PHARMACOKINETICS AND IN VITRO STABILITY
OF RETINYL PALMITATE

by

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**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Chemistry</td>
<td>2</td>
</tr>
<tr>
<td>Biological Properties</td>
<td>4</td>
</tr>
<tr>
<td>The Chemotherapeutic Application and Proposed Mechanisms of Action of Retinoids</td>
<td>10</td>
</tr>
<tr>
<td>Toxicology</td>
<td>14</td>
</tr>
<tr>
<td>Analytical Methods</td>
<td>17</td>
</tr>
<tr>
<td>STATEMENT OF PROBLEM</td>
<td>20</td>
</tr>
<tr>
<td>METHODS AND MATERIALS</td>
<td>21</td>
</tr>
<tr>
<td>Materials</td>
<td>21</td>
</tr>
<tr>
<td>Animals</td>
<td>21</td>
</tr>
<tr>
<td>Methods</td>
<td>21</td>
</tr>
<tr>
<td>Extraction of Retinoids from Plasma and Urine</td>
<td>21</td>
</tr>
<tr>
<td>Calculations</td>
<td>22</td>
</tr>
<tr>
<td>High Pressure Liquid Chromatography (HPLC)</td>
<td>23</td>
</tr>
<tr>
<td>Recovery Studies</td>
<td>24</td>
</tr>
<tr>
<td>Stability Studies</td>
<td>24</td>
</tr>
<tr>
<td>Sucrose Density Gradient</td>
<td>25</td>
</tr>
<tr>
<td>Human Studies</td>
<td>25</td>
</tr>
<tr>
<td>Mouse Studies</td>
<td>26</td>
</tr>
<tr>
<td>RESULTS</td>
<td>27</td>
</tr>
<tr>
<td>High Pressure Liquid Chromatography and Recovery of Retinoids</td>
<td>27</td>
</tr>
<tr>
<td>Stability Studies</td>
<td>33</td>
</tr>
<tr>
<td>Retinyl Palmitate</td>
<td>33</td>
</tr>
<tr>
<td>Retinol</td>
<td>33</td>
</tr>
<tr>
<td>Trans-Retinoic Acid</td>
<td>40</td>
</tr>
<tr>
<td>Cis-Retinoic Acid</td>
<td>43</td>
</tr>
<tr>
<td>Sucrose Density Gradient</td>
<td>46</td>
</tr>
<tr>
<td>Human Pharmacokinetics</td>
<td>46</td>
</tr>
<tr>
<td>Mouse Pharmacokinetics</td>
<td>53</td>
</tr>
<tr>
<td>Mouse Survival</td>
<td>57</td>
</tr>
<tr>
<td>TABLE OF CONTENTS—Continued</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>59</td>
</tr>
<tr>
<td>Method</td>
<td>59</td>
</tr>
<tr>
<td>Stability Studies</td>
<td>60</td>
</tr>
<tr>
<td>Sucrose Density Gradient</td>
<td>62</td>
</tr>
<tr>
<td>Human Pharmacokinetics</td>
<td>63</td>
</tr>
<tr>
<td>Mouse Pharmacokinetics</td>
<td>63</td>
</tr>
<tr>
<td>Mouse Survival Study</td>
<td>64</td>
</tr>
<tr>
<td><strong>CONCLUSION</strong></td>
<td>65</td>
</tr>
<tr>
<td><strong>SELECTED BIBLIOGRAPHY</strong></td>
<td>67</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Retinoid Structure</td>
</tr>
<tr>
<td>2.</td>
<td>HPLC Chromatogram of a Standard Solution of Retinoids in Ethyl Acetate</td>
</tr>
<tr>
<td>3.</td>
<td>HPLC Chromatogram of a Standard Solution of Retinoids Extracted from Plasma</td>
</tr>
<tr>
<td>4.</td>
<td>HPLC Chromatogram from a Patient Sixty Minutes after Oral Administration of 100,000 U/m² of Retinyl Palmitate</td>
</tr>
<tr>
<td>5.</td>
<td>Recovery of Retinyl Palmitate from Ethyl Acetate at 25°C</td>
</tr>
<tr>
<td>6.</td>
<td>Recovery of Retinyl Palmitate from Plasma at 25°C</td>
</tr>
<tr>
<td>7.</td>
<td>Recovery of Retinyl Palmitate from Plasma at 37°C</td>
</tr>
<tr>
<td>8.</td>
<td>Recovery of Retinol from Ethyl Acetate at 25°C</td>
</tr>
<tr>
<td>9.</td>
<td>Recovery of Retinol from Plasma at 25°C</td>
</tr>
<tr>
<td>10.</td>
<td>Recovery of Retinol from Plasma at 37°C</td>
</tr>
<tr>
<td>11.</td>
<td>Recovery of Trans-Retinoic Acid from Plasma and Ethyl Acetate at 25°C</td>
</tr>
<tr>
<td>12.</td>
<td>Recovery of Trans-Retinoic Acid Incubated at 37°C from Plasma</td>
</tr>
<tr>
<td>13.</td>
<td>Recovery of Cis-Retinoic Acid Incubated at 25°C from Ethyl Acetate and Plasma</td>
</tr>
<tr>
<td>14.</td>
<td>Recovery of Cis-Retinoic Acid Incubated at 37°C from Plasma</td>
</tr>
<tr>
<td>15.</td>
<td>Plasma Levels of Retinyl Palmitate and Retinol after Intravenous Administration of Retinyl Palmitate</td>
</tr>
<tr>
<td>16.</td>
<td>Plasma Levels of Retinyl Palmitate and Retinol after Oral Administration of Retinyl Palmitate</td>
</tr>
<tr>
<td>17.</td>
<td>Plasma Levels of Retinyl Palmitate and Retinol after Oral Administration of Retinyl Palmitate</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS—Continued

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.</td>
<td>Plasma Levels of Retinyl Palmitate in Mice after Oral and Intravenous Administration of Retinyl Palmitate</td>
<td>55</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Percent Recovery and Precision of Extraction of Retinyl Palmitate from Human Plasma</td>
<td>31</td>
</tr>
<tr>
<td>2. Percent Recovery and Precision of Extraction of Retinol from Human Plasma</td>
<td>32</td>
</tr>
<tr>
<td>3. Percent Recovery of Retinyl Palmitate following Sucrose Density Gradient Centrifugation</td>
<td>47</td>
</tr>
<tr>
<td>4. Pharmacokinetic Parameters after Intravenous Administration of Retinyl Palmitate to Cancer Patients</td>
<td>50</td>
</tr>
<tr>
<td>5. Pharmacokinetic Parameters after Daily Oral Administration of 100,000 U/m² of Retinyl Palmitate to a Cancer Patient</td>
<td>54</td>
</tr>
<tr>
<td>6. Pharmacokinetic Parameters after Oral and Intravenous Administration of Retinyl Palmitate in DBA/2 Mice</td>
<td>56</td>
</tr>
<tr>
<td>7. Mouse Survival after Oral and Intravenous Administration of 450,000 U/Kg of Retinyl Palmitate</td>
<td>58</td>
</tr>
</tbody>
</table>
ABSTRACT

A sensitive and specific high pressure liquid chromatographic method for the analysis of retinyl palmitate and other retinoids is described. After precipitation of plasma proteins with perchloric acid, the retinoids are extracted into ethyl acetate which is then analyzed for drug content by reverse-phase high pressure liquid chromatography using a µBondapak C₁₈ column. Recoveries of greater than 90% are obtained for retinyl palmitate and retinol. A 6 hour incubation in plasma with fluorescent light exposure resulted in 62% and 47% reductions in retinyl palmitate and retinol, respectively. Intravenous administration of retinyl palmitate to mice resulted in decreased survival and increased plasma drug concentrations as compared to an equivalent oral dose. In one cancer patient whose retinyl palmitate plasma disappearance kinetics were studied at 3 month intervals after daily oral dosing, the Vitamin A peak plasma levels and CXTs increased progressively while its plasma terminal elimination half life remained stable. In contrast high dose intravenous retinyl palmitate resulted in a longer plasma half life suggesting that by avoiding portal absorption, hepatic storage was reduced. The intravenous route of administration resulted in prolonged peripheral tissue levels of unbound Vitamin A which could prove more toxic than therapeutic.
INTRODUCTION

Vitamin A is a generic term used for compounds other than carotenes that qualitatively exhibit the biological activity of retinol (Goodman 1979). In recent years the term retinoid has been accepted as a general term that includes both the natural forms of vitamin A and the synthetic analogs with or without the biological activity of retinol. Vitamin A is necessary for the growth and health of higher animals. It is essential for vision, reproduction, and the maintenance of epithelial differentiation and mucous secretion. The exact nature of vitamin A in these functions has not been defined at the molecular level, except for its role in vision (Smith and Goodman 1979).

The early observations of Wolback and Howe in 1925 (Wolback and Howe 1925) on the changes in vitamin A deficient rats clearly demonstrated that vitamin A was involved in cellular differentiation and proper maintenance of epithelium (Chytil and Ong 1979). During vitamin A deficiency the columnar epithelium of many tissues are replaced with a squamous keratinizing epithelium (Wolback and Howe 1925). Supplementation of the diet with retinol reverses this metaplasia (Wolback and Howe 1933).

