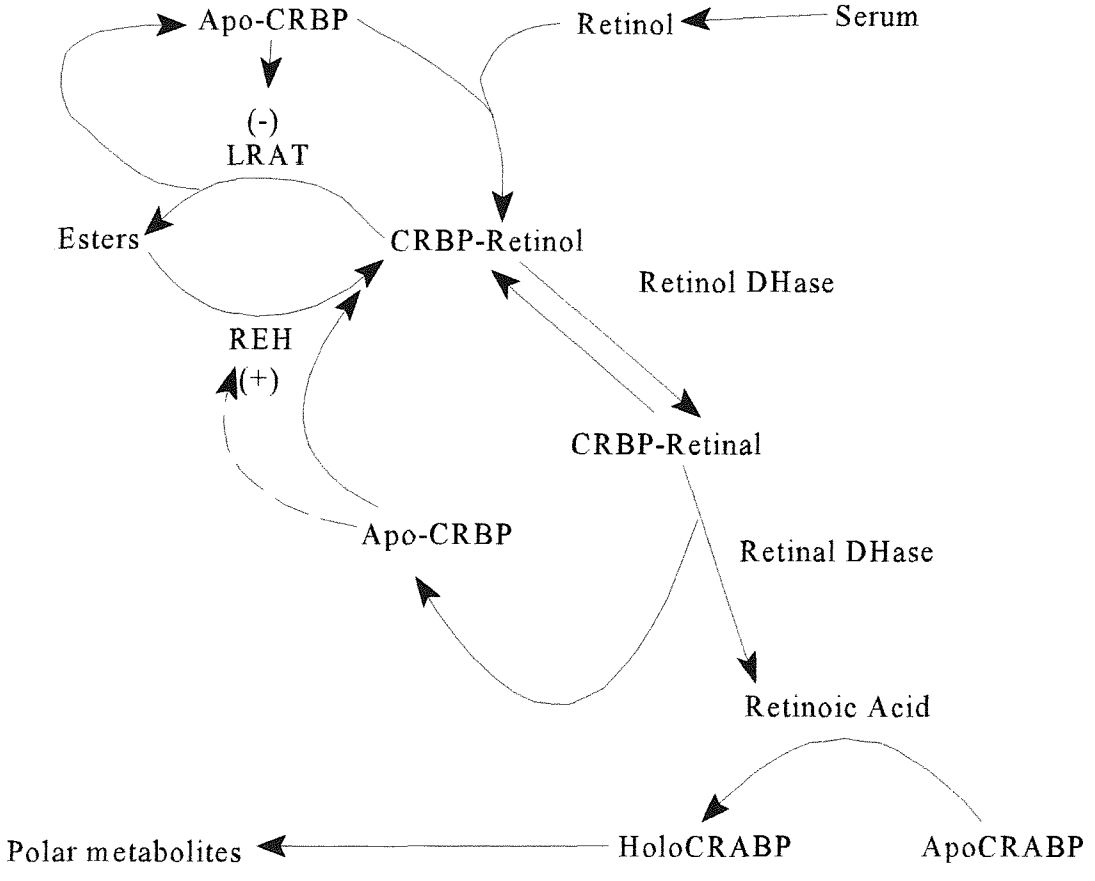

Figure 1.5 Model proposed for a pathway of retinoic acid biosynthesis and catabolism indicating the involvement of retinoid binding proteins. Extracted from NAPOLI1993.
Where Dhase - dehydrogenase; LRAT-lecithin-retinol acyl-transferase, REH- retinol ester hydrolysis

cells to the stellate cells where it is stored as the retinyl ester (28). These stores take the form of lipid droplets which also contain triacylglycerol and minor amounts of cholesteryl esters, free fatty acids, phospholipids and some unesterified retinol or cholesterol. Of the retinol within the human body 50-80% is contained within the liver, of that 90-95% is found in the stellate cells as retinyl esters (42). The remaining retinol is mainly bound to CRBP I (cellular retinol binding protein I) within the cells, bound to retinol binding protein in the blood plasma, and as unbound retinyl esters in the blood plasma. Concentrations of total retinol within the blood plasma do not change generally during the day from a normal baseline level, typically between 400-800 µg/l, although there are wide individual variations (43). Individual vitamin A status is related to the size of the liver stores. However, this measurement cannot usually be made due to the invasive nature of the techniques needed. Hence, for a normal individual the exact status of his/her vitamin A levels is unknown except when the liver stores are depleted to such a extent that plasma retinol levels drop to 300 µg/l or lower. At these levels signs of vitamin A deficiency would be apparent making retinol plasma levels unreliable as an indicator for vitamin A status. In cases of hypervitaminosis the retinol plasma levels fluctuate only mildly whereas liver stores may increase dramatically to exceed capacity. With hypervitaminosis plasma retinyl ester levels can increase up to 10-100 times their normal levels subsequent to the plasma RBP being saturated.

Retinol is released from the liver stores bound to RBP referred to as holo-RBP. Holo-RBP has been demonstrated as modulating the formation of retinyl esters while apo-RBP, the free form of the retinol binding protein, has been shown to stimulate retinyl

ester hydrolysis and inhibit retinol esterification. Ratio of holo-RBP to apo-RBP directs the retinol either into retinyl esters or to more polar metabolites. Action by holo-RBP releases apo-RBP and visa versa, hence the ratio observed is a continuous flux with relation to incoming retinol from the absorption sites (44). While the exact process of vitamin A homeostasis is not completely understood it can be reasoned from current research that RBP and CRBP I & II, and enzymatic systems such as LRAT and ARAT, as well as retinyl ester hydrolases are all interlinked within a system of balance and control (Figure 1.5). In a comparison of the amount of retinol absorbed naturally from the diet to the theoretical amount of retinol used within the body per day, a discrepancy is observed. It is found that the rate of "use" of retinol is greater than the rate at which it is absorbed. Theoretically, the retinol stores should be depleted to deficient levels over a period of time. However, this is not the case. Logically, there has to be a balance in the retinol system where by the retinol is recycled. It has been calculated that each individual retinol molecule should be recycled at least 7-13 times before it is irreversibly metabolized and eliminated (45). Therefore, organs that express mRNA activity for RBP must be considered as part of a system of checks and balances within the body. When comparing mRNA activity within different organs, the liver and the kidney exhibit the largest values. Hence it can be concluded that both the liver and the kidney play an important role in the homeostasis of retinol (45-48).

1.3.4 Transport

The stellate cells within the liver provide retinol to the blood plasma sufficiently to maintain the retinol levels. However, within the aqueous medium of plasma the retinol is vulnerable to metabolism by circulating enzymes. Retinol is protected from this fate by binding to the specific transport protein RBP and the resulting complex forms another 1:1 complex with circulating transthyretin. The transthyretin-Retinol-RBP complex helps preventing the globular filtration and the eventual metabolism of retinol by the kidney for excretion. Transthyretin is a normal non-glycosylated plasma protein, with a relative molecular mass of 54 980 (3). Of the circulating retinol 95% is as the RPB-transthyretin complex, about 5% as the RBP complex and a small amount as free retinol.

The mechanism for movement of retinol across cell membranes at the site of action is not clearly understood, although the amount transferred exceeds the known diffusion properties of retinol (49). It is therefore suggested that RPB-retinol complex is involved in this transfer, although the method is currently unknown. It is postulated that retinol uptake is mediated by RBP and a specific RBP receptor, although current evidence for the RBP receptor is contradictory (50).

1.3.5 Metabolism

Retinol is transferred between the blood plasma and the cellular binding proteins by a process that is not yet clearly understood. However, once in the cell, retinol is used

for many different processes some of which lead to irreversible metabolism to retinoic acid. Like retinol, the amounts of retinoic acid are controlled by a homeostatic mechanism, and it is believed that retinoic acid inhibits its own production (51). Retinoic acid is an important metabolite of retinol which has a key role in the regulation of cellular differentiation, as well as regulating the transcription of the genes that dictate the final nature of the cell. The postulated cellular metabolism of retinol and retinoic acid is shown graphically in figure 1.6.

The metabolic pathways shown in figure 1.6 demonstrate the interrelationship between all-trans-retinoic acid, 13-cis-retinoic acid and 9-cis-retinoic acid, all three of which have been shown to have teratogenic effects to some extent (52). Hence, excess vitamin A intake through abuse of supplements or the application of retinoid treatments could cause the saturation of the natural homeostatic controls for long enough to generate levels of teratogenic metabolites in sufficient quantities to induce malformations in fetuses.

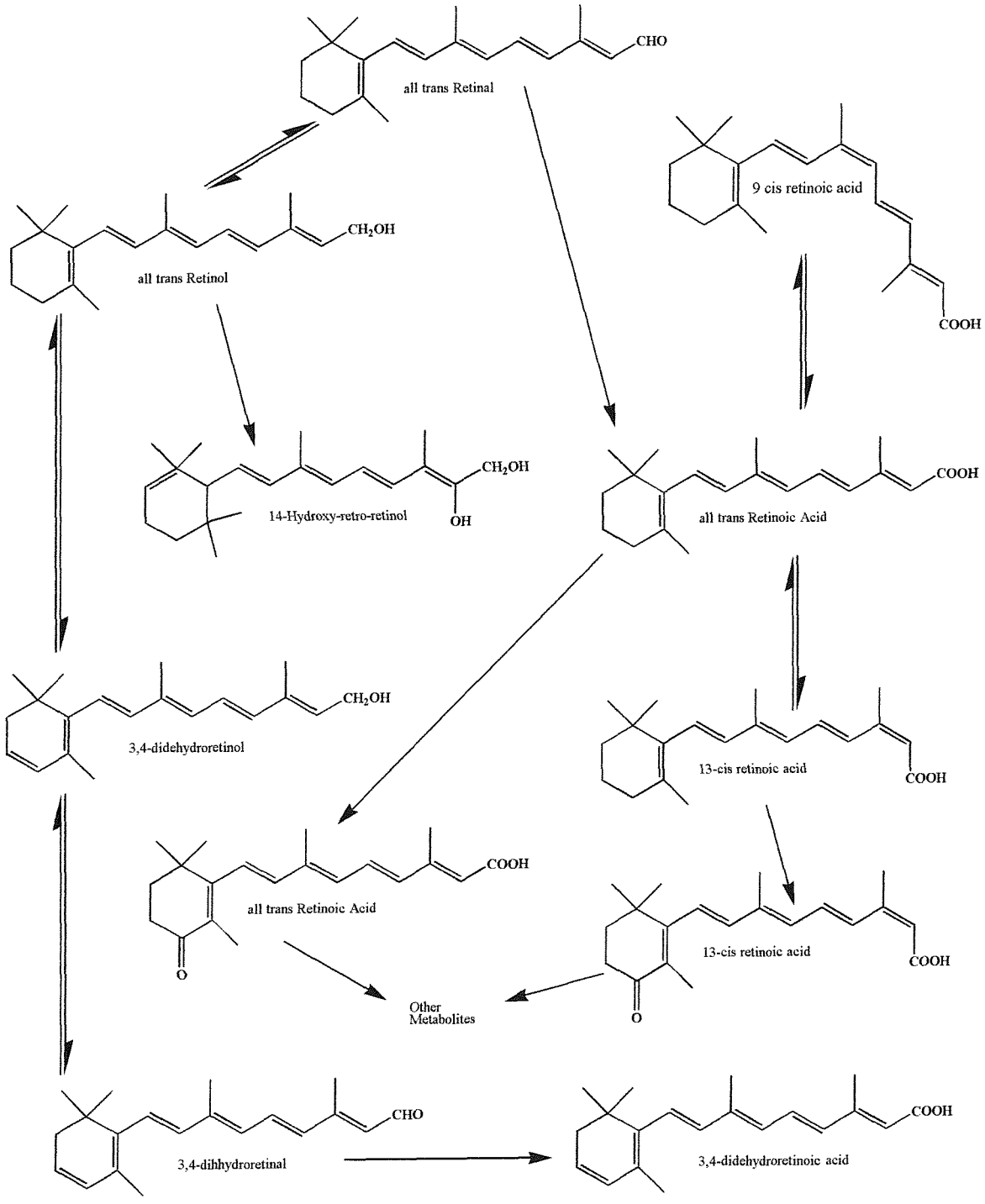

Figure 1.6 Metabolic pathway for retinol based on data published prior to 1996, adapted from (53;54)

1.4 VITAMIN A ACTIVITY

1.4.1 Vitamin A And The Eye

Retinol activity has an essential role in the function of the retina. 11-cis-retinoids are generated photochemically within the rods of the retina. These rods contain the visual pigment rhodopsin which is made up of the apoprotein ospin linked to 11-cis-retinal via a covalent shift's base linkage. The visual process then occurs by the isomerisation of the 11-cis-retinal component to all-trans-retinal via several intermediates. The complete process is performed in under 200 femtoseconds and one of the intermediates reacts with the retinal G protein transductin which transmits the information that the pigment has changed. The all-trans-retinal is then reduced by specific nicotinamide- linked retinol dehydrogenases to produce vitamin A. Regeneration of the 11-cis-retinal is carried out by phospholipid interaction with all-trans-retinol to give all-trans-retinyl esters. Further reactions converts the all-trans retinyl esters to 11-cis-retinol, the precursor for 11-cis-retinal. The complete process is still poorly understood and research continues to investigate this key biochemical cycle (55).

1.4.2 Vitamin A And Growth

Vitamin A has been linked in the growth and differentiation of epithelial tissues, in the growth of bone structures, in reproductive processes and in embryonic

development. The processes by which vitamin A affected these growth changes was not well understood prior to 1987. Theories were developed in the late 1970's and early 1980's that retinoid metabolites function as transcriptional control factors in a similar fashion to steroid hormones. Steroid hormones act via a large superfamily of nuclear receptor proteins with highly conserved structures.

In 1987 the receptor for retinoic acid was isolated independently from two laboratories (56;57). It was found to be structurally and functionally similar to the superfamily of hormone receptor proteins (58). It was also found that the receptor was activated by all-trans retinoic acid at levels consistent with *in vivo* experimental concentrations (54;59). Intensive research continued on the retinoid receptor pathways and three distinct isoforms of the receptor (α, β and γ) have been isolated for several different species (54). Retinoids are known to have a broad range in biological activity, in areas such as growth and epithelia differentiation (60), embryonic development (61-63) and spermatogenesis (64). The wide range of effects observed for the retinoid family of compounds can therefore be explained to a certain degree by the diversity of the retinoid receptor. The existence of multiple forms of the retinoid receptor raised various questions about the existence of additional receptor pathways (54). Intensive research identified an orphan receptor (these are receptors where the ligand is currently unknown) structurally different from the retinoic acid receptor (RAR), that altered transcription in the presence of all-trans-retinoic acid (65). Interestingly, the new receptor, RXR, was found to have three isoforms as well (α,β and γ) and were found not to bind all-trans-retinoic acid *in vitro*. Experimental evidence demonstrated *in vivo* that all-trans-retinoic acid expressed RXR. It was

postulated that a metabolite of all-trans-retinoic acid was actually involved. This proved to be the case when the isomer 9-cis-retinoic acid was discovered to be the ligand for RXR's (66). Further discoveries showed that 9-cis-retinoic acid also bound to RAR's as well as RXR's where as all-trans-retinoic acid only bound to RAR's. This greatly changed theories on the retinoid signalling pathway which had been suggested until then. The updated version is shown in figure 1.7 (54).

The mechanism of action for the proposed signalling pathway involves the interaction of 9-cis-retinoic acid and all-trans-retinoic acid with receptor elements. A variety of

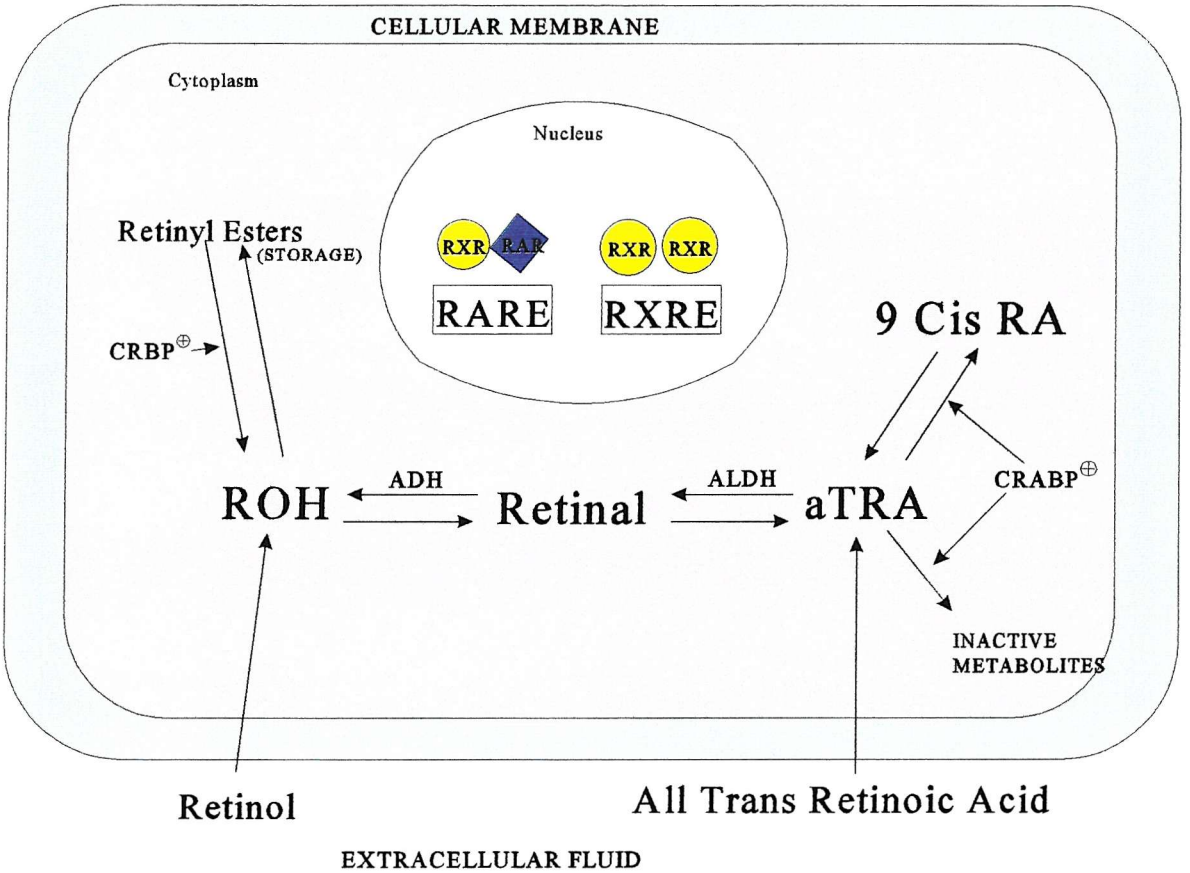

Figure 1.7: Postulated retinoid signaling pathway adapted from Mangelsdorf1994

complexes have been discovered in relation to the expression of retinol and retinoic acid in several cell lines. Complexes formed were identified as mainly RAR-RXR and

RXR-RXR heterodimers (67). Other dimers of RXR also have been discovered with response elements such as vitamin D receptors (68), thyroid receptors and several orphan receptors (69). Many and possibly all of the actions of retinoic acid occur via the receptor pathway at the gene transcription level. This is either by a change in the transcription rate or by a change in the stability of the associated mRNA responses (70). Via this mechanism retinoids of various types can exert a considerable influence on the activity and synthesis of growth factors. Research has demonstrated many areas of cellular activity in which retinoids play a vital role indicating a level of complexity not seen for any other micro nutrient or hormone. From the detailed involvement of vitamin A or retinoids in many stages of gene transcription, cell differentiation and cell growth, it can be seen that either a deficiency or a sudden excess could have a detrimental effect on growing systems.

1.4.3 Vitamin A And The Skin

The surface area of the skin in the average human adult is approximately 1.5-2.0 m^2. This makes the skin the largest organ of the body. Structurally, the skin is a surface layer consisting of several levels of different cell types. The basic structure is as shown in figure 1.8. During normal daily life the average human skin is exposed to a wide variety of external events. It is continuously under threat from external infections and from environmental pollutants. The role of vitamin A in the skin layer covers immunological responses, regrowth and repair of the cellular surface and the maintenance of a healthy skin. Analysis of cadaver skin samples has shown that

retinoic acid and its receptors can be found in various of the different cell types that make up the skin layer. Detectable levels of retinyl palmitate, retinol and 3,5-dihydroretinol are also present in the skin. Application of retinol, retinoic acid and retinyl palmitate to the skin surface has shown beneficial effects to the health and regrowth of the skin. This research has lead to the increase marketing of cosmetic products containing high levels of retinol. Such skin creams provide cosmetic effects such as the smoothing of wrinkles (photo-damaged skin) and the regrowth of dead, dry skin.

Historically, retinoids have been shown to be successful in the treatment of a wide variety of skin disorders (71). Mechanisms of action for retinoids have been investigated intensively. However, the exact nature of vitamin A's involvement in the maintenance of skin integrity is still not fully understood. It is postulated that retinoids act via retinoic acid receptors to regulate the action of a specific enzyme known as cytochrome P450 for protection of the skin barrier, and to stimulate the growth of epithelial tissues (72;73). Cytochrome P450 is a generalised name used to describe an oxidative catalytic enzyme that is known to exist in the epidermis and pilosebaceous areas of the skin (74). Of the many known variants (36+), 12 have been located within the human body and CYP1A1 is the variant expressed within the skin layer (75;76). Expression of P450 and retinoic acid receptors is very low within the separate skin layers and detection limits the degree of research able to be conducted (77). Current research focuses mainly on the development of synthetic retinoids for specific targeting of receptors in an effort to improve therapeutic value (71). Retinoid absorption across the skin barrier has been investigated. However, contradictory

evidence has been published concerning toxic (potentially teratogenic) quantities of vitamin A reaching circulation after applications of high levels of retinoids to the skin (78-80). Therefore, there was the concern of increasing systemic circulatory retinoid levels from cosmetic preparation. Anti-wrinkle creams for the repair of photo-damaged skin are marketed at levels that would be extremely toxic if absorbed into the systemic circulation. High metabolite content creams are used routinely for the treatment of skin disorders in preference to the oral dosing known to induce toxic and teratogenic side effects (81;82).

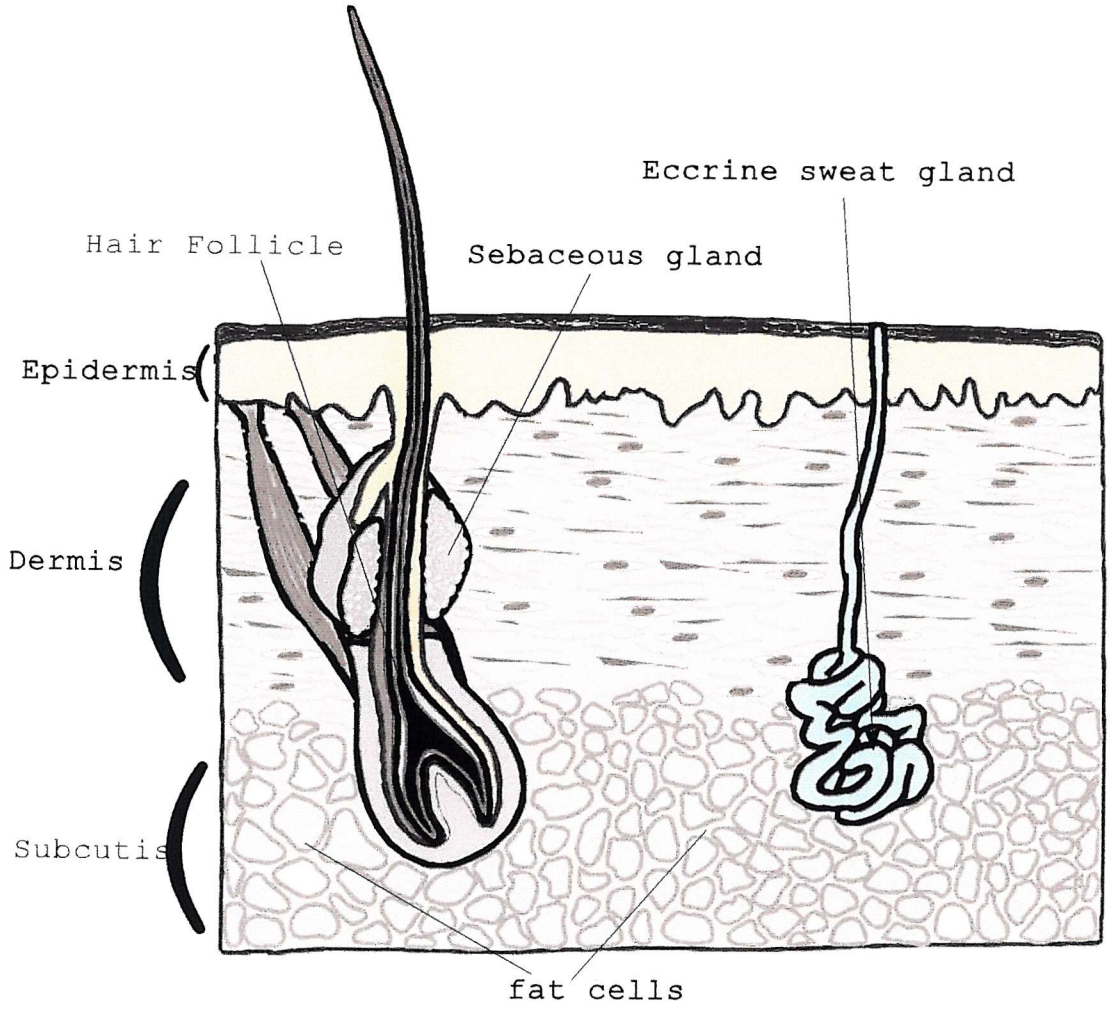

Figure 1.8 Simplistic structural diagram of a cross section of mammalian skin.

1.3.4 Vitamin A And The Function Of The Immune System

Within the human body certain functions can be categorised as being essential for the maintenance of optimal conditions. To protect these functions mechanisms exist whereby factors that disrupt the optimal condition can be eliminated, controlled or manipulated to negate the disruptive effect (83). The complete process by which the body performs this function is complex and involves many different organs and tissues within the body. The process is generalised under the title " immune system" and classified into two areas.

A/ The Hematolymphopoiesis system: - the formation and development of red blood cells and leukocytes. The normal condition requires the generation of red blood cells and leukocytes in large quantities daily. Production is limited only by the availability of healthy bone marrow and vitamin A. Within this system the all-trans-retinoic acid and 9-cis-retinoic acid signalling pathway (figure 1.6) is believed to control the proliferation and differentiation of the forming cells. The exact mechanism is currently unknown and accumulated evidence is not complete for the involvement of vitamin A metabolites in all cell types produced in the bone marrow. Techniques for determining the exact roles of vitamin A within this system are prone to interference and the effects of unknown combinations of different factors. Detailed research is currently on going in this field, but a concise theory has yet to be produced (84).

B/ the immune system:- consisting of all antibody production for humoral and cell-mediated immune responses. Evidence for the involvement of vitamin A in the function of the immune system has been derived from human and animal data in states

of deficiency. Studies have reported a decrease in the immune function in relation to the deficiency of vitamin A prior to more evident effects being observed. It has been demonstrated that vitamin A supplementation within deficient systems has restored immune function. However, it has also been observed that the immune response decreases in certain cases of hypervitaminosis. The conclusions drawn from this experimental evidence is that vitamin A and/or its metabolites play an important controlling role within the immune system. The exact nature of this role is complex and elucidation of details is proving to be subject to interference and contradictory evidence. It has been suggested that two key points indicated the role of vitamin A in the immune system. The first is the suggestion that at a critical cutoff point in vitamin A deficiency (low concentrations of circulatory retinol) the immune system ceases to function. At levels of vitamin A above the normal concentrations, the production of toxic levels of metabolites decreases the effectiveness of the immune system. The second point is that the concentration of retinol in plasma does not reflect the vitamin A status of the individual. The reason being that the depletion of the localised tissue stores can affect the accurate determination of status. Hence, although the liver store and plasma retinol indicate adequate vitamin A levels, the immune system could remain depressed. Tissue stores will regenerate within time but the process adds to the complexity (84). Research efforts continue to try to elucidate the mechanism for the involvement of vitamin A in the immune system. Some research focuses on theoretical mechanisms while the bulk on the medical uses of vitamin A in disorders of the immune system. Research to date has produced some controversial and conflicting results for a variety of cell lines.

1.4.5 Vitamin A And Infectious Or Malignant Diseases

Cancer in all its many forms is one of the major diseases of the twentieth century. Cures are continuously sought after. However, very few are known, most of which are highly invasive techniques involving drastic surgery or high doses of radiation and/or toxic chemical combinations. There is an increasing amount of evidence that consuming fruits and vegetables high in β carotene causes a decrease in the incidences of skin carcinoma. *In vitro* experimentation has consistently demonstrated that retinoic acid, retinol and even retinyl palmitate can reverse the cancerous growth of certain cell lines (85). Contradictory studies conducted in humans, however, have suggested a carcinogenic effect for β carotene. These studies conducted among male smokers have indicated a highly significant increase in malignancies for the study volunteers, to such a degree that two five year studies were abandoned after the first results were analysed (86).

In vitro research has repeatedly demonstrated an ability of vitamin A to revert cancerous cells to normal growth patterns. Based on these research data, it is believed that vitamin A can be of immense value in chemotherapy treatments. However, for unknown reasons, this research has not led to successful cures for cancer. Clinical testing of retinol, retinoic acid and several other retinoids in cancer patients has shown repeatedly a lack of the desired effect (87;88). Only in the case of acute promyelocytic leukemia has vitamin A shown an ability to reverse the malignant process. However, this is usually only a temporary remission (89). Clinical trials are currently being conducted in cancer areas such as skin cancer, head and neck cancer,

breast cancer and cervical cancer (90). Investigation are also being conducted with reference to AIDS and other disorders of the immune system (91).

1.5 HARMFUL EFFECTS OF VITAMIN A

1.5.1 Toxicity And Hypervitaminosis

The essential roles of vitamin A in so many stages in gene transcription, cell differentiation and cell growth implies that an excess or deficiency could have a detrimental effect on the organism. The storage, transport and metabolism processes function as a mechanism to control the levels of vitamin A within the body. However, in cases where the natural stores are severely depleted or in cases where the control mechanisms are substantially overloaded then growth defects, skin defects and other physical disorders can occur.

In cases of vitamin A deficiency the clinical signs are primarily xerophthalmia, but there is also an increase in morbidity and mortality. When taken in excess, vitamin A can cause three categories of hypervitaminosis; chronic, acute and teratogenic. Table 1.2 shows the dose/frequency and symptoms seen at each of the three levels of toxicity. Research has focussed on the teratogenic effects of vitamin A because of the profound adversity of malformations within newborn children. The benefits of vitamin A supplementation in a vitamin A deficient population is a proven fact. However, in a population with a well-balanced diet, the supplementation of micro nutrient compounds could be an unnecessary risk. Politically, however, people have the legal

right to consume any product freely available. Hence, research is aimed at providing data on teratogenic risks and recommendations to maintain healthy vitamin A status.

1.5.2 Teratogenicity

An embryo develops along a series of set patterns from the initial fertilization to the final new individual. This complex process involves cell proliferation, differentiation, migration and organogenesis. It has been proposed that cellular development is under the control of a series of molecules called "morphogens". A morphogen is a chemical which dictates at a genetic transcription level the structure into which the cell will develop. Research suggests that the retinol family are one of several molecules classified as morphogens (92). It is reasonable, therefore, to assume that situations of either excess or deficiency of retinol will give the possibility that cellular growth and/or differentiation could occur incorrectly or not at all (93).

Developing embryos are supplied with maternal retinol in the form of the transthyretin-RBP-retinol complex. Functionally active forms of vitamin A (such as all-trans-retinoic acid) are not supplied to the embryo (94). All active metabolites are generated within the embryonic cells. The supply of maternal retinol is closely controlled under normal conditions by the homeostasis functions described above. Hence, large fluctuations in the vitamin A status of the mother are not generally reflected within the embryo. Situations where low levels of vitamin A within the mother are reflected within the embryo are very rare, but it is known that such circumstances will lead to the development of abnormalities.

In hypervitaminosis the situation is reversed, here the retinol /retinyl ester homeostasis system can be temporarily saturated. In such conditions the embryo can be exposed to levels of retinol and its metabolites in excess of the daily requirements (93). Abnormalities may then be observed. Within different species the development of the embryo to fetus to individual follows similar patterns but on widely different time scales. Within humans the critical areas of gestation seem to be during the third to the twelfth week.

Table 1.3 shows a graphical representation of the development of the embryo in terms of the organ development and its response to teratogens. From this diagram it could be suggested that there would be an increased risk to the Central Nervous System (CNS) early in gestation (3-5 weeks) but little risk to the genitalia until the 7- 12th week. This variability in the possible malformation produced can have the effect of making retinoid teratogenicity difficult to identify and compare between different cases (95).

Another potential problem is that all of the cases observed to date have been identified retrospectively. Hence, the relationship between dose, time and duration of exposure, and resulting abnormalities (given inter-individual variations in vitamin A status) are difficult to define (96;97). Cases where chronic retinoid intake has been a part of a short term medical treatment program have demonstrated the production of teratogenic effects in humans (98). These therapeutic levels are significantly higher than from any natural source of vitamin A and cannot in reality be included in the assessment of the risk of teratogenic effects of dietary vitamin A.

Table 1.2: - Dose/Frequency relationship to observed toxicity of Vitamin A.

Toxicity	Dose/Frequency	Symptoms
Acute	>/= 100 x RDA once or twice; closely spaced dose > 500000 IU/Kg	early signs:- Nausea, vomiting, headache, vertigo, blurred vision, muscular mal co-ordination (in infants only) bulging of the fontanelle 2nd stage drowsiness, malaise, loss of appetite, physical inactivity, itching, skin exfoliation and recurrent vomiting Terminal phase coma, convulsions and respiratory failure within 1-16 days
Chronic	>/= 10 x RDA In children 2000-600000 IU/d In adults 50000-1000000 IU/d	Headache, alopecia, cracking of the lips, dry and itchy skin, hepatomegaly, bone and joint pain, liver damage
Teratogenicity	NOEL for vitamin A , the levels ingested in early pregnancy to give teratogenic effects is unknown	Fetal resorption, abortion, birth defects to all areas of the fetus depending on gestational day of dose, permanent learning disabilities

The clinical manifestation of retinoid teratogenicity has been established from animal research and supported by clinical data (52). Type of abnormalities are summarized below:

Brain Anomalies:

- Exencephaly: - a neural tube defect where closure has failed to occur in the rostral part of the tube. This leads to the development of a severe malformation of the brain. It is characterized by the protrusions of well-differentiated brain tissue in the initial stages; later stages include the degeneration of brain tissue and even in extreme cases the complete absence of the brain tissue. Such extreme cases are called anencephaly.

- Meningocele and Meningoencephalocele: - Protusion of areas of the brain from the skull near the vertex. Defined as a minor closure defect of the neural tube.

- Spina Bifida:- Defined as an unclosed neural tube of the spinal cords.

- Hydrocephalus: - Defined as an excessive amount of cerebrospinal fluid within the cranium resulting in dilation of the cerebral ventricles.

- Microcephaly: abnormal smallness of the head, usually associated with mental retardation.

Cranial Malformations:

- Microstomia with proximal shortening of the mandibles

- Chistal shortening of the mandibles, medium cleft mandible
- Shortening of the upper jaw
- Underdeveloped zygoma with exophthalmos
- Cleft palate

Clinical evidence for human teratogenicity of vitamin A has been accumulated from data derived from patients taking medication for skin disorders and from large questionnaire based studies on mothers after their baby has been born. Since the critical period for teratogenicity is early within the gestation cycle it is often difficult to assign malformations to hypervitaminosis. Hence, the nature of the malformation and the period of organogenesis when the site of malformation was created are used to classify the malformations into a positive or negative effect. Contradictory data have been published in two papers (96;97), detailing large population based investigations of deformities in new-born children compared to a selected control group with a similar background and geographical location. Rothman et al indicated that a daily dose of 10000 IU intake of vitamin A during pregnancy is sufficient to increase risk to malformations (n=22748). However, Mills et al called into question the grading of the defects by Rothman et al, asking the question of whether the defects were a direct cause of vitamin A toxicity. Mills et al concluded that a teratogenic dose was greater than the normal range consumed by the study group (n=1508). The key question to arise from these conflicting position was the relationship of dose to response. No evidence has been produced indicating that vitamin A does not produce malformations and a great deal has been accumulated from animal experimentation indicating that vitamin A in all its forms is teratogenic to a certain degree (52).

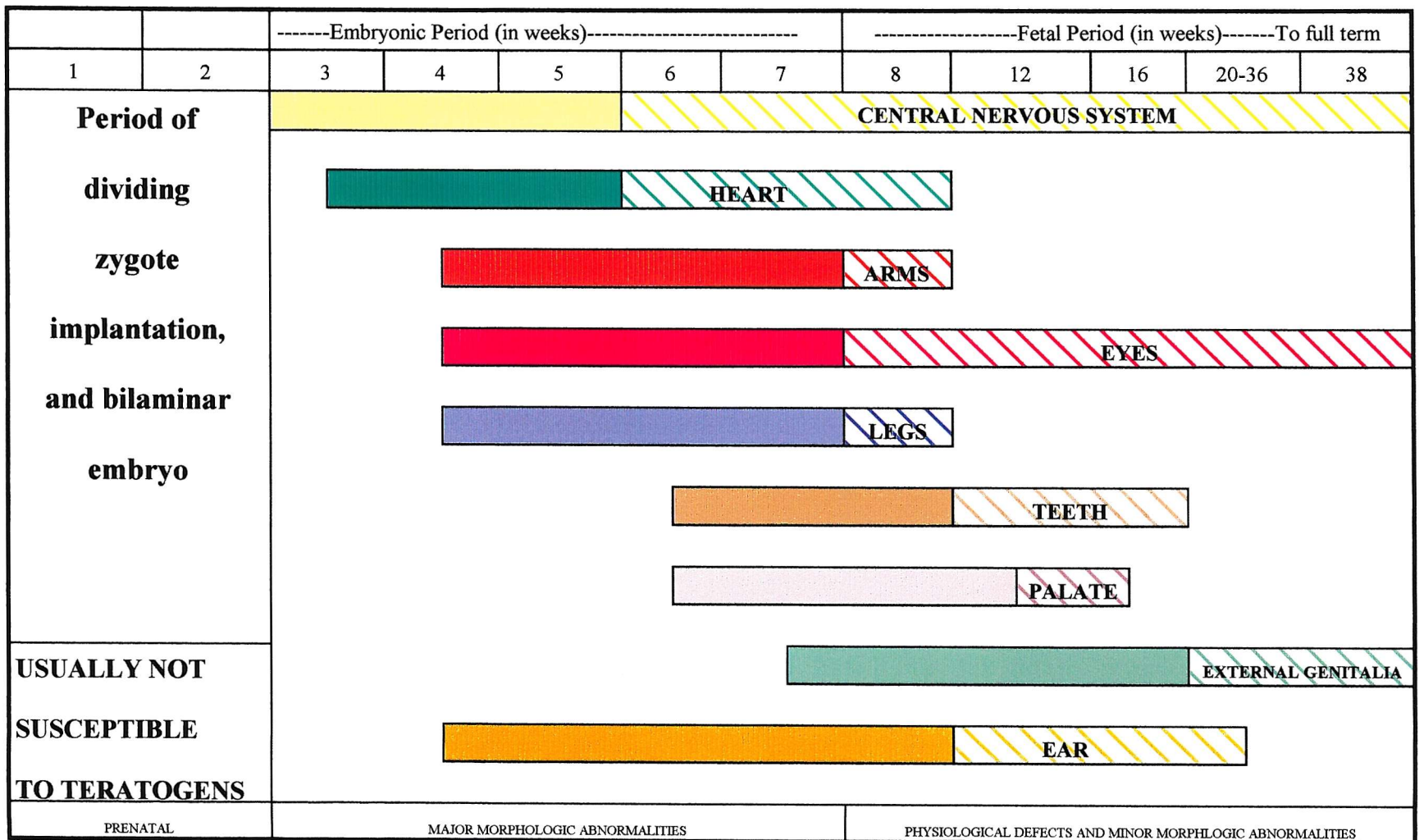

Table 1.3: - Critical time periods of possible teratogenic effects related to system development. Adapted from General Priciples of Toxicoloy,

1.6 HEALTH AND SAFETY OF VITAMIN A

Systemic circulating retinol is maintained at a relatively constant level by internal mechanisms described above. Although retinol is recycled continuously there is still a percentage eliminated by metabolic processes each day. Therefore, the average human body requires a constant update of the vitamin A stores contained in the liver and all other tissue deposits around the body. Hence, dietary sources of vitamin A are necessary for healthy and continuous life. Generalising about the average population is difficult when covering the entire human species. Dietary practices in different countries provide the necessary micro-nutrients in a variety of ways. Many countries in the third world have large groups within the population that suffer from vitamin A deficiency while the "western cultures" contain groups with an excess of intake and groups with deficiency. This difference is reflected in several cross-sectional medical studies performed in different countries where average retinol equivalents intakes were determined (table 1.4). The results demonstrate the broad range of intake that are commonly observed, although different techniques for data collection means the results are not strictly comparable.

From data such as shown in table 1.4 individual countries estimate the recommended daily allowance for members of its population. This estimate varies from country to country since there has been no effort to form an international agreement. However, considering the different nature of diets, it might be that a single RDA world wide would be of limited use since some members of the general population require more or less intake than others. Table 1.5 shows the current RDA's for several western countries with reference to 13-14 year old girls (classified as the most vulnerable group within the studied population) (99).

Table 1.4: - Estimates of average vitamin A intake per day in major "western" countries.

Country	Size	Male intake (RE)	Female intake (RE)	Range (RE)	Length of study
Germany*	2000	1010	890	----------	one week
Denmark**	The elderly population of one small town	1583	1583	805-2956	----------
Spain**		252	252	81-1231	----------
Norway**		379	379	157-556	----------
Portuguese**		3106	3106	1215-7153	----------
USA(I)***	National survey	1600	1485	(M) 284-4658 (F) 178-3995	24hr recall
USA(II)****	City of Boston Survey	1960	1860	(M) 613-5980 (F) 524-6547	3 day record
USA(III)*****	New Mexico Survey	1385	1260	(M) 583-4321 (F) 530-3921	questionaire on food frequency

Sources
* (100)
** (101)(102)
*** (103)
**** (104)
***** (105)

Table 1.5: - Selected data for the RDA (recommended daily allowance) for girls aged 13-14. Adapted from (99)

Country	Retinol Equivalents
Bulgaria	1140
Czechoslovakia	900
France	800
Germany	1000
Hungary	730
Ireland	750
Italy	730
Netherlands	800
Poland	1500
Portugal	1000
Scadinavia	800
Spain	730
United Kingdom	600
USA	800
Former USSR	1500
FAO/WHO	600

The RDA's for 13-14 year girls vary quite considerably between countries. The estimates made for the recommended daily allowances were evaluated on the basis of the amounts required to (106):

1/ recover a normal concentration range in plasma of vitamin A in deficient subjects

2/ maintain retinol plasma levels in well nourished subjects

3/ correct impaired dark adaptation, abnormal electroretinograms and follicular hyperkeratosis

Based on data derived from early depletion studies in the United Kingdom, it was indicated that for an adult male the requirements ranged from 750 to 1200 RE. Hence, an RDA was estimated at 1000 RE for men and 800 RE for women (107-109). Current (1996) recommended dietary intake values for vitamin A are shown in table 1.6. Corresponding data for Germany, USA and the European Community are also shown for comparison.

		FAO/WHO****		USA**	Germany	United***	European*
		basal	safe		****	Kingdom	Community
Infants	36524	180	350	375	500-600	350	350
Children	36556	200	400	400	600 (1-3)	400 (1-3)	400 (1-3)
	36678	200	400	500	700 (4-6)	500 (4-6)	400 (4-6)
	36804	250	400	700	800 (7-9)	500 (7-10)	500 (7-10)
Males	36869	300	500	1000	1100	600	600(11-14)
Adolescents and adults		300	600	1000	100	700	700
Females							
Adolescents and adults		270	500	800	800	600	600
	Pregnancy	370	600	800	800	700	700
	Lactation	550	950	1300	1800	950	950

Table 1.6 Recommended Daily Allowances for vitamin A based on data available 1996. Adapted from (110)
note :-
1/ basal requirements were defined as the minimal amount needed to prevent clinical signs of deficiency in nearly all healthy persons in a population.
2/ Safe level of intake additionally ensures a suitable body reserve of vitamin A for times of low intake and stress in order to sustain health for prolonged periods.
3/ Studies *, ***, **** children means where grouped differently as indicated.

The above recommended daily allowances are set with the view of adequate and safe vitamin A levels for the general population of each country. The key point not addressed with RDA data is the limit above which adverse and toxic effects are observed. Natural sources of vitamin A are consumed by the general population on a regular basis, health awareness is a commitment by all authorities. Therefore, vitamins and minerals have become a commercial venture for many companies, supplying supplements and fortifying foods to attract a greater market share. The general population usually assume that if a "little is good for you then more is better". This has led to groups in the population abusing over the counter supplements to toxic levels. As well as the supplements, foods fortified with extra vitamins and minerals (usually 100 g or ml suppling 100% RDA) add to the total vitamin content the general population is consuming daily. In animal husbandry, a general increase has been noticed in the level of vitamin A in animal livers over the past forty years through the use of growth enhancers to achieve more meat per animal (21). This fact has led to the concerns that there is a possibility that pregnant women within the general population can suffer teratogenic effects unknowingly by consumption of a normal diet. In recognition of this situation the Teratology Society of the USA issued a statement recommending that the daily dose for vitamin A should not exceed 3000RE (95). The General Medical Council of the United Kingdom also issued instructions that the consumption of liver and liver products should not be recommended to pregnant women. In response to this recommendation several courses of action were taken. Firstly, due to the simple fact that ß-carotene has never shown teratogenic effects either in animals or in humans, it was recommended that multivitamin preparations should replace retinol with ß-carotene. Secondly, the question of the minimum

teratogenic dose of vitamin A taken on a daily basis needs to be answered. Thirdly, the extent by which natural food sources supply vitamin A to the general population needs to be determined. This especially refers to the consumption of liver or liver products and the increased consumption of green leaf vegetables. Initial studies on liver consumption have given unexpected results. It has been shown in several studies that the consumption of liver does not supply the body with the same vitamin A dose as compared to a supplement preparation (111-113). It was concluded that liver could be safe for pregnant women to consume. Liver is the best natural source of essential components such as iron and folic acid necessary in pregnancy.

1.7 PHARMACOKINETICS

1.7.1 Pharmacokinetic Data Following Meal Or Supplementation

Several studies have determined the pharmacokinetic parameters for various retinoids following dosing with retinol as the palmitate. Buss et al determined the pharmacokinetic parameters of retinol and its metabolites in healthy women of child-bearing age (111). Dose regimens were with retinol as the palmitate in a liquid formulation as compared to a similar dose given as the cooked liver. Two dosing levels of 150 mg and 50 mg were used, equivalent to 500,000 IU and 167,000 IU respectively. Results obtained indicated that the bioavailability of retinol from liver was 20 fold lower than the equivalent dose as a supplement. It was suggested that the difference observed in retinoid levels indicated a difference in metabolism and absorption. Oral supplementation would supply retinol to the intestinal mucosa more

rapidly than liver where the surrounding matrix would slow the delivery of the retinol to the intestinal mucosa. Increased formation of teratogenic metabolites with the supplement dose was ascribed to a possible saturation of the transport mechanism with CRBP. Increasing levels of retinol in the intestinal mucosa would therefore be metabolised to a greater extent to all-trans- retinoic acid. Incorporation of all-trans-retinoic acid into the chylomicrons would increase the final concentrations in the blood plasma. The slower delivery of retinol as the palmitate from the cooked liver did not saturate the transport mechanism to the same degree as the supplement. Consequently lower levels of metabolites were observed.

Chen et al (112) conducted a similar study among women comparing a dosing range of 0.4 mg(1,305 IU) to 19 mg (64,200 IU) from a single meal, although 4 subjects appeared to have received a dose of 50 mg (169,474 IU). Data for the 4 subjects was excluded from the study by virtue of the fact that blood levels of the retinol metabolites measured in the plasma were lower than for the 19 mg (64,200 IU) dose. It was concluded that the vitamin A content of the meal was overestimated during analysis. Results obtained were lower than for Buss et al (111) and assay limits of detection were an important factor. All-trans-4-oxo-retinoic acid could not be determined successfully due to sensitivity problems. However, time to maximum concentration was similar to that determined by Buss et al, as were metabolite profiles. Chen et al also conducted a sixty day trial in women taking commercial vitamin supplements. Dosing regimens of 1.5 mg (5,000 IU), 3 mg (10,000 IU) and 7.5 mg (25,000 IU) per day were used. However, doses were given in different formulations and a lower bioavailability for the 7.5 mg (25,000 IU) dose in comparison to the 1.5 mg (5000 IU dose) was observed. A statistical comparison

between the supplementation study and the meal consumption study was not conducted. From these data it was concluded that the change in concentration for all-trans-retinoic acid and for 13-cis-retinoic acid after supplement dosage were similar to that determined when dosing with a vitamin A containing meal (specific meal content was not stated).

A third study was conducted by Rasmussen et al (114) with a mixed vitamin A and carotenoid meal. Dosing regimens were at 3%, 50% and 100% of RDA, (RDA for USA is 1000 RE male and 800 RE female, table 1.6). Participants were divided into two groups of mixed sex, one with individuals with an average age of 66 ± 7 years (range 54-80) and a second group with individuals with an average age of 29.2 ± 6.2 years (range 21 - 41). Retinoids examined were retinol and retinyl esters. Results obtained demonstrated a rise above a predetermined baseline for both groups at the 50% and the 100% RDA meals for the retinyl ester, with a T^{max} of 6 hours. For the 100% RDA dose the elderly group exhibited a greater change in baseline and a greater AUC in the retinyl esters in comparison to the younger group. At the 50% RDA level similar differences were observed between groups, but to lesser degree. Both retinol and carotenoids did not show any significant rise in baseline for any group. There was no significant difference found between the sexes within each group.

1.7.2 Pharmacokinetic Data Following Topical Applications

Retinoids have proved to be very successful in treating various forms of skin diseases. Historically, medication was delivered orally. However, doses required caused significant side-effects for the patient. Medical practitioners prefer the use of topical

agents to treat at location rather than the general all over treatment of oral dosing. Subsequently, dermal medication containing significant amount of retinol or retinyl palmitate came into use. Due to the nature of the skin barrier the doses contained within the topical application were higher than treatment required. Absorption across the skin barrier was presumed to be small, however only animal experiments were available. Considering the difference in skin type and nature between mice and humans, there was a concern that the retinoids could pass the skin barrier in humans to raise plasma concentration of the teratogenic retinoids. Two investigation have been published outlining the effect of various forms of retinoid topical creams on the systemic plasma concentration.

Jensen et al (79) conducted pharmacokinetics on subjects suffering from acne vulgaris. An investigation was made using a 0.05% gel containing isotretinoin (13-cis-retinoic acid) with applications made daily for 30 days. An estimated 20 g of gel was used per day per patient. Pharmacokinetics were determined throughout the study with negative results. No significant rise in baseline of any of the retinoids determined was observed during the 30 day period. Doses applied were estimated at 12 times greater than normal daily use for the gel under investigation. Some localised side effects were observed from the application of the skin gel, but plasma levels did not rise significantly.

Chen et al (78) conducted an open label non-comparative multi-dose study using a 0.1% 13-cis-retinoic acid cream. Subjects used were female patients suffering from photo-damaged skin. Study length was 42 day where each subject applied 10 g of cream to approximately 2300 cm^2 of skin area. Diet was restricted to foods known not to contain vitamin A and compliance diaries were issued to each volunteer. The dose

applied was 10 times the RDA for vitamin A. Comparisons made between the Chen et al skin study (78) and the Chen et al supplement study (112) indicated that, despite the 10 fold higher doses given in the skin study, vitamin A levels were limited to 40 -80% above the baseline. In contrast, the supplement study showed levels of 140-180% above the baseline. From the minimal systemic changes in plasma concentrations in the skin study, it was concluded that the absorption of vitamin A through the skin did not present a toxic risk to the patient.

1.8 PROJECT AIMS

The complex metabolism and transcription of retinol has raised many questions about vitamin A use in reference to its safety. Many retinol preparations are readily available to the uninformed public with little or no medical supervision. Abuse has occurred to such an extent that recommendations concerning the use of vitamin A preparations by (potentially) pregnant women have been given to medical and counselling services world-wide. The reasons for this caution is vitamin A's proven teratogenic effect on developing fetuses and the mysteries of vitamin A's importance in the mammalian physiology. In relation to the teratogenic effect of vitamin A and its metabolites this project aims to address the following questions :

1/ Does the gastric emptying of male or female adults affect the absorption of vitamin A? Can inter-individual variations be linked to variable absorption effects?

2/ What systemic levels are found after liver consumption or after consumption of vitamin A supplement products? What effect does the consumption of a similar fat

content meal have on the absorption of vitamin A supplements in comparison to a similar dose of liver?

3/ What systemic levels are found after regular dosing of transdermal vitamin A and its analogues ?

4/ What difference is observed in pharmacokinetic data after a period of regular dosing of a vitamin A supplement compared with a pre-dose baseline? Is there evidence of the build up of teratogenic metabolites in the blood plasma? What effect would long-term supplementation have on systemic levels of retinyl palmitate and retinol?

This project takes the form of four clinical studies performed to study the various factors that could influence the absorption of vitamin A and the formation of its teratogenic metabolites. They are as follows:-

- The influence of *posture and previous dosing* on the absorption of vitamin A and the formation of its teratogenic metabolites.
- The influence of *food and dosing* on the absorption of vitamin A and the formation of its teratogenic metabolites.
- Transdermal vitamin A absorption in healthy women of child bearing age.
- The influence of *multiple dosing* on the absorption of vitamin A and the formation of its teratogenic metabolites.

All clinical studies were conducted in the Clinical Investigation Suite at Southampton General Hospital, Southampton, England during the period of 1995 to 1999 within the Clinical Pharmacology Group of the University of Southampton. Studies were performed in conjunction with Hoffman La Roche Ltd (Chapter 3) and Unilever (Chapter 6).

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Chapter 2

Materials and Methods

Table of Contents

List of Tables

List of Figures

2.1 CLINICAL DETAILS

2.1.1 Subjects

Male and female subjects participated in the four clinical studies. Subjects were recruited from the staff and student population by advertisement and personal contact. Subjects were given an information sheet outlining the background and details of the study. All subjects gave written informed consent and all study protocols were approved by the local Ethics Committee. Subjects received a pre-study medical screening consisting of medical history, physical examination, 12-lead ECG and clinical laboratory determinations. Female subjects had to be non-pregnant and were on adequate contraception throughout the studies. Each subject was requested not to consume liver or liver based products 7 days prior to and during the course of the studies. To account for any other consumption of retinoids outside the study protocol, a food diary was completed by each subject during the course of the studies described in chapters 3 and 6 (figure 2.1). Each diary recorded the number of portions of specified foods consumed each day. Also recorded were the use of non-regular medications and multivitamin preparations. Studies described in chapters 4 and 5 did not use questionnaires to regulate the consumption of vitamin A during the periods between dosing. All pharmacokinetic parameters were calculated with reference to a pre-dose baseline level.

DIET QUESTIONNAIRE

NAME _____

DATE _____

STUDY A
Have you had any of the following foods in the past 7 days? Please give a number in the appropriate portion sizebox for the number of times consumed

	SMALL(<50g)	MEDIUM (50-150g)	LARGE(>150g)
LIVER	☐	☐	☐
LIVER SAUSAGE	☐	☐	☐
LIVER PASTE	☐	☐	☐
FISH (note type) _____	☐	☐	☐
CAMEMBERT CHEESE	☐	☐	☐
CARROTS	☐	☐	☐
CARROT JUICE	☐	☐	☐
SPINACH	☐	☐	☐
KALE	☐	☐	☐
SWEET POTATOES	☐	☐	☐
ENDIVE	☐	☐	☐
CHICORY	☐	☐	☐

	SMALL(<50g)	MEDIUM (50-150g)	LARGE(>150g)
APRICOTS	☐	☐	☐
APRICOT JUICE	☐	☐	☐
PEACHES	☐	☐	☐
MANGO	☐	☐	☐
CANTALOUPE	☐	☐	☐
PUMPKIN	☐	☐	☐
	☐	☐	☐

Other sources	Number of times in 7 days
BUTTER/MARGARINE	☐
COD LIVER OIL	☐
MULTIVITAMIN PREPARATIONS (GIVE MAKE AND TYPE) _____	☐

Figure 2.1: - Example of a food diary issued to the subjects for each week of the study.

2.1.2 Dosing Standards for the Clinical Studies

In each clinical study subjects were given retinol or retinyl palmitate. This was supplied in an approved safe form with known quantities. The three basic forms of dosing with retinol or retinyl palmitate were :

1. Arovit
2. Calf's liver
3. Skin cream

For each form of dosing, accurate analysis of retinoid content was performed prior to the clinical studies.

2.1.2.1 Arovit

Arovit is a liquid formulation of retinol palmitate supplied by Hoffmann-La-Roche for the purpose of these studies. It contains 45 mg retinol/ml (150000 IU/ml) as the retinyl palmitate.

2.1.2.2 Calf's liver

A whole calf's liver was purchased from a local retail source (Duke's butchers, Chandlers Ford, England). Analysis was performed on raw liver samples, a mean value of 0.311 mg retinol/gram was used for all dosing calculations (refer to section 3.1.1).

2.1.2.3 Skin creams

General purpose 6.5% w/w oil-in-water base creams were supplied by Unilever, England. Three creams were used containing either placebo, 0.236% by weight of retinyl palmitate or 0.147% by weight of retinol.

2.1.3 Treatment of Blood Samples

All blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C. The plasma layer was removed and transferred to a glass blood collection tube containing 20 µl of a 10 µg/ml BHT and a 20 µg/ml BHA solution in ethanol. All work was carried out under sodium light conditions to prevent photo-degradation of retinoids. Samples were stored in the dark at -70°C until analysis. All samples were analyzed for the following compounds:

Retinol

Retinyl palmitate

All-trans-retinoic acid

All-trans-4-oxo-retinoic acid

9-cis-retinoic acid

13-cis-retinoic acid

13-cis-4-oxo-retinoic acid

2.2 ANALYTICAL MATERIALS AND METHODS

2.2.1 Materials and Reagents

All materials and reagents used, their sources and purity are shown in table 2.1. Dosing materials where obtained from manufacturer's as donations towards each study.