A negative correlation between vitamin A intake and the incidence of malignancy in epithelial tissues, first noted in the 1920s, has been substantiated by numerous studies (Lotan 1978). These results suggested that vitamin A and its analogs inhibit carcinogenesis and that they might be employed clinically to prevent the development of neoplasms.
from premalignant lesions (Sporn, Dunlop, Newton, and Smith 1976). Vitamin A in many forms (i.e. retinol, retinoic acid, and retinyl palmitate) has been successfully used in the treatment of premalignant lesions in animal systems (Bollag 1975).

**Chemistry**

![Figure 1. Retinoid Structure](image)

The retinoid molecule (Fig. 1) can be divided into three major parts; a cyclic end group, a dimethyl substituted all-trans tetrane side chain, and a polar end group. Each region of the retinoid can be chemically modified in a number of ways resulting in an almost unlimited number of compounds. These synthetic analogs may share with retinol or retinoic acid any number of biological activities. Some analogs are both less toxic and more potent than naturally occurring retinoids, while others are inactive, indicating specific structural requirements for biological activity (Loton 1978). The future of retinoids in cancer prevention will depend on the development of these new synthetic derivatives. They should be more potent, less toxic and have an improved
therapeutic index over the natural and synthetic retinoids now available (Sporn and Newton 1979).

The parent compound trans-retinol \([3,7\text{-dimethyl}-9-(2,6,6\text{-trimethyl-1-cyclohexen-1-yl})-2,4,6,8\text{-nontetraen-1-ol}]\), an unsaturated alcohol, is found in this form only in animal tissues (Modell 1976). The main precursors of vitamin A (retinol), \(\alpha\)- and \(\beta\)-carotenes, are found only in plants. There are two forms of vitamin A; 1) vitamin A1 (retinol) is found in large quantities in the liver of saltwater fish, and Vitamin A2 (dihydroretinol) is found in the liver of freshwater fish. Synthetic retinol can be synthesized from \(\beta\)-ionone and a propargyl halide or by conversion from retinal. Pure retinol is a bright orange, viscous oil with a biological activity or potency of 33 million USP units per gram. It forms yellow prisms from propylene oxide or petroleum ether and is soluble in ethanol, alcohol, chloroform, ether, fats and oil, but is insoluble in water and glycerol. Retinol has a melting point of \(62-64^\circ\)C and a \(\text{UV}_{\text{max}}\) of 324-325 nm. Ultraviolet light inactivates retinol and in solution it exhibits a characteristic green fluorescence. The free alcohol is sensitive to air-oxidation and heat (Kutsky 1973, Windholz 1976), but in oil solutions it is quite stable (Windholz 1976). Retinol, like other retinoids can exist as cis- and trans-isomers which are interchangeable in the body to a form utilizable by the tissues (Grollman 1970).

Retinyl palmitate is an ester of retinol. In amorphous or crystalline form it has a melting point of \(28-29^\circ\)C and a \(\text{UV}_{\text{max}}\) of 325-328 nm. Retinyl esters are more stable to oxidation than the free
alcohol (Windholz 1976). Retinol is stored in the liver as retinyl esters, primarily retinyl palmitate. Retinyl palmitate is manufactured by transesterification of retinyl acetate with methyl palmitate (Morton 1970).

Retinal is an aldehyde formed by oxidation of retinol. It consists of orange colored crystals formed from petroleum ether, has a melting point of 61-64°C and a $\text{UV}_{\text{max}}$ of 373 nm. Retinal is soluble in ethanol, chloroform, cyclohexane, petroleum ether and oils, but is practically insoluble in water. The bioavailability of retinal is essentially the same as that of retinol (Windholz 1976).

Retinoic acid is formed by oxidation of retinal. All-trans-retinoic acid forms crystals from ethanol with a melting point of 180-182°C and a $\text{UV}_{\text{max}}$ (Methanol) of 343 nm (Windholz 1976).

**Biological Properties**

Vitamin A (retinol) has a number of important functions in the body. It is essential for vision, to the maintenance of differentiated epithelial tissue, and to mucous secretion. Vitamin A is also required for normal growth, especially of bone, reproduction, and embryonic development. It has a stabilizing effect on various membranes, acts to regulate membrane permeability and may be involved in micelle formation (Wasserman and Corradinora 1971, Goodman and Gillman 1980). Both retinol and retinal are capable of fulfilling these functions. Retinoic acid, on the other hand, has selective vitamin A activity. It is capable of supporting normal differentiation of epithelial tissue, but it cannot replace retinol or retinal as a visual pigment precursor (Dowing and
and Wald 1960), nor is it able to support reproduction (Thompson, McC Howell and Pitt 1964).

The function and structural integrity of epithelial cells throughout the body are dependent on an adequate supply of vitamin A. It plays an important role in the induction and control of epithelial tissue differentiation in mucus-secreting or keratinizing tissue. Retinol is required for the formation of a specific fucose containing glycopeptide and is condensed with mannose to form a glycolipid intermediate (Deluca, Schmaker and Wolf 1970). In the presence of retinol, basal epithelial cells are stimulated to produce mucus. Excess retinol leads to the production of a thick layer of mucin, the inhibition of keratinization and the display of goblet cells. In the absence of retinol, atrophy of the epithelium occurs, followed by the proliferation of basal cells at the expense of mucus cells. The new cells continue to grow and replace the original epithelium with a stratified, keratinizing epithelium (Goodman and Gillman 1975, 1980).

Vitamin A is readily absorbed from the normal gastrointestinal tract. If the amount is not much greater than the requirement, absorption is complete. If a large excess is ingested, some of the vitamin may escape into the feces (Goodman and Gillman 1975, 1980).

Preformed vitamin A is obtained in the diet primarily as long-chain fatty acid esters of retinol (Goodman 1979). These esters are hydrolyzed in the lumen of the intestine by pancreatic enzymes and within the brush border of the intestinal cell (Goodman and Gillman 1975, 1980). The resulting retinol is then absorbed into the mucosal cell.
Retinol in the mucosal cell is esterified with long-chain, primarily saturated fatty acids and incorporated into chylomicrons (Huauag and Goodman 1968). The chylomicrons are absorbed via the lymphatic system and enter the systemic circulation where lymph vessels join blood vessels (Goodman 1979). The plasma concentration of esterified retinol reaches a peak at about four hours after oral administration. The normal plasma concentration of retinol is approximately 300-700 ng/ml and can vary with age, geographical location, seasonal food intake, and socioeconomical factors (Goodman and Gillman 1975, 1980).

In the vascular compartment, most of the chylomicron triglyceride is removed by extrahepatic tissues. The chylomicron remnant which is formed is smaller, cholesterol-rich and contains almost all of the chylomicron retinyl esters. The chylomicron remnant is removed and stored by the liver. Vitamin A is associated with the hepatic cell and is found mainly in the golgi fraction and the endoplasmic reticulum (Goodman and Gillman 1980). There are several pools of vitamin A in the liver. One is filled by newly absorbed vitamin A and supplies other tissues and another serves as storage (Goodman and Gillman 1980, Goodman, Huauag and Shiratori 1965). In the liver, hydrolysis and reesterification occur, and the resulting retinyl esters, mainly retinyl palmitate, are stored within parenchymal hepatocytes (Goodman 1979, Goodman and Gillman 1980).

The population median concentration of retinol is approximately 100 μg/g of liver. Other tissues, such as kidney, lung, adrenal and intraperitoneal fat contain about 1 μg/g of retinol. There is also
selective localization of the vitamin in the retina of the eye. Administration of low doses of vitamin E markedly increase the tissue storage of vitamin A. Until hepatic saturation takes place, administration of vitamin A leads to the accumulation in the liver rather than an increase in blood concentration (Goodman and Gillman 1975, 1980).

In the normal animal retinoic acid represents only a small portion of the vitamin A in the body. Retinoic acid is absorbed through the portal system (Fidge, Shiratori, Ganainly and Goodman 1968) and does not accumulate in the liver or other tissues in appreciable amounts (Goodman 1979). Retinoic acid is rapidly metabolized and excreted in urine and bile. It is partially reabsorbed from the intestine and transported back to the liver. Unlike retinol, retinoic acid is transported in the plasma like other fatty acids, bound to serum albumin (Smith, Milch, Muto and Goodman 1973, Goodman and Gillman 1980).

Retinol is mobilized from the liver stores and delivered to peripheral tissues by a highly regulated transport system. This system involves two plasma proteins, retinol-binding protein (RBP) and pre-albumin. In the plasma, retinol circulates bound to retinol-binding protein and retinol-binding protein in turn forms a complex with pre-albumin (Smith and Goodman 1979).

Human retinol-binding protein is a single polypeptide chain with a molecular weight of approximately 21,000. It migrates as an \(\alpha\)-glogulin on electrophoresis and has a single binding site for retinol. In plasma, most of retinol-binding protein normally circulates bound to retinol. The usual blood level of retinol-binding protein is about 40-50 \(\mu\)g/ml
(Smith and Goodman 1979). Retinol-binding protein interacts strongly with prealbumin and they normally circulate in a one to one complex (Goodman 1979). Retinol-binding protein serves to solubilize the water insoluble retinol molecules and protects retinol from oxidative damage while it is being transported in the plasma (Goodman 1979). The retinol-binding protein:prealbumin complex prevents retinol-binding protein and retinol from glomerular filtration and limits the quantity of vitamin A that reaches a particular tissue (Goodman and Gillman 1975).

Evidence suggests that there are specific receptors for serum retinol-binding protein present on the surface of vitamin A requiring target cells (Heller 1975, Rask and Peterson 1976). It is hypothesized that retinol-binding protein delivers retinol to target tissues. The retinol then enters the cell (Ross and Goodman 1979), where it becomes associated with cellular retinol-binding protein (CRBP). All organs except the heart and skeletal muscle appear to contain this receptor. In the fetal heart the distribution of CRBP reflects the need of the organ for retinol (Goodman and Gillman 1980). CRBP has a high affinity for retinol, but not for retinal or retinoic acid and transports retinol to specific sites within the cell. It may also be directly involved in subsequent metabolism and or other functions of retinol within the cell (Goodman 1979). Another protein, cellular retinoic acid-binding protein (CRABP) has a high affinity for retinoic acid, but not for retinol or retinal (Chytil and Ong 1979). In the adult rat CRABP is localized in brain, skin, eye, testis, uterus and ovary, but is not found in liver (Goodman and Gillman 1980).
Alcohol dehydrogenase is capable of reversibly converting retinol to retinal in many tissues, especially in the retina (Arens and Van Dorp 1946, Wald and Hubbard 1960), but at the present time retinal is not considered to be a metabolically active form of vitamin A in functions other than vision (Deluca 1979). In addition to this reversible reaction, retinal is reduced to retinol by a NADPH catalyzed reaction in the intestine (Goodman, Huaug and Shiratori 1966). Retinol can be further oxidized irreversibly to retinoic acid (Lotan 1978). Retinol is conjugated to form a β-glucuronide by the action of glucuronyl transferase. This glucuronide undergoes enterohepatic circulation and is oxidized to retinal and retinoic acid (Goodman and Gillman 1980). Several other water-soluble metabolites of retinol are also excreted in the urine and bile (Dunagin, Zachman and Olson 1966, Roberts and Deluca 1967, Goodman and Gillman 1980). Retinoic acid can be decarboxylated, further degraded or rapidly secreted into the bile as the glucuronide. These glucuronides constitute the major metabolites (Goodman and Gillman 1975, Goodman 1979). Recently other metabolites of retinoic acid have been found. Evidence suggests that liver microsomes are involved in the hydroxylation of retinoic acid at the C-4 position, to give a 4-hydroxy-retinoic acid. This reaction requires oxygen, NADPH and is sensitive to carbon monoxide. Further oxidation of the 4-hydroxy-retinoic acid to 4-keto-retinoic acid requires NAD, is independent of the vitamin A status of the animal and is insensitive to carbon monoxide. The 4-keto-retinoic acid is further metabolized to more polar metabolites that require NADPH and are sensitive to carbon monoxide (Roberts and Frolick
1979). These further metabolites could result from additional hydrox- 
ylation at C-9, 14 or 16. Another metabolite of retinoic acid, 5,6- 
epox-retinoic acid, has been recently isolated from the small 

O-acyl esters form readily and reversibly between retinol and 
long-chain fatty acids and to a much lesser degree with retinoic acid. 
Isomerases present in many tissues can form the 11-cis- or 13-cis-
analogs of retinol and retinoic acid (Lotan 1978).

Overall the metabolism of retinol and retinoic acid is quite 
similar, although the metabolism of retinol is considerably slower than 
that of retinoic acid. The metabolism of retinoic acid is complete 
within forty-eight hours in vitamin A deficient rats, whereas after 
seven days only one half of the administered dose of retinol is 
excreted (Deluca 1979).

The Chemotherapeutic Application and Proposed 
Mechanisms of Action of Retinoids

The theory that a lower level of vitamin A in tissues results in 
higher tumor incidence, has been supported by the experiments of 
Nettesheim and Williams (1976). Groups of rats were maintained on a 
diet low in vitamin A. Following intratracheal injection of the 
carcinogen, 3-methylcholanthrene, the vitamin A-deficient rats developed 
pre-cancerous lung nodules at a rate of four times greater than in the 
control rats. A recent study in humans has also shown low levels of 
serum retinol to be associated with an increased risk of cancer. This 
association was shown to be independent of age, smoking habits, and 
serum cholesterol (Wald, Idle, Brehm and Bailey 1980).
Numerous studies have shown natural and synthetic analogs of vitamin A to be effective in the prevention and therapy of benign and malignant tumors (Sporn, Dunlop, Newton and Smith 1976). Retinoids are capable of retarding the appearance, decreasing the incidence, or inhibiting the \textit{in vivo} growth of the chemically induced tumors of the skin (Davies 1967, Bollag 1972), intestine (Chu and Malmgren 1965), lungs (Saffioni, Montesano and Sellakumar 1967, Nettesheim, Cone and Snyder 1976), mammary glands (Moon, Grubbs and Sporn 1976, Grubbs, Moon and Sporn 1977), and urinary bladder (Sporn, Squire and Brown 1976) as well as virally induced tumors (McMichael 1965, Seefter, Rettura and Padauver 1976). The growth of transplantable chondrosarcomas in rats fed retinoic acid (Heilman and Swarm 1975, Shapiro, Bishop and Poon 1976, Trown, Buick and Hansen 1976) and of mammary adenocarcinomas in mice fed retinyl palmitate were decreased (Rettura, Schitteck and Hardy 1975). The development of tumors by S91 melanoma cells injected into mice pretreated by oral or intraperitoneal administration of retinoic acid were also decreased (Bollag 1971).

There are many theories as to how retinoids exert their chemotherapeutic effect, but it is thought that they exert a physiological rather than a cytotoxic action in the arrest or reversal of the process of carcinogenesis (Sporn and Newton 1979). There is evidence that vitamin A, when administered systemically in animals has a stimulatory effect on cellular and humoral immunity (Tannock, Suit and Marshall 1972). But the most important new conceptual advance with respect to retinoids has been the discovery that retinoids can act
directly on cells in culture to suppress the process of malignant transformation induced by chemicals or radiation (Merriman and Bertran 1979). Merriman and Bertran (1979) have shown several retinoids to be effective in suppressing the transformation of a mouse fibroblastic cell line that had been exposed to the polycyclic hydrocarbon, 3-methylcholanthrene for a period of twenty-four hours. Treatment with retinoids did not begin until at least seven days after carcinogen exposure, and was partially effective even when delayed for up to twenty-one days after carcinogen exposure. The mechanism of action of retinoids in blocking transformation in these experiments is unknown, but recent studies show that retinoids are capable of blocking phenotypic cell transformation produced by sarcoma growth factor (SGF) (Todaro, Delarco and Sporn 1978).

Another area of retinoid research has related the ability of retinoids to antagonize the promoting effects of phorbol esters on mouse skin. Recent evidence shows that active retinoids are potent inhibitors of the induction of ornithine decarboxylase (ODC), which undergoes a 250-fold increase in mouse skin treated with active phorbol esters (Verma and Boutwell 1977, Verma, Rice, Shapas and Boutwell 1978). Ornithine decarboxylase is the rate-limiting enzyme in polyamine biosynthesis, and polyamine products play a role in tissue proliferation and malignant transformation (Verma and Boutwell 1977).

Verma and Boutwell applied a promoter, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) to the skin of mice, assayed ornithine decarboxylase activity and found a maximum activity at four hours after application. When retinoic acid was applied in a single dose to the skin of mice one hour prior to the promoter, there was an inhibitory
effect on the induction of ornithine decarboxylase. The response was
dose-dependent and became decreasingly effective when retinoids were
applied longer than one hour prior to administration of the TPA.
Retinoids were effective even when given by mouth one hour prior to TPA
application (Verma and Boutwell 1977, Verma, Shapas, Rice and Boutwell
1979). It appears that retinoids exert an anti-tumor activity during
the promotion and not the induction phase of tumor induction, most
likely as a result of inhibition of ornithine decarboxylase induction

It had been reported that retinoids were ineffective in
inhibiting the growth of a variety of transplantable tumors commonly used
in chemotherapeutic screens (Bollag 1971). Thus, it was assumed that
retinoids had no effect on fully transformed malignant cells until the
finding that a series of retinoids could inhibit the growth of trans­
plantable rat chondrosarcoma (Trown, Buck and Hansen 1976). Since then
it has been shown that in monolayer culture retinoids can inhibit the
proliferation of several transformed cell lines from mouse, rat and
human tumors (Lotan and Nicholson 1977). Extensive studies with
murine melanoma demonstrate that inhibition of cellular proliferation
is accompanied by a marked differentiation response, the production of
melanin (Patt, Itaya and Hakomoni 1978, Meyskens 1980). It has also
been demonstrated that retinoids inhibit the proliferation of cultured
human melanoma cell lines (Meyskens 1980). The mechanism of action of
retinoids in inhibiting the growth of transformed cells is unclear, but
retinoic acid has been shown to restore anchorage-dependent growth to a
transformed rat fibroblast cell line and to a murine cell line (Dion,
Blalock and Gifford 1978). It also has been shown that retinoids cause increased cellular adhesiveness of transformed mouse fibroblasts in monolayer (Adamo, Deluca, Akalovsky and Bhat 1979). Thus it appears that there are several transformed cell lines that still retain their regulatory mechanisms and are responsive to retinoids (Sporn and Newton 1979).