Table 2.1: - Reagent manufacturer's and reagent purity

Reagent	Source	Batch	Purity
All-trans retinol	Sigma/Aldrich (Dorset, UK)	----------	99.9%
Retinyl palmitate	Sigma/Aldrich (Dorset, UK)	----------	98.0%
All-trans retinyl acetate	Sigma/Aldrich (Dorset, UK)	Lot 59F0522	~90%
All-trans retinoic acid	Hoffman-La-Roche(Basel,CH)	Lot 229010/G	99.9%
9-cis retinoic acid	Hoffman-La-Roche(Basel,CH)	RO 04-4079	~98%
13-cis retinoic acid	Hoffman-La-Roche(Basel,CH)	Lot 308064	99.9%
All-trans-4-oxo retinoic acid	Hoffman-La-Roche(Basel,CH)	RO12-4824	~98%
13-cis-4-oxo retinoic acid	Hoffman-La-Roche(Basel,CH)	RO-22-6595	~99%
retinyl acritin	Hoffman-La-Roche(Basel,CH)	RO 13-7652	99.9%
Methanol	Merck Ltd (UK)		HPLC
Ethanol	Merck Ltd (UK)		ANALAR
n-Hexane	Prolabo (UK)		ANALAR
Ammonium Acetate	Merck Ltd (UK)		ANALAR
Sulphuric Acid	Merck Ltd (UK)		ANALAR
BHT	Merck Ltd (UK)		ANALAR
BHA	Merck Ltd (UK)		ANALAR

Note :All retinoids were stored in the dark at -70°C. No analysis were performed on a routine basis on a) Anhydroretinol; b) 9,13-di-cis retinoic acid; c) 14-hydroxy-retro retinoic acid. However, note was taken of their presence and potential interference with other retinoids was determined. Refer to section 2.3.2 for chromatographic parameters.

2.2.2 Laboratory Precautions

All work during the analysis phase was carried out in a dark room under a sodium light to protect the retinoids from light degradation. To ensure stability of all retinoids, 10μg/ml butylated hydroxytoluene (BHT) and 20μg/ml butylated hydroanisole (BHA) were used as antioxidants in all solvent solutions used in the analysis.

2.2.3 Preparation of Standard Solutions

2.2.3.1 Stock standard solutions

Stock standard solutions for retinoic acid metabolites were made by dissolving approximately 10 mg of standard powder of each compound in sufficient ethanol to give a final concentration of 1 mg/ml. Stock standard solutions for retinol and retinyl acetate were prepared by dissolving approximately 100 mg of standard powder of each compound in sufficient ethanol to give a final concentration of 10 mg/ml.

2.2.3.2 Working standard solutions

Three sets of working standard solutions were prepared :

1. a series of dilutions of the 5 retinoic acid metabolites at different concentrations.
2. a series of dilutions of retinol and retinyl palmitate at different concentrations.
3. a series of dilutions of retinol at different concentrations. This was used in the analysis of the calf's liver.

2.2.3.3 Retinoic acids working standard solutions

Aliquots of 1 ml of the ethanol stock standard solution (1mg/ml) of each of the five metabolites were combined. This solution was then diluted with ethanol (5ml) to give a concentration of 0.1 mg/ml for each metabolite. Subsequent dilutions were made to give the following working standard solutions :

- 10 µg/ml=100 ng/10µl
- 2 µg/ml=20 ng/10µl
- 0.5 µg/ml=5 ng/10µl
- 0.25 µg/ml=2.5 ng/10µl
- 0.1 µg/ml=1 ng/10µl

2.2.3.4 Retinol and retinyl palmitate working standard solutions

Aliquots of 1 ml of the ethanol stock standard solution (10 mg/ml) of retinol and retinyl palmitate were combined. This solution was then diluted with ethanol (8ml) to give a

concentration of 1 mg/ml for both retinol and retinyl palmitate. Subsequent dilutions were made to give the following working standard solutions :

–	1	mg/ml	=	10,000 ng/10µl
–	0.1	mg/ml	=	1000 ng/10µl
–	0.05	mg/ml	=	500 ng/10µl
–	0.02	mg/ml	=	200 ng/10µl
–	0.01	mg/ml	=	100 ng/10µl
–	0.005	mg/ml	=	50 ng/10µl

2.2.3.5 Retinol working standard solutions used in the analysis of calf's liver

Aliquots of an ethanol stock standard solution (10 mg/ml) of retinol were used to prepare the following working standard solutions.

-	6	mg/ml	=	0.6 mg / 100µl
-	4	mg/ml	=	0.4 mg / 100µl
-	2	mg/ml	=	0.2 mg / 100µl

2.2.3.6 Internal standard working and stock solutions

For each assay an internal standard was used to adjust for variability in the extraction process.

A. For retinoic acids :

Retinyl acritin was used as the internal standard. A stock standard solution of retinyl acritin was prepared by dissolving approximately 10 mg of standard powder in sufficient ethanol to give a concentration of 1 mg/ml. This solution was subsequently diluted with ethanol to prepare 200 ml of a working standard solution of 50 μg/ml. After subdivision into 10 ml aliquots, the working standard solution was stored in the dark at -70 °C. These aliquots were then used for all retinoic acid metabolite analysis.

B. For the analysis of retinol and retinyl palmitate.

Retinyl acetate was used as the internal standard. A 200 ml stock standard solution of 250 μg/ml was prepared and stored in the dark at 4 °C. A working standard solution was prepared for each experimental day by diluting 0.5 ml of the stock standard solution to 200 ml with ethanol. Final concentrations of retinyl acetate used were 0.625 μg/ml.

C. For the analysis of retinol in calf's liver

Retinyl acritin was used as the internal standard. A 200 ml stock solution of 0.500 mg/ml was prepared in ethanol.

2.2.4 Analysis of Samples

All plasma samples were analyzed by two reversed phase HPLC systems. Each analytical run consisted of samples from between one and three subjects, together with a series of standards to provide calibration curves for that run. All samples were analyzed in duplicate to increase the accuracy. Two separate analysis methods were used :

Analysis method 1 : for the determination of retinol and retinyl palmitate

Analysis method 2 : for the determination of the acid metabolites of retinol

2.2.5 Analysis of Retinol and Retinyl Palmitate

2.2.5.1 Analysis of plasma samples

Aliquots (0.5 ml) of subject's plasma and control plasma were spiked with 1 ml (0.75 μg) of working internal standard solution (retinyl acetate). Control plasma was additionally spiked with retinol and retinyl palmitate working standard solutions to provide calibration curves of 0-10 μg / ml. The addition of ethanol in the standard solutions also functioned to deproteinize the samples. The supernatant of each sample was removed and extracted twice with 5 ml n-hexane. The pooled n-hexane layers were evaporated at 40°C under a O_2-free nitrogen stream. The residue was reconstituted in 150 μl of ethanol and spira-mixed for 20 minutes. Samples were then transferred to HPLC vials and 150 μl of a DMSO / H_2O solution (50 : 50 v/v) was added. Aliquots (180 μl) were injected onto the

HPLC system using a 717 Plus Autosampler (Waters Associates). The column used was a Sphericlone RP18-5u-ODS-2 (150 x 4.6 mm internal diameter, Phenomenix, UK), which was maintained at 60°C in a thermostated water bath. Retinol and retinyl palmitate were eluted at a flow rate of 1.2 ml / min using a binary solvent, multiple gradient system from 55% A : 45% B to 0% A : 100% B over 20 minutes followed by 5 minutes at 0% A : 100% B. This gradient was established by two Waters M 6000A pumps (Waters Chromatography) and an automated gradient controller (Waters Chromatography). Solvent A consisted of 50% water : 25% propan-2-ol : 25% methanol. Solvent B consisted of 50% methanol : 50% propan-2-ol. The retinoids were detected at 325 nm using a Waters 490 UV-detector (Waters Chromatography). Chromatograms were recorded using a 745 data system (Spectra Physics, Waters Chromatography) and integrated for peak height.

2.2.5.2 Analysis of retinol in calf's liver

Total retinol levels in raw and cooked liver were determined by hot saponification. Aliquots (0.3 g) of finely chopped liver were mixed with 50 mg ascorbic acid, 100 µl methanol and 1 ml methanolic KOH(1M). Water - used in place of liver - was spiked with retinol working standard solutions to produce calibration curves equivalent to 0 - 1.5 mg / retinol for uncooked liver and 0 - 0.6 mg retinol / 0.3 g for cooked liver. All samples were heated to 100°C in a heating block for 30 minutes to hydrolyze retinol-esters to retinol. Samples were cooled for 5 minutes and a working internal standard solution of retinol acritin was added (100 µg in 400µl). This was further diluted with 8.5 ml of 35% ethanol.

Samples were thoroughly vortexed. Aliquots of 1ml were removed and extracted with tert-butylmethylether. The ether layer was evaporated at 40°C under an O_2-free nitrogen stream. The residue was reconstituted in 200 µl of ethanol and further diluted with 200 µl 60mM ammonium acetate. Aliquots (5 µl) were injected onto the HPLC system by an AS 3000 autosampler (Thermoseparation Products, UK). The column used was a Prodigy RP18-5µm-ODS-3 150 x 4.6 mm internal diameter (Phenomenix, UK), which was maintained at 60°C using a thermostatted waterbath. Retinol was eluted at a flow-rate of 2 ml / min using a gradient system from 15% A : 85% B to 0% A : 100% B over 15 minutes. The gradient was maintained by a CM4100 pump (Thermoseparation Products, UK). Solvent A consisted of 90% 60mM ammonium acetate buffer : 9.98% methanol : 0.02% propan-2-ol at pH 5.4 with acetic acid. Solvent B consisted of 90% methanol : 10% 60mM ammonium acetate buffer at pH 6.2 with acetic acid. Retinol was detected at 350 nm by a SM4100 UV-detector (Thermoseparation Products, UK) and data were collected electronically onto a personal computer using LC-Talk software (Thermoseparation Products, UK). Chromatograms were analyzed for peak areas.

2.2.6 Analysis of the Acid Metabolites of Retinol

Aliquots (1 ml) of subject's plasma, control plasma and control plasma spiked with working standard solutions of the acid metabolites to prepare calibration curves over the range of 0 - 100 ng / ml, were mixed with 10µl(0.5 µg) internal standard working solution

(retinyl acritin). Samples were vortexed and acidified with 150 µl 0.5M H_2SO_4 These were then extracted twice with 5 ml n-hexane. Pooled n-hexane layers were evaporated at 40°C under a gentle stream of O_2-free nitrogen and and the residue was reconstituted in 75 µl ethanol. Samples were vortexed for 10 minutes and allowed to settle for 15 minutes before transferring to HPLC vials. Samples were then further diluted with 75 µl 60mM ammonium acetate (pH 7.0). Aliquots (100 µl) were injected onto the HPLC system using a AS3000 autosampler (Thermoseparation Products, UK). The column used was a Prodigy RP18-5um-ODS-3 150 x 4.6 mm internal diameter, (Phenomenix, UK), which was maintained at 60°C using a thermostatted waterbath. The acid metabolites were eluted at a flow-rate of 2 ml / min using a multilevel gradient over 35 minutes :

0 minutes	35%A	65%B
8 minutes	38%A	62%B
15 minutes	15%A	85 %B
20 minutes	15%A	85%B
28 minutes	10%A	90%B
35 minutes	0%A	100%B

The above gradient was maintained by a CM4100 constametric gradient solvent delivery system (Thermoseparation Products, UK). Mobile phase A consisted of 89.5% of a 60mM ammonium phosphate buffer, 10% methanol with 0.5% propan-2-ol. The pH of mobile phase A was 5.4 ± 0.05. Mobile phase B consisted of 90% methanol and 10% 60mM ammonium phosphate buffer. The pH of mobile phase B was 6.2 ± 0.05.

2.3 CHROMATOGRAPHY

Three different analytical assays were performed as described above. Each sample analyzed generated a chromatogram relating the peak area of the analyte of interest to its concentration. The following chromatograms are representative examples of each type of analysis.

a/ Total retinol content in raw and cooked liver

b/ Retinoic acid metabolites in blood plasma

c/ Retinol and retinyl palmitate in plasma

A linear regression was analyzed with every chromatographic series. All standards used across the individual studies are expressed as a total mean and as an assay to assay deviation, calculated as a percentage.

2.3.1 Analysis of retinol content within liver

Figure 2.2 demonstrates a typical chromatogram of the analysis of total retinol content within liver tissue for investigation of the influence of food and dosage on the absorption of vitamin A and the formation of its teratogenic metabolites (chapter 3). Samples analyzed were either raw or lightly cooked liver. Sample concentration was calculated using parameters from linear regression analysis without weighting of a four point standard curve. Regression analysis was calculated from the known spiked concentration and a ratio

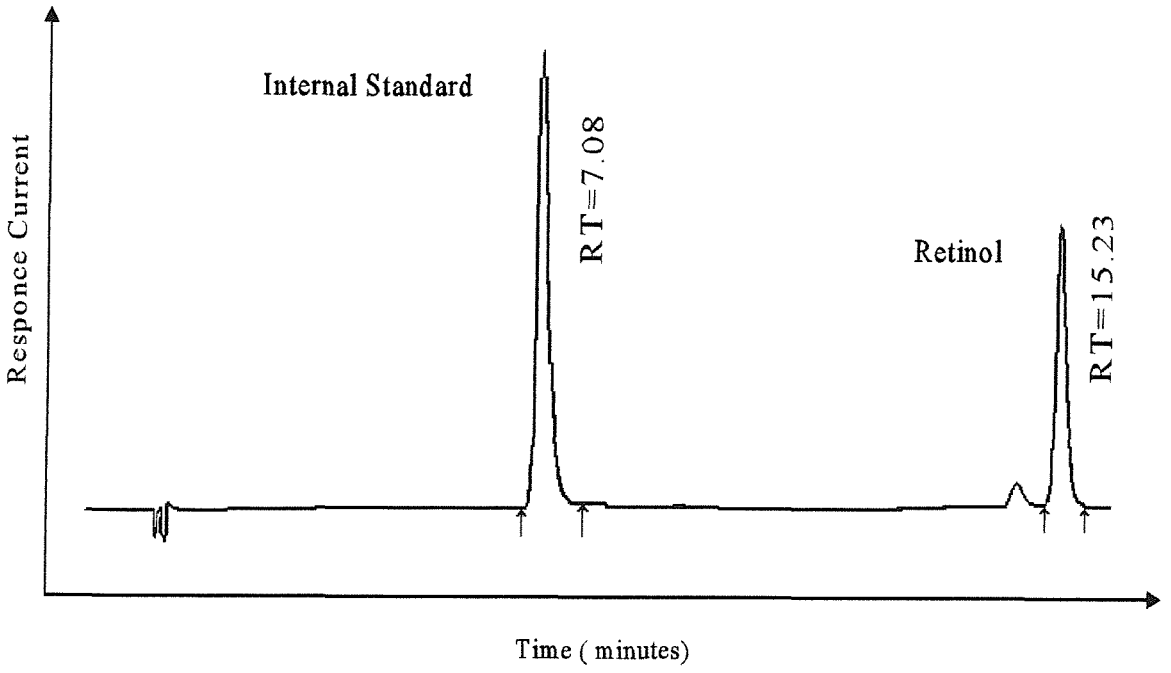


Figure 2.2 Chromatogram of a 0.4 mg/ml standard of retinol extracted from water

of the AUC for retinol (Rt 15.5 ± 0.5 min) against the AUC for an internal standard (RT 7 ± 0.3 min). Table 2.2 lists the mean concentration and standard deviation for each standard point as calculated from the linear regression analysis. Due to the levels found within the liver being less than the expected 1 mg/g, re-analysis of the liver was conducted after completion of the study with a lower standard curve. A different chromatography system was used (Thermoseparation products system as compared to a Waters chromatography system). Ratios calculated for the regression lines are not comparable due to the differences in the ratios calculated by the different systems. The mean standard curve slopes were 3.88 pre study and 1.80 post study. Assay results for raw and cooked liver were found to be in agreement between pre and post dose samples. Results for pre-

dose are shown in tables 3.1. Assay limits of detection were set at a signal to noise ratio three times baseline (Pre study 0.1 mg/g and post study 0.05 mg/g; reflecting the increase in accuracy found during the progress of analysis). Limit of quantitation was set at the lowest peak area achievable (pre-study 0.2 mg/g; post study 0.1 mg/g)

Table 2.2: - Mean standard values used in regression analysis for the raw liver retinol determinations.

0.0 std	0.1 std	0.2 std	0.5 std	0.6 std	1.0 std	1.5 std
0 ± 0.18	0.09 ± 0.001	0.22 ± 0.06	0.5 ± 0.07	0.62 ± 0.17	1.01 ± 0.1	1.49 ± 0.15
% deviation	% deviation	% deviation	% deviation	% deviation	% deviation	% deviation
-------	10	28.84	14.86	27.33	9.61	10.23

Results were calculated for samples as mg / 0.3 g liver sample. Correction for weight taken was calculated to give all results in units of mg/g liver.

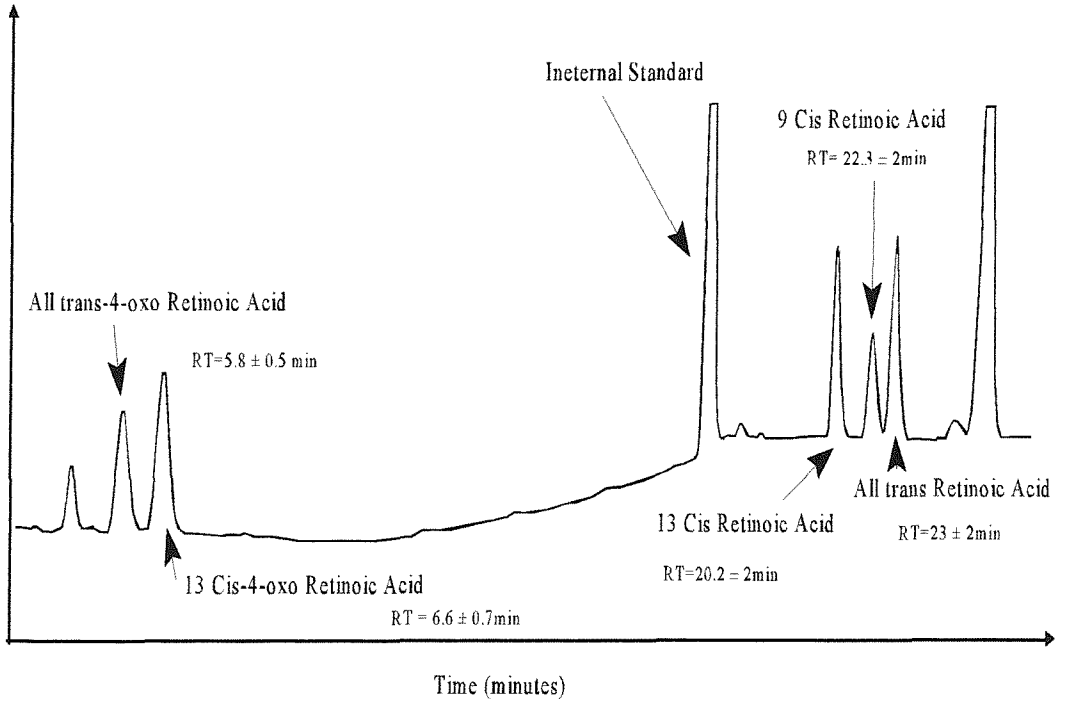


Figure 2.3: - Chromatogram of five metabolites of retinoic acid.

2.3.2 Analysis of Retinoic Acid Metabolite Content Within Human Plasma Samples

Figure 2.3 shows a typical chromatogram of the analysis of five retinoic acid metabolites found in human plasma This chromatogram is typical for all four studies. Samples analyzed were preserved with butylated hydroxy toluene and butylated hydroxy anisole, neither of which gave a response in the chromatogram. Sample concentration was calculated using parameters from linear regression analysis without weighting of a six point standard curve. Regression analysis was calculated from the known spiked concentration and a ratio of the AUC for each metabolite and an internal standard. Peak identification was made with reference to standard chromatograms and appropriate retention times; All-trans-retinoic acid (Rt = 23.0 ± 2.2 min), 13-cis-retinoic acid (RT = 20.2 ± 2.1 min), 9-cis-retinoic acid (RT 21.3 ± 1.9 min), 13-cis-4-oxo-retinoic acid (RT = 6.6 ± 0.7 min), all-trans-4-oxo-retinoic acid (5.3 ± 0.5min) and an internal standard (RT 18.5 ± 0.7 min). Table 2.3-2.6 lists the mean concentration and standard deviation for each standard point as calculated from the linear regression analysis for each of the four investigations. Figure 2.3-2.7 illustrates the standard variation seen for each metabolite for each of the investigations.

Table 2.3: - Standard calculated means based on a six point linear regression with reference to the investigation of the influence of food and dosage on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 3). All values are measured in ng/ml.

Conc^n	all-trans-4-oxo-retinoic acid	13-cis-4-oxo-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	all-trans-retinoic acid
1	1.48 ± 1.34	1.06 ± 2.50	1.20 ± 1.81	1.69 ± 2.33	1.41 ± 1.34
2.5	2.62 ± 1.82	1.91 ± 2.42	2.32 ± 1.18	2.43 ± 2.22	2.70 ± 1.70
5	4.71 ± 1.31	3.53 ± 2.41	5.35 ± 1.20	4.03 ± 3.92	5.53 ± 1.42
20	20.50 ± 4.39	18.20 ± 4.78	20.18 ± 3.37	18.41 ± 4.60	20.60 ± 4.06
100	100.01 ± 15.17	100.47 ± 10.72	100.15 ± 7.04	100.43 ± 5.61	99.85 ± 6.75

Table 2.4: -Standard calculated means based on a five point linear regression with reference to the influence of posture and previous dosing on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 4). All values are measured in ng/ml.

Conc^n	all-trans-4-oxo-retinoic acid	13-cis-4-oxo-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	all-trans-retinoic acid
1	0.17 ± 0.59	0.64 ± 0.82	0.76 ± 0.79	0.52 ± 1.62	0.51 ± 0.82
5	4.50 ± 1.09	5.61 ± 4.20	4.06 ± 1.27	3.20 ± 2.10	4.16 ± 1.96
20	18.36 ± 2.89	21.03 ± 2.73	19.88 ± 2.42	18.72 ± 2.92	18.72 ± 2.69
100	100.47 ± 6.73	99.89 ± 5.29	100.31 ± 7.34	100.66 ± 8.69	100.54 ± 8.84

Table 2.5: -Standard calculated means based on a six point linear regression with reference to the influence of multiple dosing of vitamin A and the formation of its teratogenic metabolites (Chapter 5). All values are measured in ng/ml.

Conc^n	all-trans-4-oxo-retinoic acid	13-cis-4-oxo-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	all-trans-retinoic acid
1	0.88 ± 2.49	0.20 ± 2.76	1.08 ± 2.25	1.98 ± 4.09	1.53 ± 2.28
2.5	2.89 ± 2.44	1.98 ± 3.23	2.68 ± 2.49	3.18 ± 4.00	2.73 ± 3.33
5	5.07 ± 3.02	4.67 ± 2.38	4.61 ± 2.84	5.16 ± 4.24	6.61 ± 4.84
20	24.48 ± 7.88	21.76 ± 8.41	19.55 ± 3.44	21.29 ± 5.53	19.23 ± 4.15
100	102.92 ± 20.93	104.23 ± 24.68	99.45 ± 6.11	99.99 ± 4.63	99.79 ± 6.08

Table 2.6: -Standard calculated means based on a five point linear regression with reference to the transdermal vitamin A absorption in healthy women of child bearing age (Chapter 6). All values are measured in ng/ml.

Conc^n	all-trans-4-oxo-retinoic acid	13-cis-4-oxo-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	all-trans-retinoic acid
2.5	2.08 ± 2.56	2.30 ± 1.50	2.23 ± .85	2.09 ± 1.48	2.17 ± 1.12
5	4.32 ± 2.96	4.33 ± 1.40	4.81 ± 1.07	4.35 ± 1.99	4.62 ± 1.69
20	18.90 ± 5.74	18.44 ± 4.94	20.19 ± 3.11	19.60 ± 4.54	20.53 ± 4.04
100	100.07 ± 12.94	100.11 ± 11.23	99.97 ± 9.69	100.08 ± 10.68	99.99 ± 11.77

Control plasma was found to contain about 1-2 mg/ml of different acid metabolites. This influenced the limit of detection for the added standards. Based on the % error determined from the linear regression for each standards point the limit of detection was about 0.5 ng/ml of added standard for all metabolites. All values less than this were deemed to be undetectable even if a peak was detected. The limit of quantitation was 2 ng/ml for all metabolites by reason of the more accurate 2.5 standard. However, large errors were observed between the mean data in tables 2.3 to 2.6. This is due to the nature of the regression calculated. Emphasis is given to the 100 and 20 standards while the lower end of the regression line has a decreased effect on the regression parameters.

Figure 2.4: - Regression analysis for standard variation with reference to the metabolite analysis within the investigation of the influence of food and dosage on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 3).

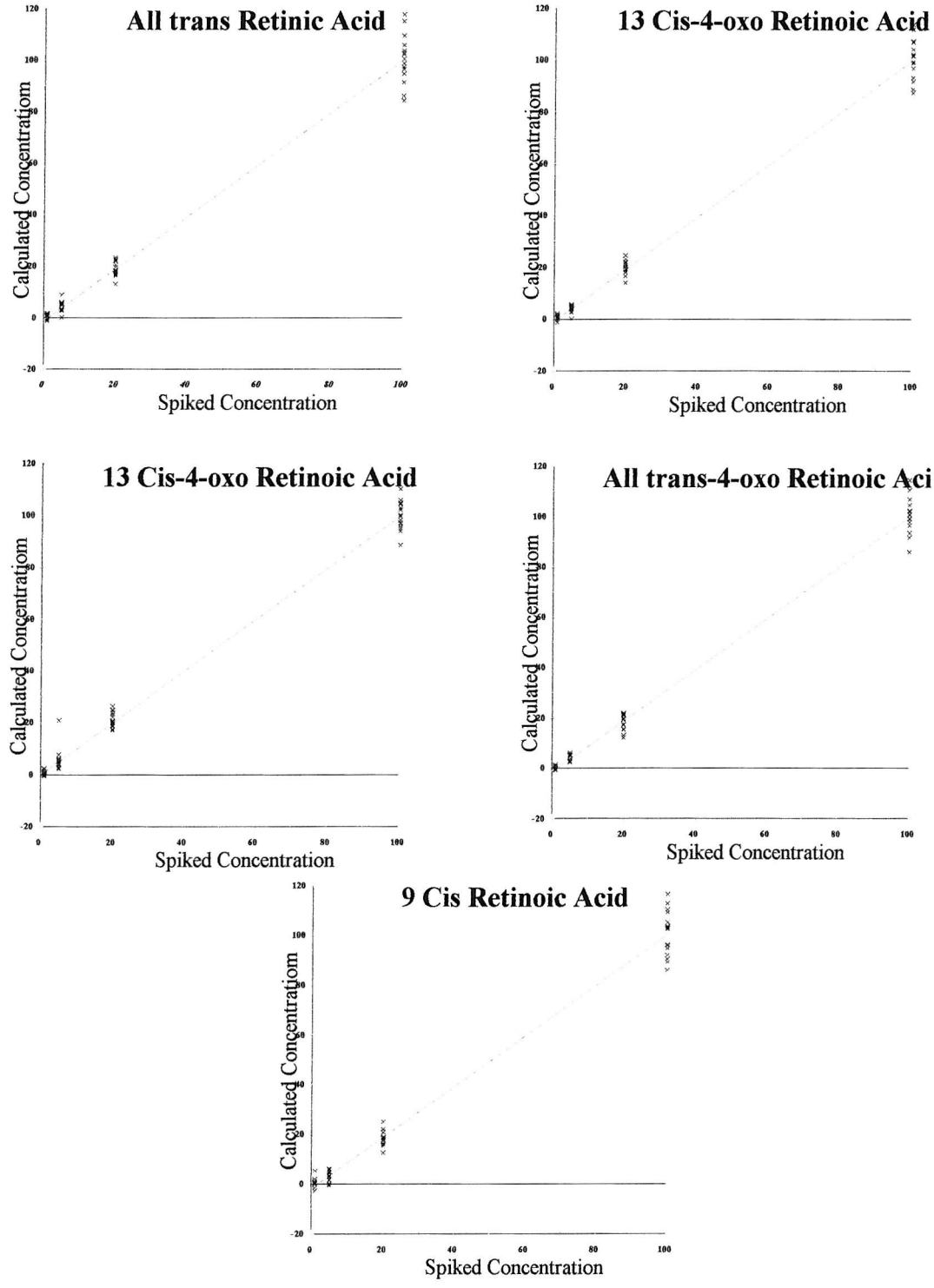


Figure 2.5: - Regression analysis for standard variation with reference to the metabolite analysis within the investigation of the influence of posture and previous dosing on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 4).

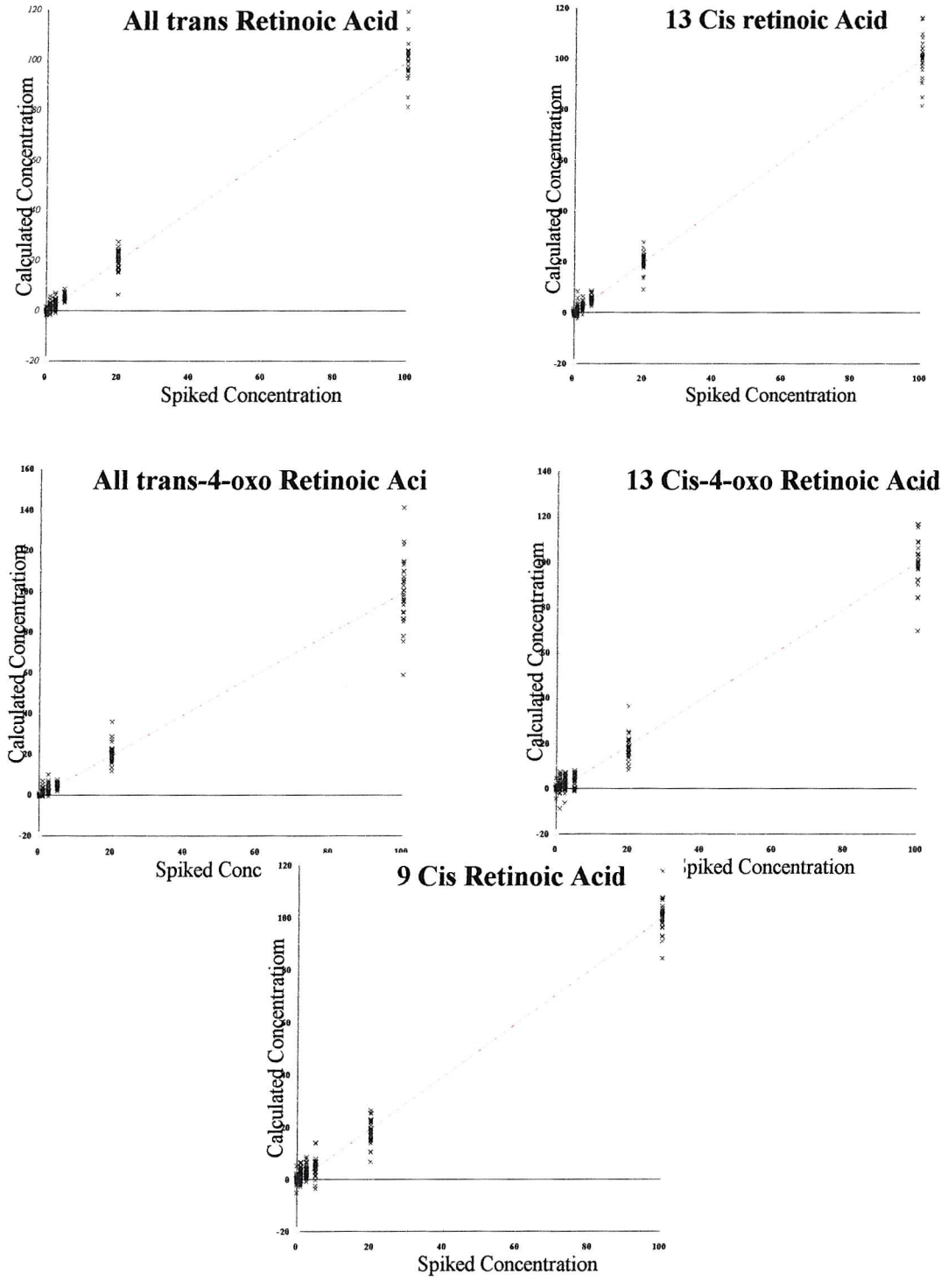


Figure 2.6: Regression analysis for standard variation with reference to the metabolite analysis within the investigation of the influence of multiple dosing of vitamin A and the formation of its teratogenic metabolites (Chapter 5).

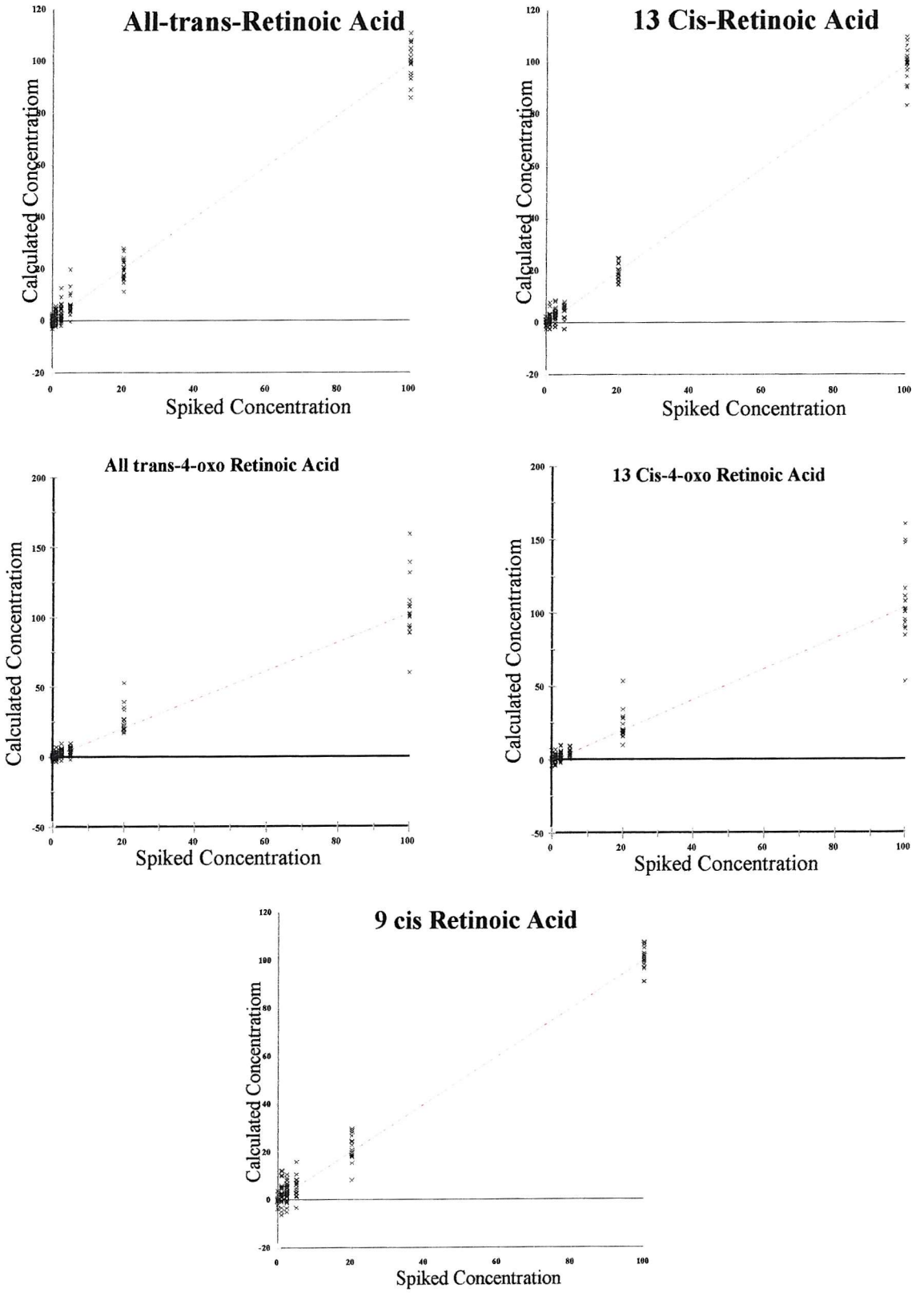
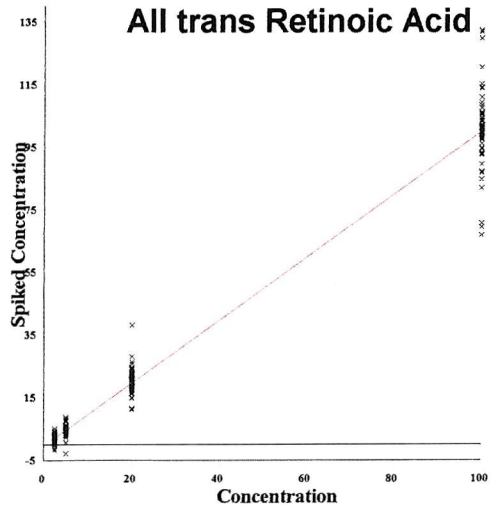
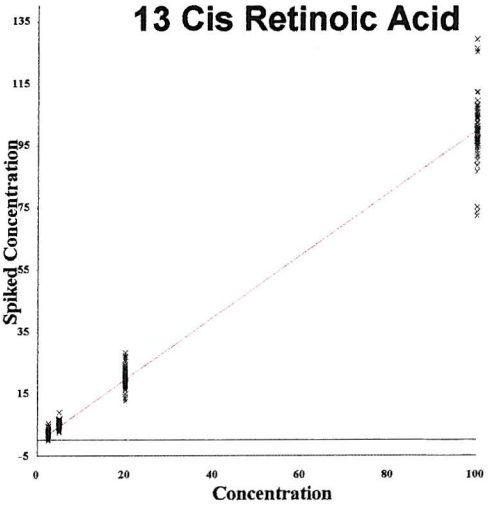
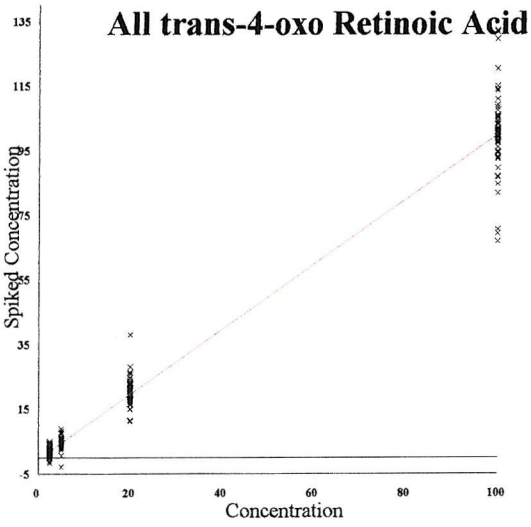
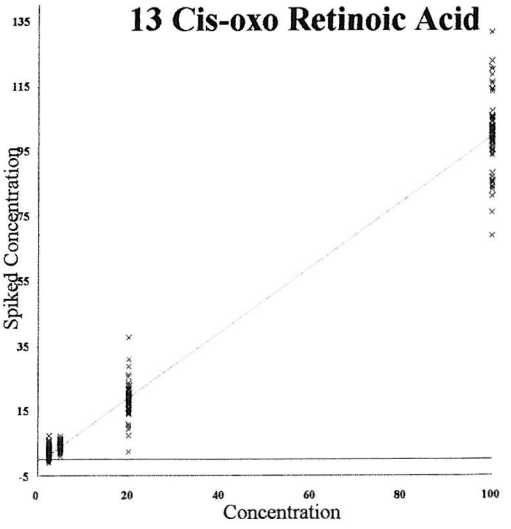


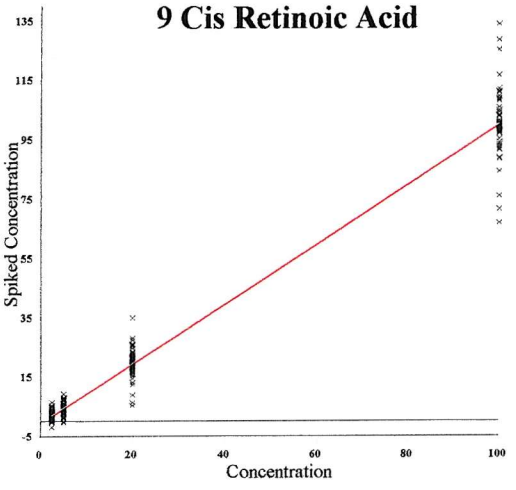
Figure 2.7: -Regression analysis for standard variation with reference to the metabolite analysis within the investigation of the transdermal vitamin A absorption in healthy women of child bearing age (Chapter 6).











2.3.3 Analysis of Retinol And Retinyl Palmitate Content Within Human Plasma Samples

Figure 2.11 demonstrates a typical chromatogram of the analysis of retinol and retinyl palmitate found in human plasma. This chromatogram is typical for all four studies. Samples analyzed were preserved with butylated hydroxy toluene and butylated hydroxy anisole, neither of which gave a response in the chromatogram. Sample concentration was calculated using parameters from linear regression analysis without weighting of a seven point standard curve. Regression analysis was calculated from the known spiked concentration and a ratio of the AUC for each component against an internal standard.

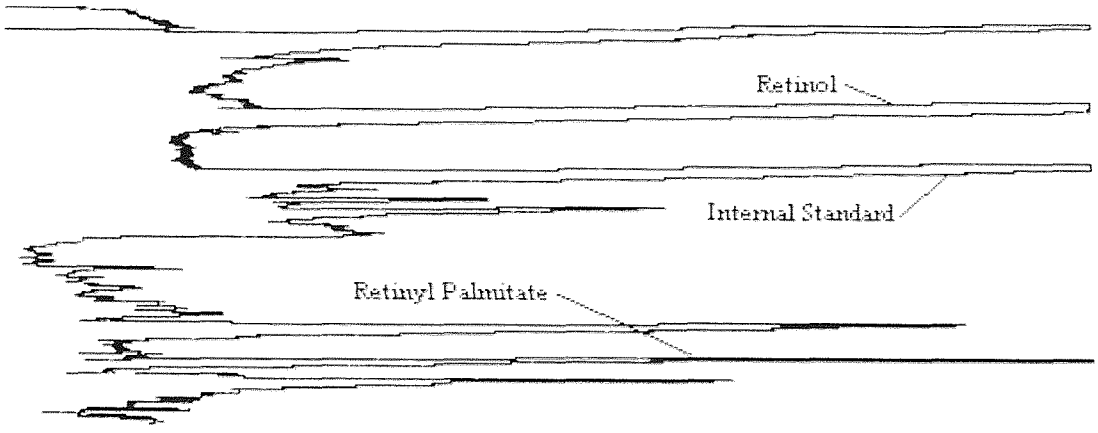


Figure 2.8: - 200 ng standard chromatogram of retinol and retinyl palmitate extracted from human plasma

Peak identification was made with reference to standard chromatograms and approximate retention times; All trans retinol (Rt = 6.7 ± 2.5 min), retinyl palmitate (RT = 23.6 ± 0.3 min) and an internal standard (RT = 10.5 ± 1.9 min).

Based on the % error determined from the linear regression for each standards point, the limit of detection was set at 100 ng/ml for retinol and at 25 ng/ml for retinyl palmitate. All values less than this were deemed to be undetectable even if a peak was detected. The limit of quantitation was set at 150 ng/ml for retinol and at 100 ng/ml for retinyl palmitate by reason of the accuracy of the 100 ng/ml retinol and the 50 ng/ml retinyl palmitate. Figure 2.11 to Figure 2.14 show the degree of variation seen among the standards when compared across the entire investigations.

Figure 2.9: Standard regression analysis for retinyl palmitate and retinol analysis within the investigation of the influence of food and dosage on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 3).

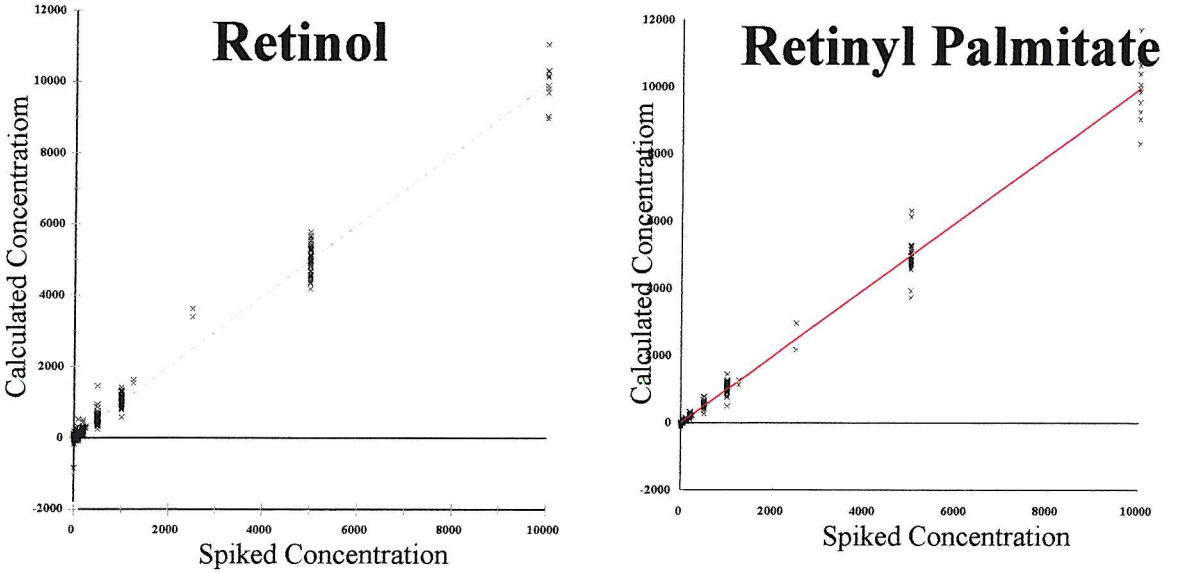


Figure 2.10: Standard regression analysis for retinyl palmitate and retinol analysis within the investigation of the influence of posture and previous dosing on the absorption of vitamin A and the formation of its teratogenic metabolites (Chapter 4).

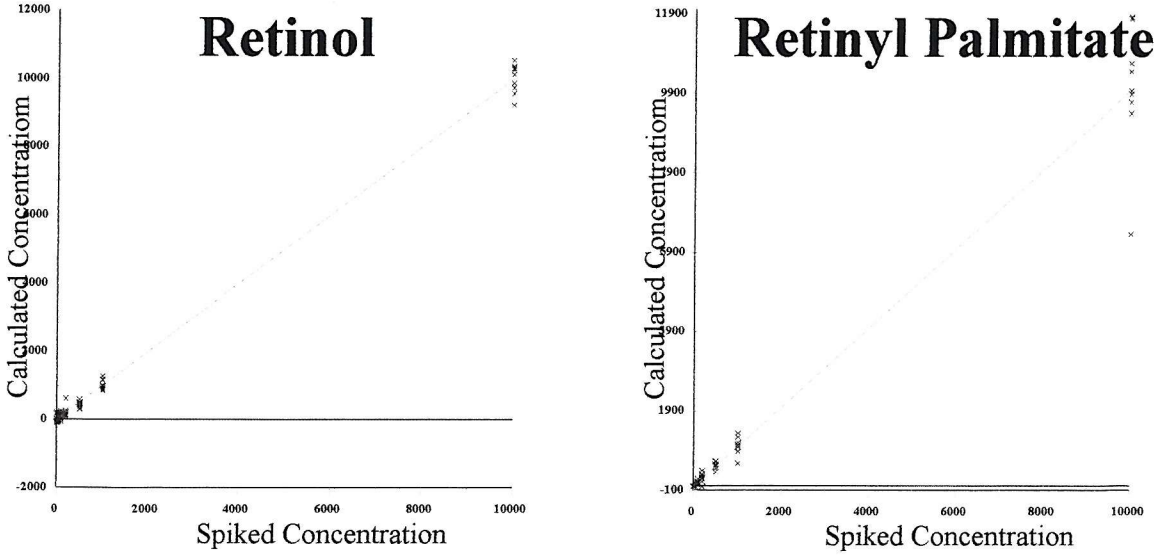


Figure 2.11: Standard regression analysis for retinyl palmitate and retinol analysis within the investigation of the influence of multiple dosing of vitamin A and the formation of its teratogenic metabolites (Chapter 5).

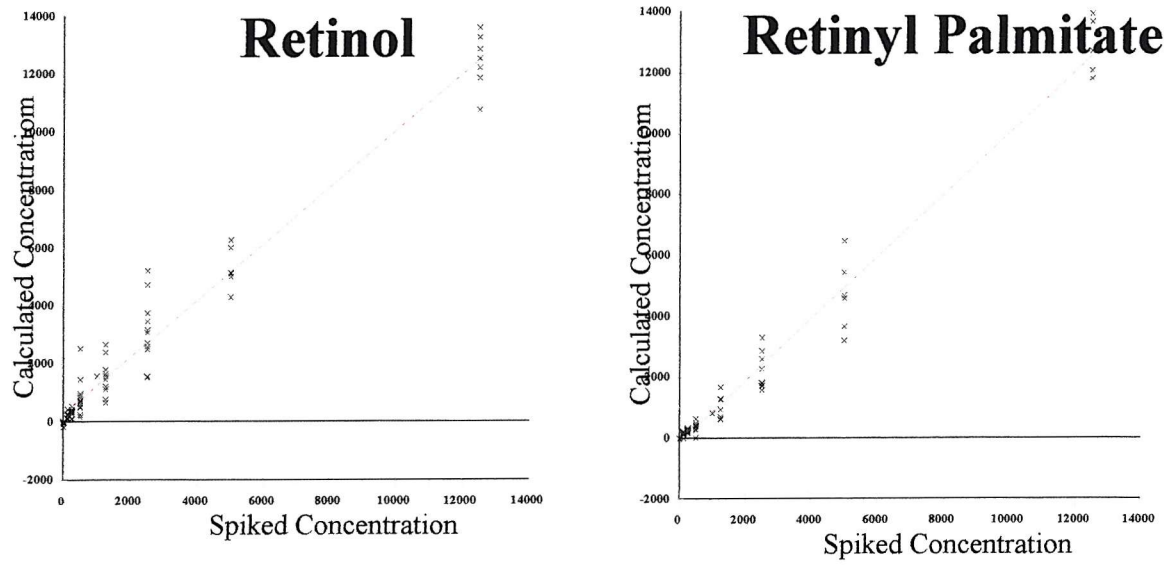
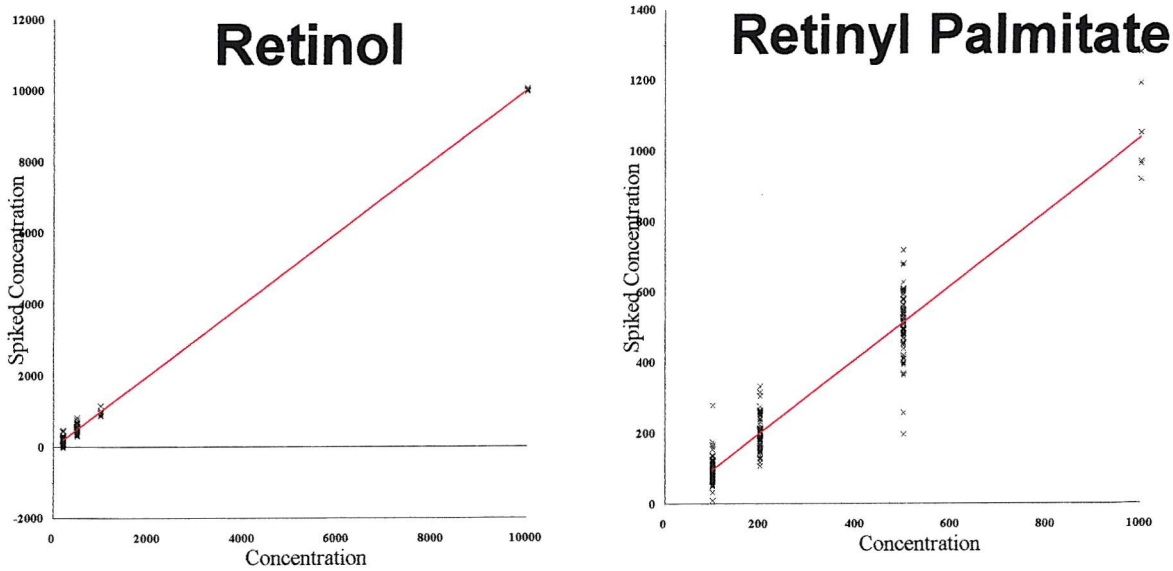


Figure 2.12: Standard regression analysis for retinyl palmitate and retinol analysis within the investigation of the transdermal vitamin A absorption in healthy women of child bearing age (Chapter 6).



2.4 DATA ANALYSIS

Chromatographic analysis was used to calculated plasma concentrations in ng/ml for all samples collected. Levels observed for the retinoic acid metabolites in some samples were at the limit of detection for the system. Due to this fact, the software used for the analysis (LcTalk version 2.03, ThermoSeparation Products, UK) was unable to accurately determined some of the peak areas at baseline levels. In order to overcome this inaccuracy each chromatogram was individually compared to a standard chromatogram and retinoic acid metabolite peaks area calculated by forced integration.

Each sample was analyzed in duplicate. The duplicate values were averaged and this mean value used to plot the concentration-time profile for each subject. This concentration-time profile was used to calculated the pharmacokinetic parameters C^{max}, T^{max}, AUC, $t_{1/2}$ and MRT for that subject. Criteria used to process the data is as follows:

1. All concentrations less than 1 ng/ml were deleted from the concentration-time profile.

2. The terminal slope of the concentration-time profile was identified and data cut back to the last point of the decay before the baseline levels out (figure 2.16).

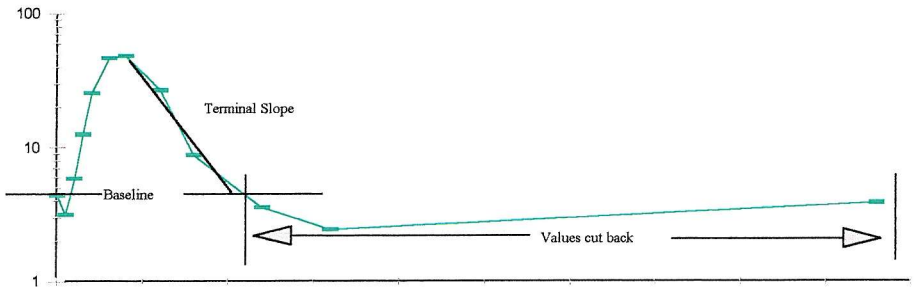


Figure 2.13: Example of data treatment for determination of the pharmacokinetic parameters

3. Baseline values were subtracted from the observed values before calculations were performed figure 2.16)

Software used to determine the pharmacokinetic parameters was WinNonLin standard, version 1.5 (Scientific Consulting Inc, USA). The individual C^{max}, T^{max}, AUC, $t_{1/2}$ and MRT were averaged and the mean values are tabulated within the relevant chapters. The mean concentration-time profiles in the same chapters were plotted form the mean data for each time point of all individual subjects.

A consequence of our method of calculation is that the tabulated mean determined for parameters such as C^{max} and T^{max} could demonstrate a ±10% difference to that observed on the concentration-time profiles shown in each chapter. Using the mean concentration-time profile to calculate the mean pharmacokinetic parameters was not considered. Large variation were observed in the pharmacokinetic parameters of the individual subjects. By averaging the individual subject concentration-time data prior to the calculation of the pharmacokinetic parameters, the variability of the individuals could not illustrated. Further more, it was considered inappropriate to calculate the data in this fashioned since it would bias the results in favour of 1 or 2 individual subjects.

The pharmacokinetic data was statistically examined using paired t-test analysis between two sets of data.

Chapter 3

The Influence of Food and Dosage on the Absorption of Vitamin A and the Formation of its Teratogenic Metabolites

Table of Contents

List of Tables

List of Figures

3.1 INTRODUCTION

In a previous investigation by Buss et al (1) the absorption of vitamin A supplement was compared to that given as a concentrated natural source (calf's liver). Two doses of 50 and 150 mg were given to healthy female volunteers in a cross-over design study. A control was used where an equivalent dose was delivered by intramuscular injection. From this study it was observed that for the 50 mg dose the concentration of retinyl palmitate was higher when given as an oral supplement in direct comparison to an equivalent dose given in the form of a liver meal. No difference was observed for the 150 mg dose and for retinol concentration. The key difference observed was for all-trans-retinoic acid concentrations in that after supplement dosing the levels were up to twenty times higher than after the liver meal dose. The conclusion drawn from these results was that the "occasional consumption" of liver as part of a normal diet would not significantly increase the teratogenic risk by vitamin A metabolites. It can also be concluded that the benefit pregnant women might obtain from such a valuable source of essential nutrients is of greater value than the small potential risk of teratogenicity. This study aims to complement the data generated by Buss et al and to repeat the observations made in comparison of supplement to liver consumption with lower doses. This dose study uses 50 and 15 mg retinol as the retinyl palmitate. Also investigated was the influence of other fatty food components on the absorption of retinol by comparison of a supplement dose on a fasting stomach and after a meat containing meal.

3.2 METHODS

3.2.1 Calf's Liver

A whole calf's liver was purchased from a local retail source (Duke's Butcher's, Chandlers Ford, England). It was then analyzed and specially prepared for consumption by subjects in these studies.

The liver was divided up into portions A-I (Figure 3.1). In addition, a portion J was

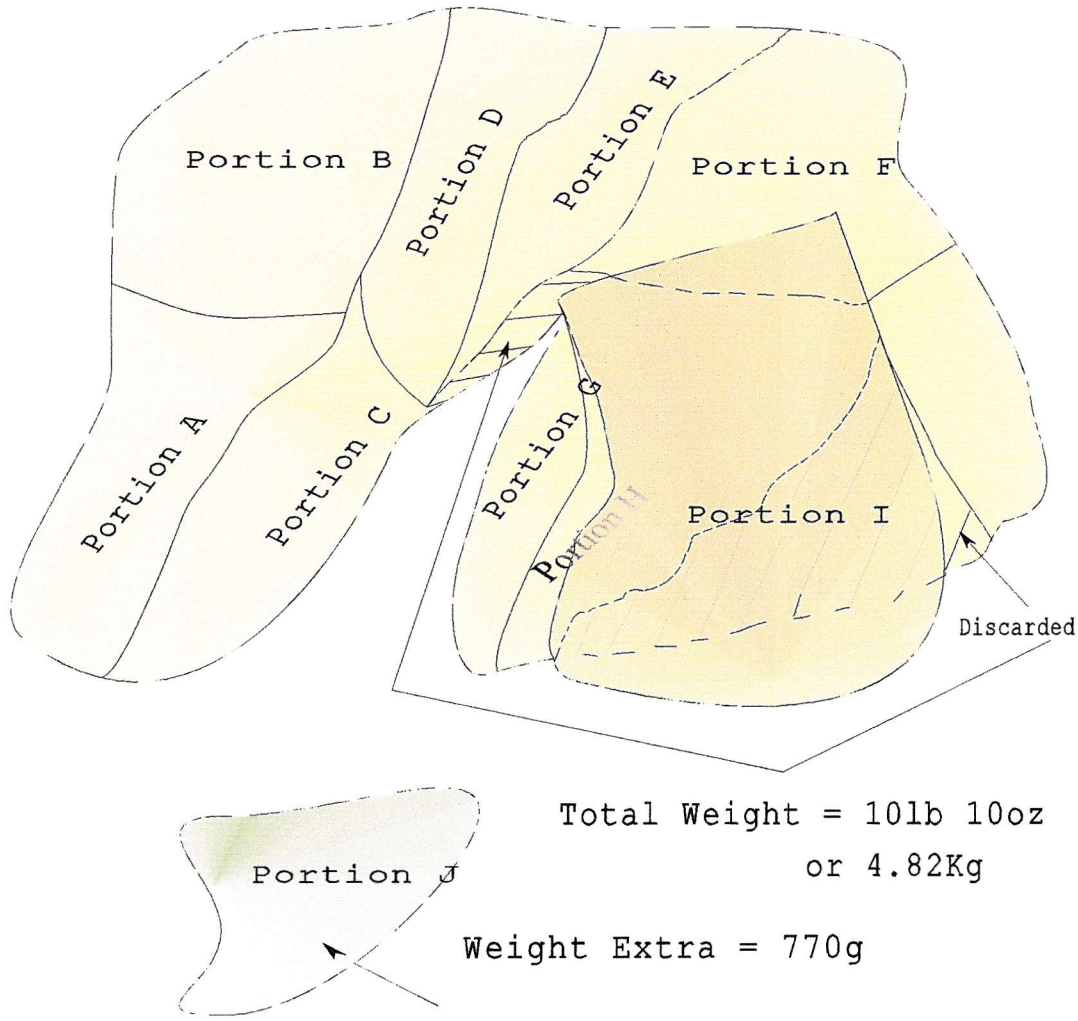


Figure 3.1: - Division of the calf's liver into storage samples used in this study

obtained from a second liver to give sufficient material for subject consumption. To analyze retinoid concentrations of the liver, samples were taken from different sites of portions A-J by trimming one or more slices from each of the different portions. A total of seventeen samples across the liver were taken (Figure 3.2). These were then analyzed for retinol content as outlined in section 2.2.5.2. Table 3.1 shows the retinol content in each of the seventeen samples.

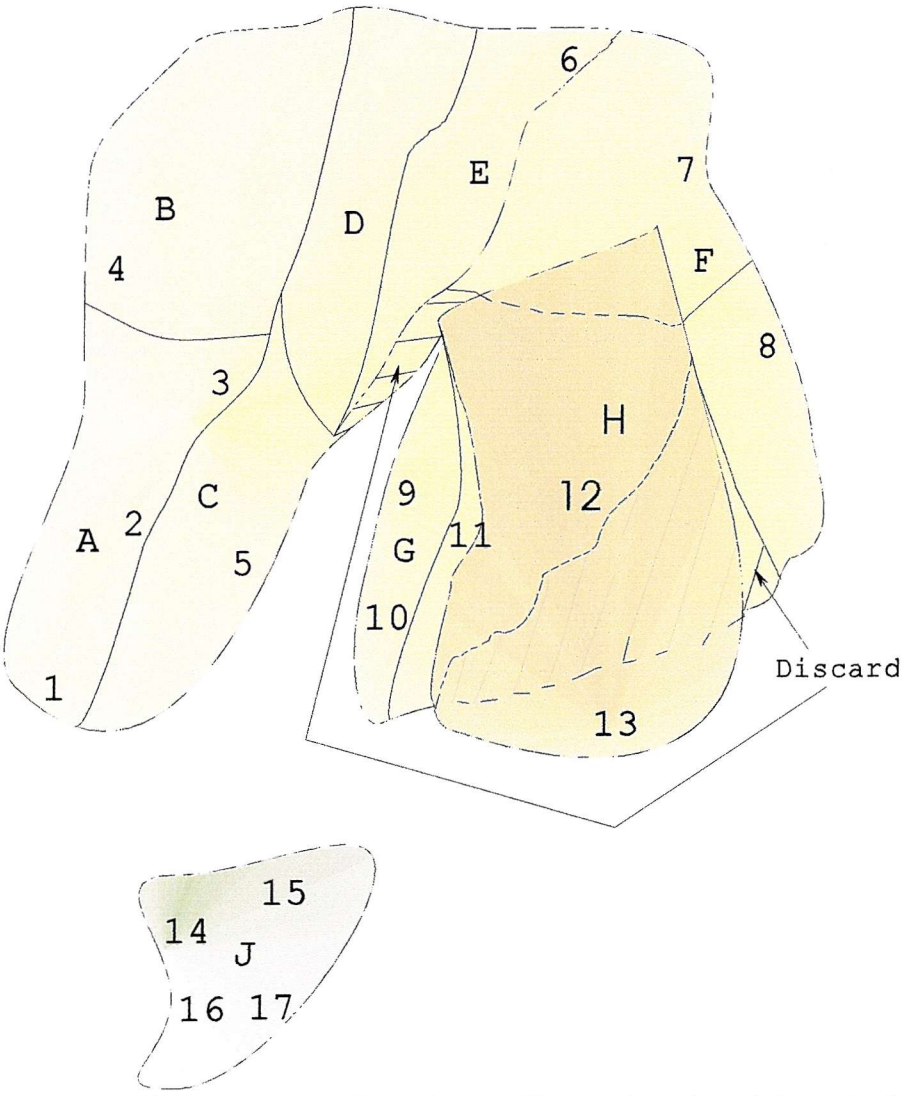


Figure 3.2: - Seventeen samples were taken from the raw liver and analyzed for retinol content.

The mean retinol content for portions A-I was 0.311 mg retinol / gram raw weight. However, the mean retinol content for portion J was 0.609 mg retinol / gram raw weight (table 3.1). Portion J was therefore kept separate and never combined with other portions when dosing subjects.

Table 3.1: - Mean concentrations of retinol in the samples removed from the dosing liver.

Liver Samples	Section Extracted from	Calculated Conc^{n}.(mg/g)
1	A	0.267 ± 0.03
2	A	0.304 ± 0.06
3	A	0.297 ± 0.05
4	B	0.285 ± 0.02
5	C	0.312 ± 0.03
6	E	0.322 ± 0.02
7	F	0.330 ± 0.07
8	F	0.249 ± 0.11
9	G	0.322 ± 0.04
10	G	0.305 ± 0.06
11	H	0.352 ± 0.06
12	I	0.315 ± 0.04
13	I	0.300 ± 0.02
14	J	0.639 ± 0.22
15	J	0.687 ± 0.13
16	J	0.452 ± 0.57 [1]
17	J	0.658 ± 0.15

[1] Sample 16 was analyzed in duplicate, the results obtained were 0.057 mg/g and 0.847 mg/g. Analysis of each re-extracted sample was repeated and similar results obtained, 0.061 mg/g and 0.901 mg/g. A mean of all results was taken and used to calculate mean concentration of liver section J.

Liver portions were frozen at -20 °C until needed. Prior to cooking, liver portions were defrosted to room temperature. They were then cut into 5-8 mm slices to give an appropriate weight necessary for the retinol dose. The total weight sliced contained sufficient retinol to dose the subjects receiving liver that day. Liver slices were seasoned with pepper and fried in vegetable oil which contained negligible amounts of vitamin A. The slices were cooled, blotted and re-weighed. The appropriate amount of cooked liver slices were then cut into equal pieces of ' liver fingers '. The ' liver fingers ' were randomly divided between the subject's plates, so that each subject received an equal amount of liver. A portion of the cooked liver was retained for analysis and validation of dose (section 3.2.5).

3.2.2 Subjects

Ten healthy female subjects participated in this study. The mean (± s.d.) age, height and weight of the subjects (table 3.2) were respectively 37.1 ± 7.8 years (range 24 - 49), 161.4 ± 9.2 cm (range 148 - 177 cm) and 63.2 ± 13.2 kg (range 50 - 84 kg). One subject (1) withdrew from the study for non-medical reasons and was replaced by a new subject for the remaining phases. Consumption of alcohol was within 14 units / week. Four subjects smoked with a maximum of 15 cigarettes per day. Two subjects suffered from asthma, taking (1, replacement) salbutamol inhaler only and (7) salbutamol and beclomethasone inhalers respectively. None of the above named medications were expected to interact with retinoids or to influence the study results.

Table 3.2: - Patient demographics.

Patient	Age	Height (cm)	Weight (Kg)	Previous medical history	Drug history	Smoking habits	Alcohol (unit/wk)	Birth control
1(ABC)	39	169	80.7	---	---	---	14	yes
1(DEF)	40	163	54.7	Asthma	Ventolin	---	---	yes
2	40	164	83.6	---	---	1	4	yes
3	44	156	50.3	---	---	15	---	yes
4	34	163	54.7	---	---	---	---	yes
5	40	160.5	54.8	---	---	---	---	yes
6	27	---	---	---	---	---	---	yes
7	37	177	70.1	Asthma	Ventolin	---	8	yes
8	49	154	51.5	---	---	10	---	yes
9	24	147.5	59.7	---	---	5-10	14	yes
10	26	174	72.9	---	---	---	---	yes

3.2.3 Study Design and Treatment

The study was designed as an open randomized six way cross-over study. Subjects attended on six separate study days, each study day was separated by a period of not less than 4 weeks (table 3.3). On each study day subjects were randomly (table 3.3) given one of the six treatments described below. Venous blood samples (10ml) were collected into heparinized tubes by use of an intravenous cannula inserted into the forearm. Blood samples were collected at the following post-treatment time period : 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 32, 48, 72, 96 and 120 hours.

Table 3.3: - Randomized subject treatment days, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal

Patient	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	Phase C	Phase A	Phase B	Phase F	Phase D	Phase E
2	Phase B	Phase C	Phase A	Phase E	Phase F	Phase D
3	Phase C	Phase B	Phase A	Phase F	Phase E	Phase D
4	Phase A	Phase C	Phase B	Phase D	Phase F	PhaseE
5	Phase C	Phase B	Phase A	Phase F	Phase E	Phase D
6	Phase A	Phase C	Phase B	Phase D	Phase F	Phase E
7	Phase B	Phase A	Phase C	Phase D	Phase F	Phase E
8	Phase B	Phase C	Phase A	Phase E	Phase F	Phase D
9	Phase C	Phase B	Phase A	Phase F	Phase E	Phase D
10	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F

The six different treatments were as follows:

A. 50 mg retinol as the retinyl palmitate in the form of 1.18 g Arovit after an overnight fast.

B. 50 mg retinol as the retinyl palmitate present in fried calf's (approximately 120.1 ±7.1 g) liver with approximately 80 g hash brown potatoes, and approximately 80 g plum tomatoes or baked beans.

C. 50 mg retinol as the retinyl palmitate in the form of 1.18 g Arovit which was given 2 ± 1 min after a meal consisting of approximately 80 g hash brown potatoes, approximately 80 g plum tomatoes or baked beans, and three beef burgers. The three beef burgers had a similar raw weight, fat and meat content as the calf's liver

D. 15 mg retinol as the retinyl palmitate in the form of 1.18 g Arovit after an overnight fast.

E. 15 mg retinol as the retinyl palmitate present in fried calf's (approximately 120.1 ±7.1 g) liver with approximately 80 g hash brown potatoes, and approximately 80 g plum tomatoes or baked beans.

F. 15 mg retinol as the retinyl palmitate in the form of 1.18 g Arovit which was given 2 ± 1 min after a meal consisting of approximately 80 g hash brown potatoes, approximately 80 g plum tomatoes or baked beans, and three beef burgers. The three beef burgers had a similar raw weight, fat and meat content as the calf's liver. The burger's were analyzed and determined to contain negligible amounts of vitamin A. Preparation and materials used for the burgers was identical to the calf's liver.

3.2.4 Sample Integrity

3.2.4.1 Pre-dose analysis of vitamin A

All pre-dose plasma samples were analyzed for endogenous levels of the following compounds:

All-trans-retinoic Acid ----------	TRA
13-cis-retinoic Acid----------	CRA
Retinyl Palmitate----------	RP
All-trans-retinol----------	Rol
13-cis-4-oxo-retinoic Acid----------	4-oxo CRA
All-trans-4-oxo-retinoic Acid------	4-oxo TRA
9-cis-retinoic Acid----------	9 CRA

Tables 3.4 and 3.5 show the pre-dose levels found for all patients across all study days for each of the seven compounds under investigation.

Table 3.4: - Pre-dose levels for phase A, B and C; 50 mg dose. All values quoted are in ng/ml

Subject	TRA	CRA	RP	Rol	4-oxo CRA	4-oxo TRA	9 CRA
1	0 0	1.64 ± 0.8	18.80 ± 10.1	892.2 ± 62.5	1.09 ± 1.9	0 ± 0	1.34 ± 1.1
2	2.39 ± 1	1.82 ± 0.4	22.87 ± 6.2	731.3 ± 97.4	3.67 ± 2.4	1.46 ± 0	0.67 ± 1
3	0 ± 0	1.95 ± 0.6	18.72 ± 6.6	757.3 ± 32.6	2.67 ± 0.7	1.04 ± 0.4	1.45 ± 0
4	0 ± 0	1.23 ± 1.0	35.59 ± 16.1	870.9 ± 41.44	1.09 ± 1.6	0 ± 0	1.34 ± 0.9
5	3.84 ± 2.4	4.0 ± 2.5	15.4 ± 3.8	517.7 ± 93.6	2.69 ± 1.5	1.37 ± 1.9	0 ± 0
6	1.58 ± 0	1.85 ± 0	14.60 ± 2.5	609.83 ± 79.9	0 ± 0	0 ± 0	0 ± 0
7	1.38 ± 0.7	4.00 ± 3.95	23.85 ± 13.5	413.73 ± 25.2	3.81 ± 1.76	2.61 ± 2.56	2.24 ± 1.06
8	1.13 ± 0.4	0 ± 0	41.77 ± 10.7	623.73 ± 47.3	2.47 ± 1.8	0 ± 0	1.94 ± 0.9
9	0 ± 0	1.49 ± 0.31	14.03 ± 12.5	570.3 ± 150.4	3.51 ± 1.4	0 ± 0	1.89 ± 1.3
10	1.63 ± 0.2	1.39 ± 0.2	19.17 ± 7.4	633.6 ± 35.4	3.27 ± 0.2	0 ± 0	2.62 ± 0.3

Note : - Values indicated by 0 ± 0 are expressed as such since the calculated value was below the limit of detection for the analytical methods.

Table 3.5: - Pre-dose levels for phase D, E and F; 15 mg dose. All values quoted are in ng/ml

Subject	TRA	CRA	RP	Rol	4-oxo CRA	4-oxo TRA	9 CRA
1	1.0 ± 0.5	1.4 ± 0.2	12.3 ± 0.7	483.3 ± 55.2	1.7 ± 0.2	0.3 ± 0.3	2.9 ± 2.1
2	0± 0	4.2 ± 0.2	25.4 ± 61.7	672.2 ± 41.6	NA	35.4 ± 48.5	3.4 ± 2.4
3	1.9 ± 0.6	1.2 ± 0.2	18.5 ± 2.7	839.1 ± 92.8	3.6 ± 1.2	3.1 ± 2.4	7.8 ± 6.0
4	1.0 ± 0.5	1.4 ± 0.2	12.3 ± 0.7	483.6 ± 55.2	1.7 ± 0.2	0 ± 0	2.9 ± 2.1
5	6.6 ± 2.0	5.6 ± 0.6	23.9 ± 4.5	508.0 ± 83.8	8.6 ± 3.3	2.2 ± 1.5	3.5 ± 1.3
6	3.0 ±2.3	3.9 ± 1.6	18.3 ± 3.7	760.2 ± 83.0	2.1 ± 0.9	1.1 ± 1.1	4.6 ± 3.4
7	2.0 ± 0.3	2.5 ± 1.1	14.2 ± 1.8	429.2 ± 9.5	4.0 ± 0.8	0 ± 0	2.0 ± 0.7
8	1.7 ± 0.1	1.3 ± 0.5	108.0 ± 45.1	798.8 ± 160.2	2.1 ± 0.4	1.0 ± 0.3	1.9 ± 0.4
9	1.1 ± 0.2	1.9 ± 0.3	37.1 ± 3.7	767.5 ± 154.5	87.8 ± 119.0	0 ± 0	1.9 ± 0.8
10	3.0 ± 0.3	1.8 ± 0.6	22.1 ± 8.7	611.2 ± 64.4	2.6 ± 1.5	3.1 ± 2.5	3.8 ± 2.2

Note : - Values indicated by 0 ± 0 are expressed as such since the calculated value was below the set limit of detection for the analytical methods.
Note: - NA is used for data that was deleted from the study due to analytical problems

Trends within the data seen in tables 3.4 and 3.5 indicate that each subject had similar baseline levels between the two sets of dose levels given. Individual variability is evident between subjects and some variability can be observed between pre-dose levels of certain subjects to a greater degree than others. Despite the pre-study day fast there is evidence of most compounds of interest being present for each subject.

3.2.4.2 Dose verification

During phases B and E as outlined in section 3.2.1 samples of the cooked liver used for each patient dose was set aside for re-analysis to confirm dose given. Table 3.6 illustrates the re-analysis results obtained for the cooked liver slices. 0.3 g of cooked liver was analyzed and a conversion factor of raw weight to cooked weight is used to calculated the concentration of the dose given. Each dose given was identified by the proportion of liver segment used in the food preparation and a mean concentration for all cooked liver portions can be compared to the original analysis as outlined in Table 3.1.

Table 3.6: - Re-analysis of cooked liver samples used for subject dosing

Phase	Subjects	Portions used	Sample Analysis	Cooked to Raw Conversion Factor	Calculated Raw Concentration
E	1&4	UNKNOWN	0.579 mg/g	1.565	0.370 mg/g
E	2	UNKNOWN	No Sample Left	1.5746	No Data
E	3	I(100 g)	0.281 mg/g	1.6384	0.171 mg/g
E	5 & 9	H (300 g)	0.267 mg/g	1.541	0.173 mg/g
E	6	J(150 g)	No sample Left	1.614	No Data
E	7	UNKNOWN	0.551 mg/g	1.653	0.333 mg/g
E	10	H(300 g)	0.567 mg/g	1.700	0.333 mg/g
B	1 & 4	E(300 g) + F(300 g)	0.636 mg/g	1.349	0.472 mg/g
B	2	A(100 g) + B(100 g)	No sample Left	1.3236	No Data
B	3 & 10	A(300 g) + D(300 g)	0.715 mg/g	1.248	0.573 mg/g
B	5 & 9	E(100 g) + B(300 g) + D(100 g)	0.894 mg/g	1.287	0.699 mg/g
E	8	H(100 g)	0.309 mg/g	1.522	0.203 mg/g
B	6	J(100 g)	0.465 mg/g	1.580	0.294 mg/g
B	7	G(300 g)	0.367 mg/g	1.416	0.260 mg/g
B	8	UNKNOWN	0.257 mg/g	1.335	0.193 mg/

3.3 RESULTS

3.3.1 General Concentration Profiles

Each subject under went six separate phases within the study. One subject withdrew from the study after phases A, B and C for non medical reasons. A substitute subject was recruited to complete phases D, E and F. However, pharmacokinetic data from the original and the substitute subjects were very different and therefore could not be compared to each other.

Subject 8 exhibited excessive levels of all metabolites for all phases. The high variation observed in the results were directly attributable to this single subject. Profiles of this subject, however, demonstrated an identical pattern to all other subjects.

3.3.1.1 Phase A: - 50 mg retinol as the retinyl palmitate taken as a liquid oral supplement on a fasting stomach.

The concentration profile after 50 mg of retinol as the retinyl palmitate is shown in figure 3.3. Post-dosing, the concentration of 13-cis-retinoic acid , all-trans-retinoic acid and all-trans-4-oxo-retinoic acid increased rapidly to a peak value (T^{max} 2.0 ± 0.8, 1.8 ± 0.92 and 2.4 ± 1.9 hrs respectively). 1- Cis-4-oxo-retinoic acid and retinyl palmitate also increased in concentration, however at a slower rate (T^{max} 6.4 ± 1.7 and 4.7 ± 1.2 hrs respectively). Retinol demonstrated a very small increase relative to the other compounds of interest, reaching a peak at a similar time to retinyl palmitate. All-trans-retinoic acid and all trans-4-oxo-retinoic acid returned to pre-dose baseline levels

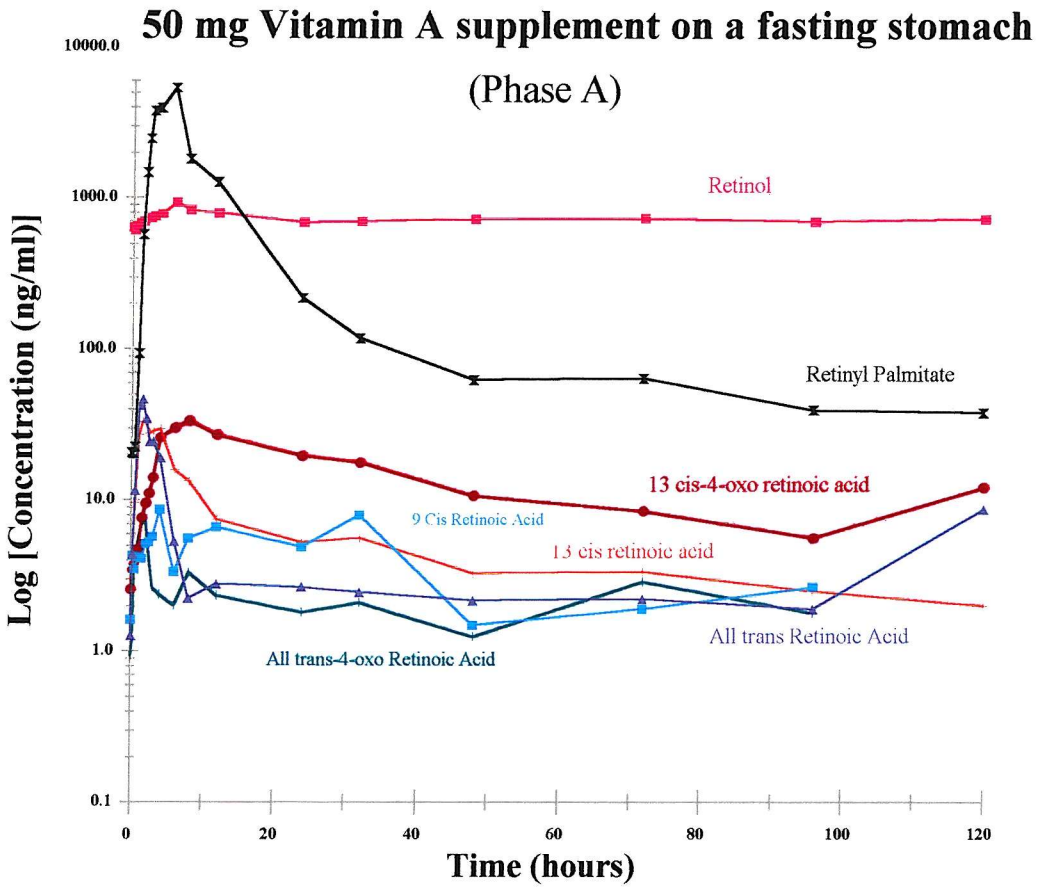


Figure 3.3: - Mean concentration data for phase A , where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

within 8 hours of the dose. 13-cis-retinoic acid and retinyl palmitate returned to pre-dose baseline levels at a slower rate, reaching baseline 10-20 hours post dose. 13-cis-4-oxo-retinoic acid decreased in concentration very slowly reaching baseline after 96 hours. All-trans-retinol remained stable on or around baseline levels after the initial slight rise in concentration. For this treatment, 9-cis-retinoic acid demonstrated fluctuating plasma concentrations without an obvious peak concentrations Pharmacokinetic parameters determined for each compound are given in table 3.7-3.11

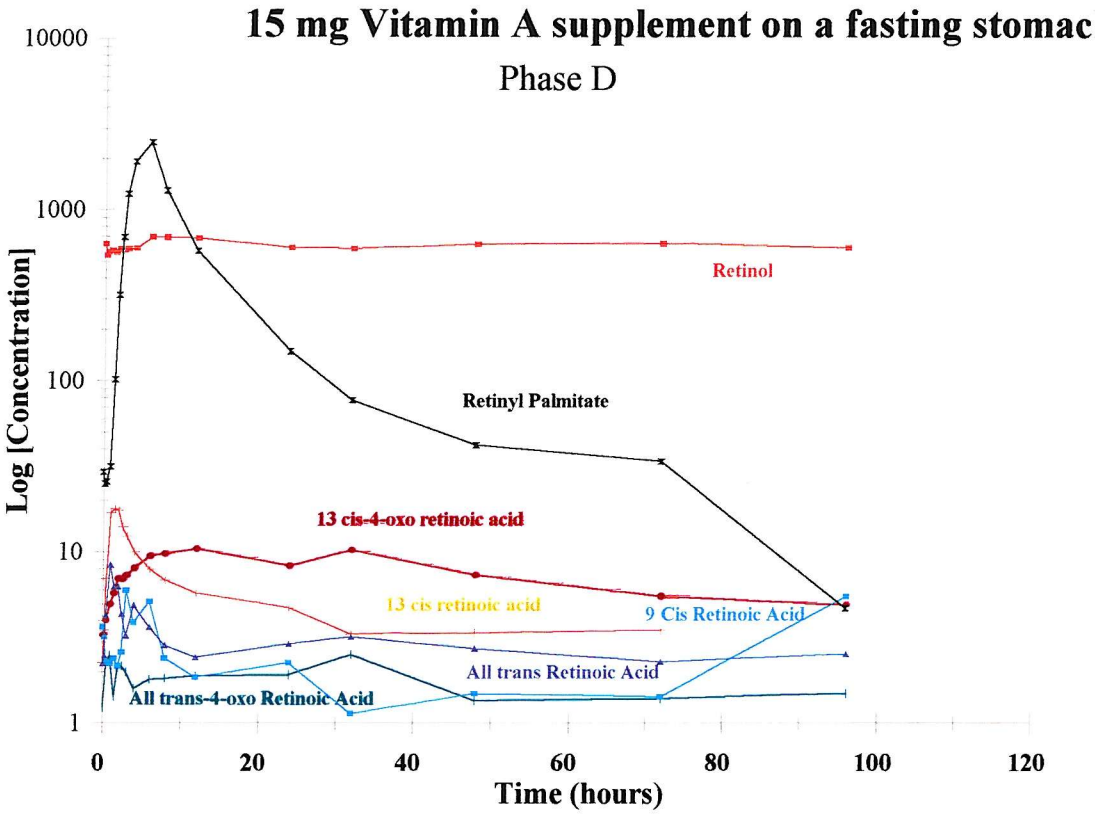


Figure 3.4: - Mean concentration data for phase D, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

3.3.1.2 Phase D: -15 mg retinol as the retinyl palmitate taken as a liquid oral supplement on a fasting stomach.

A similar time profile is observed for the 15 mg supplement given on a fasting stomach as to that of the 50 mg supplement given under identical conditions (figure 3.4). However, the levels observed for the metabolites were much lower than those observed for 50 mg supplement. Pharmacokinetic parameters for phase D were subject to variations due to the lower levels of metabolites found in the plasma. For some subjects, parameters for all-trans-4-oxo-retinoic acid and all-trans-retinoic acid could

not be determined due to the low concentrations detected in the plasma. However, higher plasma concentrations enabled pharmacokinetic parameters to be determined for 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid. Comparison of the determined pharmacokinetic parameters to those of the 50mg supplement demonstrated similar T^{max} values. In both cases it was observed that 13-cis-retinoic acid had a shorter T^{max} than 13-cis-4-oxo-retinoic acid (T^{max}_{CRA}= 1.9 ± 1.4 hrs and 12.7 ± 8.9 hrs respectively). This is consistent with previous investigations which have demonstrated that 13-cis-4-oxo-retinoic is a metabolite of 13-cis-retinoic acid.

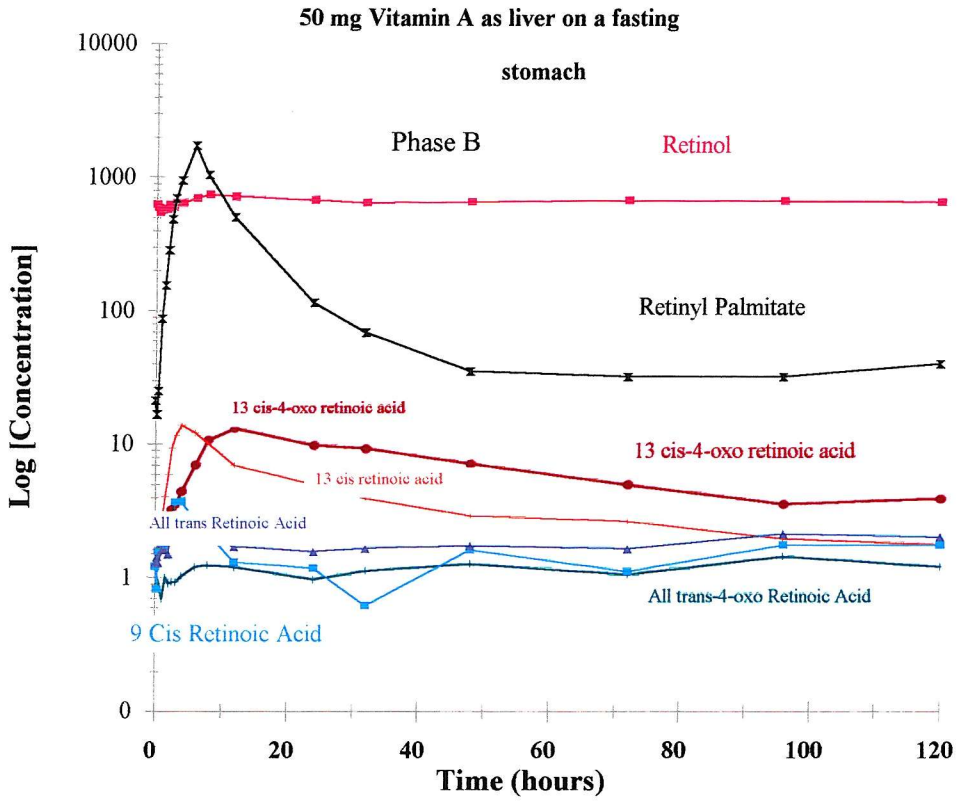


Figure 3.5: - Mean concentration data for phase B, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

3.3.1.3 Phase B and E: - 50 mg and 15 mg retinol as the palmitate taken in the form of cooked calf's liver.

The second phase of this study consisted of 50 mg (figure 3.5) and 15 mg (figure 3.6) dosing with a controlled meal containing cooked calf's liver as the source of the retinol as the palmitate. Retinyl palmitate concentrations were about 2-fold higher after the 50 mg liver dose compared with the 15 mg liver dose. In general, the levels of all the acid metabolites were reduced in comparison to the 50 mg and 15 mg supplement dosing. It was found that C^{max} data (table 3.6) of all-trans-retinoic acid, all-trans-4-oxo -retinoic

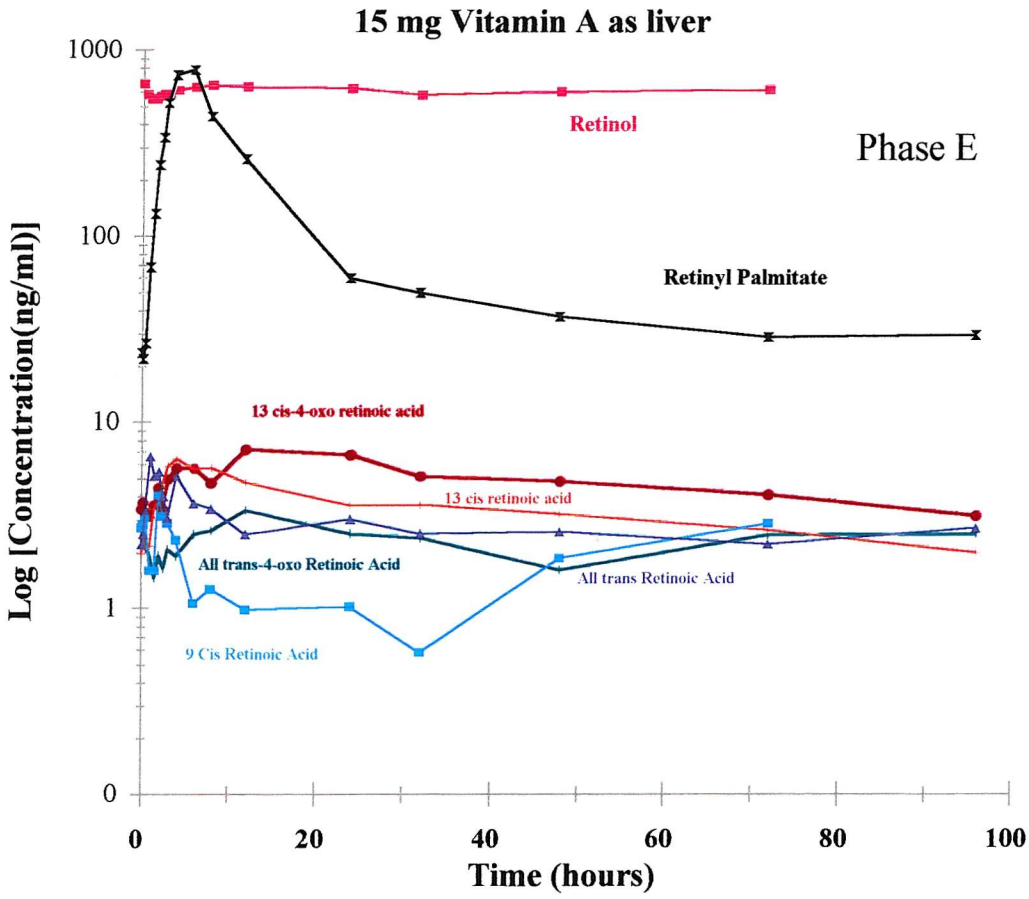


Figure 3.6: - Mean concentration data for phase E, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal

acid and 9-cis-retinoic acid for the 50 mg liver dose were very similar to those observed for the 15 mg liver dose. However, 13-cis-retinoic acid and retinyl palmitate were 1.5-2 fold higher for the 50 mg liver dose compared to the 15 mg liver dose. Retinol levels were again essentially constant at 700-1000 ng/ml ± 10% for both the 50 mg liver dose and the 15 mg liver dose. For all-trans-retinoic acid and all-trans-4-oxo-retinoic acid concentrations observed were at the limit of quantitation and baseline fluctuation effects are seen. Some pharmacokinetic parameters such as $T^{1}/_{2}$, MRT and AUC could not be determined due to the interference by the endogenous levels detected. However, 9 cis retinoic acid was detectable in both phases B and E with peak concentrations at 3-5 hrs (C^{max}_{B}= 3.3 ± 1.7; C^{max}_{C}= 2.2 ± 0.7 ng/ml respectively. This was very similar to the 15 mg supplement dosing but the 50 mg supplement dosing was 3-4 fold higher.

3.3.1.4 Phases C and F: - 50 and 15 mg vitamin A supplement following a standard cooked meal.

The concentration profile after 50 mg supplement following a standard meal is shown in figure 3.7. Post dosing, the concentrations of 13-cis-retinoic acid , all-trans-retinoic acid, retinyl palmitate and all-trans-4-oxo-retinoic acid increased rapidly to a peak value ($T^{max}_{C=}$ 2.2 ± 0.5, 6.3 ± 13.9, 2.6 ± 0.7 and 3.2 ± 2.5 hrs respectively). 13-cis-4-oxo-retinoic acid also increased in concentration, however at a slower rate ($T^{max}_{C=}$ 9.2 ± 3.1). Retinol showed a relatively small increase compared to the other compounds of interest, reaching a peak later than retinyl palmitate (T^{max}_{C} = 6.8 ± 2.2). All trans retinol remained stable at or around baseline levels after reaching the initial low rise

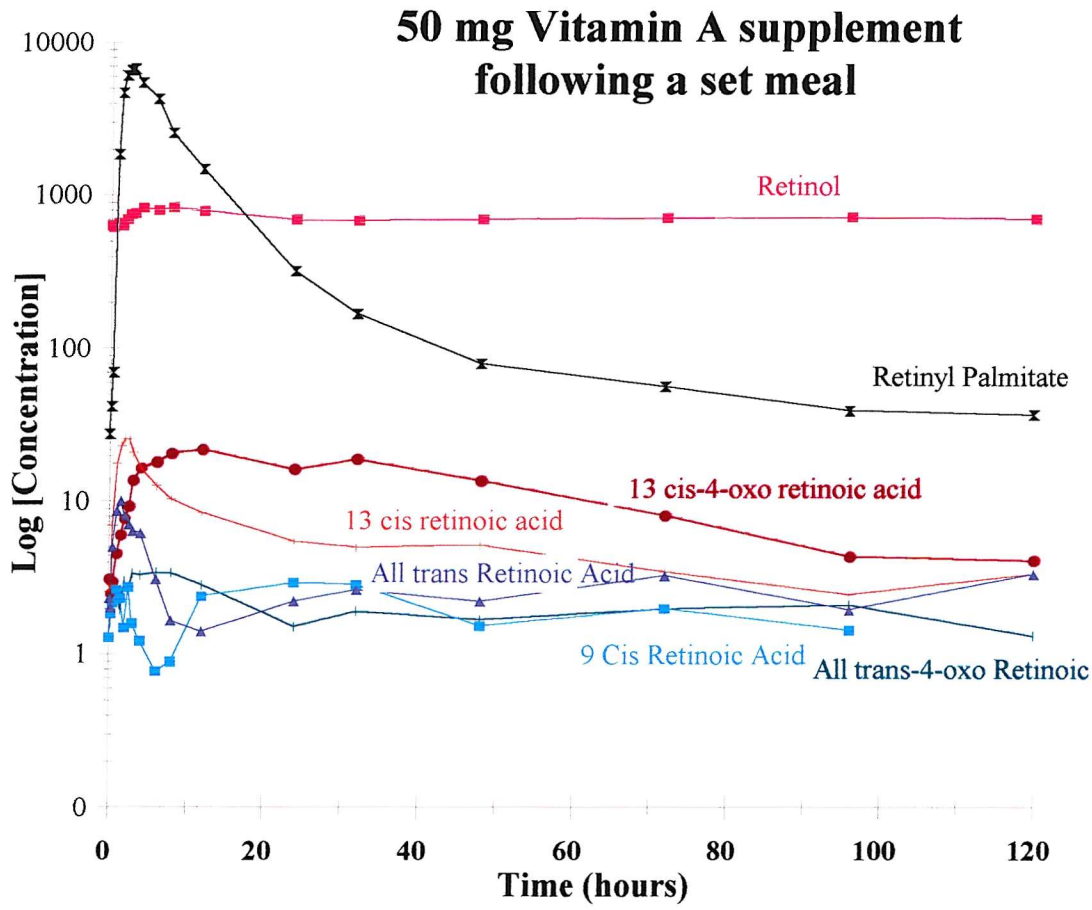


Figure 3.7: - Mean concentration data for phase C, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal

in concentration. The concentration - time profiles were similar to the 50 mg supplement on a fasting stomach as described in section 3.4.1.1. Pharmacokinetic parameters determined for each compound are given in tables 3.7-3.11. A similar profile was observed for the 15 mg supplement following a standard meal (figure 3.8). However, the levels observed for retinyl palmitate, all-trans-retinoic acid and the 13-cis metabolites were lower of those observed for 50 mg supplement. For 9-cis-retinoic acid the plasma levels observed were higher than for the 50 mg supplement. The 15 mg

supplement all-trans-4-oxo-retinoic acid plasma levels were similar to that of the 50 mg supplement. Pharmacokinetic parameters for phase F were subject to fluctuations due to the lower levels of metabolites found in the plasma. For some subjects all-trans-4-oxo-retinoic acid, 9-cis-retinoic acid and all-trans-retinoic acid parameters could not be determined due to the absence of detectable increased post-dose amounts in the plasma.

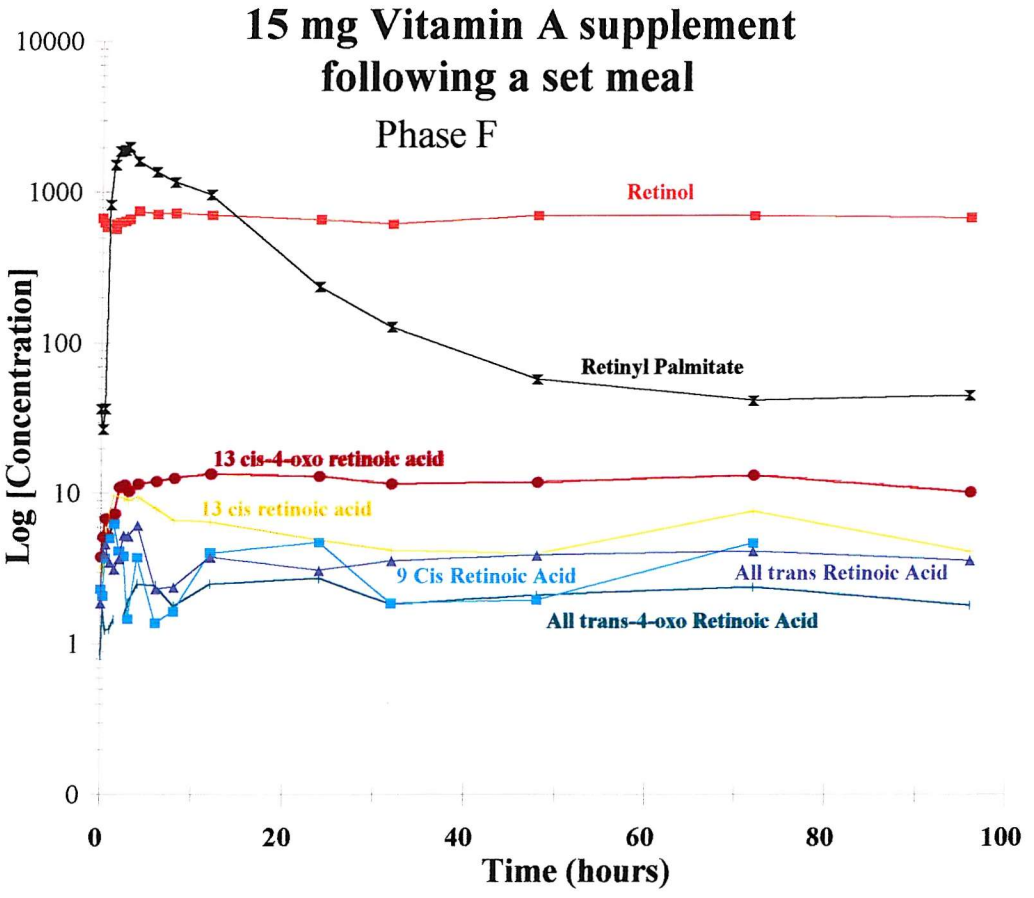


Figure 3.8: - Mean concentration data for phase F, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal

Table 3.7: - Mean pharmacokinetic parameter T^{max} for phase A; phase B; phase C; phase D; phase E and phase F. All results are recorded in hours unless otherwise indicated. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F
all-trans-4-oxo Retinoic Acid	2.4 ± 1.9	4.7 ± 4.5	3.2 ± 2.5	2.1 ± 2.1	6.3 ± 3.5	5.6 ± 3.4
13 cis-4-oxo retinoic acid	6.4 ± 1.7	10.3 ± 8.4	9.2 ± 3.1	12.7 ± 8.9	14.6 ± 14.3	20.4 ± 19.8
13 cis retinoic acid	2.0 ± 0.8	4.3 ± 1.0	2.2 ± 0.5	1.9 ± 1.4	4.9 ± 2.4	2.3 ± 1.1
9 cis retinoic acid	3.0 ± 2.5	2.4 ± 1.3	1.3 ± 0.5	6.5 ± 11.4	1.8 ± 0.8	2.4 ± 2.4
all trans retinoic acid	1.8 ± 0.9	4.3 ± 2.5	6.3 ± 13.9	1.5 ± 1.0	4.6 ± 2.9	2.3 ± 1.0
retinol	5.8 ± 2.1	7.5 ± 2.2	6.8 ± 2.2	8.3 ± 2.3	4.5 ± 2.2	13.9 ± 20.3
retinyl palmitate	4.7 ± 1.2	6.0 ± 0.0	2.6 ± 0.7	4.9 ± 1.3	6.0 ± 0.9	2.8 ± 1.9

Table 3.8: - Mean pharmacokinetic parameter C_{max} for phase A; phase B; phase C; phase D; phase E and phase F. All results are recorded in ng/ml unless otherwise indicated. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F
all-trans-4-oxo Retinoic Acid	8.6 ± 6.7	1.1 ± 0.5	3.5 ± 2.7	1.8 ± 1.0	3.0 ± 4.3	3.8 ± 5.3
13 cis-4-oxo retinoic acid	31.9 ± 30.7	9.7 ± 6.2	20.8 ± 16.2	8.2 ± 6.0	5.5 ± 7.0	11.8 ± 7.2
13 cis retinoic acid	50.6 ± 28.7	12.4 ± 4.8	24.8 ± 24.3	19.1 ± 17.2	4.8 ± 5.2	9.5 ± 7.2
9 cis retinoic acid	10.1 ± 18.4	3.3 ± 1.7	2.2 ± 0.7	4.8 ± 5.3	4.1 ± 4.6	7.5 ± 7.8
all trans retinoic acid	63.8 ± 38.1	2.4 ± 0.7	9.9 ± 8.5	7.5 ± 6.4	2.3 ± 0.5	4.8 ± 4.0
retinol	336.7 ± 207.0	140.5 ± 89.5	272.8 ± 147.6	102.0 ± 33.9	109.1 ± 89.3	139.9 ± 90.7
retinyl palmitate	6823 ± 5606	1682 ± 977	8228 ± 5703	3018 ± 1261	703 ± 478	2463 ± 2264

Table 3.9: - Mean pharmacokinetic parameter AUC for phase A; phase B; phase C; phase D; phase E and phase F. All results are recorded in hours unless otherwise indicated. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F
all-trans-4-oxo Retinoic Acid	21.5 ± 22.7	6.9 ± 6.6	40.0 ± 61.7	5.1 ± 7.6	27.6 ± 30.9	60.3 ± 134.7
13 cis-4-oxo retinoic acid	768.1 ± 508.8	318.2 ± 268.4	581.2 ± 390.5	221.3 ± 111.0	117.2 ± 160.2	236.7 ± 91.7
13 cis retinoic acid	209.6 ± 142.2	151.6 ± 62.2	183.4 ± 179.1	91.7 ± 104.3	65.7 ± 123.7	62.6 ± 58.1
9 cis retinoic acid	115.3 ± 286.4	13.7 ± 13.9	3.1 ± 1.8	33.7 ± 51.3	8.2 ± 10.9	20.5 ± 17.6
all trans retinoic acid	139.3 ± 136.9	8.6 ± 4.3	50.7 ± 85.7	26.6 ± 25.7	13.7 ± 8.4	24.0 ± 36.9
retinol	2873 ± 1261	1855 ± 1863	3467 ± 2380	1519 ± 992	1307 ± 874	2085 ± 3502
retinyl palmitate	38563 ± 33648	13446 ± 11114	54738 ± 60120	18953 ± 15284	5970 ± 6495	23165 ± 43010

Table 3.10: - Mean Pharmacokinetic Parameter $t_{1/2}$ for phase A; phase B; phase C; phase D; phase E and phase F. All results are recorded in hours unless otherwise indicated. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F
all-trans-4-oxo Retinoic Acid	1.0 ± 1.2	8.0 ± 7.5	11.0 ± 15.1	2.2 ± 2.4	14.0 ± 14.0	7.6 ± 8.5
13 cis-4-oxo retinoic acid	21.1 ± 10.8	16.9 ± 14.4	21.7 ± 10.6	28.6 ± 26.8	15.9 ± 16.9	17.4 ± 9.2
13 cis retinoic acid	5.1 ± 3.9	11.7 ± 3.3	8.7 ± 7.2	4.8 ± 4.8	14.2 ± 11.2	5.8 ± 4.9
9 cis retinoic acid	3.6 ± 4.1	2.5 ± 1.9	0.9 ± 1.0	5.4 ± 4.4	3.2 ± 2.2	7.0 ± 9.8
all trans retinoic acid	2.8 ± 3.4	3.2 ± 1.2	16.2 ± 44.1	8.2 ± 17.1	5.8 ± 5.8	6.8 ± 12.2
retinol	18.0 ± 29.2	13.0 ± 9.0	16.0 ± 15.6	15.6 ± 14.8	21.9 ± 16.0	16.7 ± 36.1
retinyl palmitate	10.6 ± 4.1	10.0 ± 6.1	10.0 ± 3.1	7.0 ± 2.8	7.5 ± 2.5	7.7 ± 1.8

Table 3.11: - Mean Pharmacokinetic Parameter MRT for phase A; phase B; phase C; phase D; phase E and phase F. All results are recorded in hours unless otherwise indicated. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

	Phase A	Phase B	Phase C	Phase D	Phase E	Phase F
all-trans-4-oxo Retinoic Acid	2.7 ± 2.7	6.5 ± 4.8	10.9 ± 14.0	2.3 ± 1.7	12.3 ± 6.4	12.5 ±14.1
13 cis-4-oxo retinoic acid	22.7 ± 8.3	21.3 ± 15.4	25.1 ± 12.7	22.5 ± 11.2	17.5 ± 14.3	21.6 ± 9.3
13 cis retinoic acid	5.5 ± 3.3	11.8 ± 4.2	8.2 ± 5.4	5.5 ± 4.1	8.4 ± 4.7	6.2 ± 3.7
9 cis retinoic acid	6.2 ± 5.0	4.1 ± 2.5	1.7 ± 1.0	7.3 ± 10.0	2.8 ± 1.2	6.2 ± 7.4
all trans retinoic acid	2.8 ± 1.8	4.4 ± 1.9	7.6 ± 15.1	7.5 ± 11.9	5.9 ± 3.6	12.2 ± 19.6
retinol	9.8 ± 2.7	12.3 ± 5.3	10.9 ± 3.9	14.0 ± 7.6	11.9 ± 4.3	14.3 ± 17.5
retinyl palmitate	9.5 ± 0.6	10.5 ± 2.6	8.9 ± 1.8	8.5 ± 1.7	10.2 ± 1.8	8.5 ± 2.0

Table 3.12a: - Statistical significance by t-test of pharmacokinetic parameters C^{max}, T^{max}, AUC, $T_{1/2}$ and MRT. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal, and NS= not significant.

Retinol	A vs B	Avs C	B vs C	D vs E	D vs F	E vs F
C^{max}	p<0.05	N.S	p<0.01	N.S	N.S	N.S
T^{max}	N.S	N.S	N.S	p<0.05	N.S	N.S
AUC	N.S	N.S	p<0.05	N.S	N.S	N.S
t½	N.S	N.S	N.S	N.S	N.S	N.S
MRT	N.S	N.S	N.S	N.S	N.S	N.S
Retinyl Palmitate						
C^{max}	p<0.01	N.S	p<0.01	p<0.01	N.S	p<0.01
T^{max}	p<0.01	p<0.01	p<0.01	p<0.05	p<0.05	p<0.01
AUC	p<0.01	N.S	p<0.05	p<0.01	N.S	N.S
t½	N.S	N.S	N.S	N.S	N.S	N.S
MRT	N.S	N.S	p<0.05	p<0.05	N.S	N.S
All-trans-retinoic acid						
C^{max}	p<0.01	p<0.01	p<0.01	p<0.01	N.S	P<0.05
T^{max}	N.S	N.S	N.S	N.S	N.S	N.S
AUC	p<0.01	N.S	N.S	p<0.05	N.S	N.S
t½	N.S	N.S	N.S	N.S	N.S	N.S
MRT	N.S	N.S	N.S	N.S	N.S	N.S
9-Cis-retinoic acid						
C^{max}	N.S	N.S	N.S	N.S	p<0.05	N.S
T^{max}	N.S	N.S	N.S	N.S	N.S	N.S
AUC	N.S	N.S	p<0.05	N.S	N.S	p<0.05
t½	N.S	N.S	N.S	N.S	N.S	N.S
MRT	N.S	p<0.05	p<0.05	N.S	N.S	N.S
13-Cis-retinoic acid						
C^{max}	p<0.01	p<0.01	p<0.05	p<0.01	p<0.01	p<0.05
T^{max}	p<0.01	N.S	p<0.01	p<0.01	N.S	p<0.01
AUC	N.S	N.S	N.S	N.S	N.S	N.S
t½	p<0.01	N.S	N.S	p<0.05	N.S	N.S
MRT	p<0.01	N.S	N.S	N.S	N.S	N.S
13-Cis-4-oxo retinoic acid						
C^{max}	p<0.05	p<0.05	p<0.05	p<0.01	p<0.05	p<0.01
T^{max}	N.S	p<0.05	N.S	N.S	N.S	N.S
AUC	p<0.01	N.S	p<0.05	p<0.05	N.S	p<0.05
t½	N.S	N.S	N.S	N.S	N.S	N.S
MRT	N.S	N.S	N.S	N.S	N.S	N.S
All-trans-4-oxo retinoic acid						
C^{max}	p<0.01	p<0.05	p<0.05	N.S	N.S	N.S
T^{max}	N.S	N.S	N.S	N.S	p<0.01	N.S
AUC	p<0.05	N.S	N.S	p<0.05	N.S	N.S
t½	p<0.05	p<0.05	N.S	N.S	p<0.05	N.S
MRT	p<0.05	N.S	N.S	p<0.01	p<0.05	N.S

Table 3.12b: - Statistical significance by t-test of pharmacokinetic parameters C^{max}, T^{max}, AUC, $T_{1/2}$ and MRT. Where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal, and N.S= not significant.

Retinol	A vs D	B vs E	C vs F
Cmax	$p<0.05$	$p<0.01$	$p<0.05$
Tmax	N.S	$p<0.01$	N.S
AUC	$p<0.01$	N.S	N.S
t½	N.S	N.S	$p<0.05$
MRT	N.S	N.S	N.S
Retinyl Palmitate			
Cmax	$p<0.05$	$p<0.01$	$p<0.01$
Tmax	N.S	N.S	N.S
AUC	$p<0.05$	$p<0.01$	$p<0.01$
t½	$p<0.01$	N.S	$p<0.05$
MRT	N.S	N.S	N.S
All-trans-retinoic acid			
Cmax	$p<0.01$	N.S	$p<0.01$
Tmax	N.S	N.S	N.S
AUC	$p<0.05$	N.S	N.S
t½	N.S	N.S	N.S
MRT	N.S	N.S	N.S
9-Cis-retinoic acid			
Cmax	N.S	N.S	N.S
Tmax	N.S	$p<0.05$	N.S
AUC	N.S	N.S	$p<0.05$
t½	N.S	N.S	N.S
MRT	N.S	N.S	N.S
13-Cis-retinoic acid			
Cmax	$p<0.01$	$p<0.01$	$p<0.05$
Tmax	N.S	N.S	N.S
AUC	$p<0.01$	$p<0.05$	$p<0.01$
t½	N.S	N.S	N.S
MRT	N.S	N.S	N.S
13-Cis-4-oxo retinoic acid			
Cmax	$p<0.01$	N.S	$p<0.05$
Tmax	N.S	N.S	N.S
AUC	$p<0.01$	$p<0.05$	$p<0.01$
t½	N.S	N.S	N.S
MRT	N.S	N.S	N.S
All-trans-4-oxo retinoic acid			
Cmax	$p<0.01$	N.S	N.S
Tmax	N.S	N.S	N.S
AUC	$p<0.01$	N.S	N.S
t½	N.S	N.S	N.S
MRT	N.S	N.S	N.S

3.3.2 Metabolite Concentration Profiles

3.3.2.1 All-trans-retinoic acid

The pharmacokinetic parameters are given in tables 3.7- 3.11 and the concentration-time profile is given in figure 3.9. After dosing, plasma levels rapidly increased to a peak concentration within 4 hour, returning to baseline levels within 8 hours.

Supplement dosing with 50 mg (phases A and C) gave higher plasma levels compared to supplement dosing with 15 mg (phases D and F) in case of a fasting stomach as well as in conjunction with a meal. The AUC and C^{max} of phases A (AUC_A=139.3 ± 136.9 ng/ml hr; $C^{max}{}_A$= 63.8 ± 38.1 ng/ml) and phases C (AUC_C= 50.7 ± 81.3 ng/ml hr; $C^{max}{}_C$=

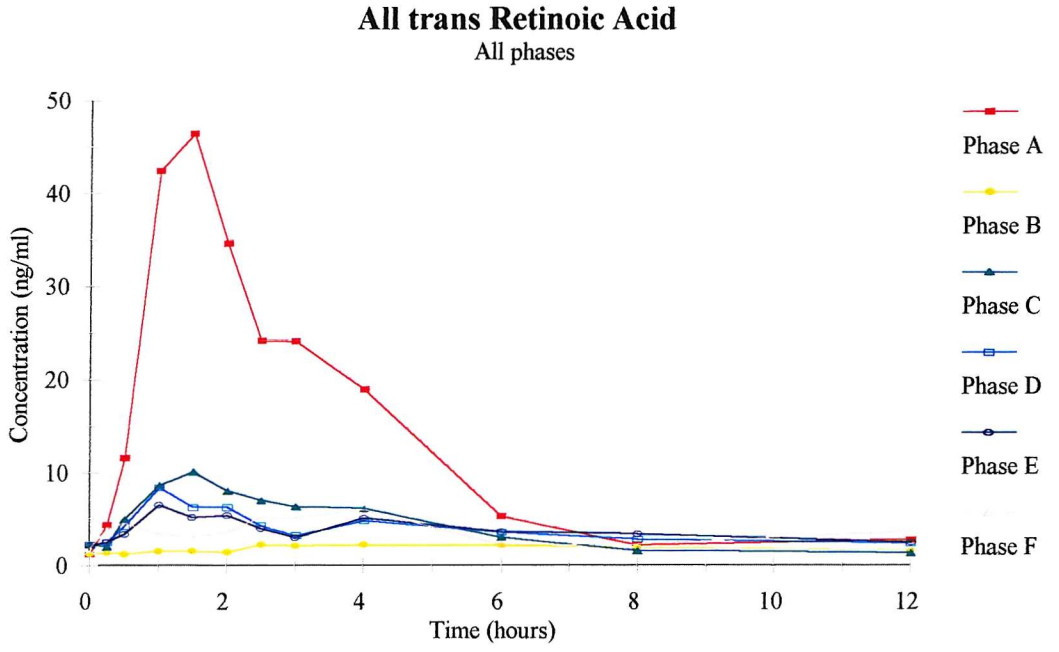


Figure 3.9 : - Mean plasma concentrations for all-trans-retinoic acid, phases A, B, C, D, E and F, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal, and N.S= not significant.

f50.7 ± 81.3 ng/ml) were higher than those of phases D (AUC_D= 26.6 ± 24.4 ng/ml hr; C^{max}_D= 7.5 ± 6.4 ng/ml) and F (AUC_F= 24.0 ± 34.8 ng/ml hr; C^{max}_F= 4.8 ± 4.0 ng/ml) respectively. The T^{max} for phases A, C and D were 1.8 ± 0.9 ng/ml, 6.3 ± 13.9 ng/ml and 1.5 ± 1.0 ng/ml respectively. Phase F had a shorter T^{max} of 2.3 ± 1.0 ng/ml in comparison to phase C but was not calculated to be significantly different.