The extensive in vivo and in vitro work with retinoids suggest that they may be effective in reversing preneoplasia in many tissues (Meyskens 1980). Because of their potent immunological stimulatory properties, their ability to induce differentiation and inhibit proliferation of many tumor types the retinoids may best be used when the tumor cell burden is low. Whether retinoids will be effective in established tumors is unknown at the present time and will require extensive clinical trials (Meyskens 1980). Initial phase II studies of 13-cis-retinoic acid in cancer patients at the University of Arizona Cancer Center have shown it to have antitumor activity against squamous cell cancers of various types (Meyskens 1981).

**Toxicology**

Intake of vitamin A in excess of requirement results in a toxic syndrome known as hypervitaminosis A. Acute poisoning in humans is characterized by nausea, vomiting and diarrhea, followed by central nervous system symptoms such as an intense headache, vertigo, irritability, drowsiness and occasionally coma. Generalized peeling of the skin begins after about twenty-four hours (Goodman and Gillman 1975, 1980, Furman 1973).
Early signs of chronic toxicity in humans include irritability, vomiting, anorexia, headache, dry pruritic skin desquamation, fatigue, myalgia, loss of body hair, nystagmus, gingivitis, mouth fissures, hepatosplenomegaly and lymph node enlargement. Intracranial pressure may be increased and neurological symptoms resembling brain tumors have been reported. The diagnosis is usually made following the appearance of tender, deep, hard swellings of the extremities and occipital region of the head. Hyperostosis in underlying bone are easily demonstrated on radiographic examination (Goodman and Gillman 1975, 1980). Plasma levels of vitamin A ranging from 300 to 2000 µg/dl are diagnostic of hyper-vitaminosis A. Treatment consists of stopping vitamin A intake (Goodman and Gillman 1980) and most signs disappear within a week.

Acute doses of more than 500,000 µg or greater than 50,000 µg daily of retinol will result in toxicity. Lesser amounts will cause toxicity in infants, which is characterized by an increased intracranial pressure, bulging fontanels and vomiting. These symptoms usually disappear within thirty-six hours after vitamin A is stopped. Toxicity of vitamin A in humans is dependent on age, dose and duration of exposure (Goodman and Gillman 1980).

In animals excess vitamin A causes an acceleration of bone and cartilage resorption and new bone formation (Hixon, Burdesshav, Denine, Steadman and Harrison 1979, Goodman and Gillman 1975, 1980). Bones continue to grow in length but not in thickness, with increased susceptibility to fracture. Vitamin A excess also causes hemorrhaging due to hypoprothrombinemia, epithelial abnormalities, changes in
cerebrospinal fluid pressure, deposition of lipid in kupffer cells, anorexia, an increase in free fatty acids and eventually to the death of the animal (Leeprute, Boonpuckanig, Bhamarapravati and Weerapradist 1973, Mallia, Smith and Goodman 1975, Singh, Singh and Venkitasubramanian 1968, Zbinden 1975). Vitamin A has also been reported to be teratogenic. Administration of vitamin A to pregnant mice during the early stages of chondrogenesis before the cartilage rudiment is established results in the inhibition of chondrogenesis in the developing embryo, causing limb malformations (Lotan 1978).

Vitamin A toxicity occurs when the level of vitamin A in the body is such that retinol begins to circulate in the plasma in a form other than that bound to retinol-binding protein. In studies of acute vitamin A toxicity in rats the serum retinol-binding protein was significantly decreased (Mallia, Smith and Goodman 1975). Most of the vitamin A circulated in the plasma is in the form of retinyl esters bound to lipoproteins of a density of less than 1.21. This nonspecific and unregulated delivery of vitamin A to tissues leads to vitamin A toxicity (Smith and Goodman 1979).

Large amounts of vitamin A activate lysosomes to release a protease, cathepsin D, that degrades the structural protein component of bone and cartilage matrix and results in a loss of mucopolysaccharide (Fell and Dingle 1963). Lipoprotein membranes exhibit increased permeability and decreased stability in the presence of excessive concentrations of vitamin A leading to mitochondrial swelling, lysosomal rupture and possible decreased cohesiveness of keratin (Logan 1972, Goodman and Gillman 1975).
Due to the potential toxicity of retinoids and the lack of knowledge about their pharmacokinetic properties it was necessary to develop a sensitive, specific and quantitative analytical procedure for their detection.

**Analytical Methods**

A phase I drug study of high dose oral and intravenous retinyl palmitate has been initiated in the section of Hematology-Oncology in patients who have become resistant to anticancer drug therapy. In order to correlate clinical retinyl palmitate toxicity with its plasma kinetics it was first necessary to develop a sensitive and specific assay for retinyl palmitate and retinol in biological fluids.

The only simple and rapid procedures available for retinoid determination have been based on either the Carr-Price antimony trichloride colormetric assay (Carr and Price 1926) or Dugan’s trifluoroacetic acid modification of the Carr-Price method (Dugan, Figerio and Siebert 1964). Neither of these methods separates the individual retinoids. A separate estimation of retinol and its esters has required lengthy chromatographic procedures (Goodman, Huaug and Shiratori 1965, Holasova and Blattina 1976, Huaug and Goodman 1968, Ito, Zile, Ahrens and Deluca 1974, Lawrence, Crain, Lotspeich and Krause 1966, Olson 1961).

Pharmacokinetic studies of retinoids have been hampered by the liability of these compounds. Retinoids are readily oxidized when exposed to air, they are sensitive to light and are thermally unstable to varying degrees (Veechi, Vesely and Oesterhelt 1973). In addition
incomplete recovery, poor resolution and artifact formation are major
difficulties in the separation of retinoids by silica gel or alumina
chromatography (Zile and Deluca 1968, Kilener-Bossaler and Deluca 1971).
Adsorption chromatography has in the past been the most widely used
procedure for the isolation of various retinoids. Recently the applica-
tion of liquid-gel partition chromatography to separate these compounds
has proven to be a major advance (Ito, Zile, Ahrens and Deluca 1974).
Synthetic mixtures of retinol, retinal and retinoic acid are well
resolved on sephadex LH-20. In addition, retinyl acetate, retinyl
palmitate, retinol and retinoic acid are well resolved on hydroxy-
alkoxypropyl sephadex. Recoveries in both cases are quantitative with
no destruction during separation (McCormic, Napoli and Deluca 1978).
The main disadvantage to liquid-gel partition chromatography is the time
required for each determination. In order to overcome this drawback,
the use of high pressure liquid chromatograph (HPLC) for rapid separa-
tion of retinoids has been investigated. Recently, HPLC on silica
columns has been used in several areas of retinoid research (Frolick,
Tavela and Sporn 1978), but the silica column strongly absorbs retinoids
and has only been used in isocratic separations of closely related
retinoid compounds. In order to develop methods which are readily
adapted to a wide variety of retinoids it was necessary to use reverse-
phase liquid chromatography. A solvent gradient system (mobile phase)
along with the reverse-phase column has proven to be rapid, quantitative,
artifact-free and highly sensitive (McCormic, Napoli and Deluca 1978).
Reverse-phase high pressure liquid chromatography is becoming the method
of choice for the analysis of natural and synthetic retinoids (Roberts, Nichols, Frolick, Newton and Sporn 1978).
Retinyl palmitate and other retinoids are known to prevent and even lead to the regression of chemically induced animal tumors and premalignant lesions in humans. However, only limited data are available concerning the pharmacokinetics and elimination of retinyl palmitate.

The objective of this project was the development of a sensitive and specific method for the analysis of retinyl palmitate and its metabolites in plasma and other biological material. The analytical method was used in order to better understand not only the pharmacokinetics and toxicity of oral and intravenously administered retinyl palmitate, but in addition the \textit{in vitro} stabilities of these compounds.
METHODS AND MATERIALS

Materials

Perchloric acid was purchased from the Mallinckrodt Chemical Company, St. Louis, Missouri. Methanol, chloroform, and ethyl acetate were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, Michigan. Trans-retinoic acid, trans-retinyl palmitate, and trans-retinol were purchased from Sigma Chemical Company, St. Louis, Missouri. Cis-retinoic acid was a gift from McNeil Laboratories, Ft. Washington, Pennsylvania. Ammonium acetate was purchased from Matheson, Coleman, and Bell Manufacturing Chemists, Norwood, Ohio. p-Bis-(O-methylstyryl) Benzene was purchased from Eastman Kodak Company, Rochester, New York.

Six to eight week old male DBA/2 mice weighing approximately twenty-five grams were purchased from the Jackson Laboratory, Bar Harbor, Maine. They were maintained on normal laboratory chow and acid water (McPherson, 1963) ad lib.

Methods

Extraction of Retinoids from Plasma and Urine

An internal standard, p-Bis(O-methylstyryl) benzene, in a ratio of approximately 1 to 3 to the retinoids, was added to 1 ml of plasma or urine in 13 x 100 mm glass test tubes. The sample was mixed well, and 1 ml of 5% perchloric acid was added. After mixing, 1 ml of ethyl
acetate:chloroform (9:1) was added. Samples were vortexed and centrifuged for 5 minutes at 3000 rpm, twice. A 0-100 microliter aliquot of the organic layer was then injected directly onto the high pressure liquid chromatograph (HPLC) (GLC-Dupont Instrument Co., Wilmington, Delaware).