Liver dosing with 50 mg (phase B) demonstrated similar plasma levels compared to liver dosing with 15mg (phase E). However, for phase B and E, levels of all-trans-retinoic acid were at the limit of quantitation. Accurate analysis was therefore difficult.

Liver (phases B and E) dosing resulted in lower plasma concentrations compared to supplement dosing. The difference was most pronounced in case of the 50 mg treatment on a fasting stomach, where C^{max} supplement dosing (C^{max}_A= 63.8 ± 38.1 ng/ml) was significantly higher than that of liver dosing (C^{max}_B= 2.4 ± 0.7 ng/ml). A similar trend was observed for the 15 mg liver and supplement doses (C^{max}_D= 7.5 ± 6.4 ng/ml; C^{max}_E= 2.3 ± 0.5 ng/ml; C^{max}_F= 4.8 ± 4.0 ng/ml, but to a smaller extent.

Supplement dosing on a fasting stomach (phases A) gave higher plasma levels compared to supplement dosing in conjunction with a meal (phases C) for the high 50 mg treatments. A similar comparison between the lower 15 mg treatments showed equal plasma levels for the two phases. The difference observed in case of the 50 mg supplement treatments showed that the AUC and C^{max} of dosing on a fasting stomach (AUC_A= 139.3 ± 129.9 ng/ml hr; C^{max}_A= 9.9 ± 8.5 ng/ml) were 2-3 fold higher than that seen in conjunction with a meal (AUC_C= 50.7 ± 81.3 ng/ml hr; C^{max}_C= 9.9 ± 8.5 ng/ml). However, the differences determined between the AUC's were not significantly different.

3.3.2.2 Retinyl Palmitate

The pharmacokinetic parameters of retinyl palmitate for all six phases are given in table 3.7 - 3.11 and the concentration-time profile is given in figure 3.10. Concentrations of retinyl palmitate showed a steady increase in plasma concentrations within the first 5 hours post-dose. All phases achieved peak concentrations by 6 hours. Plasma concentrations returned to pre-dose levels by 48 hours for all phases.

Treatments with 50 mg (phases A, B and C) resulted in higher plasma levels compared to treatments with 15 mg (phases D, E and F) for both supplement dosing and liver

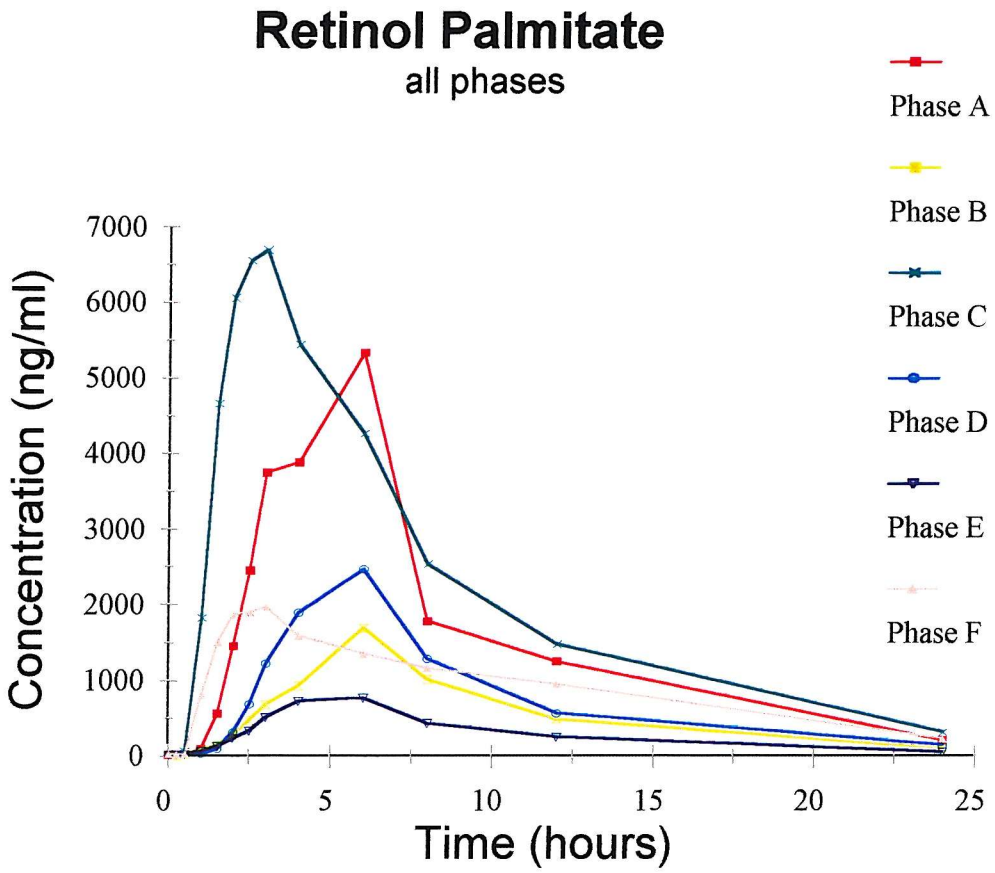


Figure 3.10 : - Retinyl palmitate plasma profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach; phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal

dosing. AUC and C^{max} values of phases A, B and C were significantly higher than those of respectively phases D, E and F. Half-life ($t_{½}$) for phases A and C ($t_{½\,A}$ = 10.6 ± 4.1; t½C = 10.0 ± 3.1) were determined to be significantly different in comparison to phases D and F ($t_{½\,D}$ = 7.0 ± 2.8; $t_{½\,F}$ = 7.7 ± 1.8). Phase B was not calculated to be significantly different from phase E for the parameters t½, T^{max} and MRT. No significant differences were found between the T^{max} and MRT values of 50 mg supplements and the 15 mg supplements.

Liver (phases B and E) dosing resulted in 65-80% lower plasma concentrations compared to supplement dosing for the high 50 mg treatments as well as the low 15 mg treatments. For the 50 mg treatments, the AUC and C^{max} of the liver dosing (AUC_B= 13446 ± 10479 ng/ml hr; $C^{max}{}_B$= 1682 ± 977 ng/ml) were significantly lower than those of the supplement dosing (AUC_A= 38563 ± 31724 ng/ml hr; $C^{max}{}_A$= 6823 ± 5606 ng/ml; AUC_C= 54738 ± 56681 ng/ml hr; $C^{max}{}_C$= 8228 ± 5703 ng/ml). A similar profile can be seen for the 15 mg treatments, where the AUC and C^{max} of phase E (AUC_E= 5970 ± 6123 ng/ml hr; $C^{max}{}_E$= 703 ± 478 ng/ml) showed lower levels compared to those of phase D (AUC_D= 18953 ± 14410 ng/ml hr; $C^{max}{}_D$= 3018 ± 1261 ng/ml) and phase F (AUC_F= 23165 ± 40550 ng/ml hr; $C^{max}{}_F$= 2463 ± 2264 ng/ml). Liver dosing also showed a significantly longer T^{max} compared to supplement dosing in conjunction with a meal. This was observed for both the 50mg treatments ($T^{max}{}_B$= 6.0 ± 0.0 hrs; $T^{max}{}_C$= 2.6 ± 0.7 hrs) as well as the 15 mg treatments ($T^{max}{}_E$= 6.0 ± 0.9 hrs; $T^{max}{}_F$= 2.8 ± 1.9 hrs).

Plasma concentrations for fasting supplement dosing (phases A and D) were similar to those for supplement dosing in conjunction with a meal (phases C and F). However, T^{max} values indicate that 50 mg supplement dosing in conjunction with a meal ($T^{max}{}_C$=

2.6 ± 0.7 hrs) achieved peak concentrations significantly more rapidly than 50 mg supplement dosing on a fasting stomach (T^{max}_A= 4.7 ± 1.2 hrs). The same conclusion can be drawn for the lower 15 mg supplement treatments (T^{max}_F= 2.8 ± 1.9 hrs; T^{max}_D= 4.9 ± 1.3 hrs).

3.3.2.3 13-Cis-retinoic acid

The pharmacokinetic parameters of 13-cis-retinoic acid for all six phases are given in table 3.7 - 3.11 and the concentration-time profile is given in figure 3.11. Peak concentrations were reached between 2-4 hours post-treatment for all phases. All levels returned to near pre-dose concentrations by 24 hours.

Again, treatments with 50 mg (phases A, B and C) gave higher plasma levels compared to treatments with 15mg (phases D, E and F) for both supplement dosing and liver dosing. C^{max} values of phases A, B and C (C^{max}_A= 48.4 ± 32.1 ng/ml; C^{max}_B= 14.5 ± 5.5 ng/ml; C^{max}_C= 27.0 ± 25.5 ng/ml) were significantly higher than those of respectively phases D, E and F (C^{max}_D= 20.3 ± 18.0 ng/ml; C^{max}_E= 6.9 ± 6.1 ng/ml; C^{max}_F= 11.3 ± 8.0 ng/ml). AUC values of phases A, B and C (AUC_A= 209.6 ± 142.2 ng/ml hr; AUC_B= 151.6 ± 62.2 ng/ml hr; AUC_C= 183.4 ± 179.1 ng/ml hr) were significantly higher than those of phases D, E and F (AUC_D= 91.7 ± 104.3 ng/ml hr; AUC_E= 65.7 ± 123.7 ng/ml hr; AUC_F= 62.6 ± 58.1 ng/ml hr). No significant differences were found in the T^{max}, $t_{1/2}$ and MRT values.

Liver (phases B and E) dosing showed lower plasma levels compared to supplement dosing for both the high 50 mg treatments and the low 15 mg treatments. For the 50 mg treatments, the AUC and C^{max} of the liver dosing (AUC_B= 151.6 ± 62.2 ng/ml hr; C^{max}_B= 12.4 ± 4.8 ng/ml) were significantly lower than the levels obtained for the supplement dosing (AUC_A=209.6 ± 142.2 ng/ml hr; C^{max}_A=50.6 ± 28.7 ng/ml; AUC_C= 183.4 ± 179.1 ng/ml; C^{max}_C= 24.8 ± 24.3 ng/ml). The same can be observed for the 15 mg treatments, where the AUC and C^{max} of phase E (AUC_E= 65.7 ng/ml/hr; C^{max}_E= 4.8 ± 5.2 ng/ml) showed significantly lower levels compared to those of phase D (AUC_D= 91.7 ± 104.3

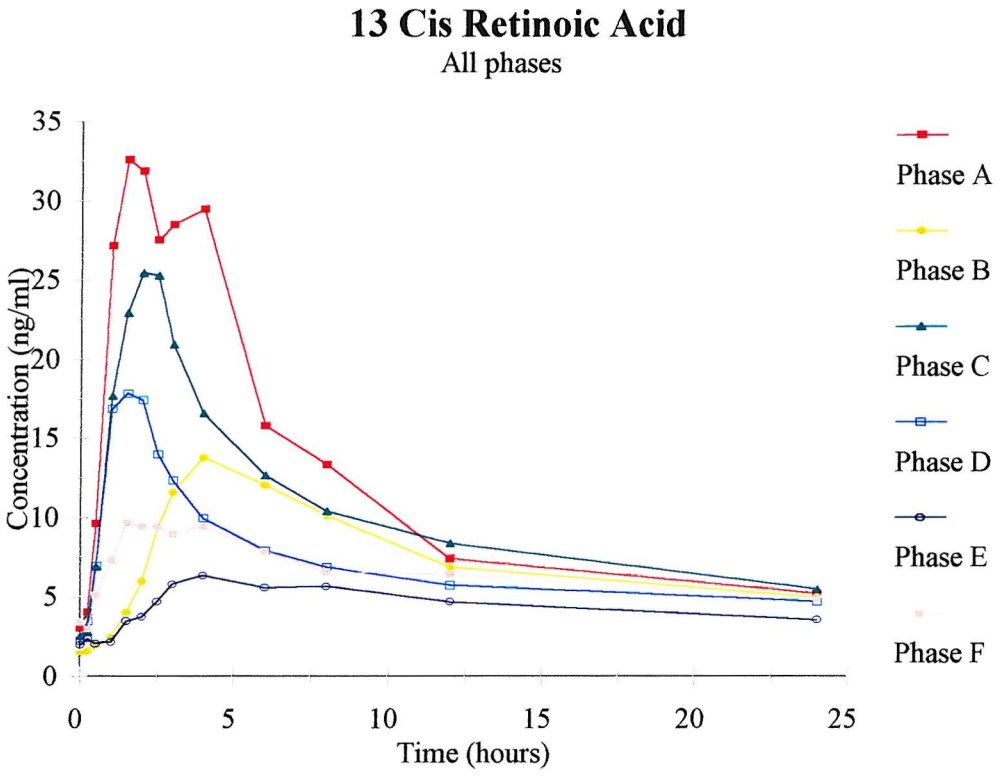


Figure 3.11: - 13 cis retinoic acid plasma concentration profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

ng/ml; C^{max}_D= 19.1 ± 17.2 ng/ml) and phase F (AUC_F= 62.6 ± 58.1 ng/ml/hr; C^{max}_F= 9.5 ± 7.2 ng/ml). Furthermore, liver treatments gave T^{max} (T^{max}_B= 4.3 ± 1.0 hrs; T^{max}_E= 4.9 ± 2.4 hrs)values which were two-fold higher than the T^{max} values obtained for the supplement treatments (T^{max}_A= 2.0 ± 0.8 hrs; T^{max}_C= 2.2 ± 0.5 hrs; T^{max}_D= 1.9 ± 1.4 hrs; T^{max}_F= 2.3 ± 1.1 hrs).

Supplement dosing on a fasting stomach (phases A and D) gave significant higher peak plasma levels compared to supplement dosing in conjunction with a meal (phases C and F) for both the high 50 mg treatments and the low 15 mg treatments (AUC_A=209.6 ± 142.2 ng/ml hr; C^{max}_A=50.6 ± 28.7 ng/ml; AUC_C= 183.4 ± 179.1 ng/ml; C^{max}_C= 24.8 ± 24.3 ng/ml; AUC_D= 91.7 ± 104.3 ng/ml; C^{max}_D= 19.1 ± 17.2 ng/ml; AUC_F= 62.6 ± 58.1 ng/ml/hr; C^{max}_F= 9.5 ± 7.2 ng/ml). No significant differences were found for the AUC, $T_{1/2}$ and MRT values.

3.3.2.4 Retinol

Plasma concentrations of retinol of each subject showed fluctuations around baseline levels across the entire study period (table 3.7-3.11 and figure 3.12). Post-dosing, retinol concentrations were slightly elevated above the baseline levels. Peak concentrations were achieved by 8 hours and returning to baseline by 20 hours post-dose. However, changes in retinol levels detected were very low compared to endogeneous concentrations of retinol. This resulted in a decreased data accuracy and

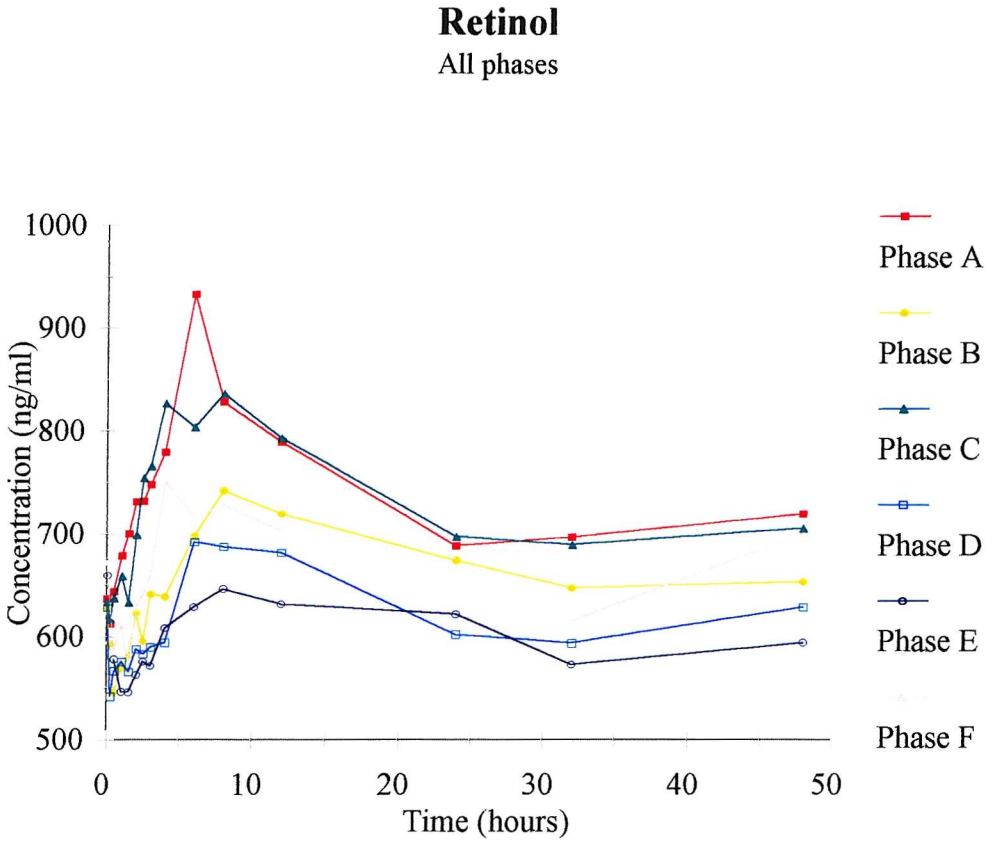


Figure 3.12: - Retinol plasma concentration profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= Phase B, 50 mg vitamin A as the palmitate in cooked liver; phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

an increased data variability. Therefore, the data and pharmacokinetic parameters obtained for retinol cannot be considered reliable. Differences found between treatments are more likely to result from data inaccuracy and variability rather than from true pharmacokinetic changes.

3.3.2.5 13-Cis-4-oxo-retinoic acid

The pharmacokinetic parameters calculated for 13-cis-4-oxo-retinoic acid are shown in tables 3.7-3.11. The plasma concentration-time profile for all phases of 13-cis-4-oxo-retinoic acid are shown in figure 3.13. Peak plasma concentrations were reached between 10-30 hours post-dosing. This was significantly slower than 13-cis-retinoic acid, which had T^{max} values of between 2.0-4.5 hours.

As with 13-cis-retinoic acid, treatments with 50 mg (phases A, B and C) demonstrated higher plasma levels compared to treatments with 15 mg (phases D, E, and F) for both supplement dosing and liver dosing. AUC values of phases A, B and C (AUC_A= 768.1 ± 508.8 ng/ml hr; AUC_B= 318.2 ± 268.4 ng/ml hr; AUC_C= 581.2 ± 390.5 ng/ml hr) were significantly higher than those of respectively phases D, E and F (AUC_D= 221.3 ± 111.0 ng/ml/hr; AUC_E= 117.2 ± 160.2 ng/ml hr; AUC_F= 236.7 ± 91.7 ng/ml hr). C^{max} values of phases A and C (C^{max}_A= 31.9 ± 30.7 ng/ml; C^{max}_C= 20.8 ± 16.2 ng/ml) were significantly higher than the Cmax of the 15 mg supplementation (C^{max}_D= 8.2 ± 6.0 ng/ml; C^{max}_F= 11.8 ± 7.2 ng/ml). C^{max} values for the liver doses did not show a significant difference between the two treatment phases (C^{max}_B= 9.7 ± 6.2 ng/ml; C^{max}_E= 5.5 ± 7.0 ng/ml). Phase F was atypical in that the plasma levels did not exhibit a

decrease after C^{max} had been achieved accounting for the significant difference in AUC observed. No significant difference was observed between equivalent 50 mg and 15 mg phases for T^{max}, t ½ and MRT.

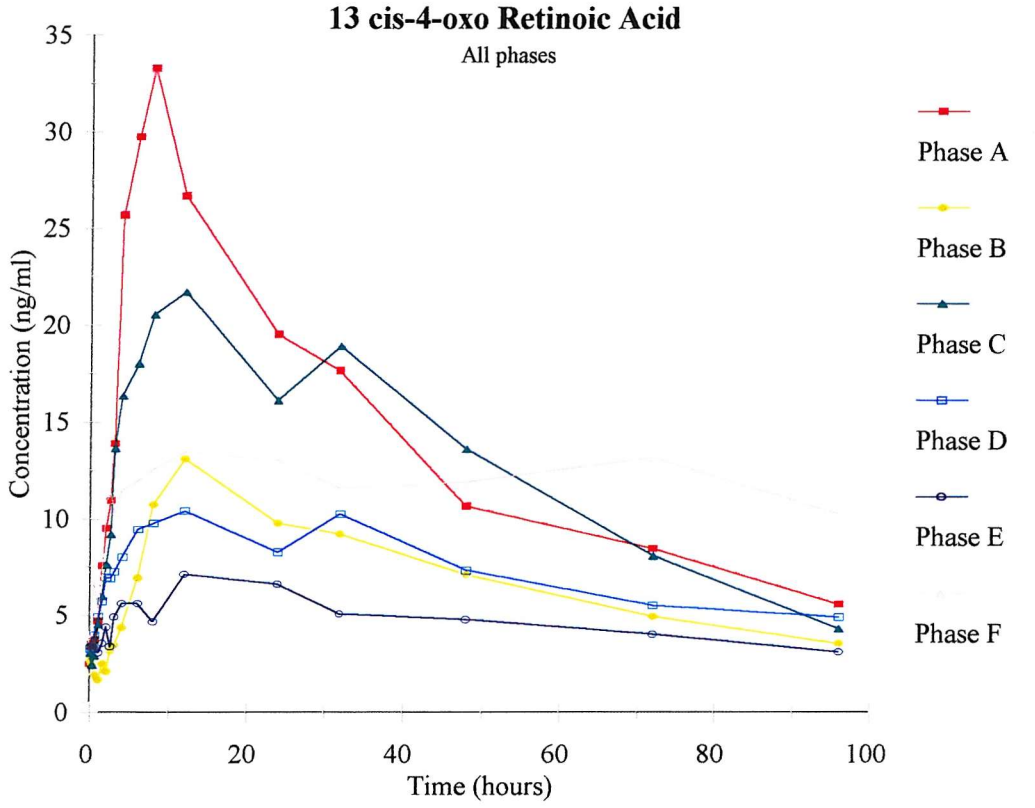


Figure 3.13: - 13 cis-4-oxo retinoic acid plasma concentration profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

Liver (phases B and E) dosing showed significantly lower plasma levels compared to supplement dosing for both the high 50 mg treatments and the low 15 mg treatments. C^{max} values of phases A, B and C (C^{max}_{A}= 31.9 ± 30.7 ng/ml; C^{max}_{B}= 9.7 ± 6.2 ng/ml; C^{max}_{C}= 20.8 ± 16.2 ng/ml) were significantly higher than those of phases D, E and F respectively (C^{max}_{D}= 8.2 ± 6.0 ng/ml; C^{max}_{E}= 5.5 ± 7.0 ng/ml; C^{max}_{F}= 11.8 ± 7.2 ng/ml).

AUC values of phases A and C (AUC_A= 768.1 ± 508.8 ng/ml; AUC_C= 581.2 ± 390.5 ng/ml) were significantly higher than the AUC of the 15 mg supplementation (AUC_D= 221.3 ± 111.0 ng/ml; AUC_F= 236.7 ± 91.7 ng/ml). There were no significant differences for AUC between supplement dosing on a fasting stomach (phases A and D) and supplement dosing in conjunction with a meal (phases C and F).

It was shown that the time taken to reach peak concentration for phase C (T^{max}_C= 9.2 ± 3.1) was significantly longer than for phase A (T^{max}_A= 6.4 ± 1.7). This was not observed for the comparison between the equivalent 15 mg treatments. No significant differences were calculated between all phases for $t_{½}$ and MRT. There occurred no significant differences for T^{max} between dosing with 50 mg or 15 mg supplement and 50 mg or 15 mg as liver.

3.3.2.6 All-trans-4-oxo-retinoic acid

Only phase A showed a clear dose-related plasma concentration-time profile. Values obtained for all other phases fluctuate around the baseline by approximately ±1.5 ng/ml (figure 3.14).

For phase A, the metabolite all-trans-4-oxo-retinoic acid achieved a C^{max} of 9.6 ± 6.9 ng/ml and a T^{max} of 2.3 ± 2.0 hours after dosing. The C^{max} value was higher than those obtained for any of the other phases. All other phases showed C^{max} values of less than 5 ng/ml. Due to the low plasma concentrations obtained, the derived pharmacokinetic parameters were subject to inaccuracy and large variations. Statistical analysis showed a significant difference between the plasma concentrations observed for dosing with

50 mg supplement dosing on a fasting stomach (AUC_A= 21.5 ± 22.7 ng/ml hrs; C^{max}_A= 8.6 ± 6.7 ng/ml) and 50 mg as liver (AUC_B= 6.9 ± 6.6 ng/ml hrs; C^{max}_B= 1.1 ± 0.5 ng/ml).

Significant differences were also observed between phases A:C and C:B for Cmax values/ Tmax, t½ and MRT showed random significances due to the low levels of all-trans-4-oxo-retinoic acid observed and the fluctuations around baseline levels.

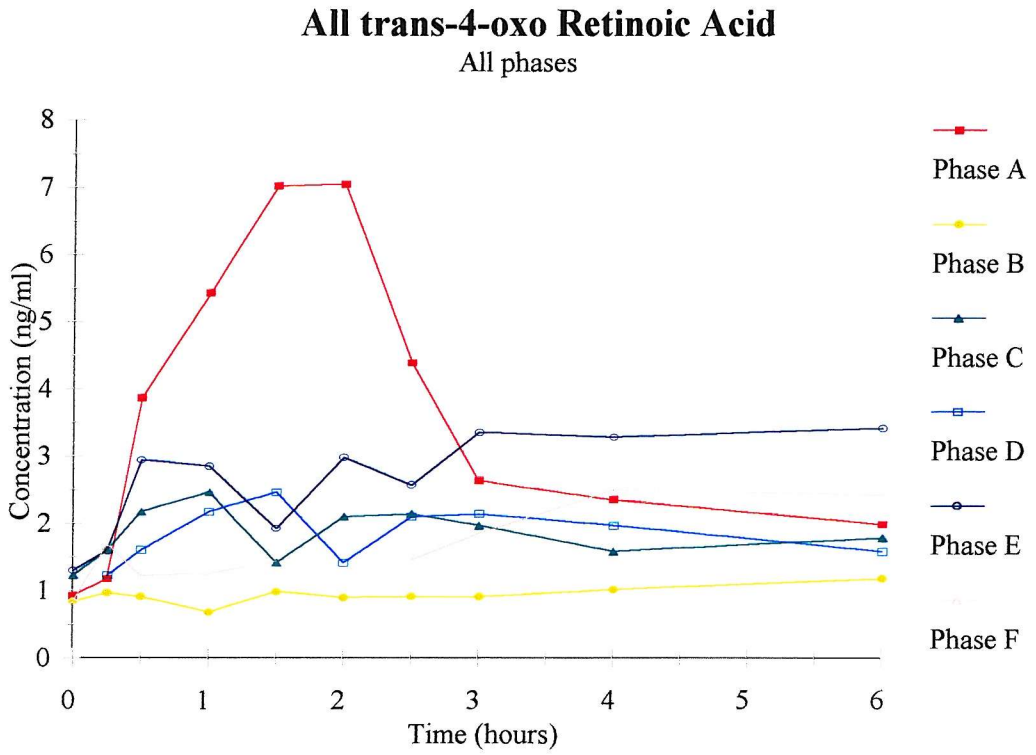


Figure 3.14: - All trans-4-oxo-retinoic acid plasma concentration profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A as the palmitate in cooked liver, phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal; phase D= 15 mg vitamin A supplement on a fasting stomach, phase E= 15 mg vitamin A as the palmitate in cooked liver; phase F= 15 mg vitamin A supplement (palmitate) following a standard cooked meal.

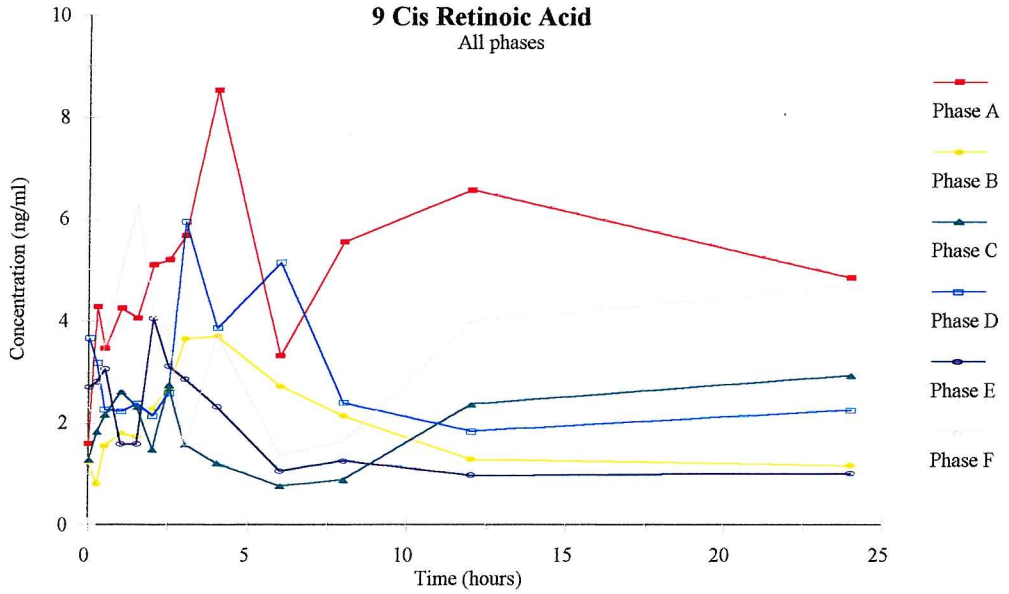


Figure 3.15: - 9 cis retinoic acid plasma concentration profiles for all phases, where phase A= 50 mg vitamin A supplement (palmitate) on a fasting stomach; phase B= 50 mg vitamin A (palmitate) in cooked liver; phase C= 50 mg vitamin A supplement (palmitate) following a standard cooked meal); phase D= 15 mg vitamin A supplement (palmitate) on a fasting stomach; E=phase E, 15 mg vitamin A (palmitate) in cooked liver; phase F= 15 mg vitamin A (palmitate following a standard cooked meal).

3.3.2.7 9-Cis-retinoic acid

All levels detected were within 3-4 ng/ml of the limit of detection (1 ng/ml), resulting in a higher degree of analytical errors. An exception to this was observed for one patient where plasma levels were observed for phases A and C in the range of 35-48 ng/ml. Results obtained were therefore subjected to a significant degree of inter-individual variability. The inter-individual variability for 9-cis-retinoic acid was significantly higher than was observed for 13-cis-retinoic acid or all-trans-retinoic acid. Figure 3.15 illustrates the detected variation of 9-cis-retinoic acid for all phases. Only phases A and B show a clear increase from the baseline, which can be assumed to be a result from the treatments given. Pharmacokinetic parameters have been calculated for 9-cis-retinoic acid, but these could be unreliable due to large inter-individual variations and analytical errors (table 3.12 a and b)

3.4 SUMMARY AND CONCLUSIONS

It is known that high doses of some vitamin A supplements and oral retinoid medical treatments can cause teratogenic effects in the human fetus. Liver is known to contain significant amounts of retinol stored in the form of retinyl palmitate. In the late 1980's and early 1990's it was discovered that the vitamin A content of animal livers in general was increasing. This was accounted for by practice of fortifying animals feeds with high dose vitamin supplement (2). Therefore, it was recognized that the regular consumption of liver or liver products could potentially cause an increase in teratogenic risk to unborn fetuses. Since the early stages of gestation is most vulnerable to teratogenic effects, the General Medical Council recommended a restriction on the consumption of liver or liver products for known pregnant women and for women intending to become pregnant.

It has been determined by Buss et al (1) that the absorption of retinol as the palmitate was different for a 50 mg dose of an oral liquid supplement under fasting conditions (AUC= 10.4 ± 5.3 μg/ml hr) compared to an equivalent dose contained within cooked liver (AUC= 5.9 ± 3.7 μg/ml hr). Previously, a 150 mg dose of supplement or liver had also been investigated. The differences observed between supplement and liver at this higher dose were less than that observed at the 50 mg dose for 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid. All-trans-retinoic acid showed a highly significant difference at both the 50 and 150 mg treatment doses between supplement and liver. At the 50 mg dose the C^{max} and AUC values of 13-cis-retinoic acid, all-trans-4-oxo-retinoic acid and 13-cis-4-oxo-retinoic acid were significantly different between the two treatments.

Higher concentrations of all-trans-retinoic acid (20 fold) and all-trans-4-oxo-retinoic acid (5 fold) were observed after 50 mg supplement dosing (AUC_{TRA}= 85.9 ± 63.4 ng ml^{-1} hr; C^{max}_{TRA}=) compared to the equivalent dose given as cooked liver (AUC_{TRA} 5.5 ± 4.6 ng ml^{-1} hr; C^{max}_{TRA}= 2.3 ± 1.0 ng/ml). 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid showed approximately 2 fold higher concentrations after the 50 mg supplement dose than the corresponding liver dose.

The current study investigated the same treatment as Buss et al (1) with a lower dose (15 mg) of vitamin A. Previously, Buss et al had shown that a 150 mg dose is essentially the same for either a supplement dose or a liver dose except for all-trans-retinoic acid. This was suggested to be due to the "overloading" of the absorption of the retinol within the gut. Essentially so much retinol was absorbed per time unit for either supplement or for liver that the plasma concentrations of retinyl palmitate and the metabolites of retinol were similar. Based on the plasma concentrations found for the 150 mg doses, it was suggested that a lower dose of 15 mg would be more informative and still be detectable with the current analytical technique (phases D, E and F). The 50 mg dose was included to provide a direct comparison to the data generated by Buss et al (phases A, B and C).

Research into the absorption of lipids in general has indicated that the amount of fat content within a given meal could cause a change in drug absorption rates (3;4). Hence, a further comparison was investigated where vitamin A oral supplement was given following a meal with similar fat content to that of the liver (phases C and F). It is known that high fat content meals can affect the rate at which gastric emptying occurs (5). The rate of gastric emptying could be the controlling factor limiting the rate at

which the matrix bound retinyl palmitate could be released and absorbed into the system. Another factor that was considered was the fact that a fatty meal would influence the absorption of lipids and lipid soluble compounds by the stimulation of bile secretions into the gut tissues as well as lipases from the pancreas. This would alter the physical conditions of the stomach and intestine in comparison to the supplement dose on a fasting stomach (phases A and D). To limit any possible effect of gastric emptying, all regimes were conducted with the subjects lying on their right side, although it had been observed that for oral supplement dosing no difference can be seen through posture effects (chapter 4).

From the data determined in this investigation it can be observed that there were large differences in plasma concentrations of the acid metabolites between individuals for all dosing regimes. The 50 mg dosing schedules showed a good similarity to the data found by Buss et al for all-trans-retinoic acid and all-trans-4-oxo-retinoic acid pharmacokinetic parameters. 13-Cis-retinoic acid and 13-cis-4-oxo-retinoic acid showed similar C^{max}, AUC and T^{max} but the $t_{½}$ were lower for both metabolites for this investigation. Overall, however, there was a good agreement between the two 50 mg data sets. The 15 mg data set showed lower C^{max} and AUC parameters, but similar T^{max} and $t_{½}$ to the 50 mg supplement dose determined in this investigation. The 15 mg data set also showed similar trends between the individual study phases in comparison to both 50 mg data sets.

Retinyl Palmitate.

Concentration data obtained for retinyl palmitate agreed with the data found by Buss et al in T^{max} and $t_{1/2}$ of phases A and B. Retinyl palmitate values for T^{max} of phases A and B were similar in both 50 mg and 15 mg treatments. However, the T^{max} for phases C and F were significantly lower in comparison to phases A and D. Plasma concentrations observed between subjects varied across a wide range (phase A= 1800 - 23000 ng/ml; phase B= 900 - 4000 ng/ml; phase C= 2200 - 18000 ng/ml). AUC and C^{max} values observed for phases A and B (50 mg dose) were higher than observed by Buss et al for a similar dose. The difference between Buss et al and this investigation can be clarified to two distinct points.

1. It is very clear with both investigations that the absorption of vitamin A is subject to large variations between individuals. A factor 10 has been observed between some subjects in the calculated AUC for retinyl palmitate. This inherent variability would cause differences in metabolite concentrations between this investigation and that reported by Buss et al.
2. The analysis of retinyl palmitate was improved between the two investigations. This investigation showed a limit of detection of 50 ng/ml. This increase in the assay sensitivity and accuracy enabled baseline levels to be determined and the terminal slope of the plasma concentration-time profile to be defined more accurately.

Phase B consisted of a dose of vitamin A with liver as the source. The absorption of retinyl palmitate seen was significantly lower than phase A and C. This trend was observed for both the 50 mg and the 15 mg doses. Retinyl palmitate bound up within

the matrix of the liver tissue could be expected to be delayed in the delivery to the intestine for absorption by the digestion of the solid material to chyme. It can be suggested that the breakdown of the liver matrix to free retinyl palmitate for hydrolysis reactions within the small intestine is insufficient to delivery the complete dose. An analysis of the faeces for each subject was not conducted and in light of the results should be considered in future investigations of this type.

With phase C, this investigation examined the effect of the food material on the absorption of the vitamin A dose. Phase C AUC and C^{max} levels agreed with those observed for phase A. This trend is also observed for both the 50 mg and the 15 mg dose. With phase C a slower lipid absorption would have been expected. T^{max} for phase C was significantly shorter than that of phase A, which was unexpected. One would expect that, for a supplement dose following a set meal, the solid food matter should be reduced to a semi-liquid state in the stomach prior to movement into the small intestine. The retinyl palmitate dose would be combined within this material and dependant on the rate of delivery of the food material into the small intestine. This would take a relatively longer time to absorb compared to an oral supplement given on a fasting stomach. However, this appears not to be the case. Therefore, it can be suggested that the meal either actually assists the delivery of the oral supplement into the intestine for absorption or increases the rate at which the dose is absorbed. It also can be suggested that the amount of absorbed is unaffected by the food material. Several reasons can be suggested to account for this difference:

1. The stomach contents were believed to be homogeneous after the

consumption of a carbohydrate meal, but when a meal contains fats and lipids it has been shown that the lipid fraction tends to accumulate at the top of the material within the stomach (6).

2. It has also been shown that meals high in fat of certain types will produce chylomicrons of differing sizes. The availability of phospholipids and tri-glycerides dictate the size of the chylomicrons formed and the smaller the size then the quicker they would be transferred to the lymphatic system to be released into the blood stream.

3. The volume of the meal was approximately 280 g providing sufficient material to extend the stomach which would increase the rate at which mixing within the stomach is mobilized. However, each individual would by definition have a different stomach capacity, hence the rate at which mixing would occur might vary significantly between subjects. Each individual would also chew the food material into different consistencies compared to other individuals. Therefore, the condition of the material being swallowed would vary quite considerable. Digestion within the stomach would take differing amounts of time prior to being ejected into the small intestine. Material eventually reaching the site of absorption could have very different properties between individual subjects and days. This could explain the inter- and intra- individual variations observed.

The availability of the retinyl palmitate to the brush border membrane and hydrolysis

to retinol would differ significantly between the different conditions(A ,B, and C). Plasma concentrations of retinyl palmitate as reflected by the AUC are variable between patients (figure 4.16) in a random manner between phases. It can be seen that a high absorption in phase A would not translate into relatively higher absorptions in the subsequent phases. This is consistent with a mechanism that is subject to large degrees of variability. The degree of mixing, phase separation and movement in the stomach are such variables.

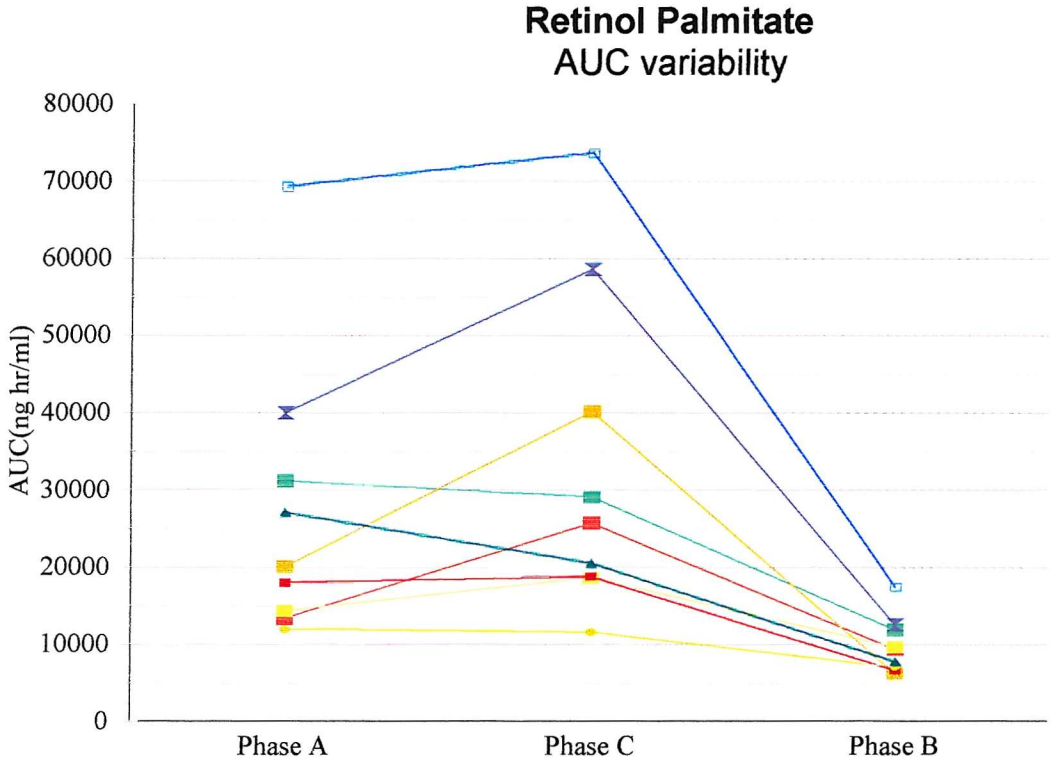


Figure 3.16 Retinyl palmitate AUC levels for phase A, phase B and phase C.

Metabolites of Vitamin A

For the current investigation it was observed that all -trans-retinoic acid and all-trans-4-oxo-retinoic acid achieved an above baseline C^{max} at similar times for both phases A

and C. However, between phases A and C all-trans-retinoic acid C^{max} values were 6 fold different (A>C), while all-trans-4-oxo-retinoic acid showed a 2 fold C^{max} difference (A>C). Phases D and F showed a similar trend to phases A and C, although the levels detected were much lower. For phase B the all-trans retinoic acid levels were less than 10 ng/ml at C^{max}. However, T^{max} for phases B and C were found to be 2-3 fold later than phase A. A similar trend observed for all-trans-4-oxo-retinoic acid. This trend was repeated in phases D, E and F.

Buss et al found 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid AUC levels of 360 ng/ml hr and 880 ng/ml hr respectively after a 50 mg dose on a fasting stomach. For this investigation, 13-cis-retinoic acid AUC for phase A were lower in comparison to Buss et al, although the levels of 13-cis-4-oxo-retinoic acid observed were of a similar high value. The 50 mg liver dose also showed reduced plasma concentrations for 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid compared to Buss et al. Overall, for the 50 mg supplement dose, 13-cis metabolite levels were comparable to the results found by Buss et al given the degree of variability between individuals observed within both investigations.

Metabolite AUC data demonstrated a consistent relationship between phases A and C. 13-Cis-retinoic acid and its primary metabolite 13 cis-4-oxo-retinoic acid achieved similar C^{max}, T^{max} and AUC values between phases A and C. There was a certain degree of variability observed between subjects, but no significant outlying values. For all trans-retinoic acid and its primary metabolite all trans-4-oxo-retinoic acid, T_{max} exhibited similar values in studies A and C. Whereas C^{max} for phase A all-trans-retinoic acid was 4-5 fold higher than phase C. However, greater AUC levels of all-trans-4-oxo-

retinoic acid were observed in phase C compared to phase A. This difference was not considered significant since it could be related to subject variability. Therefore, except for all-trans-retinoic acid, the metabolite levels observed between phases A and C were similar.

The AUC for all trans retinoic acid determined by Buss et al for dosing by liver were 22.6 ± 10.6 (150 mg) and 5.5 ± 4.6 ng ml^{-1} hr (50 mg) respectively. The values determined for 50 mg and 15 mg liver dose for this study were 16.2 ± 17.3 and 16.0 ± 18.8 ng ml-1 hr respectively. Allowing for the differences between individuals there does not seem to be the same difference in absorption as there is between dose given.

Conclusions

It is reported in Buss et al that there were no significant differences for retinyl palmitate observed for the 150 mg dose between supplement dosing and the liver dosing (1). At the 50 mg and the 15 mg dose levels a difference is observed. It can be suggested that the increased in average AUC of a supplement dose after a meal compared to that on a fasting stomach could be related to the availability of retinyl palmitate for hydrolysis and subsequent absorption. An decreased T^{max} and increased C^{max} for phase C suggests an enhance absorption profile for the retinol across the gut wall. However, phase F showed the decreased T^{max} but C^{max} was similar to phase D. Phase B and E exhibit AUC values 3-4 fold lower than either phase A, C or D, F. A conclusion that could be drawn is that the liver matrix is more difficult to break down and the subsequent availability of the retinol dose is reduced despite the evidence that food would increase bioavailibilty.

Comparison of AUC for phases A-F indicated that only phase A showed greater than average values for all-trans-retinoic acid. All other acid metabolites gave similar AUC values allowing for the variation between individuals. The possibility of an induced mechanism within the gut or liver for the metabolism of retinol and an activation of an alternative mechanism at higher concentrations could explain these results. The 15 mg dose does not saturate the initial mechanism involved and hence less acidic metabolites are generated, whereas the 50 mg supplement dose and 150 mg supplement dose conducted by Buss et al (1) activate an alternative mechanism. Hence, metabolism of the retinol is constant between the three treatments in terms of acid metabolites at a concentrations approximately 50 mg or lower. However, at concentrations greater than 50 mg metabolite formation could be enhanced. The suggested induced mechanism could be inherently different between individual subjects, contributing to the observed inter- and intra- individual variation.

In terms of teratogenic possibilities, conclusions can be drawn from the data collected by Buss et al and from data found within this investigation. Firstly, high dose supplementation of vitamin A is dangerous for an unborn child. High dose supplementation over a long period would be hazardous to the maternal subject as well. One case of note that has been reported is that of a Japanese girl who suffered extreme toxic effects from vitamin poisoning. This young woman over a period of five to six years ate a diet consisting completely of pumpkin (8). She was eventually admitted to hospital with severe vitamin A side effects. A change of diet reverted all symptoms and the young women returned to normal. However, her case does emphasize that is possible for a high teratogenic dose of vitamin A to be obtained by

a natural food source.

Secondly, from the AUC values for all-trans-retinoic acid and 13-cis-retinoic acid, it can be concluded that the potential teratogenicity of vitamin A is greater after supplementation either before food or after food in comparison to liver food sources. A comparison of fortified food sources has not been made and has to date not been reported in the literature. Metabolite all-trans-retinoic acid is reduced after consumption of a meal, but 13-cis-retinoic acid AUC remains relatively constant for all phases. Risk of teratogenicity is therefore less after a natural food source, but the risk from 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid remains relatively constant for all sources. Consumption of liver reduced the level of retinyl palmitate absorbed by approximately 3 fold. Hence, a 50 mg liver dose showed similar pharmacokinetics to a 15 mg supplement dose. Chen et al stated that doses used by Buss et al (1) were not representative of the recommended daily allowances and therefore had no clinical relevance. However, when assessing the teratogenic risk from a regular dose either as an oral supplement or as a natural source such as liver, it is necessary to use the maximum theoretical doses that could be taken. Liver is an important source of many nutrients to the potentially pregnant women. With the knowledge that the levels of vitamin A within animal livers have increased over the past 40-50 years the theoretical risk is increased. Chen et al discovered that it is relatively easy to deliver a large dose of vitamin A by natural sources without realizing it. Four subjects in the Chen et al investigation received a 50 mg dose of vitamin A without the researchers realizing (planned dose was for approximately 0.5 - 20 mg). Discovery of this fact was made during the analysis of the test meals after the study had begun. With this in mind it is

easy to understand that for the average women the possibility to consume high doses of vitamin A unknowingly is real. This, therefore, represent a higher teratogenic risk. It was noticeable from the analysis of the liver samples for this study that there exists large differences in the vitamin A content of animal livers and even variations within each liver. With the current restrictions for liver consumption, supplement tablets or capsules are being used more regularly by the average pregnant woman, although not necessarily containing vitamin A. The evidence of this study as well as that performed by Buss et al (1) and Chen et al (7) indicate that the levels of retinol absorbed after liver dosing do not present toxic levels of teratogenic metabolites to the plasma. The risk to benefit of eating liver during pregnancy is moved in the direction of benefit rather than risk. More importantly, the differences observed in the absorption between formulations of vitamin A found by Chen et al (7) would pose a more serious risk for teratogenicity than the regular consumption of a moderate amount of liver or liver products.

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Chapter 4

The Influence Of Posture And Previous Dosing On The Absorption Of Vitamin A And The Formation Of Its Teratogenic Metabolites.

Table of Contents

List of Tables

List of Figures

4.1 INTRODUCTION

Previous investigations by Buss et al demonstrated that during dosing of female human subjects the C^{max} for all-trans-retinoic acid occurred prior to the main response for retinyl palmitate when dosing orally with retinyl palmitate as the retinol. It was suggested that with retinyl palmitate being transported via the lymphatic system that there would be a delay in the systemic change observed for the retinyl palmitate. Therefore, evidence of all-trans-retinoic acid and all-trans-4-oxo-retinoic acid appearing before the retinyl palmitate maximum concentration indicated the possibility of first pass metabolism within the gut.

It has been recorded in many investigations that there is a large degree of variation between the individual subjects under investigation and even variation between the same subjects on different days of dosing. Previous investigations have shown a distinct link between drug absorption and gastric emptying. The process of gastric emptying has been shown to be affected by the position of the subject for certain types of drugs (ie prone, supine or standing). Also, Renwick et al (1) demonstrated a difference in absorption of nifedipine and paracetamol between individuals supine on either their left side or right side.

In further studies it has been demonstrated conclusively that repetitive dosing of retinyl palmitate or retinol lowers the levels of metabolites observed in the plasma. However, this lowering of response was observed with dosing schedules conducted over months. In an investigation conducted in pregnant rabbits and rats it was shown that the all-trans-retinoic acid levels achieved were consistent with previous investigations for the

first dose. However, doses administered in quick succession (3hr intervals and 5hr intervals) after this showed a substantial decrease in the levels of all-trans-retinoic acid observed. It was suggested that this could be linked to an induction of the RAR and RXR metabolic pathways within the embryo and other tissues. However, there were large variations between individual test animals.

This study will investigate two aspects. Firstly, it will investigate the possibility that the absorption through the gut of retinol dosed as the retinyl palmitate could be affected by the posture of the patient. A difference in absorption was therefore investigated between left and right side supine dosing schedules in human subjects. The second aspect of this study was to investigate the degree of variation associated with repeat dosing within a narrow time frame (24 hrs).

4.2 METHODS

4.2.1 Subjects

Five male and five female subjects were medically screened for this study. The mean±s.d. age, height and weight of the study group was respectively 24.4±0.7 years (range 21-23 years), 177.5±16.1 cm (range 147-193 cm) and 76.3±8.8 kg (range 54-88 kg). All subjects consumed alcohol, the average±s.d. consumption of which was 20.1±7.3 units/week (range 8-30 units/week). Three subjects smoked 10 cigarettes per day. One subject suffered from asthma and perennial rhinitis for which she was taking inhaled salbutamol, inhaled fluticasone, terfenadine and nasal fluticasone spray. None

of these drugs are expected to interact with retinoids or to influence the results of the study.

4.2.2 Study Design and Treatment

The study was an open study and consisted of three study days. On each study day, subjects were given a 50 mg dose of retinol as retinyl palmitate in the form of 1.18 g Arovit after an overnight fast.

Venous blood samples (10 ml) were collected into heparinized tubes by use of an intravenous cannula inserted into the forearm. Blood samples were taken at the following post-dose time intervals : 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 hours. Volunteers were subjected to different conditions for each study day :

Day 1 / Dose 1 (Phase A): Subjects were positioned in on their left side. Each subject was required to remain immobile for the first four hours of post-dose blood sampling. After four hours subjects were free to move and a standard prepared lunch was provided.

Day 2 / Dose 2 (Phase B): Day 2 was carried out two weeks after day 1. The protocol for day 2 was identical to day 1, except for the fact that subjects were required to rest on their right side for the first four hours of post-dose blood sampling.

Day 3 / Dose 3 (Phase C): Day 3 was carried out 24 hours after day 2, with an identical protocol as day 2.

4.2.3 Statistical Analysis

Concentration data was calculated using regression analysis. Pharmacokinetic parameters were determined using non-compartmental analysis. Statistical analysis was two fold. Firstly, the day 1 dose was compared to the day 2 dose using t-test analysis for the influence of posture on the absorption of retinol. Secondly, the day 2 dose was compared to the day 3 dose to determine the influence of previous dosing on the metabolism of retinol. The data used were absolute (total) concentrations and kinetic parameters were calculated after subtraction of the pre-determined baseline concentrations for each component.

4.2.4 Pre-dose Analysis Of Vitamin A

All pre-dose plasma samples were analysed for trace levels of the following compounds:

All-trans-Retinoic Acid ----------	TRA
13-Cis-Retinoic Acid----------	CRA
Retinyl Palmitate----------	RP
All-trans Retinol----------	Rol
13-Cis-4-oxo-Retinoic Acid------	4-oxo CRA
All-trans-4-oxo-Retinoic Acid---	4-oxo TRA
9-Cis-Retinoic Acid----------	9 CRA

Pre-dose samples for each phase were analysed and the levels determined are shown in tables 4.1, 4.2 and 4.3.

Table 4.1: - Pre-dose levels for phase A; 50 mg supplement with Left side Protocol (ng/ml)

Subject	TRA	CRA	RP	Rol	4-oxo CRA	4-oxo TRA	9 CRA
1	6.9	0	0	729.2	0.7	1.5	0.3
2	2.7	0	0	650.9	2.4	0	0
3	0.5	0	0	862.0	0	0	1.1
4	0.3	0.8	0	649.7	1.5	0	0
5	0.2	0.4	0	609.3	3.7	69.5	0
6	Patient Deleted due to analytical Problems						
7	1.8	0.5	0	702.7	0.1	0.2	0.3
8	4	0.8	0	833.6	2.3	0.5	1.4
9	0.2	0.8	0	991.1	0	2	0
10	2.1	1	0	601.2	1.4	1.1	0

Table 4.2: - Pre-dose levels for phase B; 50 mg supplement with Right side Protocol

Subject	TRA	CRA	RP	Rol	4-oxo CRA	4-oxo TRA	9 CRA
1	2.5	0	44.9	317.7	0.3	1.1	0.8
2	0	0	51.8	390	1	0	0
3	0.9	0	ND	356.3	2.6	0	1.8
4	0.3	1.1	29.2	611.1	1.6	0	0.6
5	1.5	1	ND	306.5	3.6	32.3	0
6	Patient Deleted due to analytical Problems						
7	1.6	0.5	ND	363.4	0.9	0.3	0.9
8	4.9	0.9	ND	617.8	4.1	0.3	4.2
9	6.6	1.2	ND	758.8	0	0	0
10	0	0	ND	559.1	2.6	1.1	0

Note: - ND represents NOT DETECTABLE: the baseline levels within these samples were below the limit of detection

Table 4.3: - Pre-dose levels for phase C; 50 mg supplement with Right side Protocol 24 hrs after phase B. The pre-dose sample for phase C also count as the 24 hour sample for phase B

Subject	TRA	CRA	RP	Rol	4-oxo CRA	4-oxo TRA	9 CRA
1	2.9	0.8	54.1	589.1	6.9	3.1	0
2	2	2.9	70.6	415	18.8	0	4.3
3	2.3	3.2	210.2	482	45.4	0	0
4	2	5.6	135.7	545.9	14.4	0.4	1
5	1	4.6	66.7	369.5	15.5	9.9	0
6	Patient deleted due to analytical problems						
7	0.7	3.5	46.3	559.3	1.7	0	2.2
8	2.6	3.2	155.2	617.6	17.8	0	2.6
9	0.4	3.6	146.5	788.5	7.6	0	0
10	0	5.6	230.6	517.2	8.3	0.5	0

From the pre-dose data the variable nature of the retinoic acid metabolites within the same patient over three study days was observable. Patient 1 showed a 2 fold difference in baseline levels between phases A and B in the all-trans-retinoic acid levels, however the opposite is seen with the all-trans-4-oxo-retinoic acid levels. Subject 5 consistently had high predose levels of all-trans-4-oxo-retinoic acid. This variability indicated the necessity to subtract predose concentrations from all subsequent pharmacokinetic determinations. All pharmacokinetic parameters determined for this study are all relative to a predose sample and reflect the change in concentration over time.