Calculations

The concentration of retinoids in plasma was calculated from the following equation:

\[ \text{\(\mu g \text{ retinoid/ml} = \frac{\text{Retinoid Response in cm}}{\text{Internal Standard Response in cm} \times \frac{1}{RWR}}} \times \frac{1}{\text{\(\mu g \text{ Internal Standard added/ml} \)}\]}

where RWR is the retinoid response over the internal standard response in cm. When they are the same concentration:

\[ RWR = \frac{\text{Retinoid Response in cm}}{\text{Internal Standard Response in cm}} \]

For mouse and patient studies, the pharmacokinetic data for orally administered retinyl palmitate was calculated from:

\[ c = A(e^{-\beta(t-\tau)} - e^{-\alpha(t-\tau)}) \]

for day one of retinyl palmitate treatment and:

\[ c = \frac{Ae^{-\beta(t-\tau)}}{1-e^{-\beta T}} + \frac{\beta e^{-\alpha(t-\tau)}}{1-e^{-\alpha T}} - \frac{A + B}{1-e^{-\alpha T}} + e^{-\alpha(t-\tau)} \]

for subsequent days of retinyl palmitate treatment. Where \( c \) is the
concentration of retinyl palmitate at a specific time point after oral administration of retinyl palmitate. A and B are constants, \( t \) is time, \( \tau \) is the delay time for drug appearance, and \( \alpha \) and \( \beta \) are elimination rate constants. The pharmacokinetic data for intravenously administered retinyl palmitate was calculated from:

\[
c = Ae^{-\alpha t} + Be^{-\beta t}
\]

where \( c \) is the concentration of retinyl palmitate at a specific time point after intravenous administration of retinyl palmitate, A and B are constants, \( t \) is time, and \( \alpha \) and \( \beta \) are elimination rate constants.

**High Pressure Liquid Chromatography (HPLC)**

A Waters (Waters Associates, Milford, Massachusetts) high pressure liquid chromatograph with two 6000 pumps, a 660 solvent programmer, a dual 440 detector, and a reverse-phase column (Bio-Sil, ODS-10, Bio-Rad Laboratories, Richmond, California) were used for the analysis of retinoids. A programmed solvent system (Waters solvent programmer 660, program 8) was used to deliver the solvent mixture. The initial eluting solvent consisted of 72% methanol and 28% ammonium acetate (1% ammonium acetate in distilled water) and ended with a final concentration of 100% methanol over 8 minutes. Retinoids were detected at 340 nm by a Waters 440 detector with a flow rate of 2 ml/min.

Alternatively, an isocratic solvent system could be used depending on the retinoid to be detected. An isocratic solvent system of 98% methanol and 2% ammonium acetate (1% in distilled water) at a
flow rate of 2 ml/min could be used for retinyl palmitate analysis. Retinol could also be analyzed isocratically using 90% methanol and 10% ammonium acetate (1% in distilled water).

Recovery Studies

The precision and percent recovery of the extraction procedure was studied using the high pressure liquid chromatographic method described above. After addition of 0.5 and 5.0 µg of retinol and retinyl palmitate to 1 ml of normal plasma along with an appropriate amount of the internal standard. Samples were extracted as previously described and quantitated by calculating the ratio of retinoids to the internal standard. The ratio of retinoid to the internal standard in ethyl acetate was used as a standard.

Stability Studies

The in vitro stabilities of 13-cis-retinoic acid, all-trans-retinoic acid, all-trans-retinol, and all-trans-retinyl palmitate in plasma were studied at 37°C and 25°C. The stabilities of these retinoids in ethyl acetate at 25°C were studied as a reference. All stability studies were done in the presence or absence of fluorescent light. Samples were wrapped in foil to protect them from fluorescent light. At designated time points 1 ml samples of plasma or ethyl acetate were obtained and an appropriate amount of internal standard was added. Plasma samples were then extracted as previously described and analyzed by high pressure liquid chromatography (HPLC).
Sucrose Density Gradient

The association of retinyl palmitate with protein was studied using a sucrose density gradient technique. In ultracentrifugation tubes 1.5 ml of 5% sucrose was layered on top of 1.5 ml of 55% sucrose. For the in vitro studies 10 µg of retinyl palmitate was incubated in 1.0 ml of saline or plasma for 1 hour at 25°C and 37°C. Plasma samples obtained from mice treated in vivo with retinyl palmitate were not incubated, but placed directly onto the sucrose gradient. After incubation 100 µl of each sample was layered onto the 5% sucrose solution. Tubes were then placed into the rotor (type 15) for centrifugation at 100,000xG for 6 hours (model L5-50 ultracentrifuge, Beckman Instruments Inc., Anaheim, California). Four fractions of 0.6, 0.75, 0.75, and 1.0 ml were taken from the top of each of the sucrose density gradients and designated as fractions 1, 2, 3 and 4, respectively. Fractions were extracted as previously described and analyzed by high pressure liquid chromatography (HPLC). Fraction 4 would be the fraction to contain any retinyl palmitate bound to protein.

Human Studies

Retinyl palmitate was administered orally or intravenously to cancer patients in single daily or multiple daily doses. Plasma and urine samples were collected at various time points after administration of the retinyl palmitate. All samples were placed on ice and protected from light. Samples were then stored at -25°C until analysis. Samples (1.0 ml) were taken at each time point, and an appropriate amount of
internal standard was added prior to extraction as previously described. They were then analyzed by HPLC for retinyl palmitate and metabolites.

Mouse Studies

**Pharmacokinetic Studies.** Eleven groups of three male DBA/2 mice were given 250,000 u/kg of retinyl palmitate by oral intubation or tail vein injection in a single dose. Mice were sacrificed at various time points from zero to twenty-four hours after administration of retinyl palmitate. Mice were anesthetized with ether, and blood was then obtained by heart puncture. Blood samples from the three mice in each group were pooled for each time point. Samples were then stored at -25°C until analysis. One ml was taken for each time point, and an appropriate amount of internal standard was added prior to extraction as described previously and analyzed by HPLC for retinyl palmitate and metabolites.

**Mouse Survival.** A study was carried out to evaluate the effects of oral and intravenous administration of retinyl palmitate on the survival of DBA/2 mice. Two control groups of five mice each were given the vehicle, TWEEN 80, either by oral intubation or tail vein injection. Another group of fifteen mice received 50,000 u/kg of retinyl palmitate by oral intubation three times a day for three days for a total dose of 450,000 u/kg. A final group of fifteen mice received the same dose of retinyl palmitate by tail vein injection. Mouse survival was observed twice daily for twenty-five days.
RESULTS

High Pressure Liquid Chromatography and Recovery of Retinoids

An HPLC chromatogram of a standard solution of retinoids in ethyl acetate is shown in Figure 2. This figure clearly demonstrates the separation and baseline resolution obtained for cis-retinoic acid, trans-retinoic acid, retinol, the internal standard, and retinyl palmitate. In Figure 3, 3 μg of retinyl palmitate and 0.8 μg of the internal standard were added to 1 ml of normal plasma and extracted. This chromatogram shows the presence of endogenous retinol, the separation and baseline resolution of retinol, the internal standard, and retinyl palmitate. Figure 4 is an HPLC chromatogram from a patient 60 minutes after oral administration of 150,000 u/m² of retinyl palmitate. This chromatogram also clearly shows retinol, the internal standard, and retinyl palmitate. There was a small amount of baseline drift in this chromatogram, due primarily to the use of the programmed solvent system. There were also several unidentified peaks which may be metabolites of retinyl palmitate.

The percent recovery and precision of extraction of retinol and retinyl palmitate are shown in Tables 1 and 2. The percent recovery and precision of extraction of retinol at 0.5 and 5.0 g/ml of plasma was 93.0% and 94.0%, respectively. For retinyl palmitate the percent recovery and precision of extraction was 96.5% and 97.5%, respectively.
Figure 2. HPLC Chromatogram of a Standard Solution of Retinoids in Ethyl Acetate.

Conditions: 72-100% methanol:ammonium acetate (1%), Program 8 (Waers 660), a flow rate of 2.0 ml/min over 10 min. Peak a is cis-retinoic acid, b is trans-retinoic acid, c is retinol, d is the internal standard, and e is retinyl palmitate.
Figure 3. HPLC Chromatogram of a Standard Solution of Retinoids Extracted from Plasma.

Conditions: 72-100% methanol:ammonium acetate (1%), Program 8 (Waters 660), a flow rate of 2.0 ml/min over 8 min. The sample was extracted as previously described. Peak a is retinol, b is the internal standard, and c is retinyl palmitate.
Figure 4. HPLC Chromatogram from a Patient Sixty Minutes after Oral Administration of 100,000 U/m² of Retinyl Palmitate.

Conditions: 72-100% methanol:ammonium acetate (1%), Program 8 (Waters 660), a flow rate of 2.0 ml/min over 8 min. The sample was extracted as previously described. Peak a is retinol, b is the internal standard, and c is retinyl palmitate.
Table 1. Percent Recovery and Precision of Extraction of Retinyl Palmitate from Human Plasma.

<table>
<thead>
<tr>
<th>µg added to 1 ml of plasma</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>96.5 ± 4.6</td>
</tr>
<tr>
<td>5.0</td>
<td>97.5 ± 5.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the percent recovery and standard deviation of samples run in quadruplicate.
Table 2. Percent Recovery and Precision of Extraction of Retinol from Human Plasma.