4.4 RESULTS

4.4.1 General Concentration Profiles

Post vitamin A oral supplement dosing plasma concentrations of all compounds of interest except for retinol demonstrated an increase in concentration relative to time. It was observed that all-trans-retinoic acid metabolites and retinyl palmitate had an initial sharp increase in concentration, peaking within the first 5 hours. The peak

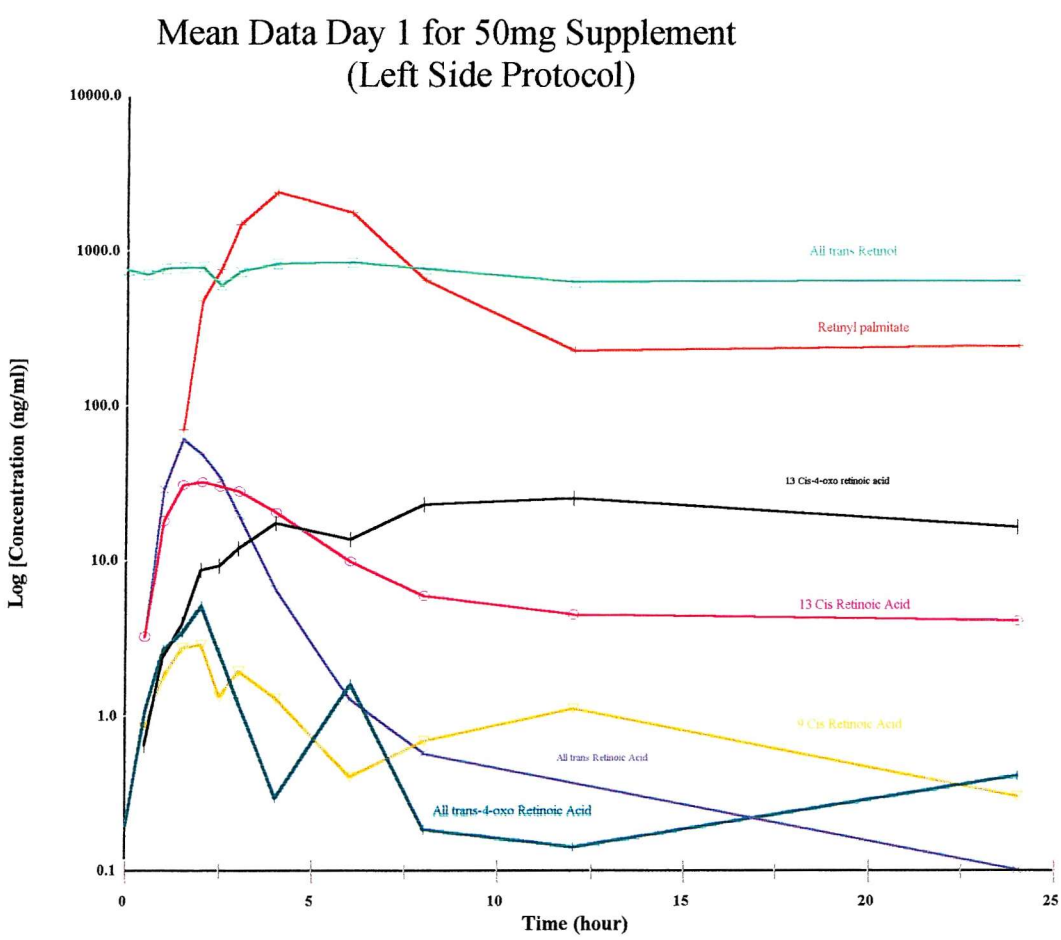

Figure 4.1: - Phase A pharmacokinetic profiles for 0 to 24 hours post dose, left side protocol

concentrations of all-trans-retinoic acid , all-trans-4-oxo-retinoic acid, 9-cis-retinoic acid and 13-cis-retinoic acid were achieved within the first 1.5 hours. Retinyl palmitate achieved peak concentrations by 5 hours, while 13-cis-4-oxo-retinoic acid achieved peak concentration by 10 hours in most cases. Levels returned from peak

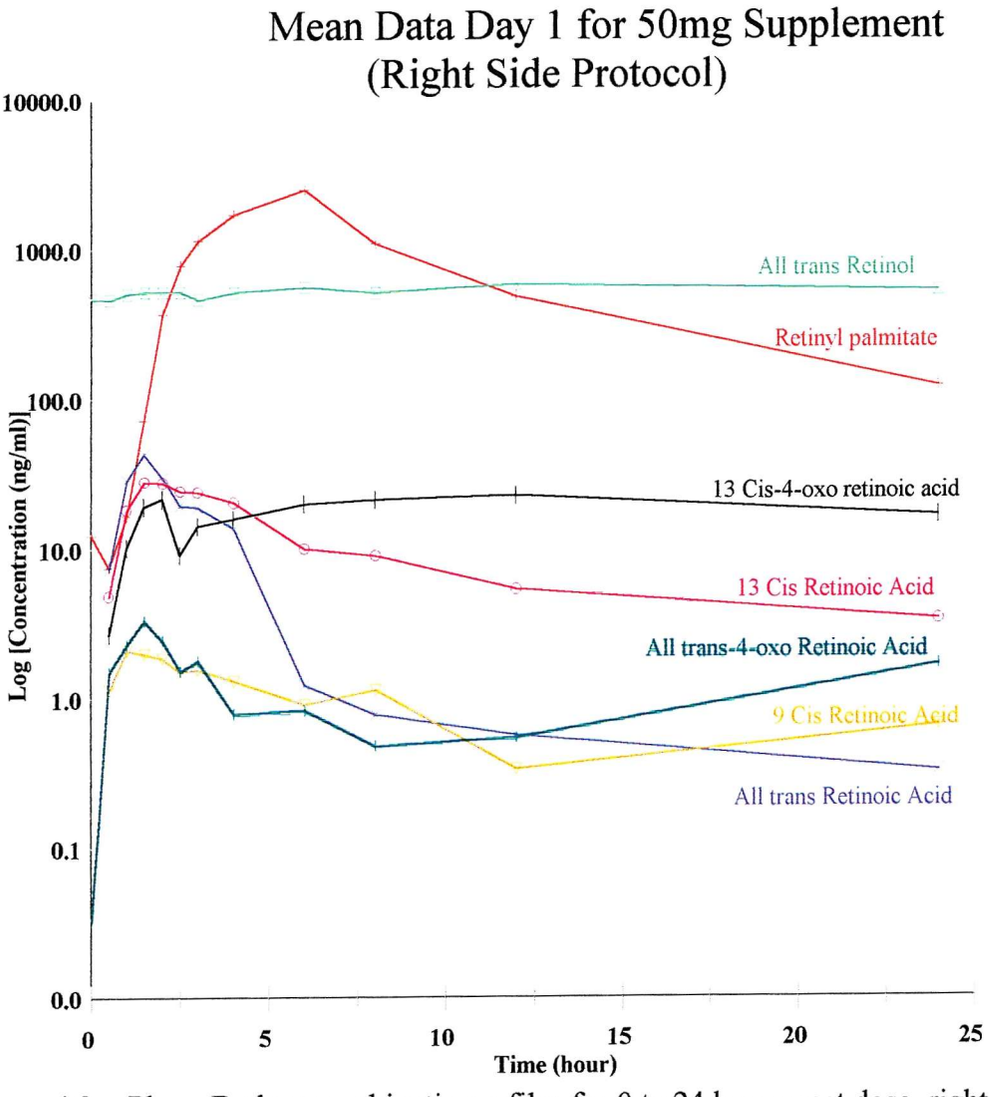

Figure 4.2: - Phase B pharmacokinetic profiles for 0 to 24 hours post dose, right side protocol day 1

concentrations to pre-dose levels by 8.5 hours for all except the 13-cis-4-oxo-retinoic acid. The 13-cis-4-oxo-retinoic acid levels remained elevated until 24 hours. All three study phases demonstrated similar profiles. Mean data for phases A, B and C are shown

in figures 4.1, 4.2 and 4.3 respectively. Table 4.4 illustrates the patient derived pharmacokinetic data of 9 patients (patient 6 was deleted from the study since the analysis of the samples was unreliable due to inconsistent and variable interference). Standard deviations from the mean are shown for all components.

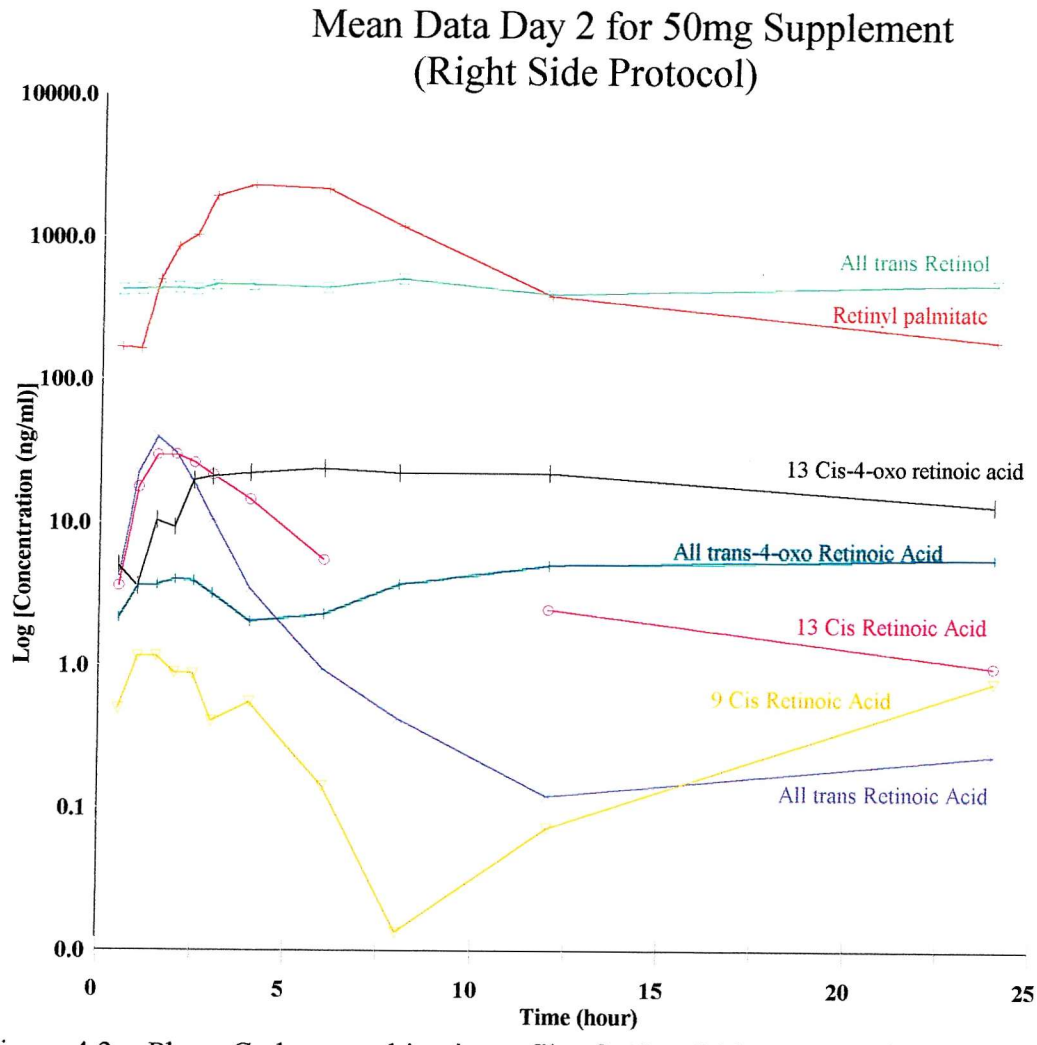

Figure 4.3: - Phase C pharmacokinetic profiles for 0 to 24 hours post dose, right side protocol day 2

Table 4.4: - Mean Pharmacokinetic Parameters for Day 1(phase A); Day 2 (Phase B) and Day 3 (Phase C)

Day 1	All-trans-retinoic Acid	13-Cis-Retinoic Acid	9-Cis-Retinoic Acid	All-trans-4-oxo Retinoic Acid	13-Cis-4-oxo Retinoic Acid	Retinol (μg)	Retinyl Palmitate
C^{max} (ng/ml)	56.2 ± 32.3	34.5 ± 11.6	3.8 ± 1.8	8.71 ± 8.22	23.71 ± 15.4	0.897 ± 0.12	2689.9 ± 1476.2
T^{max} (hr)	1.8 ± 04	1.9 ± 0.5	4.2 ± 4.5	2.3 ± 1.8	8.7 ± 2.7	7.6 ± 6.1	4.3 ± 0.9
AUC_{1440} (ng ml^{-1} hr)	97 ± 46.6	190.3 ± 63.2	21.1 ± 25.4	22.9 ± 16.0	419.7 ± 317.5	16.0 ± 3.8	12742.2 ± 6278.3
$t_{1/2}$ (hr)	0.9 ± 0.3	4.0 ± 2.0	2.7 ± 3.1	0.7 ± 0.5	36.9 ± 21.2	67.1 ± 70.1	3.8 ± 1.2
Day 2	All-trans-retinoic Acid	13-Cis-Retinoic Acid	9-Cis-Retinoic Acid	All-trans-4-oxo Retinoic Acid	13-Cis-4-oxo Retinoic Acid	Retinol	Retinyl Palmitate
C^{max} (ng/ml)	38.4±22.2	28.1±12.0	2.9±2.4	4.3 ± 3.4	24.9 ± 17.7	0.636 ± 0.1	3261.7 ± 1955.0
T^{max} (hrs)	1.8 ± 0.8	2.2 ± 0.8	6.4 ± 8.3	6.0 ± 9.0	13.3 ± 6.0	9.3 ± 6.0	4.7 ± 1.3
AUC_{1440} (ng ml^{-1} hr)	87.1 ± 50.6	182.2 ± 64.8	27.6 ± 31.5	17.4 ± 19.0	404.0 ± 302.0	13.0 ± 2.7	16831.0 ± 7864.1
$t_{1/2}$ (hr)	0.8 ± 0.4	6.4 ± 2.7	2.5 ± 1.2	4.1 ± 2.3	43.6 ± 42.7	79.4 ± 76.5	4.6 ± 0.8
Day 3	All-trans-retinoic Acid	13-Cis-Retinoic Acid	9-Cis-Retinoic Acid	All-trans-4-oxo Retinoic Acid	13-Cis-4-oxo Retinoic Acid	Retinol	Retinyl Palmitate
C^{max} (ng/ml)	37.2±17.2	31±12.8	2.35±1.92	11.43±13.57	24.6±13.7	0.592±0.111	3492.6±1581.6
T^{max} (minutes)	1.6 ± 0.2	1.9 ± 0.3	8.4 ± 9.07	6.0 ± 0.9	6.7 ± 2.4	5.2 ± 3.8	3.4 ± 1.2
AUC_{1440} (ng ml^{-1} hr)	66.2 ± 32.7	130.0 ± 62.7	9.4 ± 14.4	133.1 ± 167.7	362.8 ± 211.5	10.6 ± 3.2	20728.6 ± 9154.8
$t_{1/2}$ (hr)	0.9 ± 0.4	3.9 ± 2.3	1.4 ± 1.0	2.8 ± 3.0	31.3 ± 23.9	57.4 ± 51.5	6.3 ± 3.9

4.4.2 Metabolite Concentration Profiles

4.4.2.1 All-trans-retinoic acid

The peak concentration for all three phases of all-trans-retinoic acid are shown in table 4.4. No significant difference was observed when comparing phase A to phase B, no significant difference was observed when comparing phase B to phase C (table 4.5 statistical analysis results). Standard deviations between mean results of phases A and B showed no significant differences. Figure 4.4 illustrates patient variability between all phases. It is shown that phase A has a higher peak concentration than phases B or

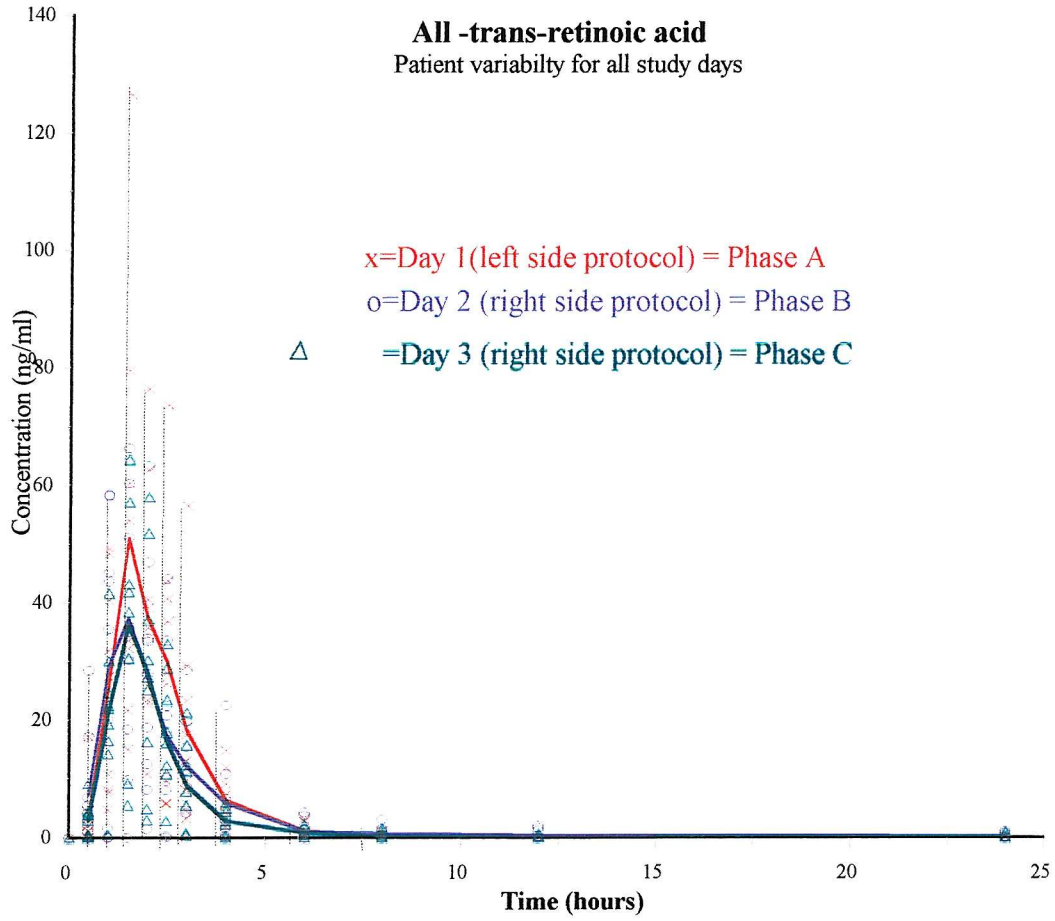

Figure 4.4: - Pharmacokinetic profile of all-trans-retinoic acid, all three study days showing patient variability.

C. However, this difference was due to the data of one patient. Phases B and C were identical in their profiles, with similar peak concentrations and decay constants. All-trans-retinoic acid levels returned to pre-dose concentrations by 8 hours (table 4.5).

4.4.2.2 Retinyl Palmitate

Concentrations of retinyl palmitate showed a steady increase in plasma concentrations within the first 5 hours post-dose (figure 4.5). All phases achieved peak concentrations by 8 hours. Plasma concentrations returned to pre-dose levels by 12 hours. Statistical analysis demonstrated no significant difference between phases A and phase B. There

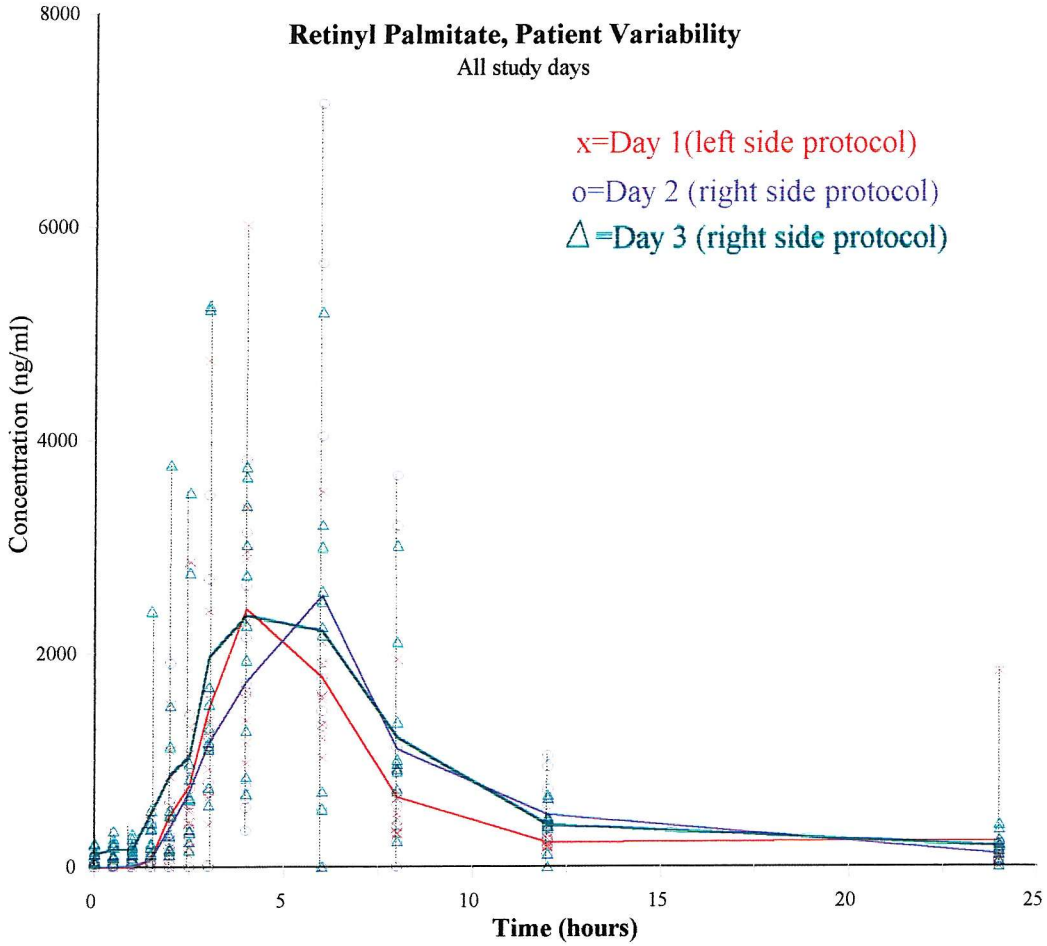

Figure 4.5: - Pharmacokinetic profile of retinyl palmitate, all three study days showing patient variability

was also no statistical difference when comparing phase B to phase C. Variability between patients in all phases were shown to be of a similar nature and the standard deviations were not significantly different (table 4.5). The variability in the concentration-time profile gave significantly different T^{max} values between day 1 and day 2. The greater T^{max} for day 1 compared to day 2 was linked to a single subject having a much higher C^{max} level at a later time compared to the other 9 subjects. Statistical analysis of the data with the subject removed gave no significant difference.

4.4.2.3 13-cis-retinoic acid

Peak concentration was reached between 2-4 hours for all patients post supplement dosing. All levels had returned to pre-dose concentrations by 24 hours. A significant difference was observed between the pharmacokinetic parameter $t_{½}$ for a comparison between phases A and B ($t_{½A}$= 4.0 ± 2.0; $t_{½B}$= 6.4 ± 2.8) and between phases B and C ($t_{½C}$=3.9 ± 2.3; table 4.5). Analysis of the concentration-time profile data showed two subjects with a 2 fold increase in $t_{½}$ compared to the other eight subjects. Calculation of the mean $t_{½}$ not including these subjects gives a value of 5.0 ± 0.9 hr for day 2. Statistically the two subjects lie outside the 3σ limit of ±2.7 hr and therefore these 13-cis retinoic acid $t_{½}$ data were rejected. Statistical analysis of the $t_{½}$ parameter after this removal indicated no significant differences between all phases. There were no significant differences determined between any of the other pharmacokinetic parameters. Mean concentration profiles demonstrated no observable differences between patient variability (figure 4.6).

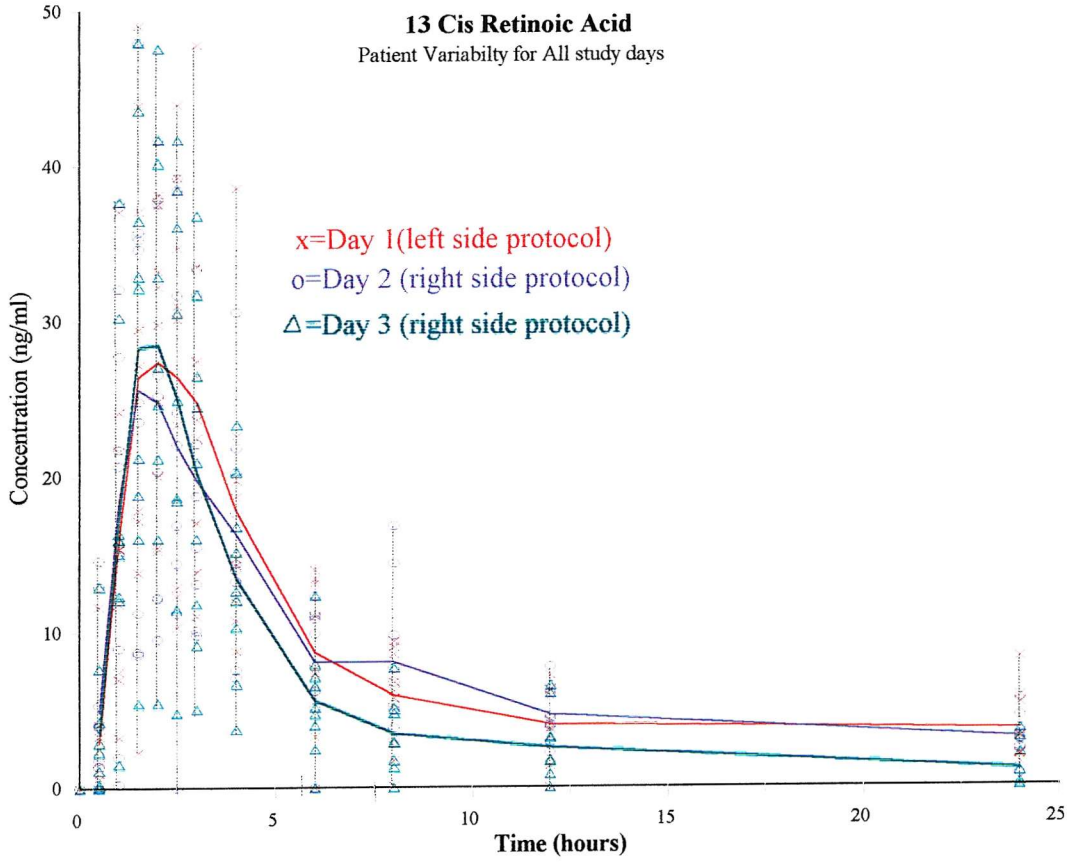

Figure 4.6: - Pharmacokinetic profile of 13-cis-retinoic acid, all three study days showing patient variability

Table 4.5: - Statistical comparisons between phases A, B and C using a paired t-test.
df = degrees of freedom, t = T number for significance at 95% confidence limits, t = 1.746 for df = 16, t = 1.761 for df = 14 and t = 1.812 for df = 10. Values greater than comparison values are significant (shown in red).

Statistical comparison T^{max}	All-trans-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	All-trans-4-oxo-retinoic acid	13-cis-4-oxo retinoic acid	Retinol	Retinyl Palmitate
Day 1 Vs Day 2	df = 16 t=0.000	df = 16 t = -1.003	df = 14 t = -0.185	df = 10 t = -0.706	df = 16 t = 0.354	df = 16 t = -0.590	df = 16 t = -0.603
Deviation from mean variability Day 1 Vs Day 2	df = 16 t = -0.760	df = 16 t = -1.021	df = 16 t = -1.445	df = 16 t = -3.374	df = 16 t = -1.824	df = 16 t = -0.272	df = 16 t = -1.794
Day 2 Vs Day 3	df = 16 t = 0.753	df = 16 t = 0.753	df = 16 t = 1.114	df = 16 t = 0.0	df = 16 t = 0.695	df = 16 t = 1.642	df = 16 t = 2.053

Statistical comparison C^{max}	All-trans-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	All-trans-4-oxo-retinoic acid	13-cis-4-oxo retinoic acid	Retinol	Retinyl Palmitate
Day 1 Vs Day 2	df = 16 t = 1.282	df = 16 t = 1.096	df = 16 t = 0.598	df = 16 t = 1.286	df = 16 t = -0.138	df = 16 t = 4.214	df = 16 t = -0.660
Deviation from mean variability Day 1 Vs Day 2	df = 16 t = 0.758	df = 16 t = -0.251	df = 16 t = -0.605	df = 16 t = 2.480	df = 16 t = -0.569	df = 16 0.606	df = 16 -0.898
Day 2 Vs Day 3	df = 16 t = 0.118	df = 16 -0.479	df = 16 t = 0.462	df = 16 t = -0.982	df = 16 t = 0.028	df = 16 t = 0.723	df = 16 0.165

Statistical comparison Auc_{1440}	All-trans-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	All-trans-4-oxo-retinoic acid	13-cis-4-oxo retinoic acid	Retinol	Retinyl Palmitate
Day 1 Vs Day 2	df = 16 t = 0.411	df = 16 t = -0.253	df = 16 t = 0.030	df = 16 t = 0.799	df = 16 t = 0.101	df = 16 t =1.858	df = 16 t = -1.050
Deviation from mean variability Day 1 Vs Day 2	df = 16 t = -0.150	df = 16 t =-0.461	df = 16 t =-0.956	df = 16 t =0.358	df = 16 t =0.057	df = 16 t = 1.359	df = 16 t = -1.255
Day 2 Vs Day 3	df = 16 t = 0.979	df = 16 t = 1.746	df = 16 t = 0.902	df = 16 t = 1.782	df = 16 t = 0.316	df = 16 t = 1.572	df = 16 t = -0.320

Statistical comparison $t_{1/2}$	All-trans-retinoic acid	13-cis-retinoic acid	9-cis-retinoic acid	All-trans-4-oxo-retinoic acid	13-cis-4-oxo retinoic acid	Retinol	Retinyl Palmitate
Day 1 Vs Day 2	df = 16 t = 0.493	df = 16 t = 0.077	df = 16 t = 0.382	df = 16 t = -2.623	df = 16 t = -0.110	df = 16 t = -0.334	df = 16 t = -1.640
Deviation from mean variability Day 1 Vs Day 2	df = 16 t = -0.502	df = 16 t = -0.469	df = 16 t = 1.132	df = 16 t = 0.358	df = 16 t = -0.110	df = 16 t = 0.039	df = 16 t = 1.030
Day 2 Vs Day 3	df = 16 t = -0.459	df = 16 t = -0.008	df = 16 t = 0.698	df = 16 t = 0969	df = 16 t = 0.433	df = 16 t = 0.674	df = 16 t = -0.673

4.4.2.4 Retinol

The plasma concentrations of retinol demonstrated fluctuations around the baseline levels calculated in tables 4.1, 4.2 and 4.3. Peak concentrations can be observed (figure 4.7) to be the result of variations in the analysis, variations in plasma concentrations during sampling and the variation between days of the study. The AUC for phase A is increased compared to phases B and C. However, the pre-dose baseline values for phase A are greater than either phase B or C. Therefore, it can concluded that the change from baseline remain relatively stable for all phases. No significant differences were observed for any other pharmacokinetic parameter or their variability.

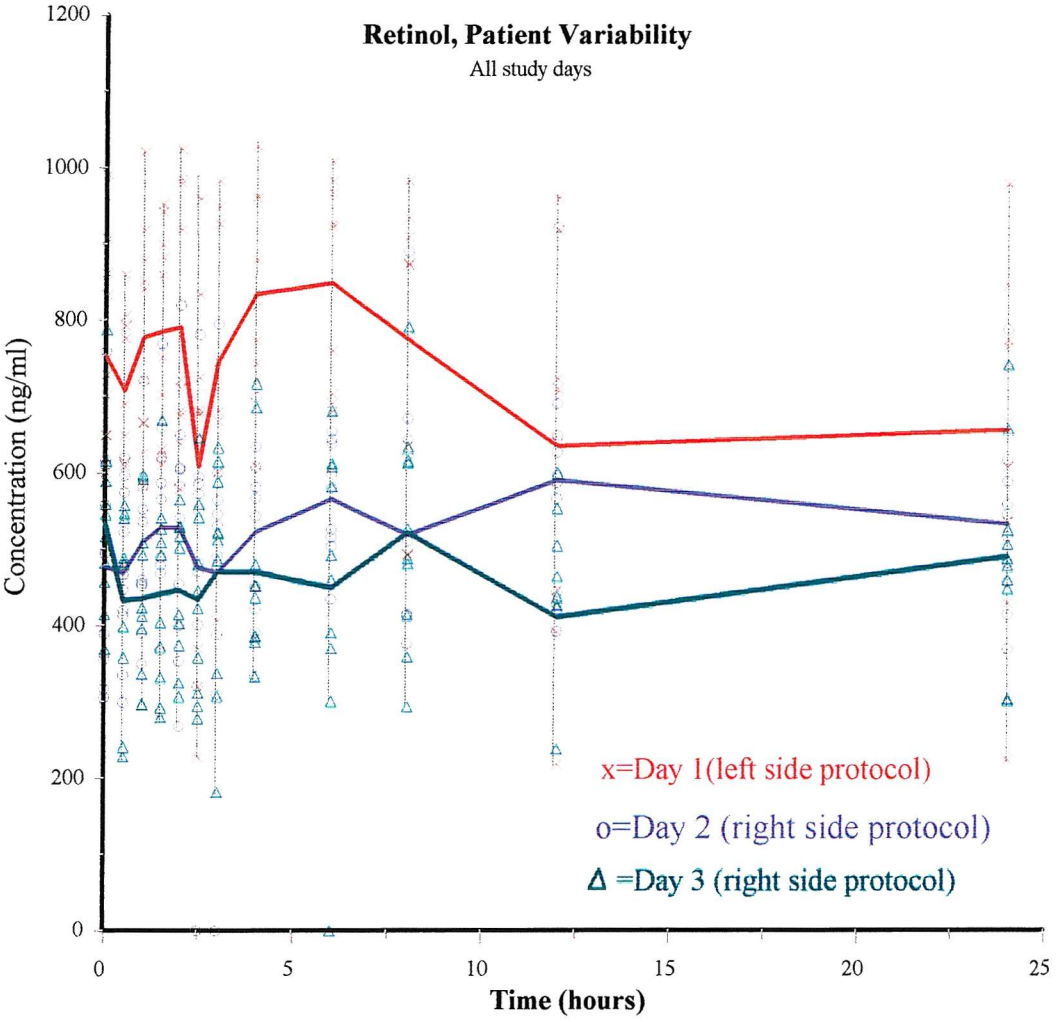

Figure 4.7: - Pharmacokinetic profile of retinol, all three study days showing patient variability

4.4.2.5 13-cis-4-oxo-retinoic acid

Plasma profiles demonstrate that 13-cis-4-oxo-retinoic acid had a similar trend for all phases. 13-Cis-4-oxo-retinoic acid achieves peak concentration between 5 - 8 hours post oral dose. Two subjects in phase B did not show a decrease in plasma levels within the twenty four experimental period. Therefore, the C^{max} for these subjects occurred at 24 hrs post dose (T^{max}). The T^{max} for these subjects were much greater than the other eight subjects and they fall outside the 3-σ limit. The T^{max} data for these subjects were therefore rejected. No significant difference in pharmacokinetic

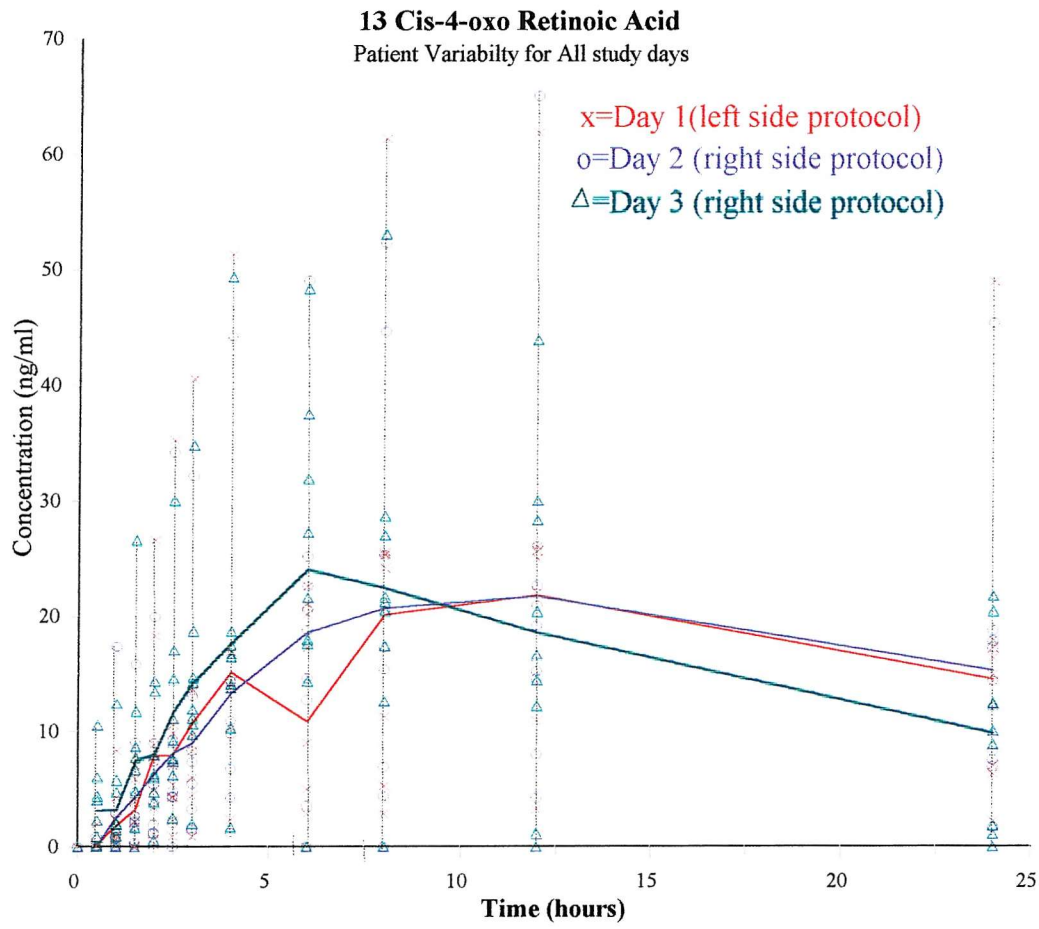

Figure 4.8: - Pharmacokinetic profile of 13-cis-4-oxo-retinoic acid, all three study days showing patient variability

parameters was observed between all phases.

C^{max} was achieved at a later time-point than that of 13-cis-retinoic acid. Elimination of 13-cis-4-oxo-retinoic acid was much slower resulting in elevated levels at 24 hours. Phase C demonstrated elevated levels prior to oral dosing. However, the subsequent change in concentration profile was seen to be almost identical to phases A and B (figure 4.8).

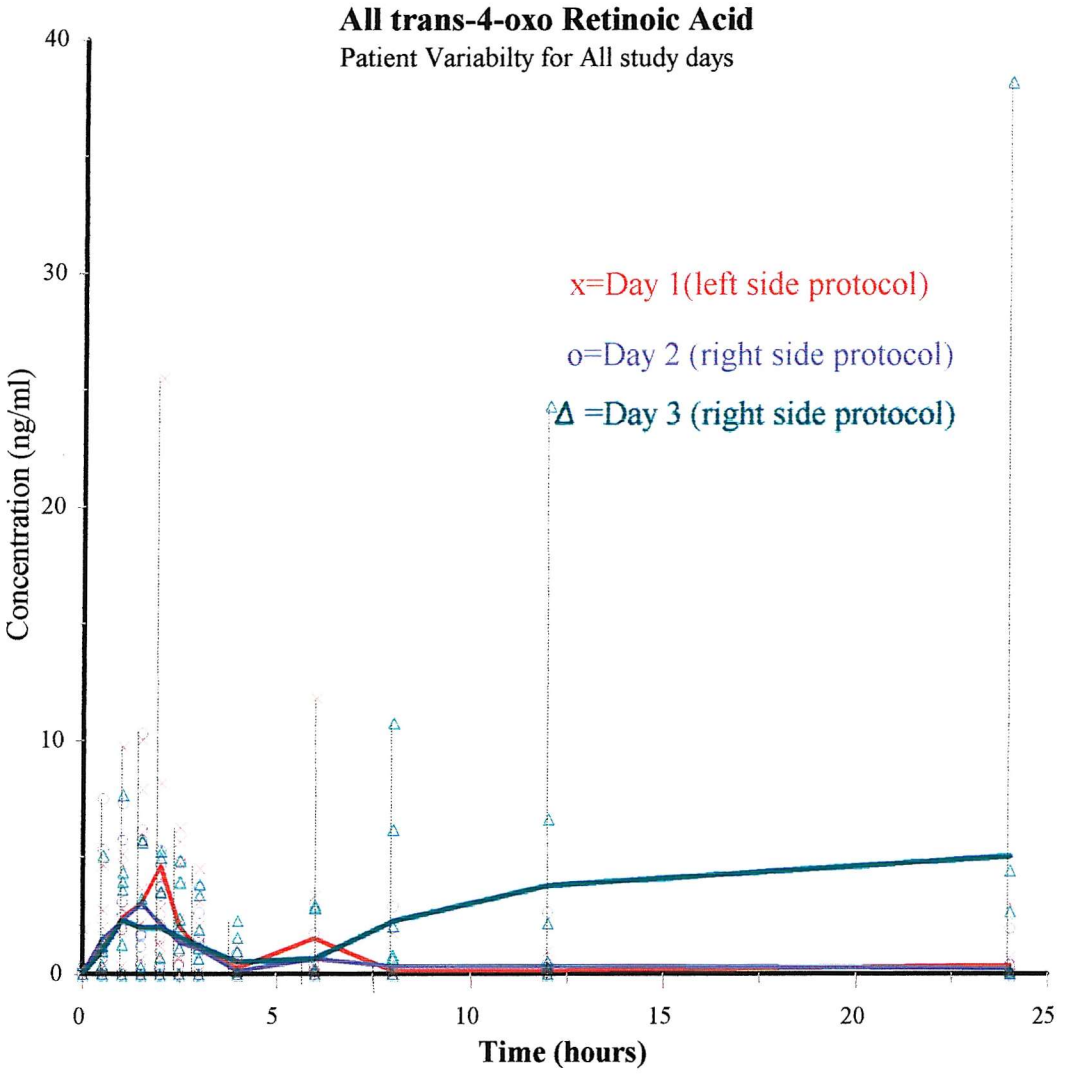

Figure 4.9: - Pharmacokinetic profile of all-trans-4-oxo-retinoic acid, all three study days showing patient variability.

4.4.2.6 All-trans-4-oxo-retinoic acid

Plasma concentration profiles showed similar peak concentrations for all phases (table 4.4). Phase A achieved a higher C^{max} than phases B and C due to the contribution of one patient with outlining results (figure 4.9). Phase C showed a significant increase in concentration after 6 hours due to high levels found in two patients.

All-trans-4-oxo-retinoic acid achieved a peak concentration within a range of 1.5 to 2 hours after dosing. By 4 hours, the concentrations had fallen to baseline levels (except for phase C). Statistical analysis showed that the AUC on day 3 was significantly different from day 2 ($P<0.05$), whereas the half life for day 1 was significantly different from day 2. In both cases it was suspected that analytical interference provided the false increase. However, this could not be determined. A significant difference was also seen between the deviations from the mean of T^{max} and the C^{max} for day 1 versus day 2. This could be attributed to the same cause mentioned above. No significant differences were observed for any other pharmacokinetic parameters (table 4.5).

4.4.2.7 9-cis-retinoic acid

It was observed from the analytical raw data that the response factor for 9-cis-retinoic acid was 50% for that of either 13-cis-retinoic acid or all-trans-retinoic acid. All levels detected where at the limit of detection (1 ng/ml) for this metabolite. Results used were therefore subjected to a significant degree of assay variability. However, figure 4.10 plots the change in concentration observed for 9-cis-retinoic acid for all three phases

against the time. Similar profiles were found for all three phases and evidence of a peak in concentration (C^{max}) between 1 and 1.5 hours (T^{max}) can be observed. The observed C^{max} is apparent in all three phases, although the concentrations are all below 10 ng/ml. This limits the degree to which these results can be considered accurate. However, the resulting profiles observed can be considered to be a reflection of the true situation although the patient variability for the 9-cis-retinoic acid was significantly higher than was observed for 13-cis-retinoic acid and all-trans-retinoic acid. There were no statistically significant differences for any of the comparisons made (Table 4.5).

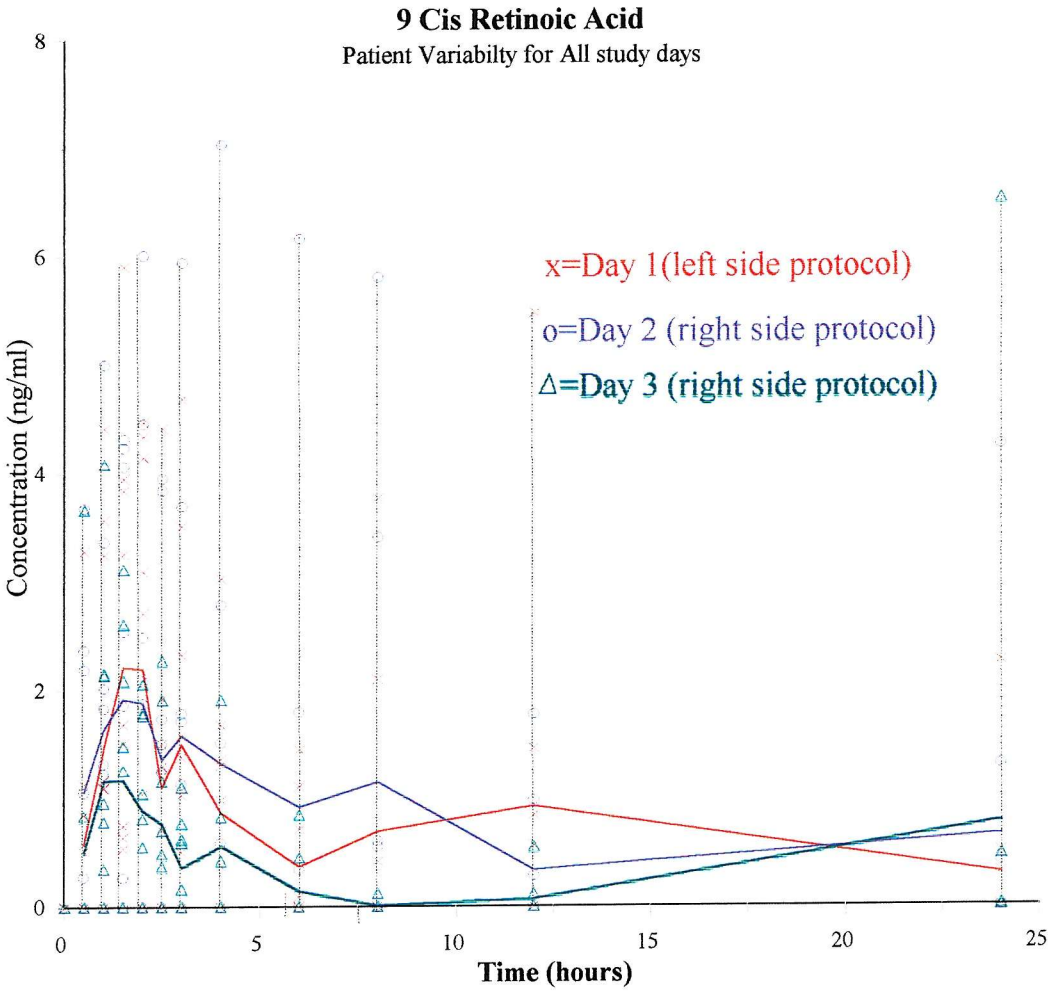

Figure 4.10: - Pharmacokinetic profile of 9 cis retinoic acid, all three study days showing patient variability

4.5 SUMMARY AND CONCLUSION

In the efforts to establish the pathways and extent of vitamin A in the human body, one problem has been consistently reported in the literature. This was the variability between individuals, even between the same individual on different days. Due to this variability and the large standard deviations, results obtained from research are difficult to interpretate. Differences in treatments can be significant in one study, but not significant in another identical study. The present study investigated two possible causes for inter- and intra- individual variability.

An important possible source of variability is the rate and extent of absorption of the oral dose, arising from the gastric and intestinal processes that occur during digestion of food material. Research on the effect of posture on the pharmacokinetics of orally administered nifedipine showed that subjects in different postures exhibited different bioavailability (1). Similar research has been conducted on paracetamol, verapamil, norverapamil, and propranolol (2;3) although only minor differences were observed. The study conducted by Renwick et al (1;3) focussed on the differences in absorption between subjects supine on their left side as opposed to their right side. This difference in posture could affect the rate of gastric emptying (4), which in turn can alter the absorption rate and bioavailability of an oral drug. Anatomically the stomach is positioned such that lying on the right side would have assistance of gravity in the passage of food material into the intestine. However, those on their left side would have the force of gravity inhibiting the movement of material into the intestine (5). Comparisons made by Renwick et al (1) demonstrated increased bioavailability for

nifedipine for subjects lying on their right side, accompanied by a reduction in the T^{max}. The conclusions drawn for the enhanced bioavailability was that nifedipine was cleared from the stomach at an increased rate which possibly saturated the absorption sites in the small intestine for a limited period.

The AUC for propranolol is also influenced by the position of the subject. The study which compared the pharmacokinetics of patients in a supine position and a tilted position (semi-upright), reported a 60% difference in the AUC in favour of the tilted position. Also, differences in the hepatic blood flow were observed between the two conditions (6).

In this study, no differences were observed in retinol (as the palmitate) absorption

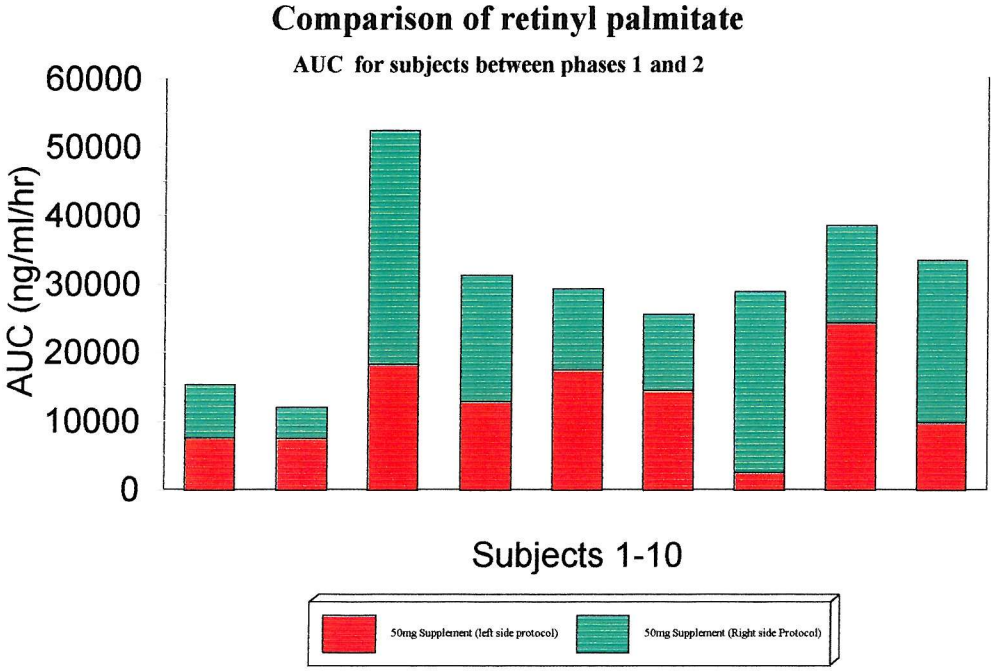

Figure 4.11: - Comparison between the absorbed amount of retinyl palmitate of individuals on different days. Each dose was given under identical conditions separated by a minimum of two weeks.

with oral dosing when lying supine on the right side or the left side (figure 4.11).

There was also no difference in the variability of the data between the different

phases. All phases showed the normal large inter- and intra- individual variability. Some subjects absorbed six times more than other subjects in similar time periods under carefully controlled dosing, but on other days could absorb four times less. It was also observed that there was no relationship between the plasma concentrations of the parent compound (retinol as retinyl palmitate) and its metabolites. The absorption range shown of retinyl palmitate is from 1026 to 6019 ng/ml between 10 randomly chosen subjects (section 4.4.2). The levels of palmitate absorbed by

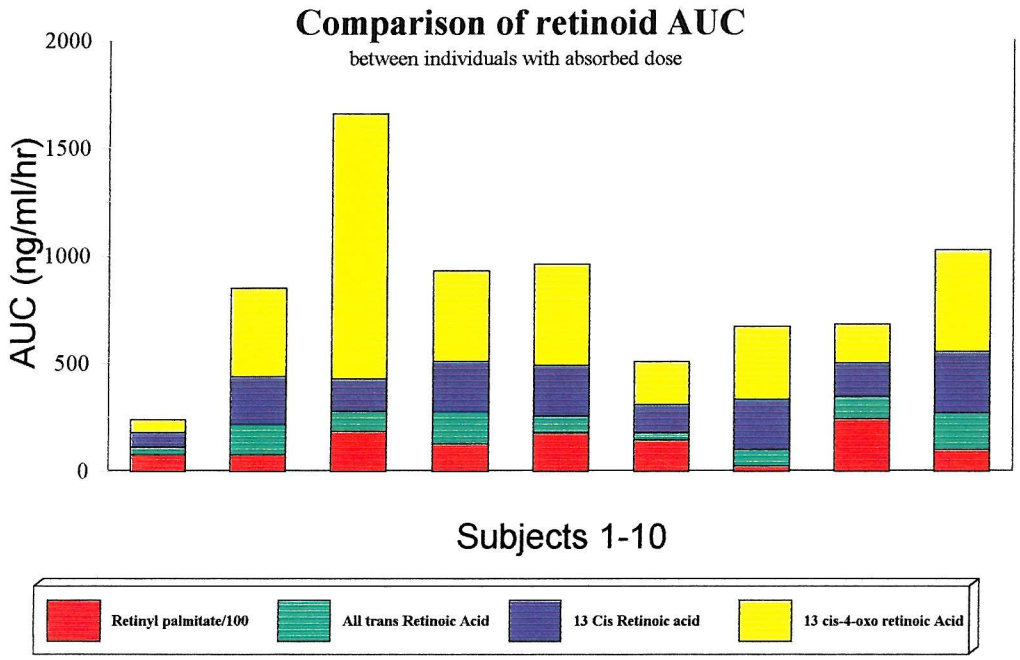

Figure 4.12: - Phase A comparison of retinoid AUC between individuals and the observed absorption of 50 mg retinol as the palmitate. Retinyl palmitate AUC shown are a graphical representation of the absorbed dose, actual AUC divided by a factor of 100. Metabolite AUC are shown as determined in ng/ml/hr.

different subjects was not reflected in the metabolite profiles determined. For example subject 5, a C^{max} for retinyl palmitate of 6019 ± 50 ng/ml was observed, whereas the major metabolite levels found were 37 ng/ml, 39 ng /ml, 1.2 ng/ml and 26 ng/ml respectively for all-trans-retinoic acid, 13-cis-retinoic acid, 9-cis-retinoic

acid and 13-cis-4-oxo-retinoic acid. However, subject 2 had a C^{max} for retinyl palmitate of 1026 ng/ml, the levels of the major metabolites measured were 74 ± 2 ng/ml, 48 ± 2 ng/ml, 5 ng/ml and 25 ng/ml ng/ml respectively. Figure 4.12 and figure 4.13 illustrate the fact that there is no link between the AUC values of retinyl palmitate and its metabolites. It is concluded that although posture affects the bioavailability of some drugs in man, the same is not true for vitamin A, and the large sources of variation between individuals remains unexplained.

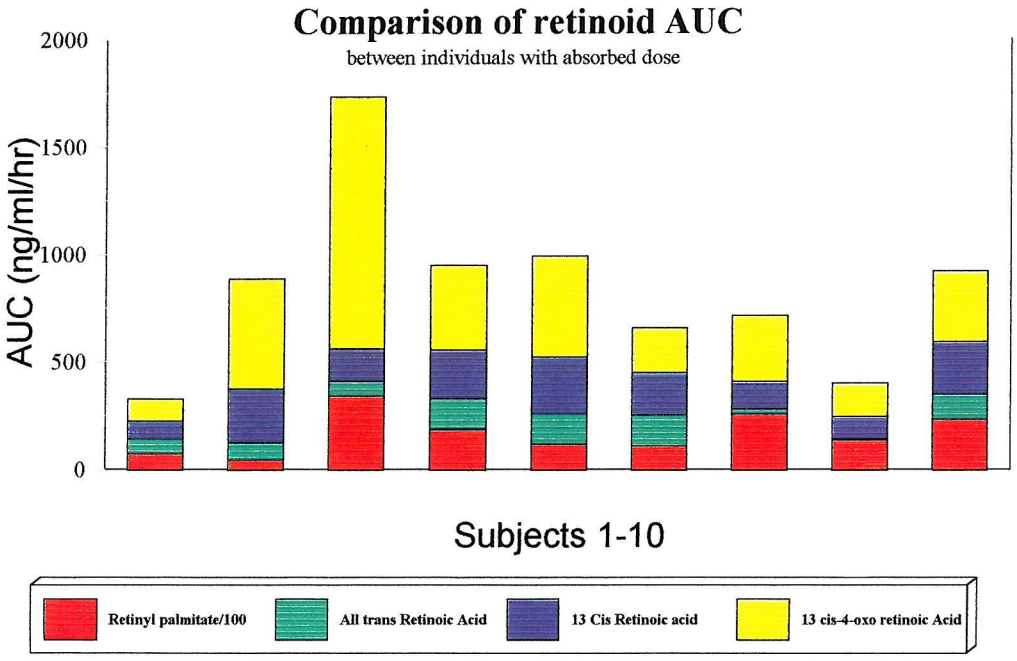

Figure 4.13: - Phase B comparison of retinoid AUC between individuals and the observed absorption of 50 mg retinol as the palmitate. Retinyl palmitate AUC shown are a graphical representation of the absorbed dose, actual AUC divided by a factor of 100. Metabolite AUC are shown as determined in ng/ml/hr.

Further comparisons made with the data generated included a male: female comparison (n=5,5). Subjects involved in the study were five adult females and five adult males with an age range of 21-22 years and 21-23 years respectively. Mean data for the determined pharmacokinetic profile of male verse female is shown in

figure 4.14. No differences were observed between the two sexes in the mean metabolite profiles measured and the amount of retinol as the palmitate absorbed. There was no significant difference in the variability of the absorption of vitamin A between male or female subjects.

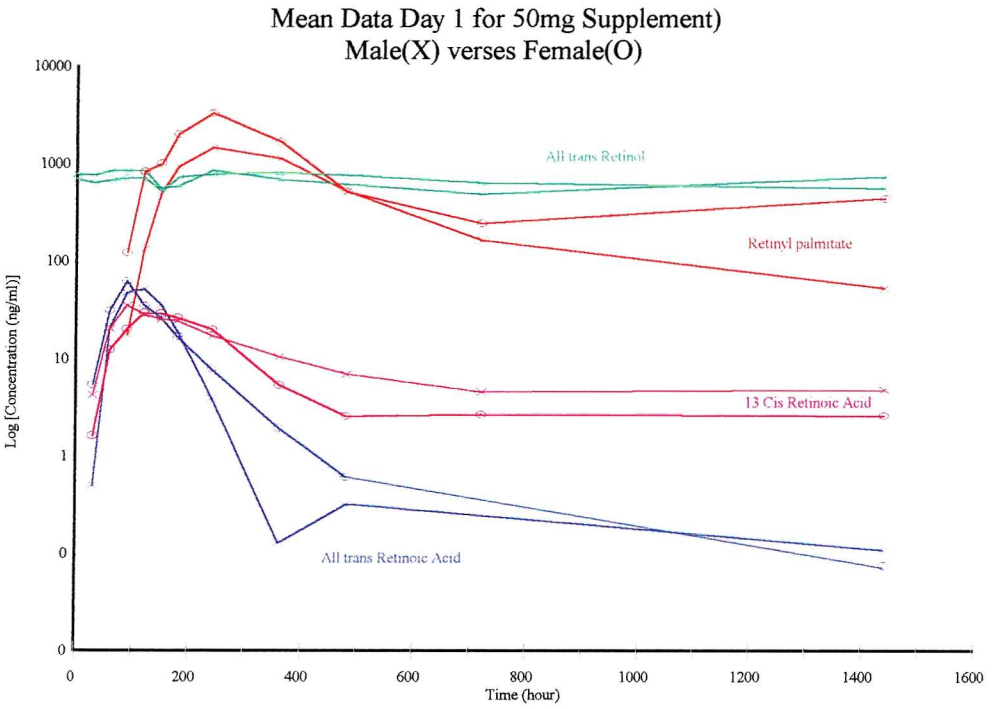

Figure 4.14 Male and female retinoid concentration profiles following a 50 mg dose of retinol as the palmitate. Protocol was left side supine. Similar data is observed for right side supine.