<table>
<thead>
<tr>
<th>µg added to 1 ml of plasma</th>
<th>Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>94.0 ± 2.3</td>
</tr>
<tr>
<td>5.0</td>
<td>93.0 ± 4.3</td>
</tr>
</tbody>
</table>

<sup>b</sup> Values represent percent recovery and standard deviations of samples run in triplicate.
Stability Studies

Retinyl Palmitate

The stabilities of retinyl palmitate incubated at 25°C and 37°C in the presence and absence of fluorescent light are shown in Figures 5-7. When incubated at 25°C in either plasma or ethyl acetate, retinyl palmitate was more stable when protected from fluorescent light. After six hours at 25°C in ethyl acetate in the presence of light there was a 40% recovery of retinyl palmitate (Figure 5a) as compared to 68% recovery at six hours in the absence of light (Figure 5b). In plasma at 25°C in the presence of light (Figure 6a) 38% of retinyl palmitate was recovered at six hours as compared to 70% in the absence of light (Figure 6b). Recoveries at six hours in plasma and ethyl acetate were similar. When retinyl palmitate was incubated at 37°C for 2, 4 or 6 hours there was 52% recovery in the presence of light and 68% recovery in the absence of light (Figure 7). The recovery at one hour of incubation was 56% in the presence of light and 70% in the absence of light (Figure 7).

Retinol

The stabilities of retinol in plasma and ethyl acetate at 25°C and 37°C in the presence or absence of fluorescent light are shown in Figures 8-10. There was little difference in retinol recovery between light exposed and unexposed samples. Recovery of retinol after six hours of incubation in ethyl acetate under fluorescent light at 25°C (Figure 8a) was 50% versus 60% in the absence of light (Figure 8b). At
Figure 5. Recovery of Retinyl Palmitate from Ethyl Acetate at 25°C.

Retinyl palmitate (5 μg/ml) was dissolved in ethyl acetate and incubated at 25°C in the presence (5a) and absence (5b) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken and directly analyzed by HPLC. Time points were done in duplicate.
Figure 6. Recovery of Retinyl Palmitate from Plasma at 25°C.

Retinyl palmitate (5.0 μg/ml) was added to plasma and incubated in the presence (6a) and absence (6b) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken, extracted as previously described and analyzed by HPLC. Time points were done in duplicate.
Figure 7. Recovery of Retinyl Palmitate from Plasma at 37°C.

Retinyl palmitate (5.0 µg/ml) was added to plasma and incubated at 37°C in the presence (○-○) and absence (●-●) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken, extracted as previously described and analyzed by HPLC. Time points were done in duplicate.
Figure 8. Recovery of Retinol from Ethyl Acetate at 25°C.

Retinol (3.0 µg/ml) dissolved in ethyl acetate was incubated at 25°C in the presence (8a) and absence (8b) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken and directly analyzed by HPLC. Time points were done in duplicate.
Figure 9. Recovery of Retinol from Plasma at 25°C.

Retinol (3.0 μg/ml) was added to plasma and incubated in the presence (9a) and absence (9b) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken, extracted as previously described and analyzed by HPLC. Time points were done in duplicate.
Figure 10. Recovery of Retinol from Plasma at 37°C.

Retinol (3.0 μg/ml) was added plasma and incubated in the presence (9a) and absence (9b) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken and extracted as previously described and analyzed by HPLC. Time points were done in triplicate.
25°C in plasma in the presence of light (Figure 9a) after six hours of incubation there was 53% recovery as compared to 63% in the absence of light (Figure 9b). In contrast, at 37°C in plasma there was a 47% recovery in both the presence and absence of light (Figures 10a and 10b).

Trans-Retinoic Acid

The isomerization of trans-retinoic acid to cis-retinoic acid and the percent recovery of trans-retinoic acid at 25°C in plasma and ethyl acetate are shown in Figure 11. The isomerization and recovery of trans-retinoic acid in the presence and absence of fluorescent light at 37°C are shown in Figure 12. Incubation at 25°C in ethyl acetate in the presence of fluorescent light caused rapid isomerization of trans-retinoic acid to cis-retinoic acid. At two hours 82% of trans-retinoic acid had been isomerized to the cis form. No further isomerization occurred during four additional hours of incubation (Figure 11). In plasma at 25°C in the presence of light, isomerization of trans- to cis-retinoic acid was slower than isomerization in ethyl acetate. At two hours in plasma at 25°C, 48% of the trans-retinoic acid had been isomerized to the cis form. In both cases the combined recovery of cis- and trans-retinoic acid was almost 100% (Figure 11). When trans-retinoic acid was incubated at 37°C in plasma under fluorescent light there was slower isomerization to the cis form (Figure 12). By two hours 28% of trans-retinoic acid was isomerized and by six hours 40% was isomerized to the cis form. Total recovery of the cis and trans was 71% showing degradation. At 37°C in plasma when protected from light at two hours, 5% of trans-retinoic acid had isomerized to the cis form, and at six
Figure 11. Recovery of Trans-Retinoic Acid from Plasma and Ethyl Acetate at 25°C.

Trans-retinoic acid (5.0 µg/ml) was added to ethyl acetate (Δ-Δ) and plasma (▲-▲) and incubated at 25°C in the presence of fluorescent light. Isomerization of trans-retinoic acid to cis-retinoic acid was measured in ethyl acetate (●-●) and plasma (o-o). At appropriate time points 1.0 ml aliquots were taken and extracted as previously described for plasma samples and analyzed by HPLC. Ethyl acetate samples were analyzed directly by HPLC. Time points were done in triplicate.
Figure 12. Recovery of Trans-Retinoic Acid Incubated at 37°C from Plasma.

Trans-retinoic acid (5.0 μg/ml) was added to plasma and incubated at 37°C in the presence (Δ-Δ) and absence (▲-▲) of fluorescent light. Isomerization of trans-retinoic acid to the cis form was measured in the absence (●-●) and presence (○-○) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken and extracted as previously described and analyzed by HPLC. Time points were done in triplicate.
hours 7% of trans-retinoic acid had isomerized to the cis form (Figure 12). Recovery at six hours was 94%.

Cis-Retinoic Acid

The isomerization of cis-retinoic acid to trans-retinoic acid and the percent recovery of cis-retinoic acid at 25°C in plasma and ethyl acetate in the presence and absence of fluorescent light are shown in Figure 13. The isomerization of cis- to trans-retinoic acid and the percent recovery of cis-retinoic acid in plasma at 37°C in the presence and absence of fluorescent light are shown in Figure 14. When cis-retinoic acid was incubated in ethyl acetate at 25°C in the presence of light for six hours 34% of cis-retinoic acid was isomerized to the trans form (Figure 13). In the presence of light at 25°C in plasma 23% of cis-retinoic acid was isomerized to the trans form. In both cases, total recovery at six hours was 100%. On incubation in plasma at 37°C in the presence of light for six hours 28% of cis-retinoic acid was isomerized to the trans form (Figure 14). In the absence of light at six hours 20% of cis-retinoic acid was isomerized to the trans form. Combined recovery at six hours were 71 and 80%, respectively.

The stability studies of cis- and trans-retinoic acid have shown that retinoic acid was subject to both isomerization and degradation when exposed to light and heat. The cis form appeared to be the more stable form of retinoic acid. However, it was also subject to isomerization.
Figure 13. Recovery of Cis-Retinoic Acid Incubated at 25°C from Ethyl Acetate and Plasma.

Cis-retinoic acid (5.0 µg/ml) was added to ethyl acetate (○-○) and plasma (●-●) and incubated at 25°C in the presence of fluorescent light. Isomerization of cis-retinoic acid to the trans form was measured in ethyl acetate (▲-▲) and plasma (▲-▲). At appropriate time points 1.0 ml aliquots were taken and extracted as previously described for plasma samples and analyzed by HPLC. Time points were done in triplicate.
Figure 14. Recovery of Cis-Retinoic Acid Incubated at 37°C from Plasma.

Cis-retinoic acid (5.0 μg/ml) was added to plasma in the presence (o-o) and absence (●-●) of fluorescent light at 37°C. Isomerization of cis-retinoic acid to the trans form was measured in the presence (Δ-Δ) and absence (▲-▲) of fluorescent light. At appropriate time points 1.0 ml aliquots were taken and extracted as previously described and analyzed by HPLC. Time points were done in triplicate.
Sucrose Density Gradient

Retinol is known to have a specific protein (retinol-binding protein) in plasma. However, retinyl palmitate is not thought to circulate with retinol-binding protein, but in association with low density lipoproteins. A sucrose density gradient was run to determine the extent of in vitro and in vivo protein binding with retinyl palmitate. The results of this sucrose density gradient are shown in Table 3. The in vitro saline samples demonstrated that any retinyl palmitate bound to protein would appear in fraction 4 of the sucrose gradient. Results from the in vitro plasma samples showed no retinyl palmitate in fraction 4, suggesting no extensive in vitro protein binding. In the in vivo 4 hour intravenous mouse sample there was 4.1% of the retinyl palmitate found in fraction 4. This study suggests that retinyl palmitate is not bound to protein in vitro and only bound to a small extent in vivo.