The second aim of the study was to examine the effect previous dosing had on the subsequent absorption and metabolite formation of a 50 mg dose of retinol as the palmitate. Previous work by Tembe et al (7) in rats and rabbits demonstrated that a previously given dose of all-trans-retinoic acid had a significant detrimental effect on a dose given within a short time period. It was observed that the AUC for a second and third dose given within three - four hours of the first dose, was significantly decreased. However, it should be noted that the dose given in the animal studies were

relatively much greater than the doses given to humans in the present investigation. Pharmacokinetics determined within acute promyelocytic leukemia patients treated with all-trans-retinoic acid showed an initial increase in plasma concentrations. As a result several patients went into remission from the treatment (8,9). However, with a continuous regular dosing schedule, all-trans-retinoic acid plasma concentrations decreased. Consequently, remission was reversed. Treatment was therefore no longer effective. Although a drug holiday will restore normal circulatory levels, further treatment with all-trans-retinoic acid proved less effective (10) unless the drug holiday was greater than 1 year.

In the present investigation, it can be concluded that repetitive dosing under the time frame considered does not affect the pharmacokinetics associated with each retinol palmitate dose. Hence, a situation as observed by Tembe et al (7) in animal models was not apparent in a human model at this dose. Therefore, it can assumed that short term multiple dosing has little effect on the large inter- individual variation observed in most human and animal studies with retinoid pharmacokinetics.

4.5 REFERENCES

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Chapter 5

The Influence Of Multiple Dosing On The Absorption Of Vitamin A And The Formation Of Its Teratogenic Metabolites.

Table of Contents

List of Tables

List of Figures

5.1 INTRODUCTION

Retinoids play an important role in the treatment of some medical illnesses. All-trans-retinoic acid and 13-cis-retinoic acid are commonly used in cancer therapy and in certain diseases of the skin (1;2). One fact that has been observed with the prolonged oral use of all-trans-retinoic acid and 13-cis-retinoic acid is that plasma levels of the active component decrease over time by as much as 80%. The effectiveness of the treatment is therefore negated since the time periods required for such a significant decrease is relatively short (1-6 weeks) (3).

Retinol and/or retinyl palmitate are taken regularly by large group of the general population within commercial supplementation or fortified foods. It is unknown whether prolonged exposure to such supplementation would cause a decrease in retinol or retinoid metabolites plasma levels. In a previous study, retinol was dosed daily at 5 times the RDA over a period of eight weeks. Analysis of the plasma concentrations of retinol showed that the baseline levels of the subjects tested did not change outside of normal variability. This study did not report on the effects of the various teratogenic metabolites of vitamin A. Within pharmacokinetic investigations of vitamin A on acute promyelocytic leukemia remission it has been shown that repeated dosing of all-trans-retinoic acid resulted in lowering of the overall plasma concentrations (3-5). This effect reduced the impact of treatment on acute promyelocytic leukemia patients and in some case remission was reversed. Such an effect for retinol dosing could, therefore, reduce the availability of the active metabolites of vitamin A for differentiation or cellular growth.

The aim of this investigation was to study the effects of a repeated dose of retinol palmitate on the subsequent plasma levels of vitamin A and its active metabolites. It has already been previously shown that identical doses of retinol palmitate within 24 hours of each other do not change the pharmacokinetics typically observed. Therefore, for this investigation a period of 8 days was used to investigated the effects of multiple dosing on the plasma levels of vitamin A and its metabolites.

5.2 METHODS

5.2.1 Subjects

The study population was derived from healthy young males between the age of 18 and 60 years. The mean ± s.d. age, height and weight of the study population was respectively 25.4 ± 6.7 years (range 21 - 41 years), 176.9 ± 6.7 cm (range 168 - 188 cm) and 77.3 ± 11.9 (range 63 - 94 kg). One subject smoked 10 cigarettes per day and alcohol consumption was within 20 units per week for all subjects. One subject had mild asthma for which he took salbutamol inhalers as required. No instances of salbutamol intake were needed during the study period. One subject regularly took multivitamin preparations which was stopped one week prior to the study. None of the above was expected to interfere with the study results.

Note:- A total of ten subjects participated in this study. One subject was excluded due to analytical problems; refer to pgs 196-197

5.2.2 Study Design and Treatment

Subjects were asked to take 50 mg of retinyl palmitate in the form of 1.18 g Arovit on each day for eight days after an overnight fast. Subjects attended the Clinical Investigation Unit on day 1 and 8 when blood samples were taken at the following post-dose time intervals : 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 24 hours. On these 2 days, subjects were required to remain fasted for the first 4 hours of post-dose sampling. A standard lunch was served afterwards.

5.1.3 Compliance and Dose Administered

Day 1 / Dose 1: A oral administration of 50 mg retinol was given as the palmitate at time 0 on day 1. Dose compliance was 100%.

Day 2 - 7: Sealed pre-weighed containers were supplied to each subject on day 1 containing 50 mg retinol as the palmitate. Each container was wrapped with aluminum foil for protection against light degradation and labeled for each day of use. Subjects were required to consume one retinol dose for each day between the two pharmacological study days. The used containers were returned to the study organizer on day 8 and re-weighed to verify dose given. Compliance based on this evidence was assumed to be 100%.

Day 8 / Dose 8: A oral administration of 50 mg retinol was given as the palmitate at time 0 on day 8. Dose compliance was 100%.

5.3 RESULTS

5.3.1 General Concentration Profiles

In the pharmacokinetic profiles for day 1 (figure 5.1) and day 8 (figure 5.2) all components of interest except for retinol increased with time to a maximum concentration before returning to baseline levels. Dosing on day 8 followed a similar pattern to that for day 1, although higher C^{max} concentrations were reached. However, the change from baseline to C^{max} concentrations was similar to that observed for day 1. On both treatment days it was observed that all-trans-retinoic acid and 13-cis-retinoic acid had an initial sharp increase

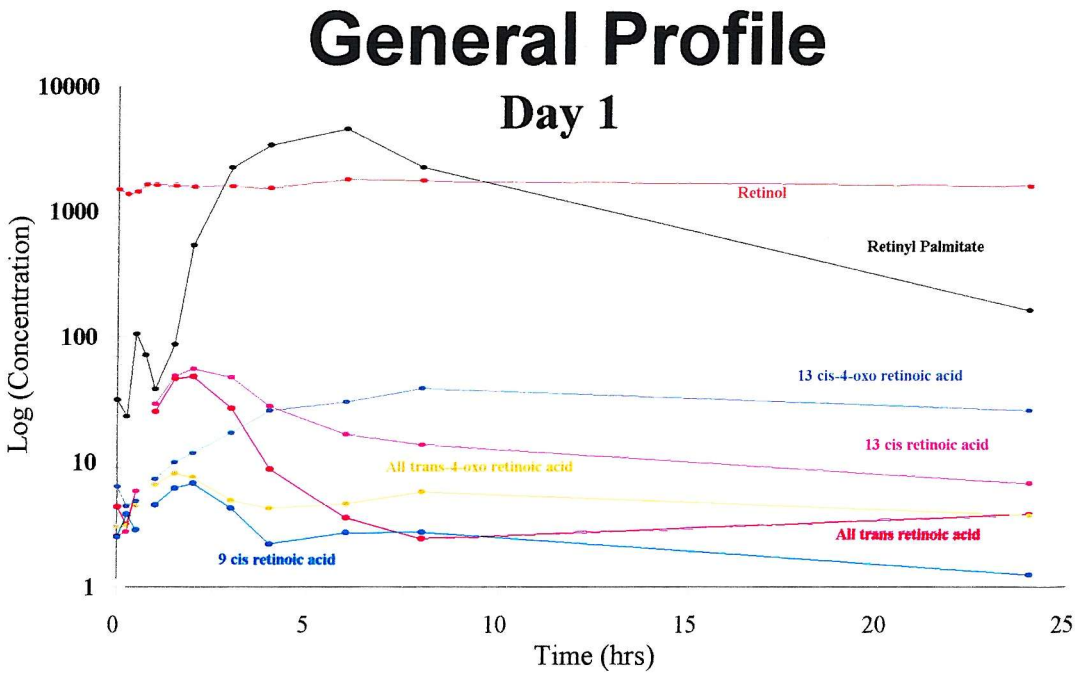

Figure 5.1: General concentration time profile for pharmacokinetics performed on day 1 of the multi-dose experiment.

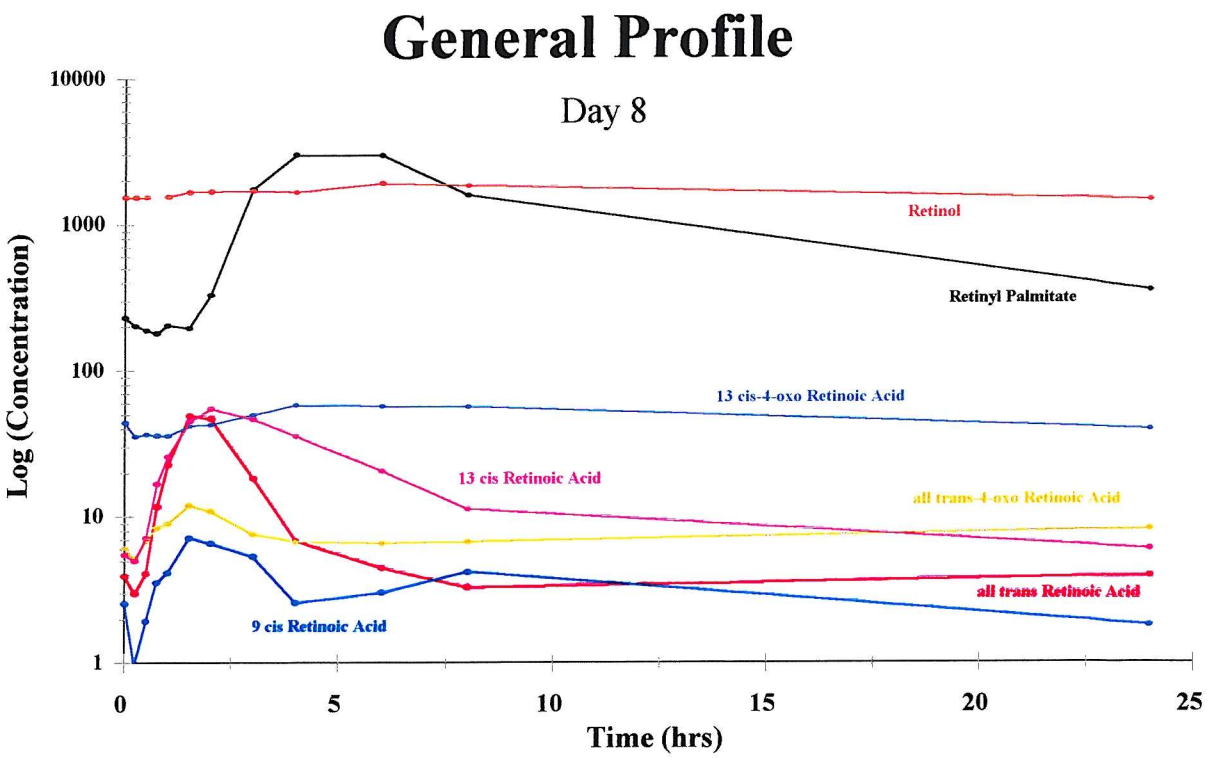

Figure 5.2: General concentration time profile for pharmacokinetics performed on day 8 of the multi-dose experiment.

occurring within 0.5 to 1 hr of the dose being administered. This increase reached a maximum by 2.5 hrs in most cases. All-trans-retinoic acid returned to baseline by the 5 hour mark, whereas 13-cis-retinoic acid was still elevated at 30-40% of the C^{max} concentration by the 24 hour mark. Retinyl palmitate showed a sharp increase in concentration reaching C^{max} values between 4-6 hrs post dose. On day 1, the C^{max} concentration were approximately 100 fold higher than the pre-dose baseline levels. On day 8 the mean retinyl palmitate values reached similar concentrations to that of day 1. However, on day 8 retinyl palmitate pre-dose values ranged from 150 ng/ml to 250 ng/ml, which were approximately 2-fold higher than those observed for day 1. Therefore, the change in concentration after dosing is less for day 8 as compared to day 1. From the maximum, retinyl palmitate showed a steady decrease to the 24 hour mark. Plasma levels

did not reach baseline values in the time frame used for this investigation. It should be noted that, all-trans-retinoic acid had returned to baseline levels when the retinyl palmitate reached C^{max}. No further rise in baseline for all-trans-retinoic acid was observed for either day. Polar metabolites of all-trans-retinoic acid and 13-cis-retinoic acid also rose from initial low baseline values to a peak concentration prior to decreasing back to baseline levels. All-trans-4-oxo-retinoic acid achieved a peak concentration at approximately 2.5 hours, but at only 15% (± 10%) of the peak concentration observed for all-trans-retinoic acid. In the day 1 profile, 13-cis-4-oxo-retinoic acid increased steadily for 8 hours post dose, reaching a steady baseline that did not decrease by much after 24 hours. In the day 8 profile, the pre-dose levels of 13-cis-4-oxo-retinoic acid was significantly higher in comparison to day 1. Plasma concentrations of 9-cis-retinoic acid were also determined. It was observed that the concentration-time profiles and peak concentrations were similar to that seen for all-trans-4-oxo-retinoic acid. Mean pharmacokinetic parameters for day 1 and day 8 are shown in table 5.1 and 5.2.

Table 5.1: Mean pharmacokinetic parameters for 9 male subjects after a 50 mg oral supplement dose retinol as the palmitate on a fasting stomach.

Day 1	C^{max}	T^{max}	AUC	$t_{1/2}$	MRT
All-trans-retinoic acid	59.1 ± 29.5	1.8 ± 0.4	90.6 ± 45.8	1.2 ± 0.4	2.3 ± 0.4
13-cis-retinoic acid	63.3 ± 20.5	2.0 ± 0.4	219.4 ± 86.1	2.8 ± 2.3	3.3 ± 1.5
retinyl palmitate	7170 ± 3752	5.6 ± 1.3	47053 ± 30364	4.1 ± 0.7	6.9 ± 0.6
retinol	2105 ± 1160	4.7 ± 2.9	6811 ± 9998	100 ± 75	10.7 ± 2.5
9-cis-retinoic acid	9.2 ± 2.9	1.5 ± 0.8	25.0 ± 26.0	5.6 ± 4.8	3.4 ± 1.9
All-trans-4-oxo-retinoic acid	9.6 ± 2.9	2.4 ± 1.4	17.6 ± 7.2	3.2 ± 3.2	3.2 ± 1.8
13-cis-4-oxo-retinoic acid	34.6 ± 12.9	7.1 ± 1.6	532.0 ± 285.7	26.0 ± 7.9	10.3 ± 3.1

Table 5.2: Mean pharmacokinetic parameters for 9 male subjects after seven days of dosing with 50 mg oral supplement retinol as the palmitate on a fasting stomach .

Day 1	C^{max}	T^{max}	AUC	$t_{1/2}$	MRT
All-trans-retinoic acid	53.7 ± 31.3	1.6 ± 0.3	82.3 ± 52.0	1.1 ± 0.3	2.3 ± 0.5
13-cis-retinoic acid	62.3 ± 15.1	2.3 ±0.8	197.0 ± 93.9	2.3 ± 2.7	3.4 ± 1.4
retinyl palmitate	4766 ± 5605	5.0 ± 1.9	29174 ± 26530	3.2 ± 2.1	6.2 ± 2.0
retinol	2188 ± 1176	4.1 ± 2.4	10123 ± 19438	222 ± 277	9.0 ± 3.3
9-cis-retinoic acid	8.8 ± 1.7	1.7 ± 0.9	14.9 ± 8.8	3.8 ± 2.9	2.7 ± 0.9
All-trans-4-oxo-retinoic acid	13.8 ± 7.7	1.8 ± 1.4	21.8 ± 16.2	2.6 ± 1.6	2.8 ± 1.0
13-cis-4-oxo-retinoic acid	31.5 ± 14.7	5.0 ± 2.6	358.2 ± 359.7	29.8 ± 10.9	10.7 ± 1.0

5.3.2 Metabolite Concentration Profiles

5.3.2.1 All-trans-retinoic acid

A concentration-time profile was determined for all nine subjects on day 1 and day 8 of the investigation. Mean data for all subjects are shown in figure 5.3 for both treatment days. It can be observed that there was an almost identical profile for the mean data between the two treatment days. The AUC and C^{max} parameters determined for day 1(AUC_{day1}= 90.6 ± 45.8; C^{max}_{day1}= 59.1 ± 29.5) and day 8 (AUC_{day8}=82.3 ± 52.0; C^{max}_{day8}= 53.7 ± 31.3) were within ±7% of a mean between the two. Statistical analysis showed no

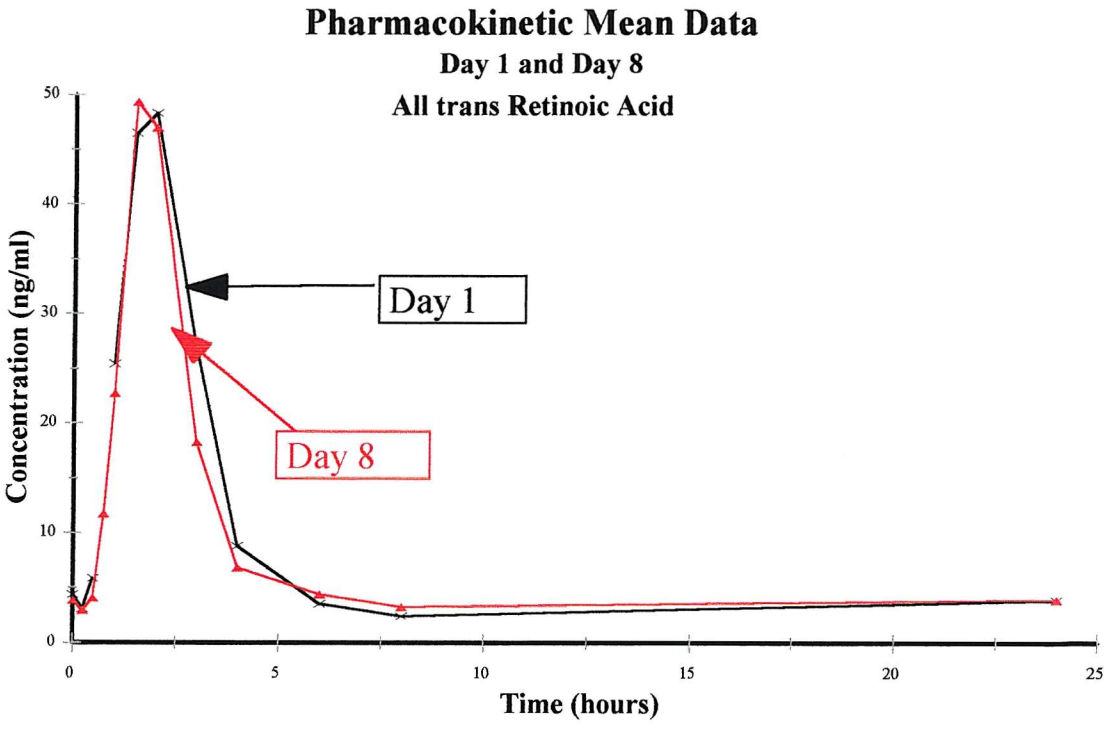

Figure 5.3: Comparison of day 1 and day 8 concentration-time profiles for all-trans-retinoic acid.

significant difference between these pharmacokinetic parameters. Histograms of AUC and $t_{1/2}$ are shown in figures 5.4 and 5.5 respectively. Plotted according to subject age, it can be observed from figure 5.4 that six subjects showed a 44.4 ± 19.8% drop in AUC between day 1 and day 8. The remaining three subjects showed a gain of 41.7 ± 10.9% between day 1 and day 8. The mean of the differences for all nine subjects showed a 10% difference between day 1 and day 8. Previously, also no difference was observed in the 24 hour repeat dosing study between day 1 and day 2 (chapter 4). Other parameters determined were T^{max}, $t_{½}$ and MRT, no statistical difference could be observed between day 1 and day 8 for these parameters. Values found for day 1 and day 8 are directly comparable to data found previously (tables 4.4 and 3.7-3.11) when dosing with 50 mg of retinol as the palmitate.

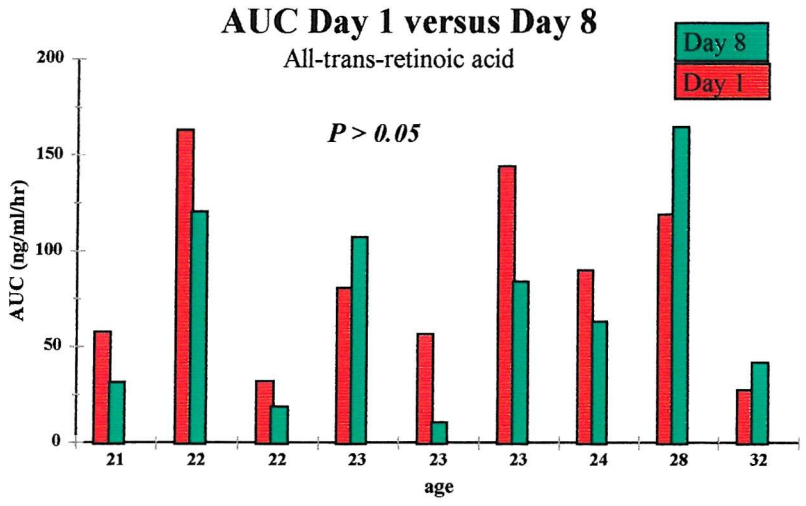

Figure 5.4: Comparison of day 1 and day 8 AUC values for all-trans-retinoic acid.

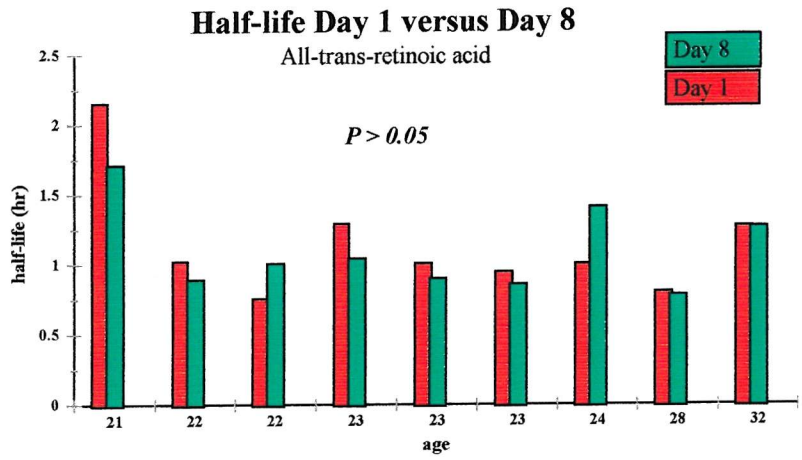

Figure 5.5: Comparison of day 1 and day 8 half-life values for all-trans-retinoic acid.

5.5.3.2 Retinyl Palmitate

In the analysis of the data generated by the nine subjects investigated, it was found that one subject did not exhibit plasma- time profiles similar to the other subjects. Peak concentrations at C^{max} compared to all the other subjects was 20-30 times lower. Upon investigation this subject was found to be on a regular vitamins and minerals supplement medication. This medication contained vitamin A in 100% RDA doses. The subject stopped the medication one week prior to the start of the trial. However, in light of the investigation aims and the results observed, it can be concluded that one week was

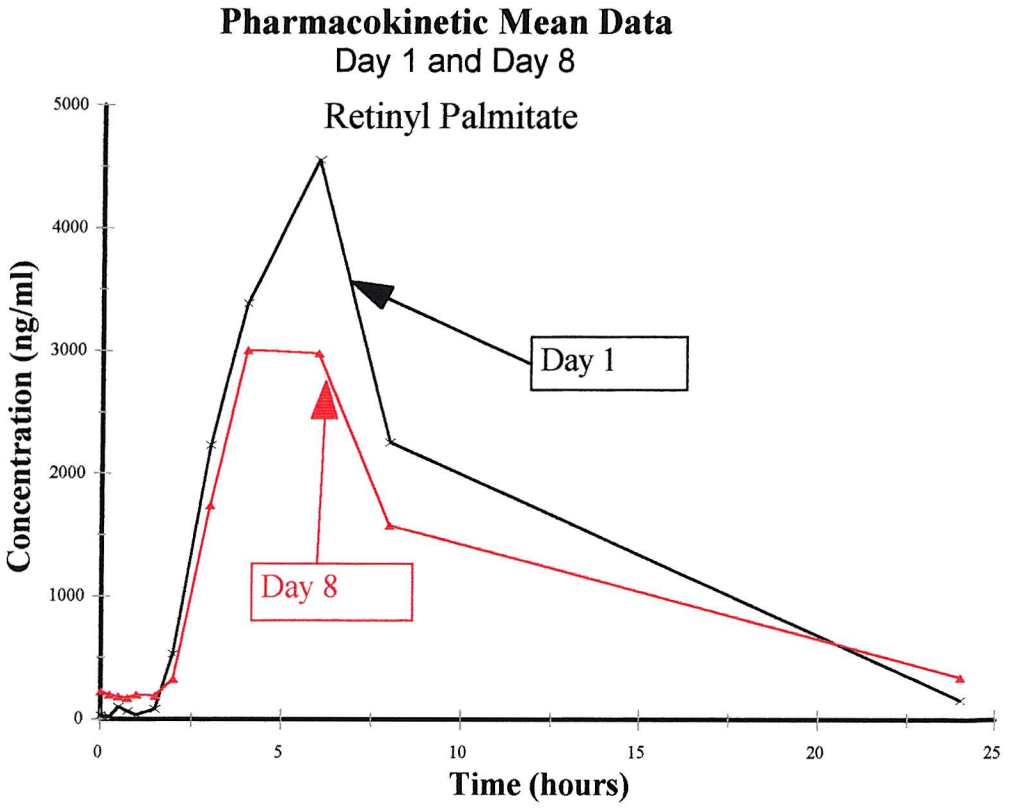

Figure 5.6: Comparison of day 1 and day 8 concentration-time profiles for retinyl palmitate.

insufficient to return homeostatic mechanisms to normal. Therefore, the retinyl palmitate data was considered to be abnormal for this subject. The results of this subject was, therefore, excluded from the investigation. Interestingly, the levels of the metabolites for this subject while lower than the average, followed the general profile expected, however, lower than the average.

Time period	0	0.25	0.50	0.75	1.0	1.5	Mean
Day 1 (ng/ml)	43.4	33.1	248.5	145.7	69.1	75.3	102.7
Day 8 (ng/ml)	253.6	177.1	160.5	173.3	196.3	164.0	188.8

Table 5.3: Plasma concentrations of retinyl palmitate within the first 1.5 hours post dose on day 1 and day 8

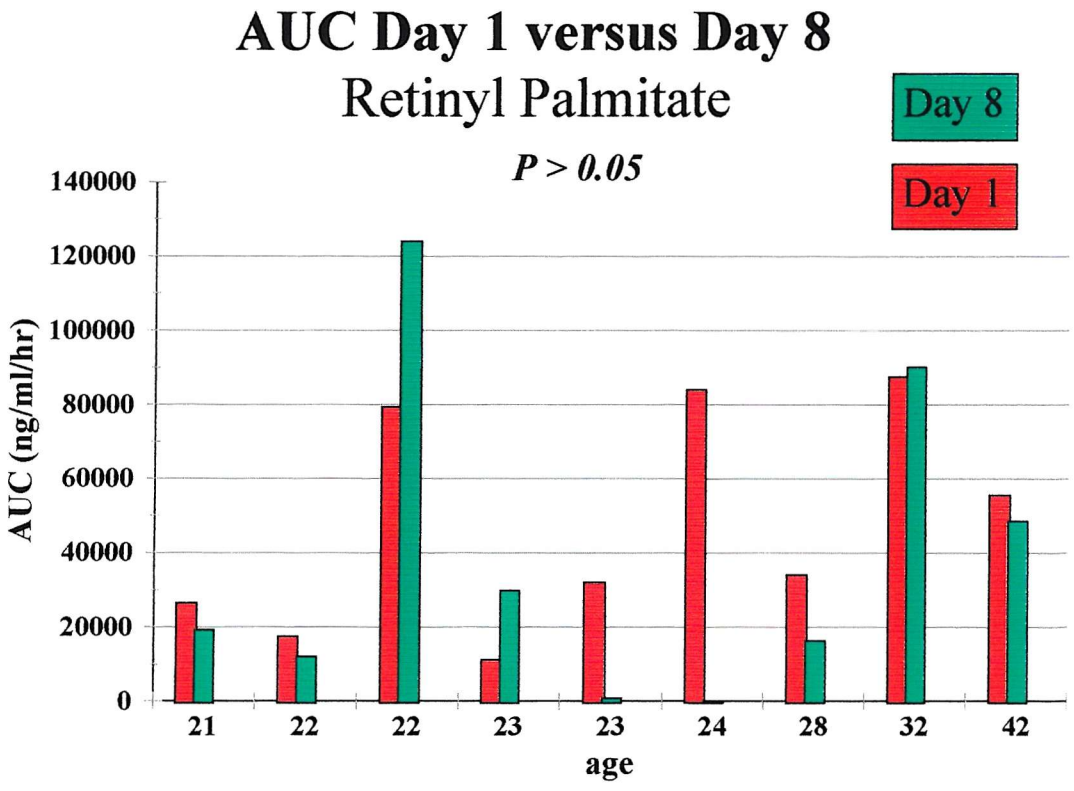

Figure 5.7: Comparison of day 1 and day 8 AUC values for retinyl palmitate.

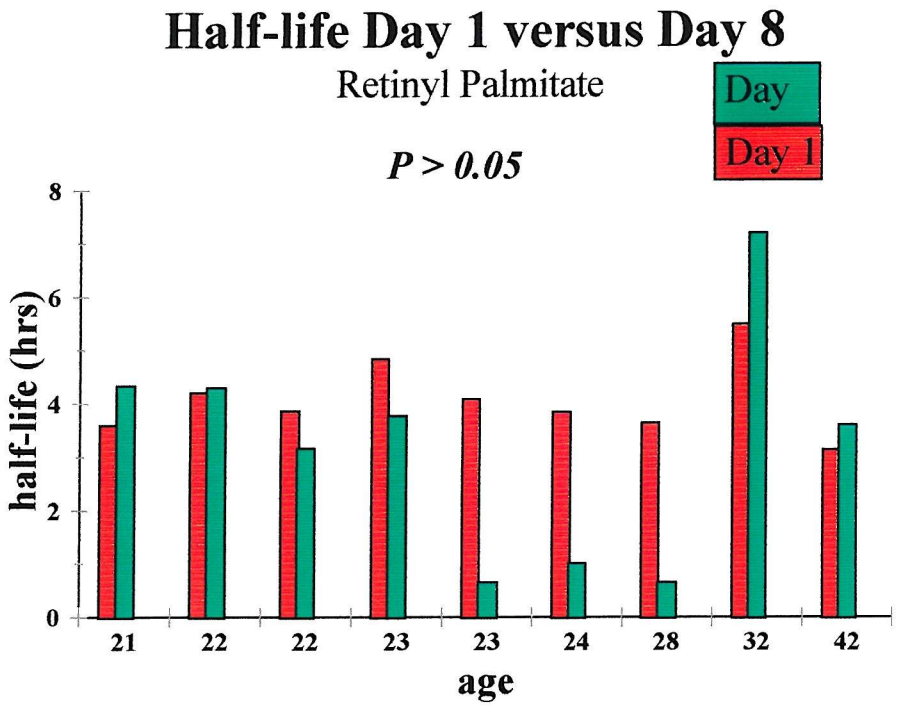

Figure 5.8: Comparison of day 1 and day 8 half-life values for retinyl palmitate.

The mean concentration-times profiles for the remaining 9 subjects were similar for day 1 and day 8, although day 1 appeared graphically to be greater than day 8 (figure 5.6). Comparison of the first 1.5 hours of data (baseline levels) between day 1 and day 8 is shown in table 5.3. Plasma concentrations for the day 8 appears to be elevated compared to day 1. The baseline levels shown in table 5.1 indicate that on day 1 baseline average is 102.7 ng/ml whereas on day 8 the baseline average is 188.8 ng/ml. Assay accuracy is limited to the lowest standard detectable, this is 50 ng/ml (Chapter 2, section 2.). Therefore the % difference between day 1 and day 8 is not conclusive evidence of accumulation since the average between the two (145.7 ± 50 ng/ml) covers the range in which the two baseline values fall. Statistical analysis of the data in table 5.1 indicate the

the difference is not significant. Pre-dose values averaged at 0.5 -1.0% of the observed C^{max} (C^{max}_{day1}=7170 ± 3752; C^{max}_{day8}= 4766 ± 5605). The C^{max} values vary significantly from as low as 200 ng/ml to as high as 13500 ng/ml. After the 2 hour mark the baseline begins to rise rapidly to peak at 3 - 6 hours after dosing for both treatment days (T^{max}_{day1}=5.6 ± 1.3; T^{max}_{day8}= 3.2 ± 2.1). Following C^{max} concentration the plasma concentration decays down to approximately 30% C^{max} in the 24 hour period investigated. The profiles observed for day 1 and day 8 retinyl palmitate concentration shown in figure 5.6 suggest that day 8 showed a lower absorption compared to day 1. However, statistical analysis of the C^{max} values (t-Test) indicate that there was no significant difference between the two dosing days after the baseline values have been subtracted ($P > 0.05$). Closer investigation of the day 1 and day 8 data showed that the AUC values for two individuals were very different on day 8 compared to day 1. Figure 5.7 shows the AUC data for all 9 individuals plotted against age. For two subjects the AUC for day 8 (AUC_{sub1}= 3976 ng/ml/hr; AUC_{sub3}= 2369 ng/ml/hr) was 15-30 fold lower than the AUC for day 1 (AUC_{sub1}= 83841 ng/ml/hr; AUC_{sub3}= 32769 ng/ml/hr). The two subjects in question were not taking any supplement medication and were both non-smoking. Although each subject was asthmatic, other subjects had similar medical histories but did not exhibit the same difference between day 1 and day 8. Correspondingly the half-life for these subjects is also reduced by up to 30% of day 1 ($t_{1/2}^{day1}{}_{sub1}$= 3.81; $t_{1/2}^{day8}{}_{sub1}$= 1.06; $t_{1/2}^{day1}{}_{sub3}$= 4.08; $t_{1/2}^{day8}{}_{sub3}$= 0.65: figure 5.8). Only one other subject showed a lower half life on day 8 as compared to day 1. This subject also showed a 50% decrease in AUC between day 1 and day 8. All other subjects showed AUC and half-life values that are similar between day 1 and day 8.

5.5.2.3 13-cis-retinoic acid

All nine subjects demonstrated a consistent concentration time profile between day 1 and day 8. Graphical representation (figure 5.9) of the concentration time data showed almost identical profiles for the two treatment days. The initial baseline values for both treatment days were the same within given limits. Post dosing, both concentration time profile illustrate a rapid rise from the baseline values to peak between 2-3 hours (T^{max}_{day1}= 2.0 ± 0.4 hrs; T^{max}_{day8}= 2.3 ± 0.8 hrs). Peak concentrations reached were similar for both treatment days (C^{max}_{day1}= 63.3 ± 20.5 hrs; C^{max}_{day8}= 62.3 ± 15.1). Calculated AUC values

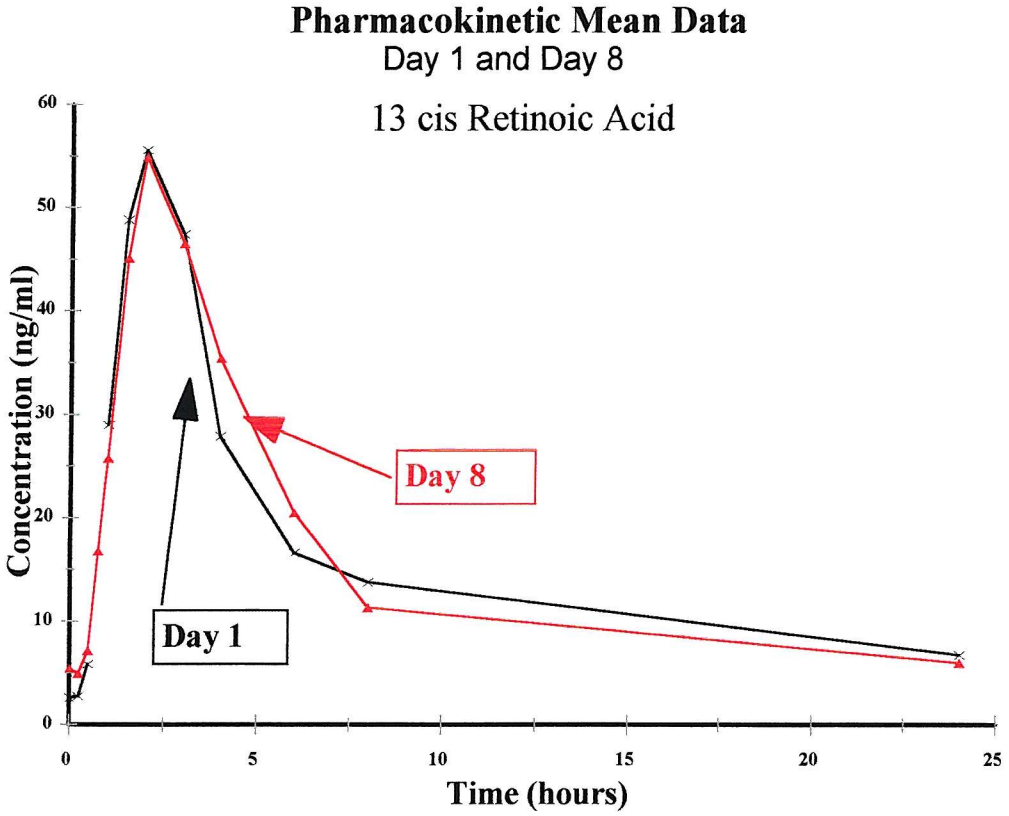

Figure 5.9: Comparison of day 1 and day 8 concentration-time profiles for 13-cis-retinoic acid

for all nine subjects are plotted in figure 5.10. All subjects exhibited consistent AUC values for both treatment days (AUC_{day1}= 219.4 ± 86.1ng/ml/hrs; AUC_{day8}= 197.0 ± 93.9 ng/ml/hr), although there was some variation between individual subjects. The variability did not seem to correspond to subject age. Half-life values calculated from baseline subtracted data showed little difference between day 1 and day 8 ($t_{½}^{day1}$= 63.3 ± 20.5 hrs; $t_{½}^{day8}$= 62.3 ± 15.1) figure 5.11). AUC and C^{max} values found for all subjects were similar to that previously determined (table 4.4; tables 3.7-3.11). No significant difference between the pharmacokinetic parameters T^{max}, C^{max} or MRT determined for 13-cis retinoic acid was found between day 1 and day 8.

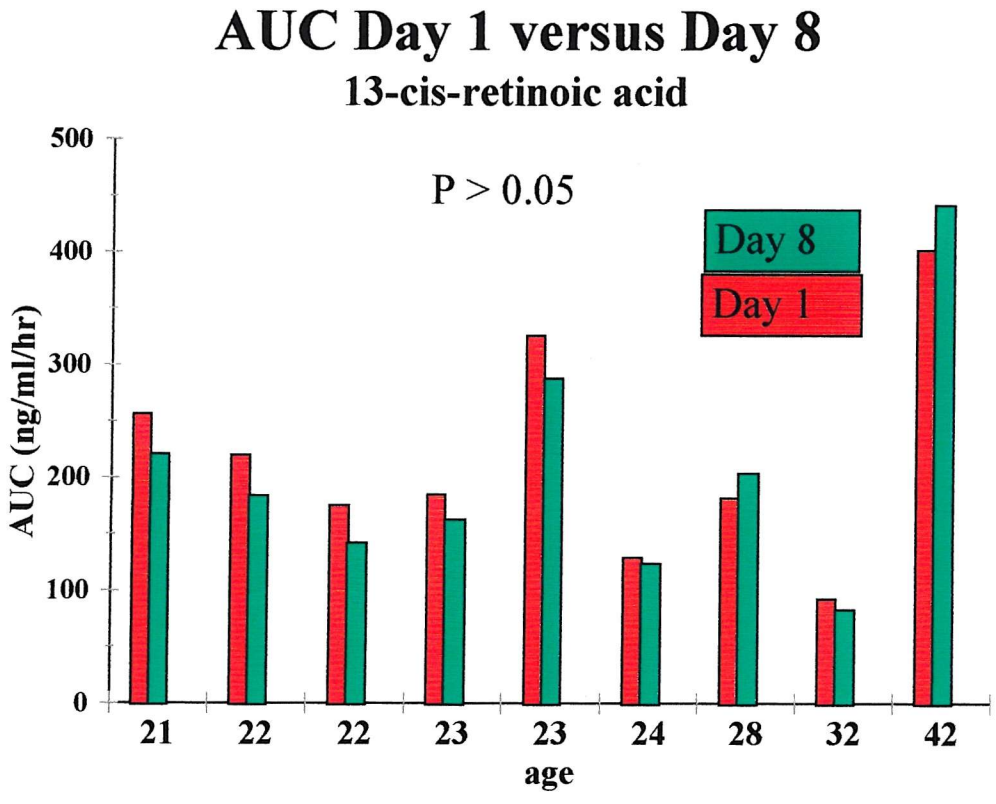

Figure 5.10: Comparison of day 1 and day 8 AUC values for 13-cis-retinoic acid.

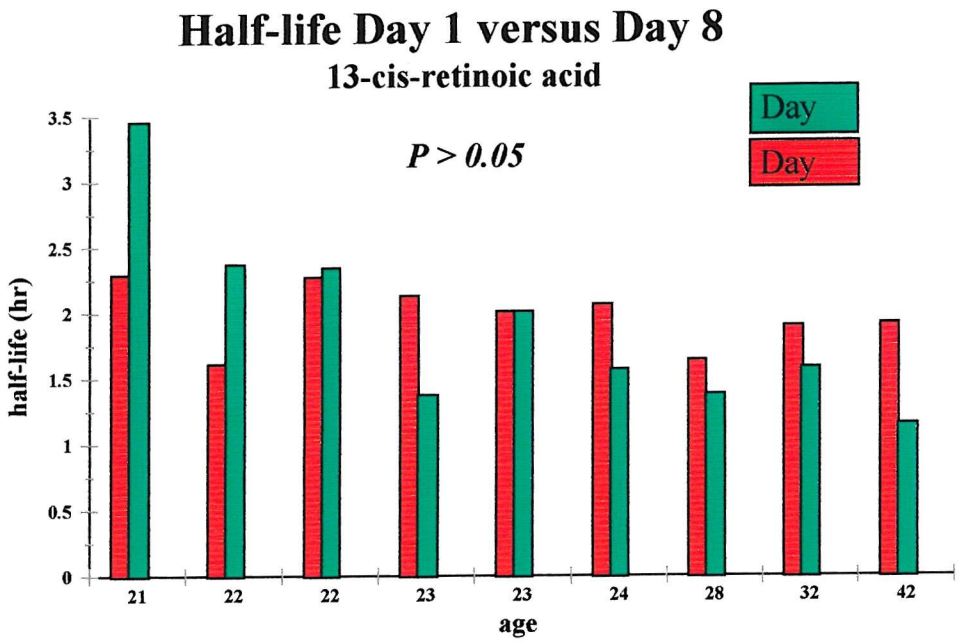

Figure 5.11: Comparison of half-life values for 13-cis-retinoic acid.

5.3.2.4 Retinol

All-trans retinol plasma concentration has previously been found to be minimally affected by the dosing of individuals with retinol as the retinyl palmitate, either orally or by skin absorption (Chapters 3 - 4; Chapter 6). The effect of dosing with 50 mg of retinol as the palmitate everyday for 8 days also does not appear to effect the retinol content observed within the plasma (figure 5.12). No observable differences can be seen between the pharmacokinetic parameters determined for retinol on day 1 and day 8. AUC values

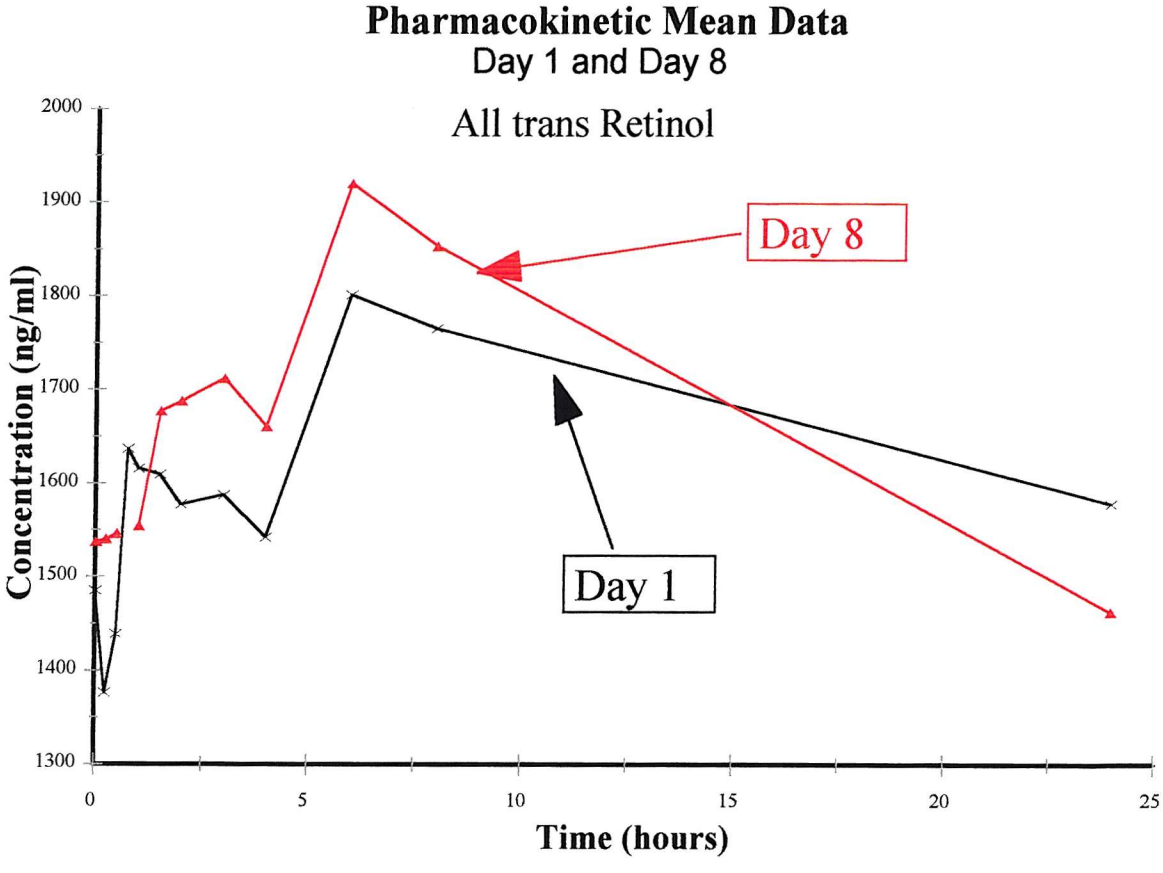

Figure 5.12: Comparison of day 1 and day 8 concentration-time profiles for retinol.

determined for 7 subjects were similar between day 1 and day 8 (AUC_{day1}= 6811 ± 9998 ng/ml/hr; AUC_{day8}= 10123 ± 19438 ng/ml/hr)(figure 5.13). Two subjects showed a difference in AUC between day 1 and day 8, one with a 70% drop and one with a 70% gain. Considering the endogenous background levels (typically 6000-10000 ng/ml) of retinol within plasma this variation between treatment days is not considered to be caused by the investigated factor. Similar variations within the same subject have been noted previously (chapter 3 and chapter 4). Figure 5.14 shows the half-life parameter determined for day 1 and day 8. This parameter is subject to experimental error due to the minimal change in concentration seen after oral dosing with retinol. Accurately determining a change of such a low level within a large background is not possible. Therefore, the pharmacokinetic data calculated after baseline subtraction were subject to overestimation

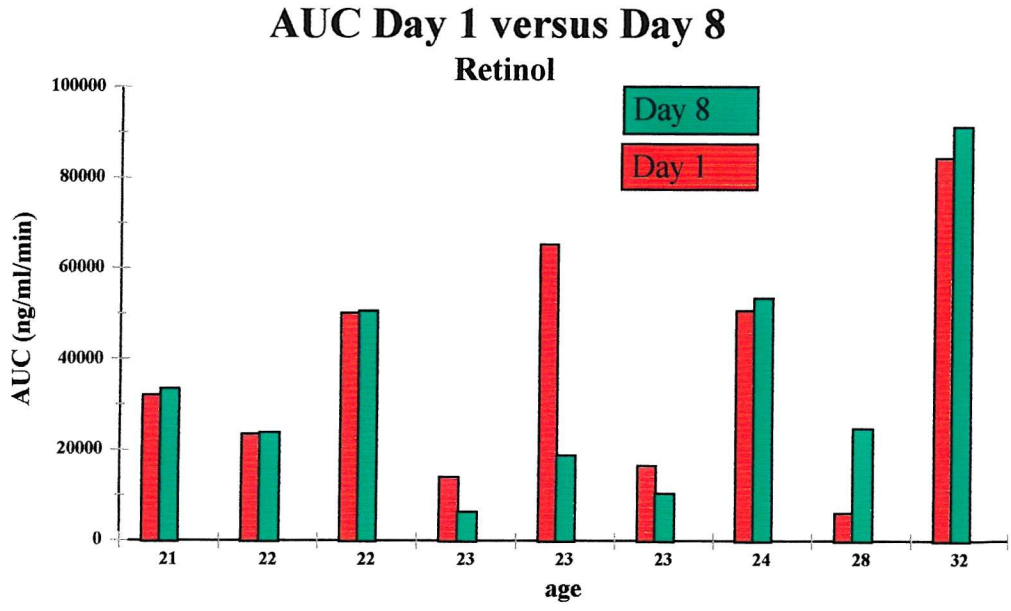

Figure 5.13: Comparison of day 1 and day 8 AUC values for retinol.

or underestimation due to baseline fluctuations affecting the slope of the terminal regression (figure 5.14). Statistical analysis of the data produced indicated no significant differences between that calculated for day 1 and that calculated for day 8.

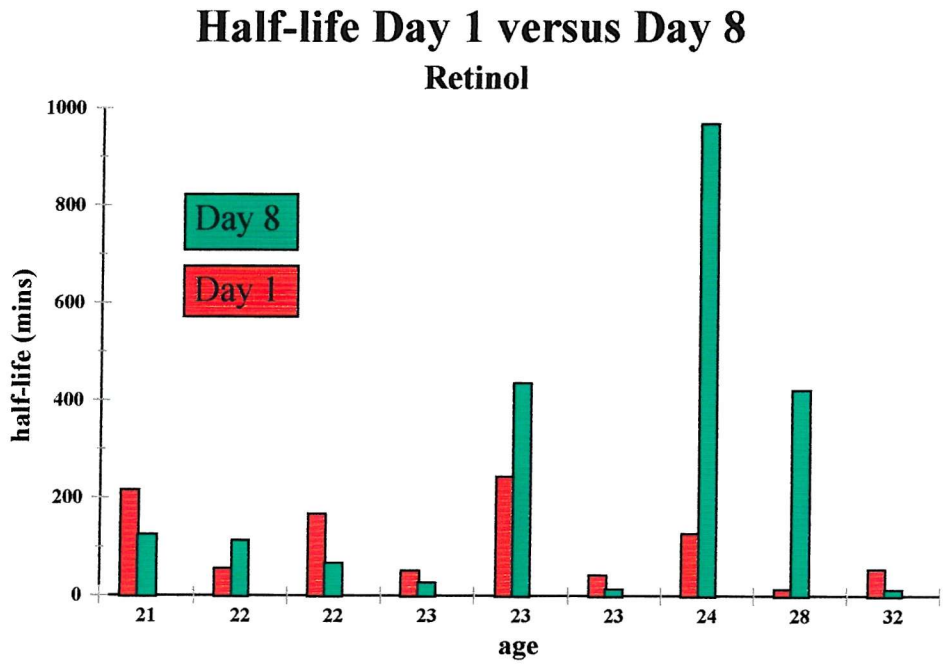

Figure 5.14: Comparison of day 1 and day 8 half-life values for retinol.

5.3.2.5 13-Cis-4-oxo-retinoic acid

The metabolite 13 cis-4-oxo retinoic acid is known to accumulate within the plasma and tissues of a human subject for long periods after dosing with retinol as the retinyl palmitate. Figure 5.15 shows the plasma concentration-time curve for 13-cis-4-oxo-retinoic acid prior to the baseline subtraction. A 47% difference is observed between the AUC of the two dosing day (AUC_{day1}=358.2 ± 359.7; AUC_{day8}=532.0 ± 285.7) prior baseline subtraction. However, statistical analysis of the pharmacokinetic data determined after baseline subtraction demonstrated no significant difference between the two dosing days. Figure 5.16 shows the plasma concentration-time curve for 13 cis-4-oxo retinoic acid after baseline subtraction. It can be observed that after subtraction of the baseline values for day 8 AUC is reduced in comparison to day 1 AUC. This is the opposite to what was observed in the data without baseline subtraction. A histogram plot of AUC data for day 1 and day 8 is shown in figure 5.15. This shows that AUC between treatments day for 13-cis-4-oxo-retinoic acid varies between individual subjects. It can be observed that four subjects had $AUC_{day1} > AUC_{day8}$, while 3 subjects had $AUC_{day1} = AUC_{day8}$ and two subjects had $AUC_{day1} < AUC_{day8}$. There was no correlation between age and the differences between the mean AUC for day 1 and day 8. Statistical analysis (t-test) indicate that there were no significant differences between the two treatment days for AUC and C^{max} parameters. Figure 5.18 is a histogram plot of the calculated half-life values for all nine subjects. Given that the data varied significantly between individuals, the half-life values for each subject was consistent between the two treatment days ($t_{½\ day1}$= 26.0 ± 7.9; $t_{½day8}$= 29.8 ±

10.9). Statistical analysis of the pharmacokinetic parameters $t_{½}$ and MRT indicate no significant difference between the two treatment phases.

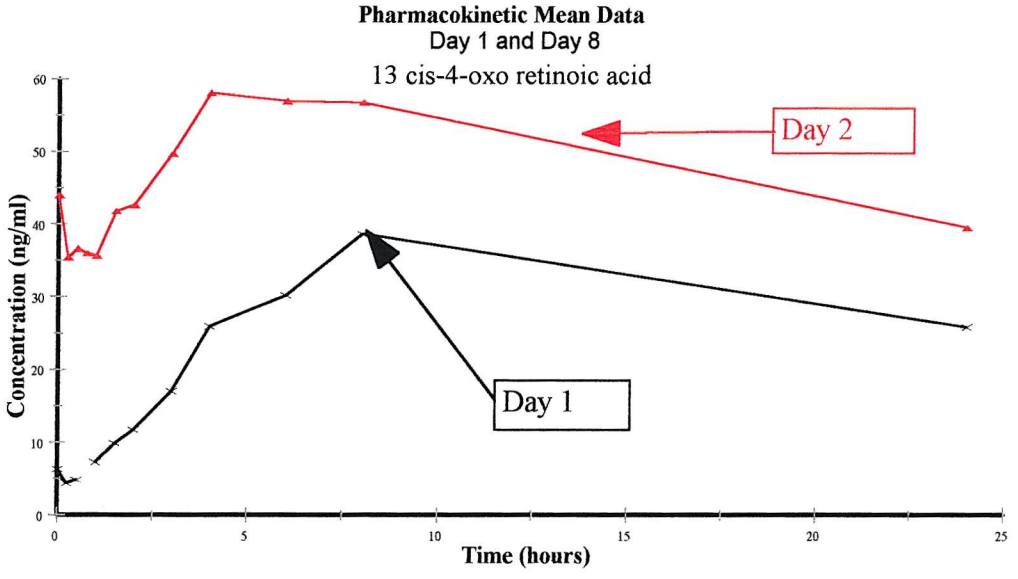

Figure 5.15: Comparison of day 1 and day 8 concentration-time profiles for 13-cis-4-oxo-retinoic acid before baseline subtraction

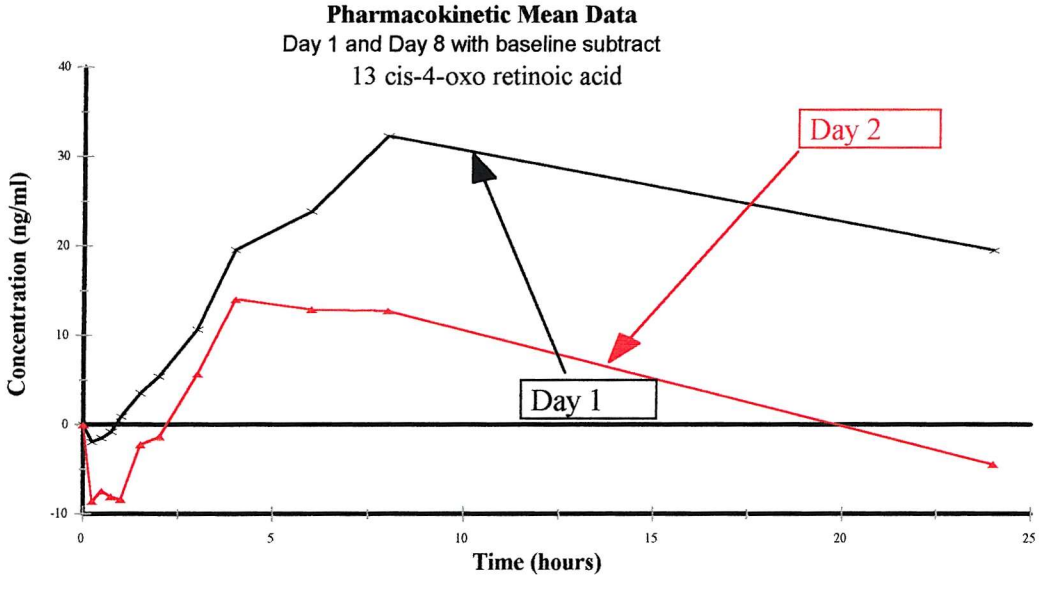

Figure 5.16: Comparison of day 1 and day 8 concentration-time profiles for 13-cis-4-oxo-retinoic acid after baseline subtraction

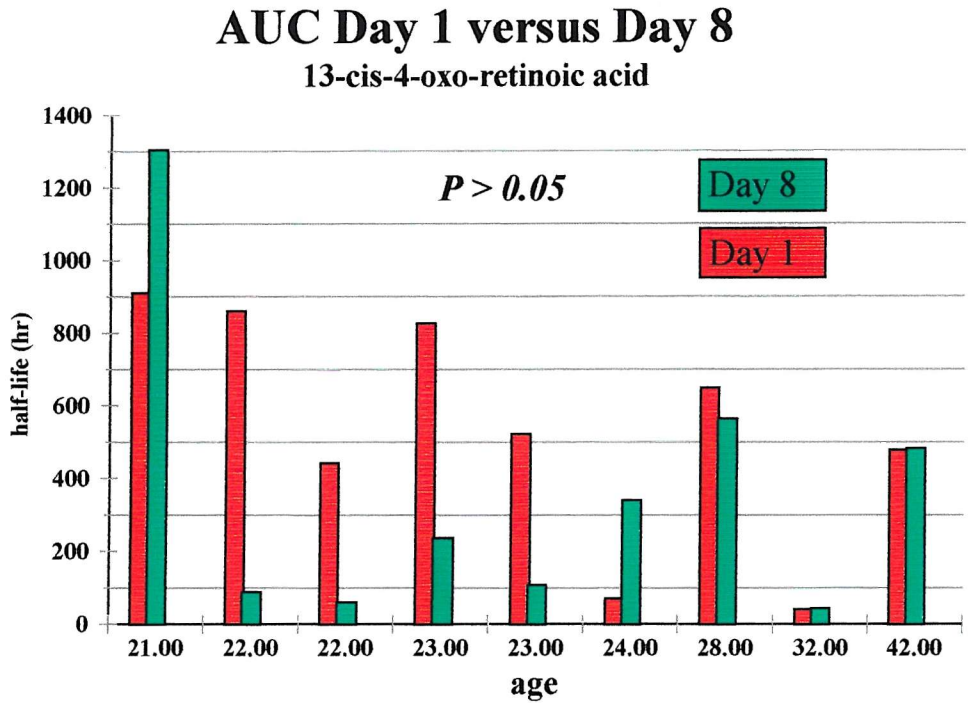

Figure 5.17: Comparison of day 1 and day 8 AUC values for 13-cis-4-oxo-retinoic acid.