Human Pharmacokinetics

Shown in Figure 15 are the plasma disappearance curves for retinyl palmitate and retinol after intravenous administration of 400,000 U/m² of retinyl palmitate to a cancer patient. Retinyl palmitate showed a biphasic plasma disappearance curve with a rapid distribution phase and a slower elimination phase. Retinol appears to be formed rapidly from retinyl palmitate after intravenous administration. Levels of retinol were maximum at 5 minutes then decreased until approximately 6 hours at which time they became constant at about 900 ng/ml. The baseline or zerotime retinol plasma level was also 900 ng/ml.
Table 3. Percent Recovery of Retinyl Palmitate Following Sucrose Density Gradient Centrifugation.*

<table>
<thead>
<tr>
<th>Fraction from the top</th>
<th>Volume (ml)</th>
<th>37°C Plasma</th>
<th>37°C Saline</th>
<th>25°C Plasma</th>
<th>25°C Saline</th>
<th>4 hr. IV in vivo&lt;sup&gt;a&lt;/sup&gt; mouse plasma sample</th>
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</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
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</tbody>
</table>

* Samples were incubated one hour and 100 µl was applied to the sucrose gradient (1.5 ml 5% and 1.5 ml 50%) and centrifuged 6 hours at 100,000 xg.

<sup>a</sup> Mice received 250,000 U/kg IV by tail vein injection.
Figure 15. Plasma Levels of Retinyl Palmitate and Retinol after Intravenous Administration of Retinyl Palmitate.

Plasma levels of retinyl palmitate (○-○) and retinol (●-●) after intravenous administration of 400,000 U/m² of retinyl palmitate. 1.0 ml aliquots of each time point were taken, extracted as previously described and analyzed by HPLC.
The pharmacokinetic parameters for two cancer patients after intravenous administration of retinyl palmitate are shown in Table 4. The average half-life ($t_{1/2}$) for retinyl palmitate was 421.2 min, and the average area under the retinyl palmitate plasma disappearance curve (CXT) normalized for dose was 2279.82 µg·min/kg. Plasma samples for patient B were also analyzed for retinol. In this patient the $t_{1/2}$ for retinol was 71.8 min, and the CXT was 551.3 µg·min/kg.

Figures 16 and 17 show the plasma disappearance curves for retinyl palmitate and retinol after daily oral administration of 100,000 U/m² of retinyl palmitate to a cancer patient. Figure 16 shows the plasma disappearance curve from the first day of treatment. The peak plasma level of retinyl palmitate was seen at 5 hours, and there was no evidence of retinyl palmitate in the plasma until approximately 2 1/2 hours after its administration. After the retinyl palmitate plasma level peaked, there was a rapid decline in plasma levels with no retinyl palmitate detectable at 24 hours. Retinol plasma levels remained fairly constant at all time points.

Shown in Figure 17 are retinyl palmitate and retinol plasma disappearance curves for the same patient as shown in Figure 16 ninety-one days after therapy was begun with daily oral administration of 100,000 U/m² of retinyl palmitate. The peak retinyl palmitate concentration of 12.6 µg/ml occurs at approximately 3 hours. At 24 hours the retinyl palmitate plasma concentration was 240 ng/ml as compared to the zero time plasma concentration of 188 ng/ml. The retinol plasma level at time zero was 659 ng/ml, but increased to
Table 4. Pharmacokinetic Parameters after Intravenous Administration of Retinyl Palmitate to Cancer Patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>$\alpha$(min$^{-1}$)</th>
<th>$\beta$(min$^{-1}$)</th>
<th>$t_{1/2}$(min)</th>
<th>Normalized CXT (µg·min/kg)</th>
<th>Dose (U/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0317</td>
<td>0.0034</td>
<td>203.0</td>
<td>1938.82</td>
<td>350,000</td>
</tr>
<tr>
<td>B*</td>
<td>0.0134</td>
<td>0.0011</td>
<td>639.4</td>
<td>2620.82</td>
<td>400,000</td>
</tr>
<tr>
<td>Average</td>
<td>0.0226</td>
<td>0.0023</td>
<td>421.2</td>
<td>2279.82</td>
<td></td>
</tr>
</tbody>
</table>

Retinol$^a$

| B*      | 0.3811               | 0.0097              | 71.8           | 511.3                       |             |

$^*$ Patients shown in Figure 15.

$^a$ Calculations for retinol were based on:

$$C = \text{Constant} + AE^{-\alpha t} + B^{-\beta t}$$
Figure 16. Plasma Levels of Retinyl Palmitate and Retinol after Oral Administration of Retinyl Palmitate.

Plasma levels of retinyl palmitate (●●) and retinol (○○) after the first oral (Day 1) dose of 100,000 U/m² of retinyl palmitate. 1.0 ml aliquots of each time point were taken and extracted as previously described and analyzed by HPLC.
Figure 17. Plasma Levels of Retinyl Palmitate and Retinol after oral Administration of Retinyl Palmitate.

Plasma levels of retinyl palmitate (●-●) and retinol (○-○) 91 days after treatment with 100,000 U/m² of oral retinyl palmitate daily was begun. 1.0 ml aliquots of each time point were taken and extracted as previously described and analyzed by HPLC.
1.3 µg/ml at 3 hours after retinyl palmitate administration. By 24 hours retinol plasma levels returned to 703 ng/ml.

Table 5 shows the pharmacokinetic parameters for the retinyl palmitate plasma disappearance curves illustrated in Figures 16 and 17. The average $t_{1/2}^\beta$ was 72.2 minutes. The CXT increased from 644.6 µg·min/kg on 10/25/79 (Day 1) to 1806.1 µg·min/kg on 3/13/80 (Day 91). The plasma retinol $t_{1/2}^\beta$ was 123.0 minutes and the CXT was 180.5 µg·min/kg.

**Mouse Pharmacokinetics**

Plasma levels of retinyl palmitate after intravenous and oral administration of 250,000 U/kg of retinyl palmitate to DBA/2 mice are shown in Figure 18. The plasma disappearance curve after intravenous administration of retinyl palmitate was monophasic with a peak retinyl palmitate plasma concentration of 500 µg/ml at 5 min. After oral administration there was no evidence of retinyl palmitate in plasma until approximately 30 minutes. Retinyl palmitate plasma levels peaked at about 3 hours with a concentration of 10.0 µg/ml. Shown in Table 6 are the pharmacokinetic parameters for retinyl palmitate in DBA/2 mice. Retinyl palmitate administered orally had a $t_{1/2}^\beta$ of 66.2 minutes and a CXT of 1628.6 g·min/kg, whereas after intravenous administration the $t_{1/2}^\beta$ was 83.9 minutes and the CXT was 62720 µg·min/kg (i.e. almost 40 times larger than after oral dosing).
Table 5. Pharmacokinetic Parameters after Daily Oral Administration of 100,000 U/m² of Retinyl Palmitate to a Cancer Patient.

<table>
<thead>
<tr>
<th>Date</th>
<th>( \alpha (\text{min}^{-1}) )</th>
<th>( \beta (\text{min}^{-1}) )</th>
<th>( t_{1/2} (\text{min}) )</th>
<th>CXT (( \mu g \cdot \text{min/kg} ))</th>
<th>Peak (( \mu g/ml ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/25/79</td>
<td>0.0120</td>
<td>0.0087</td>
<td>80.0</td>
<td>644.6</td>
<td>2.88</td>
</tr>
<tr>
<td>(Day 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/18/80</td>
<td>0.0129</td>
<td>0.0108</td>
<td>65.5</td>
<td>1806.1</td>
<td>12.55</td>
</tr>
<tr>
<td>(Day 91)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>0.0125</td>
<td>0.0097</td>
<td>72.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Retinol(^a)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10/25/79</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3/13/80</td>
<td>0.0064</td>
<td>0.0056</td>
<td>123.0</td>
<td>180.5</td>
</tr>
</tbody>
</table>

\(^a\) Calculations for retinol were based on:

\[ C = A(e^{-\beta t} - e^{-\alpha t}) + \text{constant} \]
Figure 18. Plasma Levels of Retinyl Palmitate in Mice after Oral and Intravenous Administration of Retinyl Palmitate.

Plasma levels of retinyl palmitate after oral (o-o) and intravenous (•-•) administration of 250,000 U/kg of retinyl palmitate to DBA/2 mice. 1.0 ml aliquots of each time point were taken and extracted as previously described and analyzed by HPLC.
Table 6. Pharmacokinetic Parameters after Oral and Intravenous Administration of Retinyl Palmitate in DBA/2 Mice.

<table>
<thead>
<tr>
<th>Route</th>
<th>α (min⁻¹)</th>
<th>( t_{1/2} ) (min)</th>
<th>CXT (µg·min/Kg) for 250,000 U/Kg</th>
<th>Peak Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>0.0105</td>
<td>66.2</td>
<td>1628</td>
<td>10.0</td>
</tr>
<tr>
<td>Intravenous</td>
<td>0.0083</td>
<td>83.9</td>
<td>62720</td>
<td>500.0</td>
</tr>
</tbody>
</table>

Mice were given 250,000 U/Kg of retinyl palmitate by oral intubation or intravenously by tail vein injection.
Mouse Survival

The survival of DBA/2 mice after oral and intravenous administration of 50,000 U/Kg of retinyl palmitate three times daily for three days are shown in Table 7. No animals died in either control group or in the group of mice treated orally with retinyl palmitate during the twenty-five days following the last dose of retinyl palmitate. However, five of the fifteen mice treated intravenously were dead by day 25. Using the Wilcoxon and Log rank tests there was a significant difference between the survival of mice given oral and intravenous retinyl palmitate (p = 0.017), showing increased toxicity for the intravenously administered drug.
Table 7. Mouse Survival after Oral and Intravenous Administration of 450,000 U/Kg of Retinyl Palmitate.