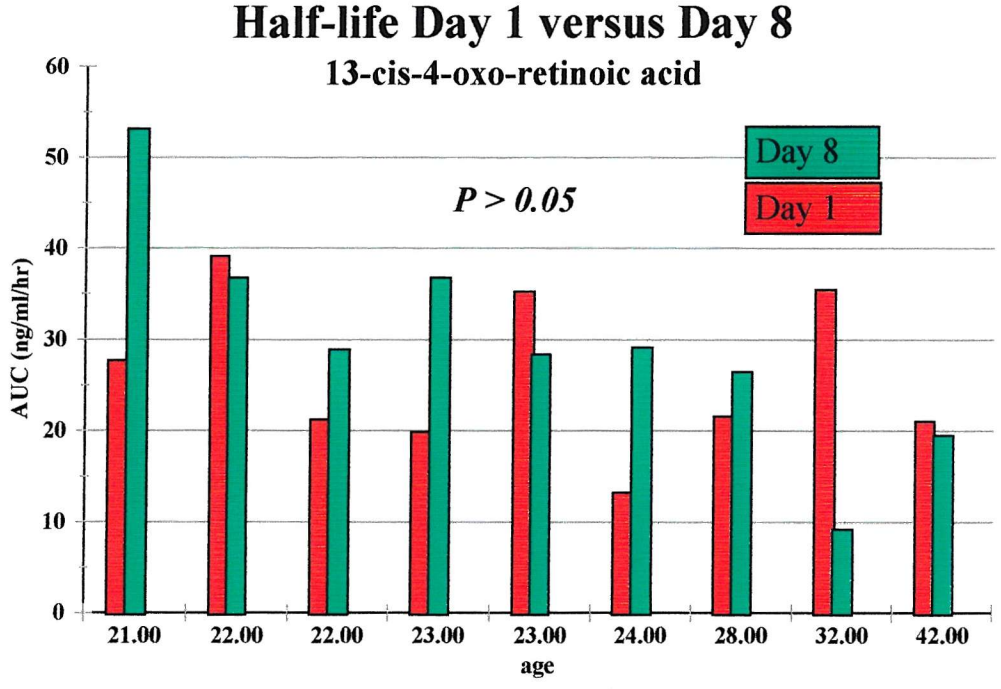

Figure 5.18: Comparison of day 1 and day 8 half-life values for 13-cis-4-oxo-retinoic acid.

However the T^{max} values did show a significant difference between day 1 and day 8. T^{max} of day 8 was significant less than T^{max} of day 1 ($P < 0.05$, mean $T^{max}_{day1} = 7.1 \pm 1.6$ and $T^{max}_{day8} = 5.0 \pm 2.6$). However, one subject had a T^{max} of 0, this outlining value lowered the mean sufficiently to give a false significance to the difference. Statistical analysis with this subject removed indicated no significant difference between the two treatment days.

5.3.2.6 All-trans-4-oxo-retinoic acid

All-trans-4-oxo-retinoic acid is a rapidly cleared polar metabolite of all-trans-retinoic acid. It has been shown previously to appear quickly after oral dosing with retinol as the palmitate within the plasma. The observed T^{max} is similar to all-trans-retinoic acid but C^{max} is approximately 5 fold lower ($C^{max}_{day1} = 9.6 \pm 2.9$; $T^{max}_{day1} = 2.4 \pm 1.4$ hrs; $C^{max}_{day8} = 13.8 \pm 7.7$; $T^{max}_{day8} = 1.8 \pm 1.4$ hrs as compared to $C^{max}_{day1} = 59.1 \pm 29.5$; $T^{max}_{day1} = 1.8 \pm 0.4$ hrs; $C^{max}_{day8} = 53.7 \pm 31.3$; $T^{max}_{day8} = 1.6 \pm 0.3$ hrs). For data without baseline subtraction day 8 C^{max} appears to be greater than day 1 C^{max}. However, once baseline subtraction had occurred the C^{max} for both day 1 and day 8 are very similar (figure 5.19). Statistical analysis of the baseline subtracted data for T^{max} and C^{max} indicated no significant differences between day 1 and day 8.

It is noticeable from these results and previous results (chapter 3 and chapter 4) that all trans-4-oxo retinoic acid exhibits a -small second rise 6 - 12 hours after dosing. This second rise is relatively small compared to the initial C^{max} but could effect the baseline

levels of all-trans-4-oxo-retinoic acid present on a subsequent day.

The AUC calculated for all-trans-4-oxo-retinoic acid is variable between individuals and between day 1 and day 8. Figure 5.20 illustrates the variability between the individual subjects and the variability observed for each subject between dosing days. Statistical analysis of the data indicated no significant difference between day 1 (AUC_{day1}= 17.6 ± 7.2 ng / ml) and day 8 (AUC_{day8}= 21.8 ± 16.2 ng/ml). A similar pattern is observed for the half-life values ($t_{½\ day1}$= 3.2 ± 3.2hrs^{-1}; $t_{½\ day2}$= 2.6 ± 1.6hrs^{-1}; figure 21) and the MRT values (MRT_{day1}= 3.2 ± 1.8hrs; MRT_{day2}= 2.8 ± 1.0hrs).

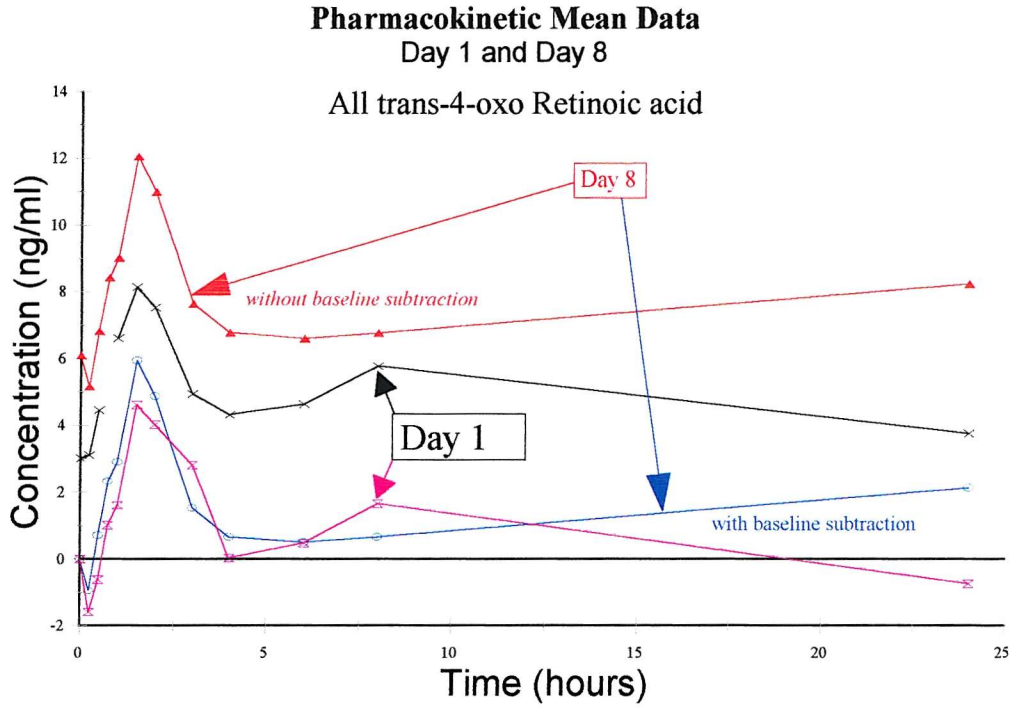

Figure 5.19: Comparison of day 1 and day 8 concentration-time profiles for all-trans-4-oxo-retinoic acid. Profile without baseline subtraction (day 1 -----; day 8 -----): profile with baseline subtraction (day 1 ----; day 8 ----)

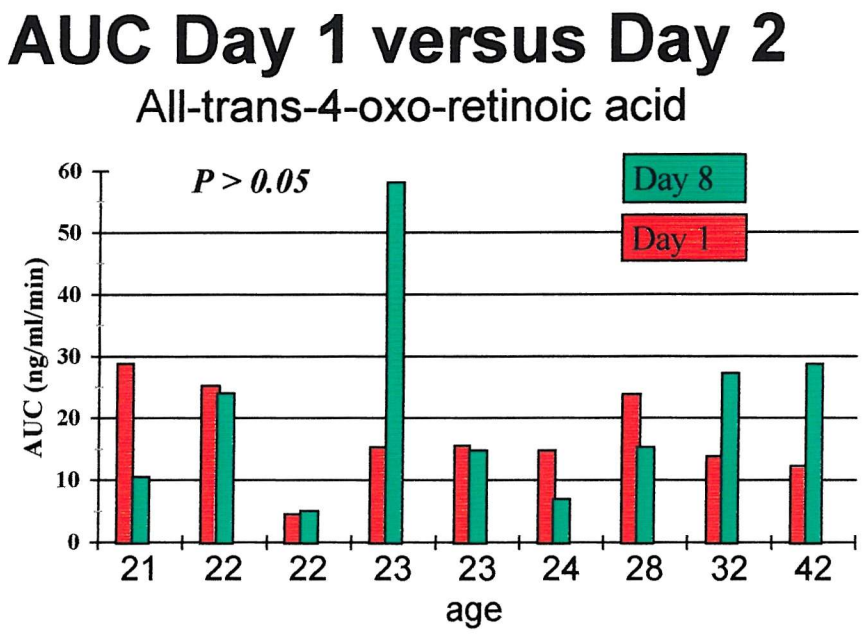

Figure 5.20: Comparison of day 1 and day 8 AUC values for all-trans-4-oxo-retinoic acid.

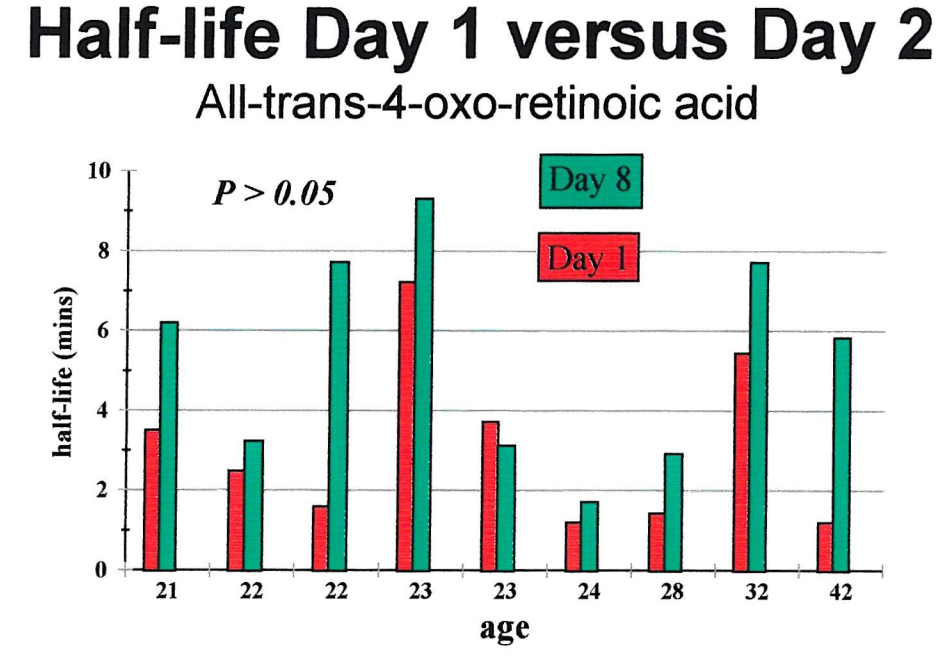

Figure 5.21: Comparison of day 1 and day 8 half-life values for all-trans-4-oxo-retinoic acid.

5.5.2.7 9-Cis-retinoic acid

9-Cis-retinoic acid exhibits a time profile similar to that of all-trans-retinoic acid and all trans-4-oxo retinoic acid (T^{max}_{day1}= 1.5 ± 0.8 hrs; C^{max}_{day1}= 9.2 ± 2.9 ng/ml; T^{max}_{day8}= 1.7 ± 0.9 hrs; C^{max}_{day8}= 8.8 ± 1.7ng/ml). Plasma levels found after dosing with retinol as the palmitate were similar to that seen for all-trans-4-oxo-retinoic acid (section 5.3.2.6) and 5 fold lower than that of all-trans-retinoic acid (section 5.3.2.1). Peak concentration for the metabolites occurred between 1 and 2 hours post dosing for both dosing days. Also, similar to all-trans-4-oxo-retinoic acid there is evidence of a second rise in the baseline between 4-8 hours after the initial dosing. This is the period where 13-cis-retinoic acid and

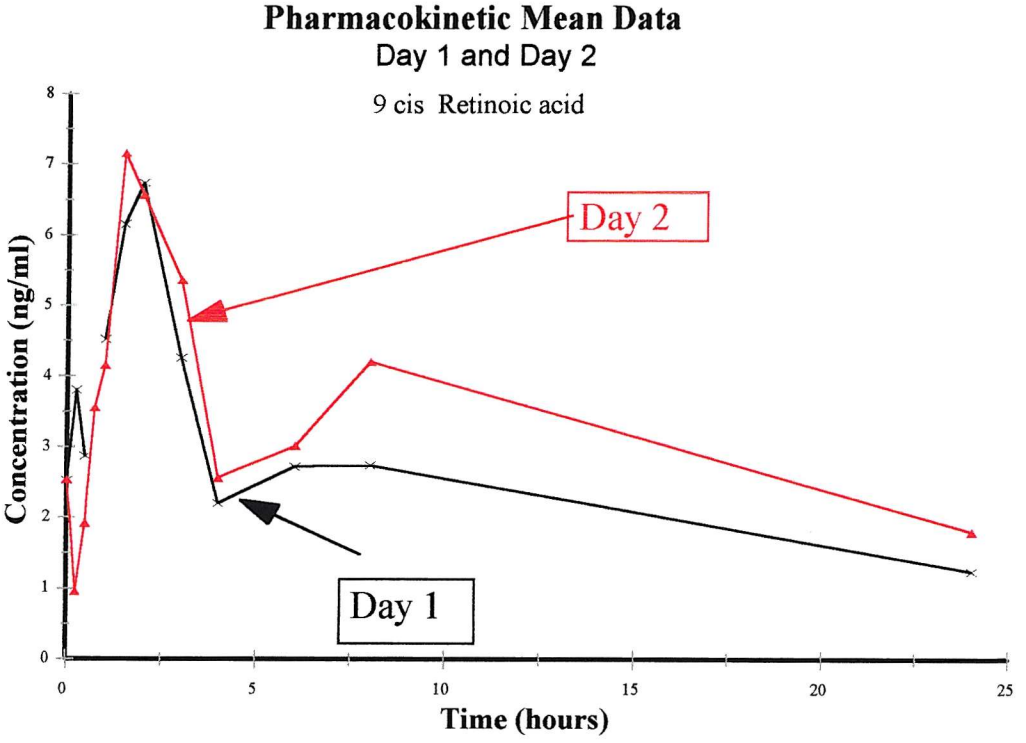

Figure 5.22: Comparison of day 1 and day 8 concentration-time profiles for 9-cis-retinoic acid.

13-cis-4-retinoic acid dominate the concentration time profile (figure 5.1 and figure 5.2). However, 9-cis-retinoic acid plasma concentrations for this period are very low, at or just above the limit of detection for this metabolite. Hence, the pharmacokinetic parameters determined are subject to large calculation error and should be consider indications of

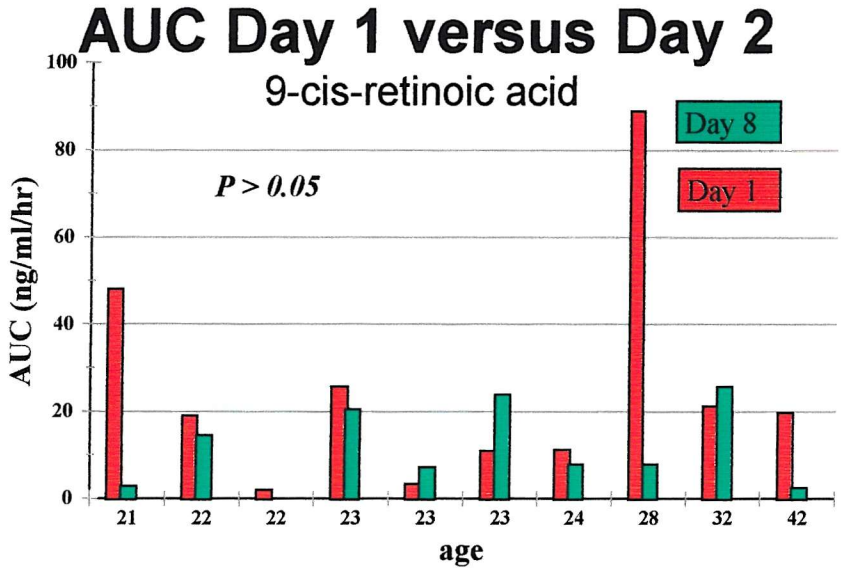

Figure 5.23: Comparison of day 1 and day 8 AUC values for 9-cis-retinoic acid.

trends rather than actual trends. Figure 5.23 shows a direct comparison of the calculated AUC parameter for individual subjects on day 1 and day 8 of the investigation. Allowing for the variability of the results due to the very low levels found, the AUC's are similar between both days (AUC^{day1}=25.0 ± 26.0; AUC^{day8}= 14.9 ± 8.8 ng/ml/hr) except for two individuals. The two large differences observed can be related to the plasma concentration found, in each case C^{max} values were only approximately twice the limit of detection (2 ng/ml). After the subtraction of the baseline value pharmacokinetic parameters were

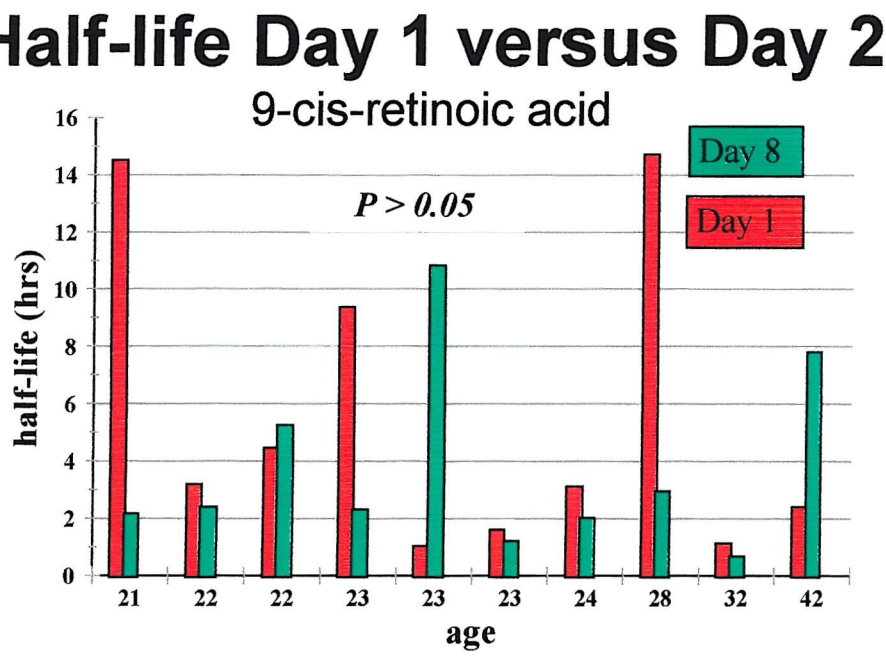

Figure 5.24: Comparison of day 1 and day 8 half-life values for 9-cis-retinoic acid.

calculated on 4-5 positive points including the pre-dose and the C^{max} values. Statistical analysis of the C^{max}, T^{max}, AUC, $t_{½}$ and MRT values indicate no significant difference between day 1 and day 8 treatments.

5.4 SUMMARY AND CONCLUSIONS

The objective of the investigation was to discover if the repetitive oral dosing of retinol as the palmitate would affect the mechanisms by which retinol and its metabolites are handled with in the human system. It has been reported several times within literature that

the repetitive oral and I.V. administration of high doses of all-trans-retinoic acid or 13-cis-retinoic acid caused a decrease in the drug circulatory levels (6-8). This effect was observed after a relatively short time period and reversal of clinical effects were noted (9). All-trans-retinoic acid and 13-cis-retinoic acid are both utilized within the chemotherapy of specific cancer types and for intensive treatment of dermal disorders (10). A decrease in the systemic drug levels caused reversal of remission in the treated cancer patients (3). For example, in the treatment of acute promyelocytic leukemia all-trans-retinoic acid is very successful in giving complete remission when either given as sole treatment or as part of chemotherapy. In newly diagnosed patients all-trans-retinoic acid is approximately 100% successful in giving complete remission. However, in patients with a relapse within 1 year of being treated with all-trans-retinoic acid, the remission rate is drastically reduced to approximately 10% (5). With relapsed patients that were treated with all-trans-retinoic acid over one year ago, the subsequent treatment could give complete remission but not with the same success rate. In effect, a resistance to retinoic acid treatment has been established. Efforts to overcome this problem have led to investigations in drug holidays, combination therapy and drug reversal of the retinoic acid resistance (11-13).

In an animal investigation, three consecutive oral doses of all-trans-retinoic acid were given at three hours interval. It was found that the second and third doses did not give a rise in plasma concentrations (14). Since a deficiency as well as an increase of all-trans-retinoic acid or 13-cis-retinoic acid in the plasma can give morphological effects during pregnancy, it was questioned whether retinol or pre-formed retinol could produce the same

effect in humans. Also, in an animal model daily chronic retinoic acid dosing has been reported to have the effect of decreasing retinol plasma concentrations (15). However, it has been noted that the rat model and other animal models do not accurately reflect the human model with respect to vitamin A and inconsistencies have been observed with vitamin A pharmacology (16).

A longitudinal investigation amongst elderly supplement users showed that regular daily higher doses of vitamin A (>10 000 IU) gave retinol plasma concentrations that did not decrease over a five year period (22). However, the data shown indicated that retinyl palmitate levels decreased by approximately 40% over the five year period. The control group and subjects taking <10 000 IU per day showed little or no change in the plasma concentration of retinol or retinyl palmitate. This report did not investigate this decrease, but rather concentrated on the fact that toxic levels of retinol or retinyl palmitate were not generated by sustain high dose supplementation.

In this study, patient compliance to the dosing on day 1 and day 8 appeared to be 100%. Therefore, a similar dose was delivered to each subject on both treatment days. However, retinol plasma concentrations did not increase or decrease significantly in the time period investigated. The possibility of high dose consecutive supplementation of vitamin A affecting the normal circulatory retinol levels has not been observed. Plasma concentrations of retinyl palmitate peaked at typical values (C^{max}= 4.5 - 7.2 μg/ml; T^{max}= 5-6 hrs) in comparison to the single dose pharmacokinetics previously reported. All the

metabolites of vitamin A analyzed were detected within the plasma both prior to and after dosing with retinyl palmitate. Since vitamin A is an endogenous compound the pharmacokinetic parameters investigated were calculated from the change in the initial baseline values. The peak plasma concentrations (C^{max}) observed for the first dose matched that observed in previous investigations (tables 3.7-3.11 and table 4.4) as well as those reported in the literature for single dose pharmacokinetics of retinyl palmitate and retinol (table 5.4).

After eight days of dosing with 50 mg of retinyl palmitate, the metabolite levels observed immediately after a dose were similar to that observed for the first dose. Only 13-cis-4-oxo-retinoic acid appeared to reach a steady state level and demonstrated a lower change in AUC after the eight day period. Investigations reported in literature suggested that with multiple all-trans-retinoic acid dosing, the time taken for a decrease in plasma levels to be observed is between 3-7 days (11;12). This investigation used the 7 day value to compare with the in literature reported investigations. Dose delivery was verified with the retinyl palmitate levels observed in the plasma. However, no significant difference was found between plasma levels at day 1 and day 8. Nine subjects showed a significant increase above the baseline for retinyl palmitate plasma concentrations. However, the tenth subject showed a much reduced retinyl palmitate plasma concentrations after dosing in comparison to the other nine subjects. There was no difference between the two treatment days for this subject and the circulatory retinol levels did not vary by more than the assay limitations. The metabolite profile for this subject was, strangely, similar to the

other nine subjects, although values observed were on the low end of the range of variability seen. This subject was the only individual to have been on a regular over the counter supplement for vitamins and minerals prior to this investigation. This subject had been taking this form of supplementation over a 2-3 year period. The results observed for this subject are indicative of a reduction in plasma levels, but could also simply be due to inter-individual variation. The results for this subject do suggest the possibility that a much longer period of regular supplementation compared to a control group could give the results initially speculated.

It should be noted that there are important differences between the present study and studies reported in literature, which could explain the different results obtained. Firstly, most of the repetitive dosing investigations in literature involved subjects suffering from severe and acute forms of cancers. These subjects do not generally reflect the normal population and with combination drug therapies many of the subject's systems are atypical compared to the normal healthy individual. Most studies only reported on the pharmacokinetics of all-trans-retinoic acid and 13-cis-retinoic acid metabolites of vitamin A. Table 5.4 summarizes the C^{max} and T^{max} parameters for some of these investigations.

Table 5.4: Pharmacokinetic parameters reported in literature in investigations in single and multi-dose vitamin A supplementation. APL- acute promyelocytic leukemia; NSCLC -non small cell lung cancer; HIV/KS- human immunodeficiency virus positive/ Kaposi's sarcoma; HDAP- histologic diagnosis of adenocarcinoma of prostate;CC-histological confirmation of cancer; TRA- all-trans-retinoic acid; CRA- 13-cis-retinoic acid.

Diagnosis	Dosage (mg/m2)	N	C^{max} (ng/ml)	T^{max} (hrs)	Comments	Ref
APL*	45	15	380 (30-2550)		Treatment TRA; 1 patient with plasma conc^{n} <3 ng/ml at all times	(17)
APL*	45	13	301		Treatment TRA;	(18)
Solid Tumors*	60	2	742, 400			
NSCLC*	45	31			Only 1 patient with AUC <250	
APL**	45	20			Treatment TRA;	(19)
predominantly NSCLC**	45	19			At least 5 patients with AUC <150	
HIV/KS*	40	8	272 (151-725)		Treatment TRA;	(7)
Breast cancer	50	17	274 (17-831)		One patient AUC could be determined since plasma conc^{n} were to low	
HDAP**	50	17	day 1: 295 ±215 day 14: 146 ± 116 day 21: 292 ± 188	3.9 ± 1.9 6.1 ± 1.8 4.0 ± 1.2	Treatment TRA; 3 times daily for 1-14 days every 22 days	(11)
APL	30	4	20-741	1-2	Treatment TRA; Boys aged 6 -15years	(13)

Diagnosis	Dosage (mg/m2)	N	C^{max} (ng/ml)	T^{max} (hrs)	Comments	Ref
HIV/KS**	40	13	day 1: 330 ± 60 day 7: 60 ± 12 day 21: 340 ± 61 day 77: 335 ± 63		Treatment TRA; Orally dosed, divided into 3 doses per day on every other week for 12 weeks. Significant difference between day 1 and day 7. (P=0.018 Wilcoxon signed rank test)	(20)
Healthy Women*	150 mg (≈83mg/m^2)	10	87.3 ± 60.6	2.0 ± 0.2	Treatment Retinyl Palmitate; Pharmacokinetics for retinyl palmitate, all-trans-retinoic acid, 13-cis-retinoic acid, and 4-oxo metabolites reported. Only all-trans-retinoic acid is shown here.	(21)
	50 mg (≈28mg/m^2)		49.0 ± 34.3	1.5 ± 0.3		
CC**			Day 1	2 (median)	Treatment TRA;	(9)
	20	3	90 ± 60			
	30	8	300 ± 240	(range 1-3)	Children; stratified by age	
	40	6	360± 330		≤12 and >12	
			Day 3		dose delivered orally 3 times daily to total 60 mg/m^2 dose on every three days out of 5 for 4 weeks.	
	20	2	15 ± 9			
	30	2	60 ± 60			
	40	2	21 ± 3			
			Day 22			
	20	2	120 ± 30			
	30	6	270 ± 120			
	40	5	300 ± 150			

* - Single dose pharmacokinetic investigation ** - multi-dose pharmacokinetic investigation

Also, most studies in literature dose with all-trans-retinoic acid, while in the present study subjects were dosed with retinyl palmitate. Dosing with different forms of vitamin A could have large effects on the plasma concentrations of vitamin A and its metabolites.

When dosing with retinyl palmitate it is suspected that absorption through the gut wall is followed by transport to the blood via the lymphatic system. The T^{max} for the parent compound, retinyl palmitate, is 4.1 ± 0.7 hrs and 3.2 ± 2.1 hrs for day 1 and day 8 respectively. This is similar to that observed in literature investigation (T^{max}= 4.9 ± 1.4) (21). However, for all-trans-retinoic acid and 13-cis-retinoic acid T^{max} occurs at 1.2 ± 0.4 and 2.8 ± 2.3 respectively, prior to that of retinyl palmitate. To generate these levels of the primary metabolites of retinol within the blood so quickly the T^{max} values indicate that the metabolism should occur before retinol is re-esterified into retinyl palmitate and released into the lymphatic system. This suggests a first pass metabolism within the gut wall for the hydrolyzed retinyl palmitate absorbed through the gut wall. The subsequently produced all-trans-retinoic acid is then transported to the liver via the blood and not the lymphatic system. 13-Cis-retinoic acid is produced slightly later than all-trans-retinoic acid since it is the product of all-trans-retinoic acid's isomerisation in the 13 cis form.

When dosing with all-trans-retinoic acid, it can be suggested that there is also a the first pass metabolism within the gut wall, which is induced by high concentrations of all-trans-retinoic acid for its own elimination. All-trans-retinoic acid is already suggested of being able to control its own cellular levels by inhibition of retinol metabolism and induction

of its own metabolism. Increased metabolism of all-trans-retinoic acid within the gut due to its high concentrations, would explain the significant decreased plasma levels observed in literature when dosing daily with all-trans-retinoic acid. Treatment based on intermittent dosing schedules have been shown to return all-trans-retinoic acid plasma concentrations to initial levels. Also, treatment with drug combinations have been shown to return all-trans-retinoic acid plasma levels to normal values. In this investigation, plasma levels of all-trans-retinoic acid after dosing with 50mg of retinyl palmitate (AUC_{day1}= 90.6 ± 45.8 ng/ml hr; AUC_{day8} 82.3 ± 52.0 ng/ml hr) do not reach the same concentrations seen when dosing with chronic all-trans-retinoic acid (AUC range 250 to 2400 ng/ml hr). It can be suggested that treatment with retinyl palmitate does not produce the same high all-trans-retinoic acid levels within the gut wall in order to induce the metabolism of all-trans-retinoic acid, giving rise to its decreased plasma levels after multiple dosing reported in literature.

In conclusion, it has been shown that multiple-dosing with supplementation containing retinyl palmitate will not change the homeostatic control of retinol within the circulatory system. This is in contrast to what has been observed for all-trans-retinoic acid treatments in literature. The period in which the investigation was conducted was too short to confirm any long-term effects of chronic high dose supplementation. The one individual within this study who regularly took 100% RDA supplementation did demonstrate different pharmacokinetics to the other subjects. It is suggested that a similar investigation with a control group and a known supplement user group should be conducted. The difficulty

would be ensuring that the bioavailibilty of the dose would the same for each individual in the supplement user group. Different manufacturers formulate their products differently and the bioavailibilty can be significantly different.

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Chapter 6

Transdermal Vitamin A Absorption in Healthy Women of Child Bearing Age.

Table of Contents

List of Tables

List of Figures

6.1 INTRODUCTION

More than 40 years ago retinol was introduced to the pharmaceutical world in dermal formulations for the treatment of certain skin disorders (1;2). From the original form of retinol other natural metabolites and synthetic retinoids have been discovered to have notable effects on such skin diseases as actinic keratoses, Darier's disease and pityriasis. In 1986 Kligman et al demonstrated that the topical application of all-trans retinoic acid reduced the amount of damage caused by photo aging (3). Photo aging is the natural process whereby the skin is damaged by UV radiation and bright sunlight. Lesions are formed and heal naturally, but eventually leave small alterations in the skin surface. This alteration is generally known as "wrinkles". The concept that topical retinoids could smooth out wrinkles was accepted into the cosmetic industry very quickly. The market for such products has proved to be immensely profitable. Therefore, products over the last decade have come to contain more and more retinol or retinoid based molecules in the composition. Creams are currently available in doses of hundreds of thousand IU's and are all over the counter products.

From the original work with oral 13-cis-retinoic acid (isotretinoin) it is known that regular high doses of these compounds can cause malformations in unborn children if taken during the early gestation periods. With the increase usage of high dose topical applications, concern was expressed over the possibility that teratogenic concentrations of vitamin A metabolites could reach systemic circulation. However, little data is available on the absorption of vitamin A across the skin and the levels of teratogenic metabolites found in plasma after topical applications. Two studies

reported on the systemic plasma concentration following topical application of retinoid creams. In a study conducted by Jensen et al, 20g of a 0.05% isotretinoin (13-cis-retinoic acid) gel was applied for 30 days on patients suffering from acne vulgaris (4). Some localized side effects were observed from the application of the skin gel, but no significant rise in the systemic retinoid levels were found. A second study was conducted by Chen et al, who applied 10g of a 0.1% isotretinoin cream for 42 days on patients suffering from photo-damaged skin (5). It was found that retinoid plasma levels found were limited to 40-80% of the baseline values.

This study was designed to investigate the risk of teratogenic metabolites being formed in the plasma of healthy women during and after regular applications of a fixed amount of high dose vitamin A containing skin creams. Doses applied and study period were respectively higher and longer than those of the two studies mentioned above. Furthermore, this study dosed with retinol and retinyl palmitate, the two most common forms of vitamin A found in cosmetics and over the counter products.

6.2 METHODS

6.2.1 Skin Creams

General purpose 6.5% w/w oil-in-water base creams were supplied by Unilever. They contain preservatives, emulsifiers, viscosity agents, sequestrants and neutralizing agents. Three creams were used. They contain either/or retinol, retinyl palmitate or a placebo. The creams were supplied in sachets, each containing 10 g of cream.

Creams were analyzed after packing into sachets and at the end of the study. No significant decrease in retinoid content was found.

6.2.2 Subjects

Thirty-six female subjects participated in this study. The means and standard deviations for age, height and weight of the study group were 33.3±9.1 years (range 19-47 years), 165.3±6.4 cm (range 155-181 cm) and 65.1±12.6 kg (range 51 - 94 kg). Patient demographics can be found in table 6.1. Smoking was 20 cigarettes/day or less and the maximum alcohol consumption was 30 units/week. Four subjects suffered from asthma for which they took salbutamol and steroid inhalers. One subject took diclofenac for back pain due to ankylosing spondylitis. Another subject took vitamin supplements which were stopped prior to the study. None of the medications above were expected to influence the study results. Prior to the study, each subject underwent a standardized grading of skin condition for dryness, oedema, redness, vesication, wrinkles and greasiness.

Table 6.1: Demographics for 36 subjects enrolled for the study on transdermal absorption of vitamin A

Mean age : 33.3±9.1 (range 19-47)
Weight: 65.1±12.6 (range 50.5-93.5)
Height: 165.3±6.4(range 155-181)
No of asthmatics = 4 (medication- salbutamol and steriod inhalers)
Other medication = 1 (Diclofenac taken once by one subject)
Vitamin supplementation = 1 subject (stopped prior to start of study)
Smoking = 11 subjects (range of 1-20 per day)
Alcohol consumption = 25 subjects (range 1-30 units per day); 4 subjects (range unknown)

6.2.3 Study Design and Treatment

The study was designed as a semi-blinded non-randomized placebo-controlled study. Subjects were required to apply 10g of cream to the trunk and thighs daily for a period of 56 days. Three test creams were supplied by Unilever, identified as skin creams A, B, C :

- Cream A : contained 0.236% by weight of retinyl palmitate (equivalent to 15mg/ 10g cream)
- Cream B : contained no retinoids
- Cream C : contained 0.1475 by weight of retinol (equivalent to 15mg/ 10g cream)

Clinical investigators were not aware of the identity of the creams during the study. All sachets were weighed before and after use. Each subject was given a unique trial number. The thirty-six subjects were divided into three treatment groups of twelve subjects. Skin grading was used as a guide to subject allocation so that each skin type was evenly represented in each of the three treatment groups (A, B&C). For practical purposes the subjects were further sub-divided into 3 study groups, in which four subjects received cream A, four subjects received cream B and another four received cream C. Table 6.2 shows the skin grading of the subjects and the treatment allocation.

Table 6.2:- Skin grading of each volunteer under 6 categories and assigned treatment group for each individual.

Patient Number	Redness	dryness	Oedema	Vesication	Wrinkles	Greasiness	Study Group	Treatment group
1	0	0	0	0	0	0	1	A
2	0	0	0	0	0	0	1	B
3	0	0+	0	0	0	0	1	A
4	0	1	0	0	0	0	1	A
5	0	0	0	0	0	0	3	C
6	0	0	0	0	0	0	2	C
7	0	0	0	0	0	0	3	C
8	0	0/+	0	0	0	0	3	A
9	0	0	0	0	0	0	2	C
10	0	0/+	0	0	0	0	3	B
11	0	0	0	0	0	0	2	C
12	0	0+	0	0	0	0	1	B
13	0	0+	0	0	0	0	2	B
14	0	0	0	0	0	0	1	C
15	0	0	0	0	0	0	1	A
16	0	0+	0	0	0	0	1	C
17	0	0	0	0	0	0	2	B
18	1	0	0	0	0	0	1	C
19	0	1	0	0	0	0	1	B
20	0	0	0	0	0	0	2	B
21	0	0	0	0	0	0	3	A
22	0	1	0	0	0	0	1	C
23	0	0	0	0	0	0	1	B
24	0	(+)	0	0	0	0	3	B
25	0	0	0	0	0	0	3	C
26	0	1	0	0	0	0	3	A
27	0	0	0	0	0	0	2	A
28	0	0	0	0	0	0	2	A

Patient Number	Redness	dryness	Oedema	Vesication	Wrinkles	Greasiness	Study Group	Treatment group
29	0	(+)	0	0	0	0	3	C
30	0	0	0	0	0	0	2	A
31	0	0	0	0	0	0	2	B
32	0	(+)	0	0	0	0	2	A
33	0	0	0	0	0	0	2	C
34	0	0	0	0	0	0	3	B
35	0	0	0	0	0	0	3	B
36	0	0	0	0	0	0	3	A

Clinical observations and examinations were made of the subject's skin condition during week 1, 4, and 8 of the study. A graded assessment of dryness, oedema, redness, vesication, wrinkles and greasiness was recorded. General dermatological condition was observed and note taken of any unusual changes to the skin. A single blood sample was taken in the morning of days 1, 2, 3, 7, 14, 21, 28, 35, 42, 49, 56, 57, and 63. A more detailed blood profile was taken on days 0, 28, and 56. On these days, samples were taken at 0, 2, 4, 6, and 18 hours after application of skin cream applied on that day. All empty cream sachets were collected and re-weighed. The weight of creams applied was calculated and recorded.

6.2.4 Compliance

Weight of cream sachets before and after use were determined. Each treatment group results for twelve subjects were averaged over a 57 day period. Group A (0.236% by weight retinol palmitate) complied closely with the protocol with an average application of 9.8-10.4 grams of cream during the study period. Group B (placebo) experienced some clinical effects from the cream applications and general a lower amount was applied, range 8.7-9.8 grams. Group C (0.1475% by weight retinol) demonstrated close compliance with protocol with a range of 9.2-10.3 grams cream applied. Figure 6.1 illustrates the close compliance of all subjects with the study

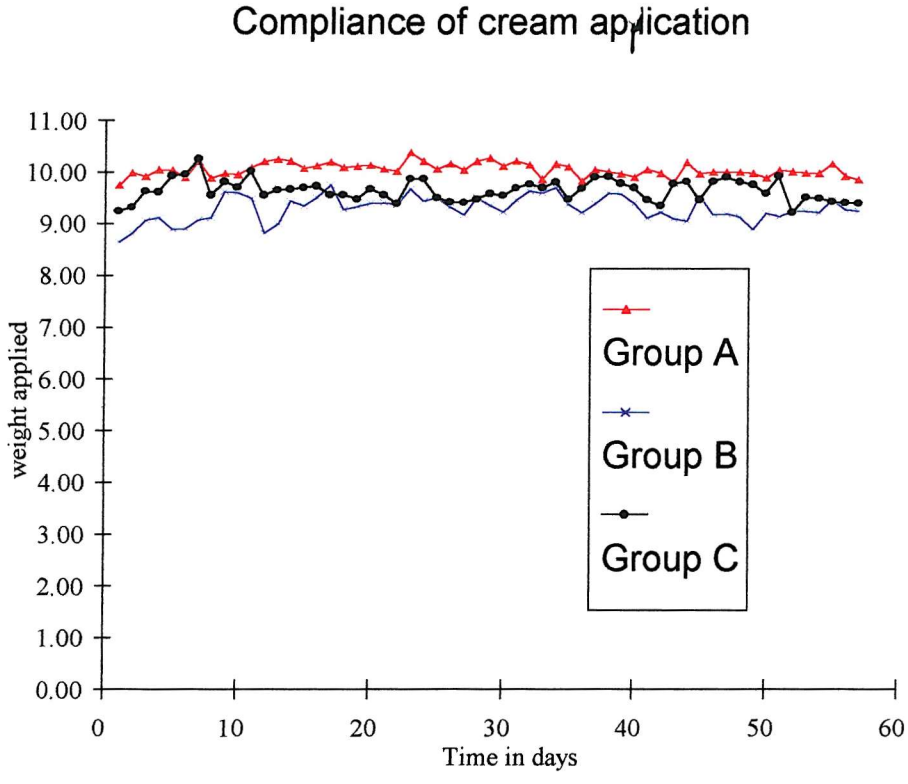


Figure 6.1 Sachet application of cream for this study was controlled by weight before and after application. Mean data for group A (0.236% by weight of retinol palmitate) subjects (n=12); group B (placebo) subjects (n=12); and group C (0.1475%) subjects (n=12).

protocol over the 57 days of cream application. Of the 2052 sachets applied by all groups only 50 were either not returned or returned without the top of the sachet. Of these 50, one subject in group B failed to return 16 sachets correctly and two subjects in group C failed to return 10 and 9 sachets respectively. Therefore, three individuals account for 70% of the non-returned sachets indicating good compliance with study protocol for 92% of the volunteers.

As part of the study protocol a comprehensive diary was kept each week by each subject listing approximate portions of selected foods know to contain retinol, retinyl palmitate or ß-carotene. Diary compliance was 99% complete, only one diary was not returned (subject 7; final week of study). Of the twenty one entries in the diary for food only 3 were consumed by study volunteers on a regular basis They were fish (various types), carrots 100-150 g portions and butter/margarine. For the fish entries, each subject was required to note the size and type of fish consumed. Each diary was examined for sources of vitamin A and the appropriate plasma sample checked for variation to the trend. No examples were observed of consumed food affecting the plasma concentrations of any of the compounds under analysis.

In addition to food, health treatments were also a required part of the diary information, which included the use of multivitamin supplements and non-regular forms of self medication. Types of multivitamin preparations taken were verified as containing only non-vitamin A related supplements or inorganic micro-nutrients. Regular usage of any form of medication was checked against the determined plasma concentrations of all compounds of interest and corresponding variations noted. No medically related variations were observed during the course of this study.

During the course of the entire study no patient withdrew for medical reasons or from patient voluntary withdrawal. Overall compliance with the study protocol was considered to be excellent.

6.2.5 Pre-dose Analysis of Vitamin A and Metabolites in Plasma

Prior to the initialization of the study a blood pre-dose sample was taken to determine initial plasma concentrations for each individual within the study. The sample was taken one week after subjects were requested to initiate dietary restrictions and 1 hour before the first cream was applied to the body. Results from the duplicate analysis of each sample were averaged. Mean results for each group and for each compound under investigation are shown in table 6.3.

Table 6.3: - Analytical concentrations determined for the pre-treatment sample taken on day 1 of the study protocol. Values recorded are expressed as ng/ml

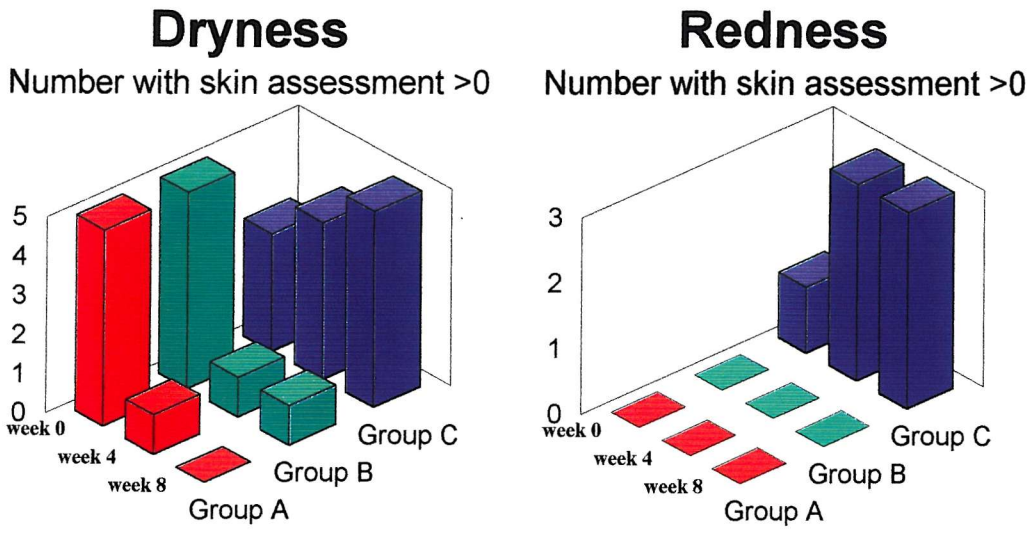
	Group A	Group B	Group C
all-trans-retinoic acid	1.42 ± 0.56	1.33 ± 0.89	1.83 ± 1.16
13-cis-retinoic acid	1.28 ± 0.33	1.28 ± 0.68	1.82 ± 1.20
retinyl palmitate	22.5 ± 12.5	23.9 ± 13.9	28.2 ± 14.2
retinol	699 ± 223	699 ± 164	761 ± 166
9-cis-retinoic acid	0.45 ± 0.65	0.37 ± 0.53	0.29 ± 0.40
13-cis-4-oxo-retinoic acid	0.92 ± 2.42	1.45 ± 2.25	1.11 ± 2.35
all-trans-4-oxo-retinoic acid	1.40 ± 2.79	0.61 ± 0.87	1.04 ± 1.29

6.3 RESULTS

6.3.1 Observed Effects of Creams on the Skin

Treatment group C were noticeably affected by the routine application of the cream and dermatological conditions deteriorated in the later stages of the study. A summary of reported dermatological conditions is given in figure 6.2 for redness and dryness. No effect was observed for the parameters oedema, vesication, wrinkles and greasiness during the entire course of the study.

Figure 6.2: - Skin assessments for weeks 0, 4 and 8 in the application of topical skin creams to the trunk and thighs of women of child bearing age. Where group A (0.236% by weight of retinol palmitate) subjects (n=12); group B (placebo) subjects (n=12); and group C (0.1475%) subjects (n=12).



6.3.2 Precursor and Metabolite Concentration Profiles

6.3.2.1 Retinyl palmitate

Mean plasma concentration profiles for retinyl palmitate are shown in figure 6.3. Low pre-dose levels of retinyl palmitate were detected in 35 out of the 36 subjects in the study. The range of pre-dose concentration was from 8.3 to 59.0 ng/ml. The mean pre-treatment plasma concentrations were 22.5 ± 12.5, 23.9 ± 13.9 and 28.2 ± 14.2 ng/ml for groups A, B and C respectively (table 6.4). The study protocol included a "wash out" period of one week with continued monitoring of the plasma levels and skin condition at the end of treatment. The mean concentration of retinyl palmitate within the samples analyzed during the last week of the study were 22.6 ± 11.1, 25.0 ± 12.2 and 24.5 ± 11.9 for groups A, B and C respectively. Comparison of day 57 levels with the pre-treatment levels demonstrated that no significant increase in plasma concentrations of retinyl palmitate occurred across the study period (table 6.4). Plasma profiles showed relatively random baseline fluctuations across the complete study period (figure 6.3). Monitored days during the study protocol occurred on week 0, 4 and 8, and plasma concentration profiles showed an increase in the plasma levels above the baseline fluctuations (figure 6.3, graphs a, b and c respectively). Increases in concentration peaked within 2-3 hours of skin application and returned to baseline within 2 hours. Mean data was derived from 12 subjects per group. However, the changes from baseline observed could be attributed to a small number of individuals within each group. The increases observed for individual subjects did not exceed 100 ng/ml in most cases, although one subject demonstrated an increase during the

observation on day 28 (figure 6.3, graph b) of 225 ng/ml. Ratio of pre-treatment concentration to post treatment concentration reduced the variability for the individual subjects. However, plasma concentration profiles remained similar to the raw data (figure 6.4). Treatment group B (placebo) showed a similar profile to the other treatment groups, indicating that the clinical variations in retinyl palmitate did not arise from the retinoids present in the applied skin creams (A & C).

Table 6.4: - Mean plasma concentrations for retinol, retinyl palmitate and all metabolites measured for day 0 (pre-treatment) and day 57. Statistical comparisons were by paired t-test.

	Pre-treatment			Day 57		
	A	B	C	A	B	C
all-trans-retinoic acid	1.4±0.6	1.3±1	1.8±1.2	1.1±0.2**	1.3±0.2*	1.5±0.9*
13-cis-retinoic acid	1.3±0.3	1.3±0.7	1.8±1.2	1.3±0.3*	1.3±0.9*	1.5±1.5*
13-cis-4-oxo-retinoic acid	0.9±2.4	1.5±2.3	1.1±2.4	2.1±1.4*	1.9±1.0*	1.6±1.2*
all-trans-4-oxo-retinoic acid	1.4±2.8	0.6±0.9	1.0±1.3	2.4±5.5*	0.6±0.8*	2.3±0.8*
9-cis-retinoic acid	0.5±0.7	0.4±0.5	0.3±0.4	0.2±0.4*	0.1±0.2**	0.2±0.3*
retinyl palmitate	23±13	24±14	28±14	23±11*	25±12*	25±12*
retinol	699±223	699±164	761±166	686±163*	684±206*	828±166*

* Statistical analysis by t-test indicated no significant differences between pre-treatment and day 57

** Statistical analysis by t-test indicated a significant difference of $p<0.05$.

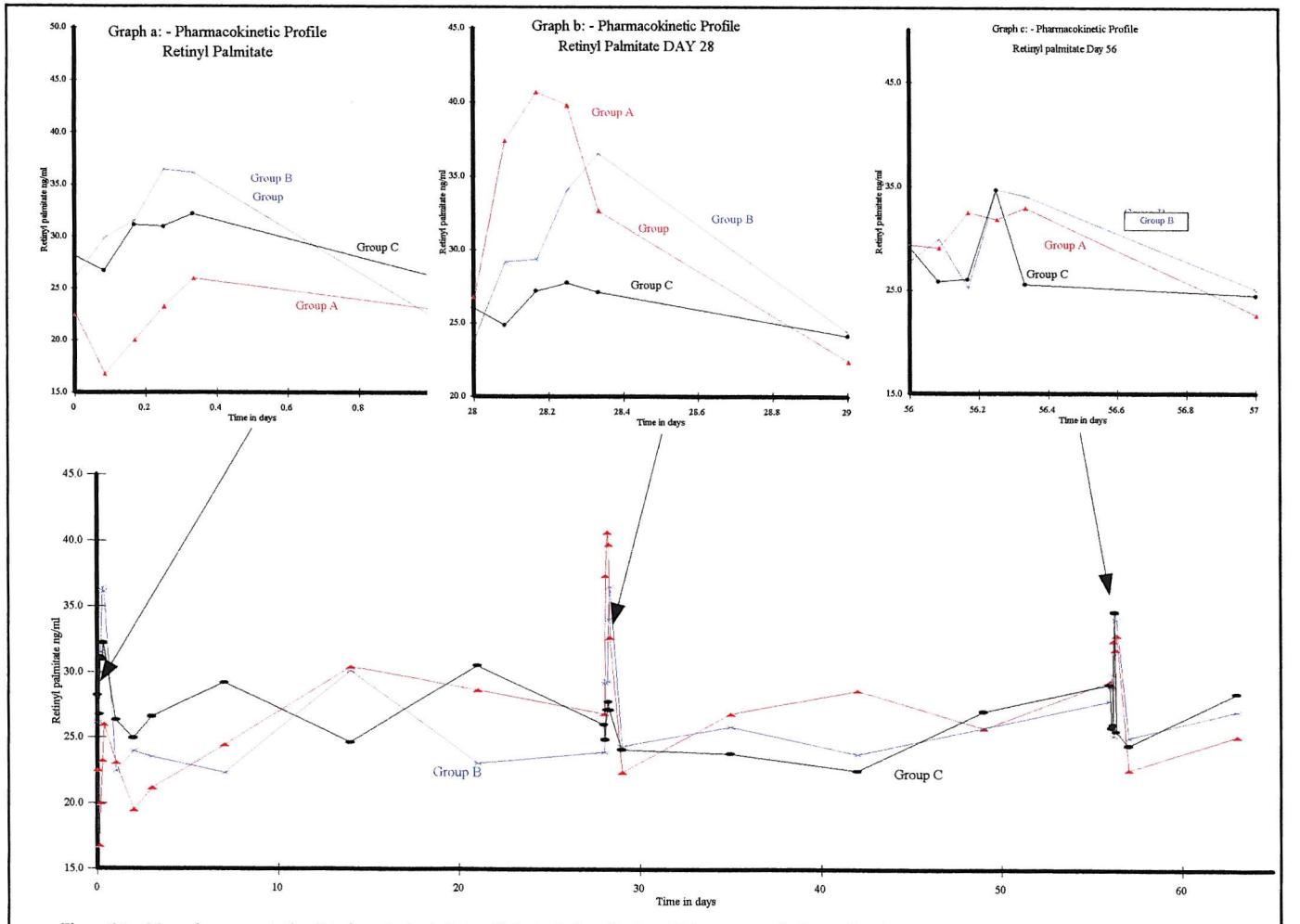


Figure 6.3: - Mean plasma conctration data for retinyl palmitate with the topical application of skin creams to the human female torso. where group A= 0.236% of retinol palmitate (w/w%), group B = placebo and group C = 0.1475% retinol (w/w%).

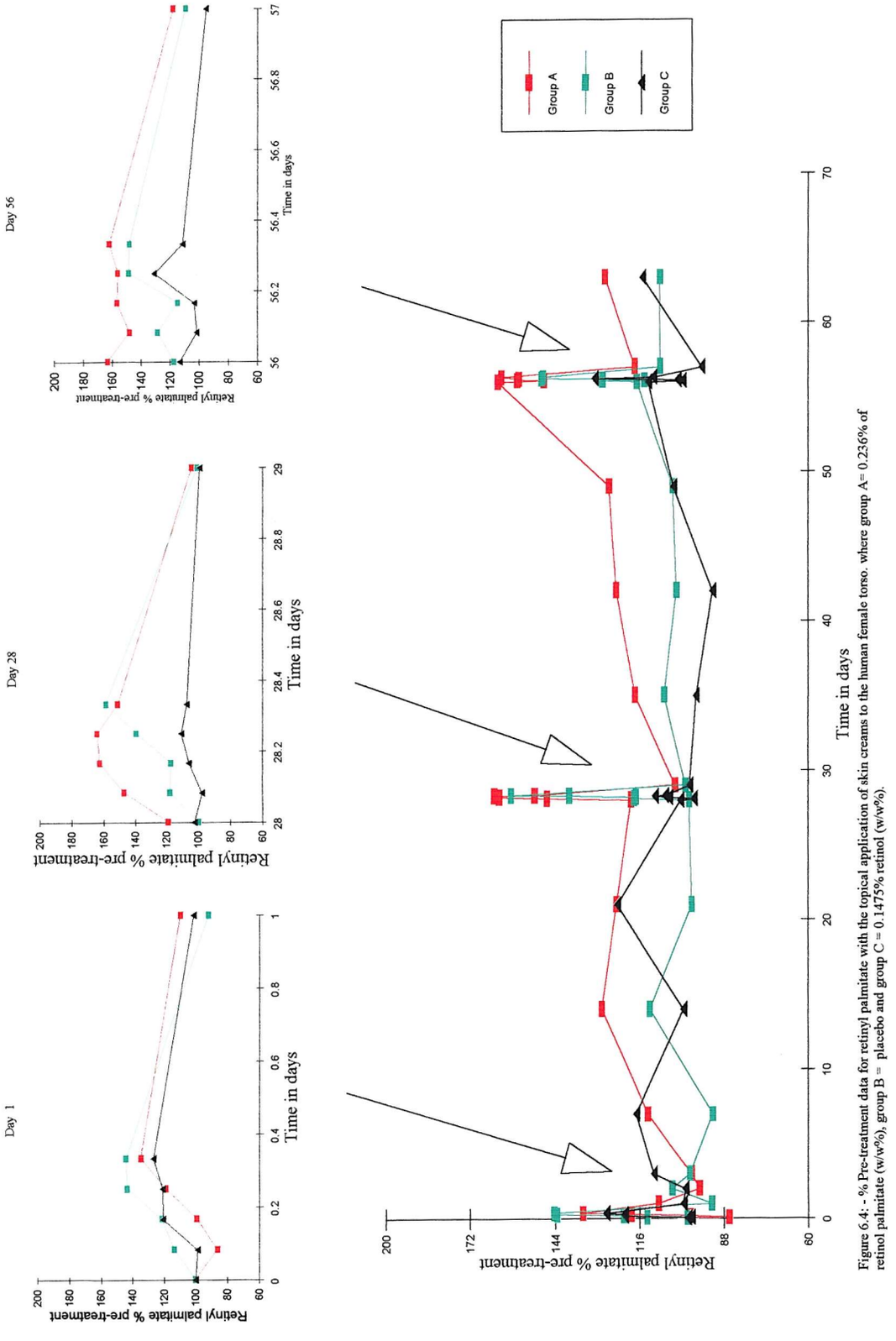


Figure 6.4: - % Pre-treatment data for retinyl palmitate with the topical application of skin creams to the human female torso. where group A= 0.236% of retinol palmitate (w/w%), group B = placebo and group C = 0.1475% retinol (w/w%).