<table>
<thead>
<tr>
<th>Group</th>
<th># Mice Started</th>
<th># Mice Surviving at Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-oral +</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Control-intravenous+</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Oral</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Intravenous</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>

Mice were given 50,000 U/Kg of retinyl palmitate by oral intubation or intravenously by tail vein injection three times daily for three days.

+ Controls received the vehicle, Tween 80.
DISCUSSION

Method

The method that was developed for analysis of retinoids uses a simple one step extraction procedure from plasma followed by reverse-phase HPLC analysis. With this extraction procedure recoveries of greater than 90% were obtained for all retinoids tested, showing that there was no extensive binding of retinoids with plasma components. Also, there was no extensive degradation of retinoids during the extraction procedure. The programmed solvent system of methanol and ammonium acetate allowed for a wide variety of retinoids to be separated in a single chromatogram, but there were problems associated with this programmed solvent system. The major problems were chromatographic and created problems in peak interpretation. These were due primarily to the high methanol concentration needed to elute retinoids from the C\textsubscript{18} reverse-phase column. An isocratic solvent system may be more practical for routine analysis of retinoids from plasma samples. With the isocratic solvent system analysis time could be shortened because there is no need to reequilibrate the column after each injection. A C\textsubscript{8} reverse-phase column may also help in allowing the reduction of the methanol concentration needed to elute the retinoids from the column and would allow more flexibility in the solvent system.
Stability Studies

Retinoids have been reported by numerous investigators to be both light and temperature labile. Stability studies were carried out with and without fluorescent light at 25°C and 37°C in order to see if decomposition would be a major problem in the analysis of retinoids. Retinyl palmitate recovery was unchanged by incubation in either ethyl acetate or plasma at 25°C (Figures 5a,b, 6a,b), but recovery was decreased by fluorescent light exposure at 25°C and 37°C. Additionally, more degradation was seen when the incubation temperature was increased (Figures 5a,b, 6a,b, 7). All disappearance curves for retinyl palmitate were biphasic, suggesting two separate phases in retinyl palmitate degradation. No degradation products were seen in the chromatograms. This could be due to the loss of any number of double bonds in the retinoid structure resulting in loss of detection at 340 nm. Another possible explanation could be that the degradation product(s) could be detected by the HPLC method used.

As was the case with retinyl palmitate, there was no difference seen in the degradation of retinol incubated in ethyl acetate and plasma at 25°C (Figures 8a,b, 9a,b). There was only a slight difference in the degradation of retinol when incubated with and without light at 25°C or 37°C (Figures 8a,b, 9a,b, 10a,b). At 37°C in both light and dark there was a more rapid degradation of retinol than when incubated at 25°C (Figure 10a,b). Retinol has been reported to be extremely sensitive to oxidation by air. This may account for the apparent lack of a significant increase in degradation when retinol was exposed to light. Thus, retinol may be more sensitive to oxidation by air than to...
degradation by light. Incubation at 37°C did increase retinol degradation, but again, there was little difference in retinol incubated with light and without light at 37°C (Figure 10a,b). As with retinyl palmitate, the disappearance curves for retinol were all biphasic. Again, no degradation products were detected suggesting loss of detection at 340 nm or some other modification of the retinoid structure.

Trans-retinoic acid, when incubated at 25°C in ethyl acetate in the presence of fluorescent light isomerizes to cis-retinoic acid more rapidly than when incubated in plasma at 25°C with light (Figure 11). In both cases the majority of trans-retinoic acid was isomerized to cis-retinoic acid. When trans-retinoic acid was incubated without light at 37°C (Figure 12) there was very little isomerization to cis-retinoic acid. In the presence of light at 37°C (Figure 12) there was rapid loss of trans-retinoic acid and isomerization to the cis form. It appears that at 25°C in light there was isomerization of trans-retinoic acid to cis-retinoic acid and also some loss in recovery of retinoic acid. The loss of recovery of retinoic acid could be due to loss of detection at 340 nm or some other modification in the retinoid structure.

Cis-retinoic acid, when incubated at 25°C (Figure 13) isomerizes to trans-retinoic acid to a much lesser degree than trans-retinoic acid isomerized to the cis form demonstrating that cis-retinoic acid is the more stable isomer of retinoic acid. At 37°C (Figure 14) there was increased isomerization of cis- to trans-retinoic acid and also some loss of recovery of the retinoic acid. As with the other retinoids there were no degradation products seen for retinoic acid. This was
probably due, again, to loss of detection at 340 nm or some other modification in the structure changing its chromatographic properties.

These stability studies demonstrate the instability of retinoids to certain physical conditions. Care must be taken to limit the degradation of these compounds during analysis. Ideally, work with retinoids should be carried out in the dark or under argon light, and solutions should be kept in amberized glassware and on ice to reduce their degradation during sample collection and analysis. A recent article (McCormick, Napoli, and Deluca, 1980) has suggested that all stock solutions of retinoids be stored in degased methanol at -80°C under N₂.

Sucrose Density Gradient

A specific system of proteins is known to exist for the transport of retinol in plasma. Retinyl palmitate, however, is thought to circulate in plasma in association with lipoproteins. A sucrose density gradient was run to see if retinyl palmitate was bound to protein in vivo or in vitro. The results of these gradient studies suggest that retinyl palmitate is not bound to protein in vitro and bound only to a small degree in vivo. The system of transport proteins in plasma serves to protect tissues from the toxic effects of free vitamin A. When retinyl palmitate is administered orally it is broken down to retinol. Intravenous administration, however, places the retinyl palmitate directly into the bloodstream by-passing the physiological transport system. In other word, the intravenous route of administration is not the physiological route and could lead to greater tissue
toxicity and anticancer activity due to the direct tissue effects of unbound vitamin A. It will be important to determine which route of administration would have the greatest value in cancer chemotherapy.

**Human Pharmacokinetics**

Intravenous administration of retinyl palmitate to human cancer patients produced a biphasic plasma disappearance curve suggesting a two compartment system for the deposition of this drug. This indicates that retinyl palmitate is distributed beyond the plasma to other compartments within the body. Retinol appears to be formed rapidly from retinyl palmitate after intravenous injection and then its concentration decreases rapidly to reestablish a constant plasma level. The shape of retinyl palmitate plasma disappearance curve after oral administration of retinyl palmitate suggests a marked hepatic extraction during its first pass through the portal system.

There was only a small increase in retinol plasma levels after oral administration of retinyl palmitate. However, with repeated oral doses of retinyl palmitate there was a progressive increase in plasma retinyl palmitate and retinol probably due to hepatic saturation. Use of radiolabeled retinyl palmitate would be helpful in detecting the formation of metabolites.

**Mouse Pharmacokinetics**

In the mouse high dose retinyl palmitate administered intravenously produced a monophasic plasma disappearance curve in contrast to humans where the intravenous plasma disappearance curve was biphasic,
This was probably due to different modes of handling the retinyl palmitate between the two species. In contrast, orally administered retinyl palmitate underwent similar plasma elimination kinetics in both mice and patients. The CXT and peak concentrations were higher for intravenous than oral retinyl palmitate, but the half-lifes were approximately the same.

**Mouse Survival Study**

The survival of mice given high dose intravenous retinyl palmitate was significantly less than that of mice given the same dose orally. This decreased survival duration was probably due to the greater CXT and peak levels of intravenous retinyl palmitate which by-passes the physiological transport system. Additionally, high dose oral retinyl palmitate, absorption may not be complete and large quantities may be lost into the feces.
CONCLUSION

The method described for the analysis of retinoids involves a one-step extraction procedure from plasma or urine and direct analysis of the organic layer by high pressure liquid chromatography. The procedure can be used for the study of the pharmacokinetics of retinyl palmitate in animals and humans. The method can also be used to assess the relationship of vitamin A blood levels to toxicity. The speed, sensitivity, and specificity of the method allows for the rapid analysis of many samples.

The in vitro stability studies of retinoids show their sensitivity to both light and temperature. This lability requires that a great deal of care be taken during the collection and analysis of retinoids. Ideally, analyses should be carried out in the dark or under an argon light, but if neither of these are practical, samples and standards should at least be wrapped in aluminum foil. Samples and standards should also be kept on ice to prevent decomposition. Samples containing retinoids should always be capped, and standards should be stored in degassed methanol at -80°C to prevent decomposition.

The finding that retinyl palmitate is apparently not bound to plasma proteins helps to explain its greater toxicity following high dose intravenous administration in comparison to oral administration. When administered intravenously, retinyl palmitate by-passes transformation to retinol in the intestine, and the physiological
transportation mechanism for retinol. This system of transport
proteins serves to protect tissues from vitamin A toxicity.

The CXT (area under the plasma disappearance curve) for intra-
venous retinyl palmitate is higher than for oral administration of
retinyl palmitate. This indicates that intravenous administration
produces greater plasma levels of retinyl palmitate than oral adminis-
tration. The other pharmacokinetic parameters for the two routes are
variable and need further evaluation. Daily oral administration of
retinyl palmitate produces increasing retinyl palmitate and retinol
concentrations in the plasma. The most likely explanation for this
would be saturation of liver stores allowing retinyl palmitate and
retinol to remain in the plasma.

Since high dose intravenous retinyl palmitate was more toxic
than the equivalent oral dose, it will be important to determine in
patients if the intravenous route of administration will also cause
greater toxicity as compared to the oral route.
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