6.3.2.2 Retinol

Plasma concentration of retinol were determined by HPLC analysis in the range of 200-500 ng/ml. Endogenous levels of retinol range from 400-1200 ng/ml and an increase within the 200-500 ng/ml range was subject to some assay variability. Retinol concentration profiles exhibited a consistent pattern between individuals (figure 6.5). The analytical error was found to be within ±10% for all patients except for some instances where high endogenous levels affected the analytical result. The mean pre-treatment plasma concentrations of retinol were 699 ± 223, 699 ± 164 and 761 ± 166 ng/ml for groups A, B, and C respectively (table 6.4). The range of concentrations were 642-784 ng/ml, 661-748 ng/ml and 727-828 ng/ml for groups A, B, and C respectively over the 63 days of the protocol. Figure 6.5 shows the plasma concentration profiles for retinol plasma concentration across the entire study period. Graphs a, b and c illustrated the intensive week 0, week 4 and week 8 monitoring respectively. Final plasma concentrations determined on day 57 were 686 ± 163, 684 ± 206 and 828 ± 166 for groups A, B and C respectively. No observable increases in concentration were observed above the baseline in comparison with initial pre-treatment by statistical analysis (table 6.4). No differences were found between treatment groups.

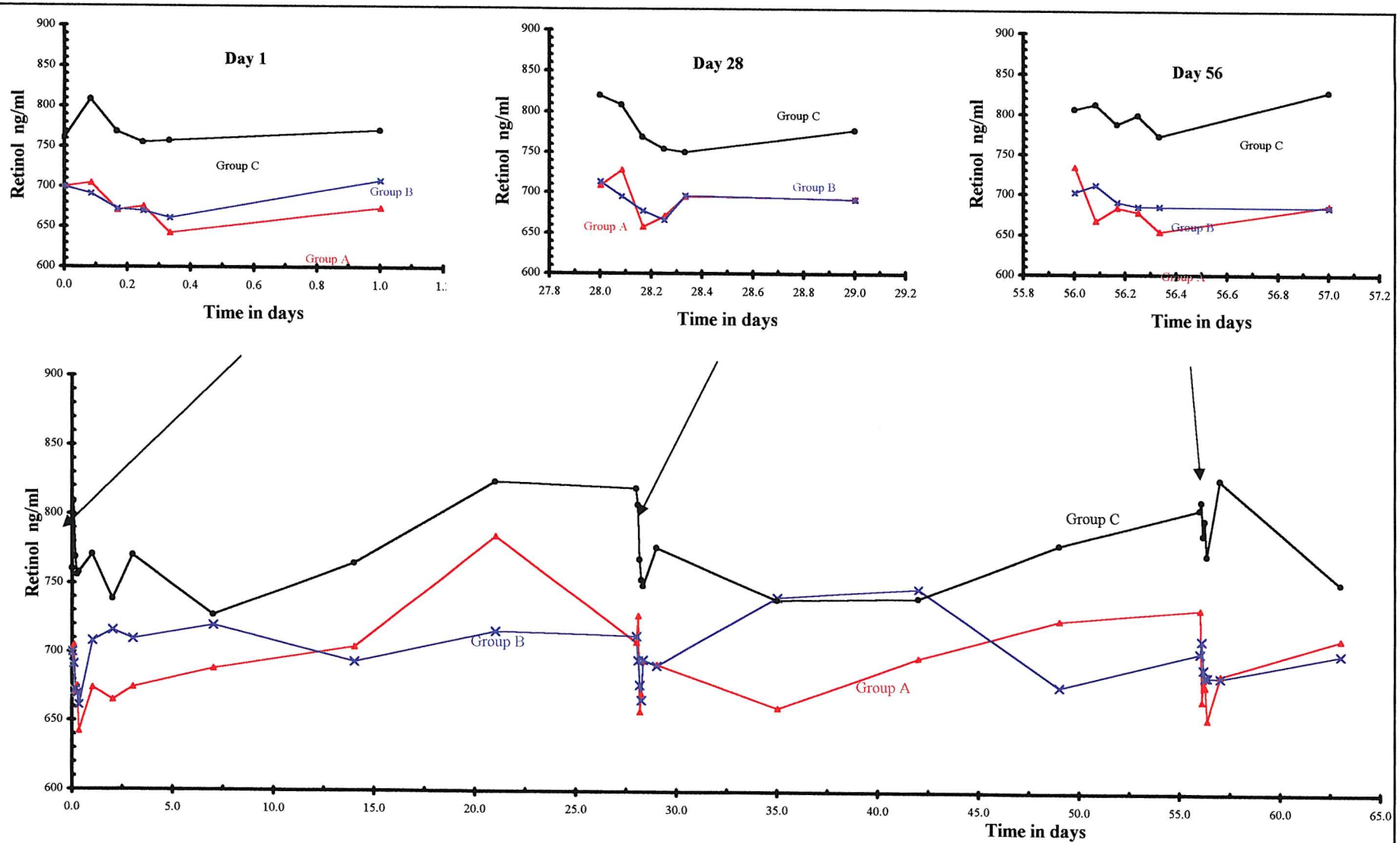


Figure 6.5: - Mean plasma concentration data for retinol following topical applications of skin creams to human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B= placebo, group C= 0.1475% retinol (w/w%).

6.3.2.3 All-trans-retinoic acid

Table 6.4 shows the mean plasma concentrations pre-treatment and after 57 days of skin cream applications for all-trans-retinoic acid. Final mean plasma concentrations determined on day 57 were 1.07 ± 0.23, 1.27 ± 0.55 and 1.50 ± 0.86 ng/ml for groups A, B and C respectively. Within in each group the pre-treatment range of concentrations were 0.72-2.78 ng/ml, 0.71-3.64 ng/ml and 0.77-4.59 ng/ml respectively. No increases in concentration were observed above the pre-treatment baseline for the complete study protocol for groups B and C. Pre-treatment mean plasma concentration of Group A was significant higher ($p<0.05$) in comparison to that of day 57. This difference was linked to the higher values found in two subjects on the pre-treatment analysis compared to the values found on day 57 for the same individuals. Figure 6.6 shows the plasma concentration profiles for all-trans-retinoic acid across the entire study period. Graphs a, b and c illustrates the intensive week 0, week 4 and week 8 monitoring respectively. Each intensive investigations of week 0, week 4 and week 8 demonstrated little variations from the pre-treatment baseline.

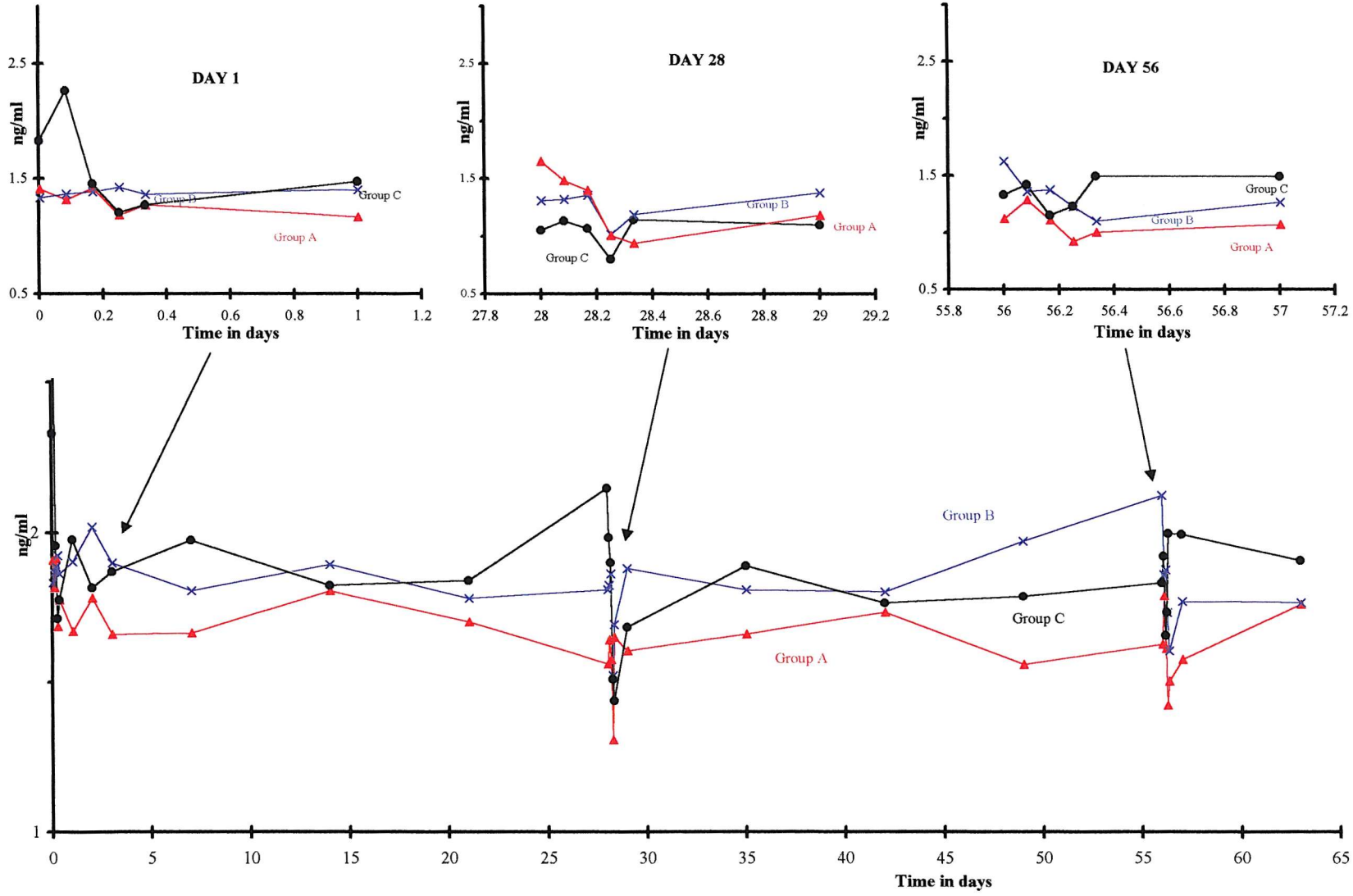


Figure 6.6: - Mean plasma concentration datafor all trans retinoic acid following topical application of skin cream to the human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B=placebo and group C=0.1475% retinol (w/w%)

6.3.2.4 13-Cis-retinoic acid

The mean pre-treatment plasma concentrations of 13-cis-retinoic acid for groups A, B, and C respectively were 1.28 ± 0.33, 1.28 ± 0.68 and 1.82 ± 1.20 ng/ml (table 6.4). The range of concentrations were 0.58-1.92 ng/ml, 0.31-2.56 ng/ml and 0.42-4.78 ng/ml for groups A, B, and C respectively. Final plasma concentrations determined on day 57 are compared with pre-treatment levels for groups A, B and C by t-test statistical analysis (table 6.4). No significant increases in concentration were observed above the pre-treatment baseline for the complete study protocol. Figure 6.7 shows the plasma concentration profiles for 13-cis-retinoic acid across the entire study period. Graphs a, b and c illustrates the intensive week 0, week 4 and week 8 monitoring respectively. No observable increases in concentration were observed above the pre-treatment baseline for the intensive investigations of week 0, week 4 and week 8.

6.3.2.5 13-Cis-4-oxo-retinoic acid

Figure 6.8 shows the plasma concentration profiles for 13-cis-4-oxo-retinoic acid across the entire study period. Graphs a, b and c illustrates the intensive week 0, week 4 and week 8 monitoring respectively. The mean pre-treatment plasma concentrations of 13-cis-4-oxo-retinoic acid was 0.92 ± 2.42, 1.45 ± 2.25 and 1.11 ± 2.35 ng/ml for groups A, B, and C respectively (table 6.4). Final plasma concentrations determined on day 57 were compared to pre-treatment levels by t-test analysis for groups A, B and C (table 6.4). No significant differences in mean concentration were observed above the pre-treatment baseline for the complete study protocol in all groups. No observable

differences in concentration were observed above the pre-treatment baseline for the intensive investigations of week 0, week 4 and week.

6.3.2.6 All-trans-4-oxo-retinoic acid

Figure 6.9 shows the plasma concentration profiles for all-trans-4-oxo-retinoic acid across the entire study period. Graphs a, b and c illustrates the intensive week 0, week 4 and week 8 monitoring respectively. The mean pre-treatment plasma concentrations of all trans-4-oxo retinoic acid was 1.40 ± 2.79, 0.61 ± 0.87 and 1.04 ± 1.29 ng/ml for groups A, B, and C respectively (table 6.4). Final plasma concentrations determined on day 57 were compared with pre-treatment levels by t-test statistical analysis for groups A, B and C (table 6.4). No significant differences in concentration were found above the baseline for the complete study protocol of all groups. No observable increases in concentration were observed above the baseline for the intensive investigations of week 0, week 4 and week 8.

6.3.2.7 9-cis-retinoic acid

Figure 6.10 shows the plasma concentration profiles for 9-cis-retinoic acid across the entire study period. Graphs a, b and c illustrates the intensive week 0, week 4 and week 8 monitoring respectively. The mean pre-treatment plasma concentrations of 9-cis retinoic acid was 0.45 ± 0.65, 0.37 ± 0.53 and 0.29 ± 0.40 ng/ml for groups A, B, and C respectively (table 6.4). Final plasma concentrations determined on day 57 were 0.24 ± 0.36, 0.12 ±.0.22 and 0.14 ± 0.23 ng/ml for groups A, B and C respectively. No significant increases in concentration were observed above the baseline for the

complete study protocol for groups A and C. No observable increases in concentration were observed above the baseline for the intensive investigations of week 0, week 4 and week 8. Group B showed a significantly higher mean pre-treatment plasma concentration compared to that of day 57 ($p<0.05$). However, plasma concentrations found in all individuals were subject to assay interference due to low concentrations being detected. The significant difference observed can be associated to three individuals where the interpretation of chromatograms was made difficult by unstable baselines and broad peak shapes. Data was included since no justifiable reason could be given to exclude these subjects based on analytical problems.

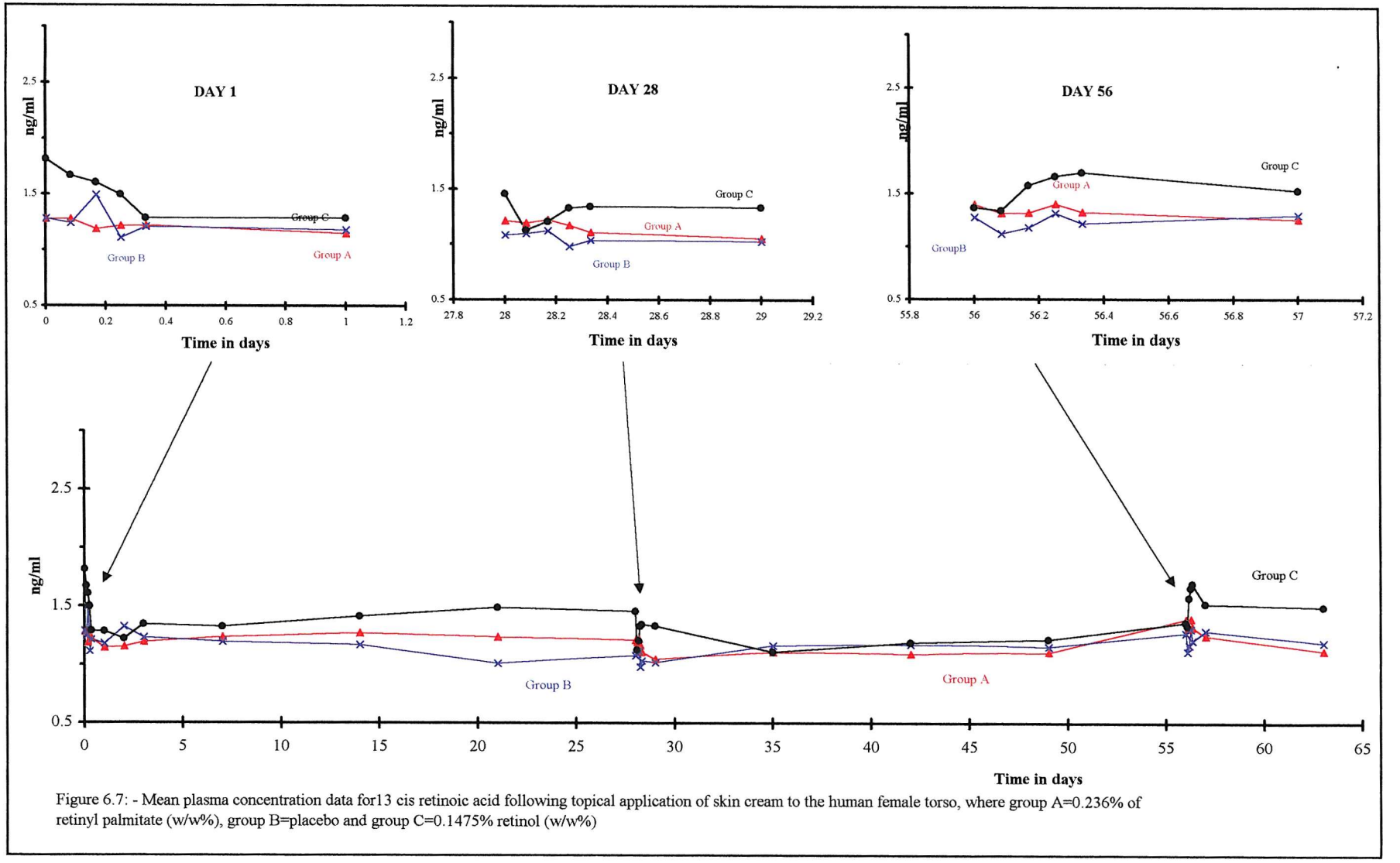


Figure 6.7: - Mean plasma concentration data for13 cis retinoic acid following topical application of skin cream to the human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B=placebo and group C=0.1475% retinol (w/w%)

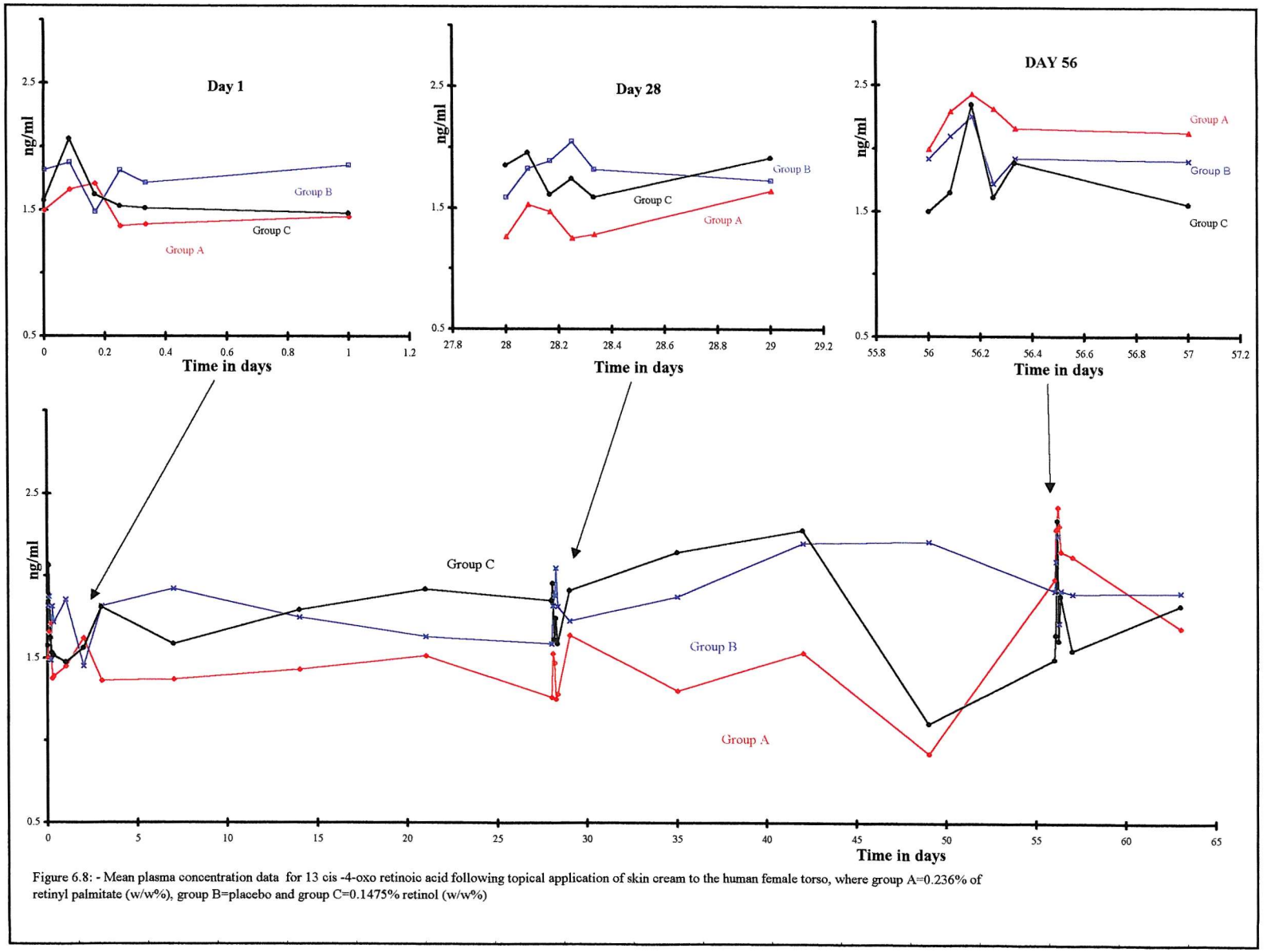


Figure 6.8: - Mean plasma concentration data for 13 cis -4-oxo retinoic acid following topical application of skin cream to the human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B=placebo and group C=0.1475% retinol (w/w%)

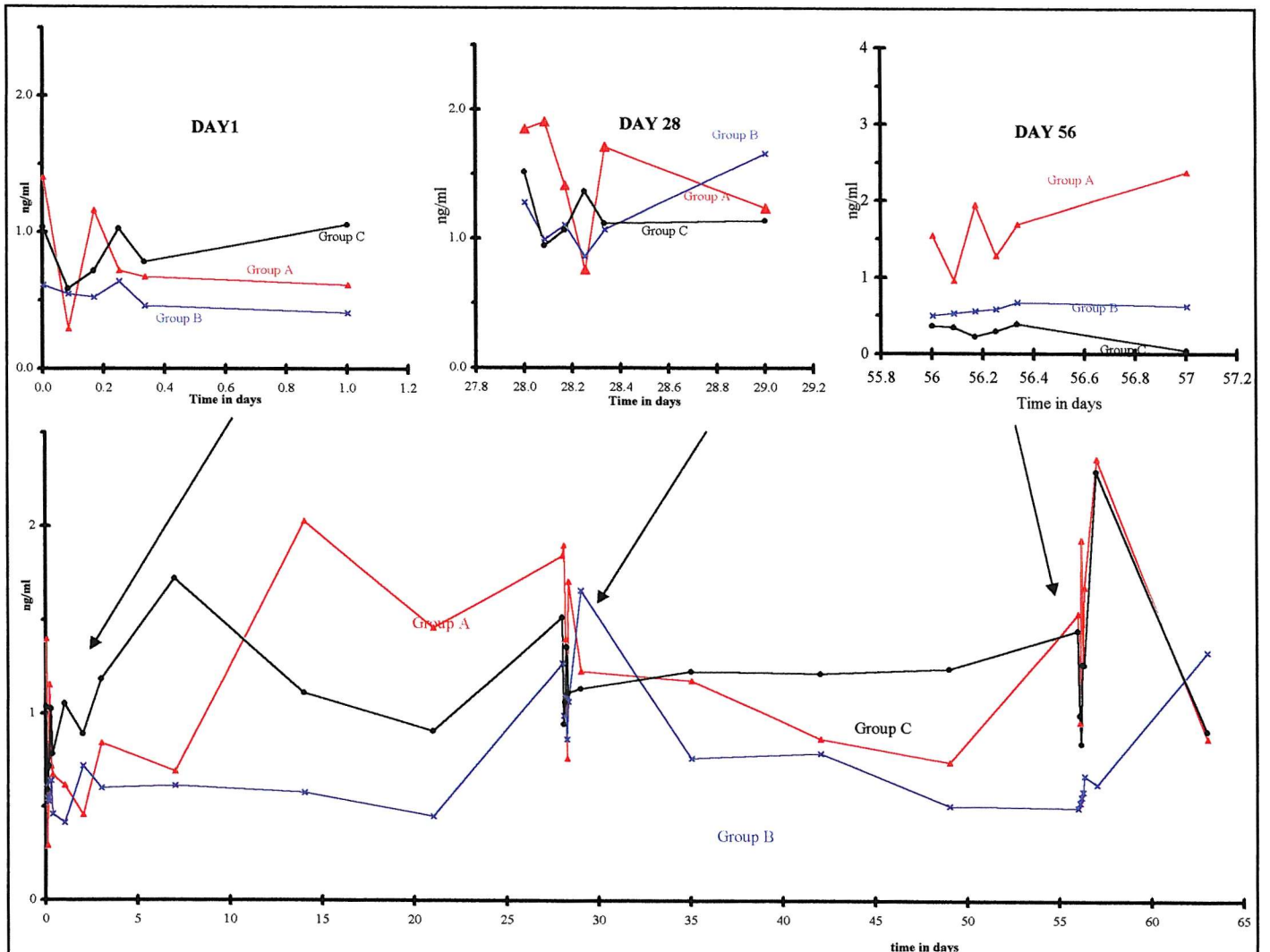


Figure 6.9: - Mean plasma concentration data for1all trans-4-oxo retinoic acid following topical application of skin cream to the human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B=placebo and group C=0.1475% retinol (w/w%)

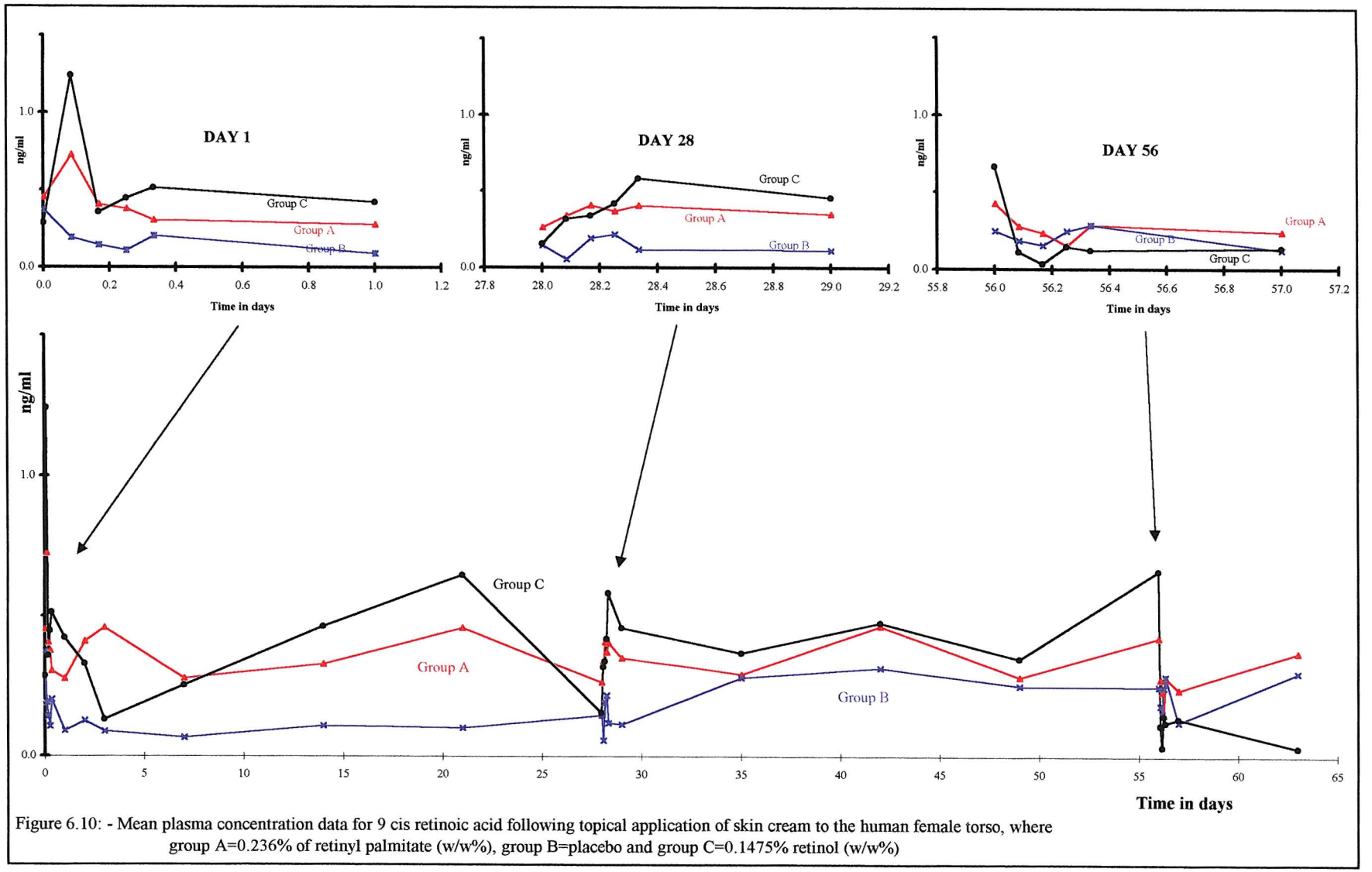


Figure 6.10: - Mean plasma concentration data for 9 cis retinoic acid following topical application of skin cream to the human female torso, where group A=0.236% of retinyl palmitate (w/w%), group B=placebo and group C=0.1475% retinol (w/w%)

6.4 SUMMARY AND CONCLUSIONS

The aim of this study was to investigate the systemic changes in plasma concentrations for retinol and its metabolites after the intensive application of cosmetic based skin creams containing retinol or retinyl palmitate. In order to maximize possible absorption, the largest surface area practical was defined in the study protocol, i.e the trunk of the body (chest, stomach, back and sides) and the thighs (front and back). Subjects were requested to not use face, arms or lower legs for skin cream application. Total surface area between individuals was therefore kept at a relatively constant amount. Compliance with respect to the dietary restrictions was found to be good. Completion of food diaries was 99.9%. No unexplained rises in the plasma concentration were observed related to the consumption of foods known to contain qualities of vitamin A pre-cursors.

For the purposes of this investigation the content of vitamin A within each skin cream was as high as technically possible. Levels used were 10-100 fold higher than most commercially available skin care products and hair treatment products. Any absorption, therefore, would be magnified and detectable in the analysis of the plasma samples. In the case of group C the content of retinol was sufficient to cause skin irritation in many subjects in the later stages of the study protocol. The effects seen included itchy folliculitis on the shoulders and the site of the waistband, persistent rashes on stomach and thighs, increased redness and dryness of the affected skin areas and minor cases of eczema observed at the site of the umbilicus or the back of the thighs. No discernable increases were observed in relation to the plasma

concentrations of the retinol or metabolites during the instances of these complaints. Effects observed were considered to be localized toxicity of retinol and increased sensitivity of the skin to tight clothing. Very few problems were observed within groups A and B. All irritation observed could be related to the quantity of cream necessary to be applied and to the rubbing motion used by the subjects. Subjects were advised to apply the cream across a broad area and to use a rubbing motion in the direction of the body hairs to spread the cream across the entire surface of the trunk and thighs. Problems with the application of the skin creams within groups A and B were quickly resolved once advice was taken on how to apply and rub the cream on the body.

The plasma concentration data obtained for retinol and all its metabolites were at endogenous levels throughout the total study period. In terms of assay sensitivity this proved to be at the limit of detection for the system. Chromatogram analysis was regularized to the manual identification of minor peaks with comparison to standard chromatograms assayed within 3 hrs of each sample. Peak retention times for the initiation and ending of the rise in the baseline above a signal to noise level of 3 were recorded. Integration of peak areas were then forced to the retention times determined to generate the peak areas. All data generated were quality controlled and objectively verified by an independent member of the study team. The data generated and shown in figures 6.3 to 6.10 indicate that there was negligible systemic availability of retinol or its precursors and negligible metabolites of vitamin A within the plasma during the total study protocol.

Plasma concentrations were reassessed at day 63 after initialization of the study, one week after stopping the application of the skin creams. No decrease or change in comparison to the pre-treatment baseline was observed and the problem associated with group C cleared up quickly with the cessation of the cream application.

It can be concluded high dose topical application of vitamin A does not present a risk of teratogenicity. This in agreement with results reported in literature.

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Chapter 7

General Discussion

Table of Contents

List of Tables

List of Figures

7.1 BACKGROUND

In the research into the attributes and the function of vitamin A it has been shown conclusively that the acid metabolites of retinol present in the blood and tissues of most mammalian animals are teratogenic. There have been a number of reviews of the data accumulated to date of all the scientific evidence confirming this fact (1;2). The question that is difficult to answer concerns the levels of these metabolites which produce a teratogenic effect as compared to those which produce the beneficial effects associated with vitamin A. Research into this question is limited to the teratogenic work in animals and pharmacokinetic studies in animals and humans (3;4). These investigations are complication by the fact that there are observed differences in teratogenic sensitivity of different species to the effects of retinoid dosing. Some species will tolerate very high dosing without teratogenic effects while others express teratogenicity at very low levels (5). The metabolism of vitamin A between animal species, including the human species, has also been shown to be different (table 7.1).

Table 7.1: Lowest reported teratogenic dose of vitamin A and its primary metabolites after oral administration in several animal species. Where P = predominate metabolite seen, S= secondary metabolite seen, M= minor metabolite seen. Adapted from Armstrong et al and Dolk et al (1;6)

	Lowest teratogenic dose (mg/kg/d)			Main metabolic pathway					
Species	Retinol	TRA	CRA	TRA	4TRA	14H	CRA	4CRA	9,13CRA
Mouse	25	4	100	P	S	S	M	S	
Rat	45	6	75			P	M		
Rabbit	2.5	6	5	P	S	S	P		S
Monkey	N.D.	7.5	5	P	S	S	P	S	
Human	0.5 - 1.5 (tentitive)	N.D	0.5	P	S	M	P	S	M

7.2 ANIMAL EXPERIMENTS

The metabolism of vitamin A is different in different animal species. The rat will predominantly metabolise vitamin A to the 14-hydroxy-4,14-retro retinol (7), whereas in mice the metabolic pathway of vitamin A has been shown to be via all-trans-retinoic acid, all-trans-4-ox-retinoic acid and 14-hydroxy-4,14-retro-retinol (8). The rabbit metabolises vitamin A in essentially the same manner as humans, although 13-cis-retinoic acid is predominately metabolised to 9,13 di-cis-retinoic acid (9). Within the human species this is metabolised to the 13-cis-4-oxo-retinoic acid metabolite (10;11). Of all the animal species tested it has been shown that the primate (specifically cynomolgus monkey) and the rabbit appear to metabolise vitamin A in essentially the same way as the human species (12).

In relation to the teratogenic potential of vitamin A, it has been established through animal investigations that the metabolites of vitamin A have different relative potencies. It has been shown that on average the potencies are in the following order : all-trans-retinoic acid > 13-cis-retinoic acid > 9-cis-retinoic acid > all-trans-4-oxo-retinoic acid > 13-cis-4-oxo-retinoic acid. However, the relationship does alter slightly for each separate species. Table 7.1 shows the relative teratogenic concentrations necessary for all-trans-retinoic acid to show an effect (6). Typically, this value can be approximated to 5 mg/kg/d for all species including humans. 13-Cis-retinoic acid has been shown to have up to 20-50 fold differences between species. For humans, limited data is available for the estimation of

a minimum teratogenic dose of 13-cis-retinoic acid. However, in comparison to the other animal species it can be seen that the human species has the highest sensitivity to teratogenic effects of 13-cis vitamin A metabolites. The human species has been shown to be at least 10 fold more sensitive to 13-cis-retinoic acid effects than either the rabbit or the monkey.

7.3 HUMAN TERATOGENICITY

Since the introduction of retinoid treatments for various diseases of the skin, teratogenic effects within a certain number of children have been observed. Table 7.2 shows some of the reported cases of teratogenicity that have been observed with treatment using all-trans-retinoic acid. Effects have also been observed with 13-cis-retinoic acid in relation to treatments for acute cystic acne.

Table 7.2: Known cases of vitamin A teratogenicity within the human population. Adapted from Miller, R.K et al, 1998.

Ref	Face/Head	Cardiac	G.U.	Other
-13	Tiny ear canals, "dysmorphic face"	Transposition		Ectopic neurons
	high palate			
	Absent right ear, cleft lip and palate	Hypoplastic left		
		ventricle		
	Bilateral cleft lip			
	Microcephalus			
	Absent right ear and canal			

Ref	Face/Head	Cardiac	G.U.	Other
	Absent left auditory canal: VATAR syndrome Hypoplastic left ear Low set deformed ears; Micrognathia: micro-opthalmia			
	Cleft lip and plate, "cheek/jaw" anomalies, left anophthalmia			
	Cleft plate	Heart defect		
-14			Left double collecting system, ectopic insertion	
-15			Urinary tract anomalies	
-16	Goldenhars - preauricular appendage, atresia of ear canals, hemifacial atrophy			Cranial nerve palsy
-17	Microcephaly			Small adrenals; multiple CNS
-18	Low set ears dysmorphic face		Large single kidney; abnormal genitalia	Partial sirenomelia
-17	Low set ears : micrognathia			Multiple bony abnormalities.

These cases have confirmed human susceptibility to the teratogenic effects of the metabolites of vitamin A (19). In the known cases, treatment generally consisted of a high daily dose of the retinoid compound for several weeks. In the case of 13-cis-retinoic acid the plasma levels were still detectable months after treatment was stopped. Therefore, concern was expressed over the increased teratogenic risk associated with the elevated levels of 13-cis-retinoic acid and 13-cis-4-oxo-retinoic acid that persist long after

treatment was finished (20;21). Control measures were introduced to limit treatment with retinoids to female patients and female patients had to be on an adequate form of birth control for up to 1 year after the last dose had been taken.

Further studies on the question of vitamin A's teratogenic potential have all been conducted via retrospective case control studies involving either questionnaires or telephone interviews. Five such surveys are reported within literature conducted over a six year period. Miller et al summarized the data for these investigation (table 7.3) (22). There are several disadvantages to this form of investigation. Since the data is usually collected after the pregnancies, the researchers have to rely on the memory of individual mothers. This can be unreliable, especially in the circumstances of malformed children since the crucial period is very early in the gestation stages. Very few mothers would be able to accurately remember what they ate during that period. The focus of these investigations is usually the use of vitamin A supplements and multi-vitamin tablets. Crucial data could therefore be missed in the investigation giving bias to the results. The Rothman et al investigation was more detailed in terms of vitamin A dose and in the accuracy of the data gathered (23). Data was gathered from potential mothers in a national prenatal screening and only data of the three months prior to and the three months after the last menstrual period were investigated. However, Rothman et al treated the data obtained in some questionalable ways. The classification of the malformations seen has been criticized intensively and the assumption made about actual dose can be considered unreliable. However, the results obtained by Rothman et al do give a general indication that vitamin

A could be teratogenic in dosing levels of approximately 10000 to 20000 IU per day during the crucial gestation periods. Efforts to repeat these results have not been successful even if Rothman's malformation classification was used (24).

Table 7.3. Human studies concerning exposure to vitamin A during pregnancies. From Miller et al. (22).

Ref	Study Design	Population	Results	Comments
-25	Case-control within a surveillance system	11,293 nonchromosomal malformation cases 11,193 control	Exposure to 10000IU or more Exposure to 40000IU or more	Large screening program: some malformations of doubtful importance.
-26	Case-control within a surveillance system	2658 cranial neural crest-related defects. 2609 controls with other malformations	Exposure by lunar month 1st month OR = 2.5 (1.0,6.2) 2nd month OR = 2.3 (0.9, 5.8)	Well-characterised malformations: no data on vitamin dose available.
-27	Prospective from open prenatel screening program	22748 pregnancies 339 (1.5%) with malformations	Total intake > 15000IU Prevalence ratio (vs < 5000IU) Cranial neural crest defects 3.5 (1.7, 7.3)	Included data on dietary exposure: misclassification of craniel neural crest defects
-28	Case-control within a population based surveillance system	4918 malformation cases. 3029 contols	multivitamins alone All defects OR = 0.94 (0.86, 1.03) Cranial neural rest defects OR =0.86 (0.76, 0.97) Multivitamins and vitamin A tablet All defects OR = 0.60 (0.28, 1.29) Cranial neural crest defects OR = 0.69 (0.24, 1.91)	High power to detect differences: no data on vitamin intake
-29	Case-control within a population-based surveillance system	Cleft study 9.25 cases. 871 controls Heart study 254 cases 561 controls	Presumed exposures > 10000IU or = 0.55 (0.21, 1.5) clefs or = 0 (0, 2.2) Heart defects	Interviewed an average of 3.5 yrs after delivery: only estimated data on vitamin A doses

7.4 LIVER SOURCES

Related to the question of the amount of vitamin A that can be taken safely are issues of the sources of vitamin A. The amount that the general population consumes as well as the deviation of intake across cultural divides, make this estimate difficult (30). Several studies have been conducted in different groups of the population within various countries of the world (31). From the data generated by these questionnaire based studies, an estimation of the daily allowance for vitamin A has been made (32). However, each country has different views and the recommended daily allowances set can differ by up to 100% between countries (table 1.4) (33-35).

All sources of vitamin A must be taken into account when considering the potential benefits and risks. One of the prime sources of vitamin A to the general population is liver or liver products. However, early in the 1990's evidence was presented to the UK Department of Health indicating that the mean levels of retinyl palmitate in animal livers from Finland had reached higher levels than previously thought. It had been discovered that per 100g of liver the palmitate content ranged from 27000 μg (cow) to 62000 μg (pig). With the daily recommended allowance in the UK set at 750 μg, consumption of some animal livers or their products present a potential risk of large amounts of retinol reaching the systemic circulation. Once in systemic circulation or during first-pass metabolism, teratogenic metabolites of vitamin A are produced. Depending on the amount of the administered dose, dangerous levels of all-trans-retinoic acid and 13-cis-retinoic

acid are potentially possible.

In response to the evidence presented, the Department of Health investigated the retinoid content of animals slaughter within the United Kingdom. The last survey of retinoid content in animal livers was conducted in 1982 by the laboratory of the government chemist in a series of unpublished results (36). In 1978 Paul and Southgate published a series of analysis of animal livers (37), and in 1960 values can be found in the third edition of McCance et al's "The composition of foods" (38). With the wide range of values found in each liver type, the significance of the change in liver concentration over the past 30 years is difficult to assess. Figure 7.1 shows the tentative comparison of the mean data for all four investigations. The general trend observed is that for such animals as ox, lamb,

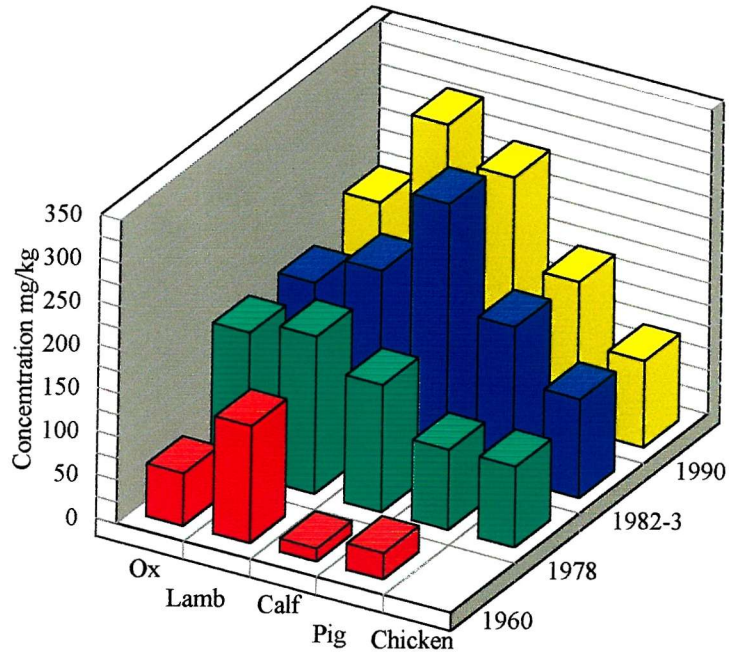

Figure 7.1: Relative concentrations of vitamin A in animal livers over a thirty year period.

calf and pig the levels determined shows an increase over the time period (36). However, the number of livers analysed is small (n=10±5 for each species) in comparison to the number of animals used for meat production each year across the world. Yet, with the known teratogenic effects of vitamin A and the increased use of supplementation among the general population, concern was raised as to whether it was sensible for potentially pregnant women to consume liver or liver products (39;40).

With the increasing concentration of retinyl palmitate in animal livers, it was believed that a teratogenic dose could be delivered within one meal containing approximately 70 g of liver. Hence, an investigation was conducted by Buss et al (41) to compare the absorption and metabolite profile in females of child-bearing age given an oral liquid supplement or calf's liver. It was demonstrated that there was a significant difference in the absorption from liver compared to that of an oral supplement and especially the extent of formation of all-trans-retinoic acid. Liver produced significant lower levels of retinyl palmitate and retinoic acid metabolites as compared to the supplement. This study confirms the findings of Buss et al. It also showed that lower doses of vitamin A gave a similar difference between liver and oral supplements. Therefore, the consumption of a meal containing liver or liver products gave a lower the systemic availability of retinol and its metabolites in comparison to an equivalent dose as a oral liquid supplement on an empty stomach.

7.5 EFFECTS OF POSTURE AND FOOD

With the comparisons between right side and left side supine positions it was thought that the stomach emptying processes could affect the movement of retinol into the small intestine. The stomach consists of three parts: the corpus (or body), the antrum and the fundus. The two main parts are the corpus and the antrum, with the fundus operating as part of the main body. The stomach is designed for several purposes. The first is the storage of food until in can be passed into the duodenum. The second is to prepare the food material for digestion by mixing the material with secretions generated within the stomach until it is in a semi-fluid state, chyme. Within the stomach the rate of emptying is determined by the intensity of the peristaltic waves. However, several factors control the rate at which the antral peristaltic mechanism occurs (42) :

A/ As the stomach fills with food the corpus expands. This does not create excess pressure to emit chyme into the duodenum, but the expansion cause nerve signaling to be triggered which increases the peristaltic pumping mechanism. The greater the extension the more intense the wave forms are until the stomach begins to empty. The contraction of the corpus will reduce the wave forms again.

B/ Certain types of food have the effect of releasing the hormone gastrin, the effect of which is to increase the acidity and volume of the gastric secretions. It also

stimulates the antral peristaltic pump.

C/ In the duodenum the chyme is processed at a specific rate. Changes in the conditions within the duodenum of either volume or acidity controls the rate at which the chyme is processed. Movement can be increased or decreased depending on the circumstances.

d/ Certain types of food cause the release of hormones from the intestine, which increase the force of the contractions within the body of the stomach and decrease the amount of material released into the intestine. One of the food types known to cause this are fats. Hence, for foods containing large quantities of fats, the mechanism increases the power and duration of the mixing process, but might delay the absorption.

In the fasting state the stomach antrum remains open to a small degree, but there is no stimulation by expansion and hormone release. Dosing with the oral liquid formulation on an empty stomach would maximise any differences due to posture. However, this study demonstrated that posture and simple gastric emptying had no effect.

With the consumption of a fat containing meal shortly before the oral dose is given, a curious fact emerged. The T^{max} for retinyl palmitate was significantly quicker when vitamin A supplement was dosed in conjunction with a meal as compared to dosing on an

empty stomach (T^{max} 4.8 ± 1.2 hrs as compared to 2.8 ± 0.8 hrs). This was initial not expected. An echo planar imaging study of the stomach during the consumption of liquid meals has shown the potential for the lipids to separate from the aqueous phase (43). Lipid fractions were observed to collect above the aqueous phase and hence entered the intestine later. Subsequently, absorption was slowed. However, in our study, after dosing of a vitamin A supplement in conjunction with a meal, more solid material would be present in the stomach. This would create very different conditions in comparison to the liquid soups used in the Echo planar trial and to the oral vitamin A supplement on an empty stomach. Liquified lipids would probably move down the stomach more quickly than the food material. However, when Arovit was given with food, mixing would not be homogeneous and the supplement would filter around the heavier food particles. Expansion of the stomach would increase the degree of antral peristaltic motion. Hence, it could be suggested that the oral liquid supplement after a meal has an increased rate of entering the intestine compared to that of the fasting stomach. Therefore, it would be observed that the absorption of the retinyl palmitate would be at similar amounts between regimes, but the delivery to the intestine would have been quicker in the dose given following a meal.

Another explanation could be the fact that the meal would provide greater quantities of glycerides and phospholipids to the stomach mixture. Tri-glycerides and phospholipids are instrumental in the formation of oily droplets within the gastric system, which contains various lipophilic compounds within their structure (44). An increase in the quantity of

such material would promote the formation of oily droplets during the transfer from stomach to intestine. With a greater number of droplets available, their interaction with the brush border membrane would increase. Presumably the time taken for absorption of the contents of these droplets would therefore decrease. The tri-glycerides and phospholipids released would be absorbed and be incorporated into the formation of chylomicrons. With the fat content in the meal affecting the size of the chylomicrons formed, then the delivery of the retinyl palmitate to the blood stream should be enhanced (45). One investigation into this question has been reported using eight healthy males. In this study individuals were given four vitamin A containing test meals on different occasions containing different amounts of added triglycerides. Statistical analysis of the pharmacokinetics of retinyl palmitate between the four triglyceride concentrations exhibited no significant differences. It was concluded from the data generated that a significant amount of vitamin A is absorbed in the presence of trace amounts of triglycerides (46). Different levels of triglycerides did not alter the absorption profile for retinyl palmitate, but did affect the ratio of retinyl ester types reaching the chylomicron remnants.

7.6 VARIABILITY OF VITAMIN A ABSORPTION

During this study it was also observed that there were marked differences between individuals in the absorption and metabolism of a similar dose under controlled

conditions. Similar observations were made by Chen et al (47) and by other researchers (48;49). It was suggested that, since vitamin A is a lipophilic compound, the mixing with the gastric secretions could be different between dosage forms. Chen et al also observed a marked absorption difference between the two supplement formulations used. It was seen that one 5,000 IU containing vitamin A supplement formulation gave 13-cis-retinoic acid levels that were higher than a 10,000 IU dose of a second formulation and similar to a 25,000 IU dose of the second formulation. The inherent variability between vitamin supplement formulations and natural sources of retinoids makes comparison between studies difficult. However, one suggestion is that the mechanisms of movement for partly digested food from the stomach to the intestine (antral peristaltic contractions) could effect the bioavailability of vitamin A. Different formulations could mix with gastric secretions to different degrees, depending upon the carrier material used for preparing the formulation.

Another factor to consider is the supply of bile acids and pancreatic secretions for lipid digestion. Different individuals will produce bile acids in different proportions and quantities, thereby affecting the absorption of fatty acids and lipid soluble compounds such as vitamin A. Different food sources (such as liver, fruits and green vegetables) could be digested more slowly in the intestine decreasing the time for retinol to be released for the absorption process.

Another possible cause of variability is the total vitamin status of each individual. Each person would have a vitamin A store within the liver. This retinol store could influence

the plasma levels of both retinol and CRBP I. Those with a vitamin A content lower than others would absorb more of the retinol found in a food source. Those with a healthy liver store might not need to absorb such large quantities of vitamin A, or if they did then rapid metabolism could clear the plasma of the extra absorbed. To compare between patients of similar vitamin A status would be difficult since the circulatory concentration of retinol is relatively constant despite the size of the dose given (50). Placing a selected group of individuals on a controlled diet with adequate vitamin A supplementation for an equilibrium period could ensure similarities between individuals.

Posture, previous dosing and multiple dosing did not affect vitamin A pharmacokinetics in this study and therefore could not account for the large inter- and intra-individual variations seen in this study and in literature.

7.7 MULTIPLE DOSING

It has been observed in patients undergoing treatment for various forms of cancer that prolonged exposure to all-trans-retinoic acid causes a decrease in the levels of circulating retinoids. Pharmacokinetics performed with patients in these conditions demonstrated lower absorption and metabolite profiles following long-term administration (51-53). Although this would not reflect normal situations in healthy individuals, it would suggest that an elimination pathway for retinoid metabolism is induced by the continuous dosing with elevated levels of the metabolites of retinol. In such patients it was demonstrated that

a brief drug holiday would restore retinol plasma concentrations to normal levels. However, the risk of reversal of remission during such periods was high, and thus limits the usefulness of vitamin A and its metabolites in the treatment of various forms of cancer. In our study, multiple dosing did not show a decrease in the plasma levels of retinol and its metabolites. However, our study cannot be compared to the above mentioned studies conducted amongst cancer patients. Our main interest was to investigate the pharmacokinetics of vitamin A and its metabolites following multiple dosing by the general public, rather than following multiple dosing due to medical treatment. The most common form of multiple vitamin A dosing is the regular usage of vitamin supplements. Therefore, our study used different conditions as compared to the studies mentioned above:

(1) our study involved healthy patients.

(2) in our study subjects were dosed with retinyl palmitate instead of the retinoic acid metabolites used in the treatment of cancer.

(3) in our study much lower doses were used.

It should be noted that our study only demonstrated the effect of short-term multiple dosing (1 week). A long-term multiple dosing vitamin A supplement study could give significant different results.

7.8 SAFETY CONSIDERATIONS

As has been demonstrated, liver is not the most efficient source of vitamin A, but it is a very good source of nutrients such as iron, B_6 and folic acid which are beneficial to pregnant women. High concentrations of the teratogenic metabolites do not occur after a dose delivered by liver (at the levels studied). Significant lower concentrations were found after liver dosing compared to supplement dosing. Also, supplement dosing in conjunction with a meal showed a significant faster absorption of vitamin A. These factors must be taken in consideration in the assessment of the teratogenic potential of vitamin A. In terms of the relative safety of vitamin A, a greater risk would be associated with the unsupervised use of supplements, since the bioavailability of vitamin A can vary significantly between different formulations. Doses contained within some readily absorbed supplements could prove to be teratogenic if taken on a regular basis above manufacturer's recommendations. The crucial periods of teratogenicity is during the early stages of pregnancy when the pregnancy might be unknown. The formation of many of the key components of developing organs and the basic structure of the final organism is controlled in part by the signalling pathway of retinol to retinoic acid. Therefore, the risk of teratogenic effects is increased by virtue of the fact that the critical periods of embryonic development occur during the early stages of pregnancy, since the mother may not be aware of the pregnancy in order to take precautions. Also, it has been repeatedly reported by dieticians that oral retinoid medical treatments are continuing to be prescribed

without adequate verification of contraception (54-57). Questionnaires to general medical practitioners from Canada, Scotland and Sweden have shown that a significant number do not realise the importance of adequate contraceptive control during oral retinoid treatment. In the USA, the BUAS survey indicated that 900 pregnancies with retinoid exposure were born between 1989 and 1998. This report only had data from 38-40% of all the cases within America where women of child-bearing age were treated with a retinoid product. This report was treated with concern by the pharmaceutical industry, which embarked on a campaign to increase the awareness of the inherent dangers of retinoid treatments for patient and doctors alike.

This lack of awareness also extends to the use of supplements during pregnancy. One study conducted in India dosed pregnant women with high levels of vitamin A to investigate the clinical effects of maternal serum retinol levels dropping in the final trimester of the pregnancy. While vitamin A is important to a pregnant woman and her developing child, it must be asked if supplementation is safe. The pharmacokinetics of retinyl palmitate has shown that the larger the dose given, the more the formation of teratogenic levels of metabolites. Smaller doses will restore the basic vitamin A stores in a similar manner to a single mega-dose, but this is logistically impractical. There are no reports available on vitamin A teratogenicity within the developing countries. Vitamin A deficiency is common within these countries and supplementation is recognised as a means to combat this. However, if the western-world data indicate such a high proportion of pregnancies exposed to retinoid medical treatments and indicate some supplement abuse cases, what

is the long term effect of this policy of supplementation in the third world ?

In the final assessment of teratogenic risk, the most susceptible group or groups should be considered. Most obviously the risk is relevant to women of a child-bearing age, but research has shown that girls in the 13-14 age group are more prone to supplement abuse than other women except for the elderly. Girls of 13-14 years old are in the stages of growing up. Fashions are pre-eminent in their lives. The desire to look good and feel good could lead to the regularised control of their diets and the use of health supplements. Pregnancy among this higher risk group is not uncommon due to changing morals of western life. Also, among this group awareness of what is healthy and what is not is limited. Liver products tend not to be consumed by this segment of the population, but other sources of vitamin A are regularly taken. A Japanese case of toxicity from over-eating pumpkins is such an example. Health foods, vitamin supplements and other dietary considerations are aimed at the general population through advertising. The young female is particularly susceptible to this form of advertising.

The use of supplements containing high levels of vitamin A by healthy pregnant women in the western culture should be considered needless. However, the consumption of liver during pregnancy would provide other beneficial nutrients, but with lower risks than supplementation. Individuals might overdose on supplements, unknownly or in the mistaken belief of beneficial effects. The overall conclusion for this investigation is that liver does not represent a teratogenic risk under normal eating habits. Regular applications of cosmetic creams do not present a teratogenic risk. It has also been shown that normal

regular use of supplements do not present a teratogenic risk. However, of the forms of vitamin A investigated, the use of supplements can give an element of risk due to public unawareness and the potential unknowing abuse of freely available products. It is therefore of great importance to inform the general public and to increase its awareness about the risks and benefits of vitamin A.

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