GROWTH PROMOTING ACTIVITY AND METABOLISM OF SELECT HETEROAROTINOIDS IN VITAMIN A-DEFICIENT RATS

By

MELISSA SIMMS-KELLEY

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Kansas State University

Manhattan, Kansas

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Thesis Approved:

Eldon C Nelson
Thesis Adviser

Franklin R. Leach

Robert J Matts

Andrew Mort

K D Berlin

Wayne B. Powell
Dean of the Graduate College

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LIST OF ABBREVIATIONS

9-*cis*-RA	9-*cis*-Retinoic Acid
ADH	Alcohol Dehydrogenase
AF-2	Carboxy Terminal Activation Domain
ALDH	Aldehyde Dehydrogenase
ARAT	Acyl:CoA Retinol Acyltransferase
Arg	Arginine
^{14}C	Radioisotope of Carbon
CRABP I	Cellular Retinoic Acid Binding Protein I
CRABP II	Cellular Retinoic Acid Binding Protein II
CRBP 1	Cellular Retinol Binding Protein I
CRPBII	Cellular Retinol Binding Protein II
DBD	DNA Binding Domain
DOS	Disk Operating System
DR	Direct Repeat
ED_{50}	Effective Dose at 50% Maximum Activity
FT1	Feeding Trial 1
FT2	Feeding Trial 2
GC/MS	Gas Chromatography/Mass Spectrometry
Gln	Glutamine
HAc	Acetic Acid
HN-10	HN at 10 µg/rat/day
HN-100	HN at 100 µg/rat/day
HN-200	HN fed at 200 µg/rat/day

HN2-10	HN2 fed at 10 μg/rat/day
HN2-100	HN2 fed at 100 μg/rat/day
HN2-200	HN2 fed at 200 μg/rat/day
HPLC	High Preformance Liquid Chromatography
HRE	Hormone Response Element
HS-10	HS fed at 10 μg/rat/day
HS-100	HS fed at 100 μg/rat/day
HS-200	HS fed at 200 μg/rat/day
HS2-10	HS2 fed at 10 μg/rat/day
HS2-100	HS2 fed at 100 μg/rat/day
HS2-200	HS2 fed at 200 μg/rat/day
HS-LM1	HS Liver Metabolite 1
HS-LM2	HS Liver Metabolite 2
HS2-LM1	HS2 Liver Metaboile 1
HS2-LM2	HS2 Liver Metabolite 2
HS2-LM3	HS2 Liver Metabolite 3
HS2-SM1	HS2 Serum Metabolite 1
HS2-SM2	HS2 Serum Metabolite 2
LBD	Ligand Binding Domain
LC/MS	Liquid Chromatography/Mass Spectrometry
LRAT	Lecithin:Retinol Acyltransferase
MTD	Maximum Tolerated Dose
NK	Natural Killer
N-CoR	Nuclear Co-Repressor
ODC	Ornithine Decarboxylase
O-DHA*-LM1	O-DHA* Liver Metabolite 1
O-DHA*-LM2	O-DHA* Liver Metabolite 2

O-DHA*-LM3	O-DHA* Liver Metabolite 3
O-DHA*-KM1	O-DHA* Kidney Metabolite 1
O-DHA*-KM2	O-DHA* Kidney Metabolite 2
O-DHA*-KM3	O-DHA* Kidney Metabolite 3
O-DHA*-KM4	O-DHA* Kidney Metabolite 4
O-DHA*-KM5	O-DHA* Kidney Metabolite 5
O-DHA*-KM6	O-DHA* Kidney Metabolite 6
P450	Cytochrome P450
PDA	Photodiode Array
RA	All-*trans*-Retinoic Acid
RA-10	RA fed at 10 μg/rat/day
RA-100	RA fed at 100 μg/rat/day
RA-200	RA fed at 200 μg/rat/day
RAG	Retinoyl-β-glucoronide
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RBP	Retinol Binding Protein
ROG	Retinyl-β-glucoronide
RXR	Retinoid X Receptor
RXRE	Retinoid X Response Element
SDR	Short Chain Dehydrogenase
SMRT	Silencing Mediator for Retinoid and Thyroid Receptor
SPE	Solid Phase Extraction
t-RA	All-*trans*-Retinoic Acid
TOC	Trachea Organ Culture
TPA	12-O-tertadeconylphorbol-13-acetate

TRR	Transthyretin
μCi	microCurie
UGT	UDP-glucuronosyl transferase

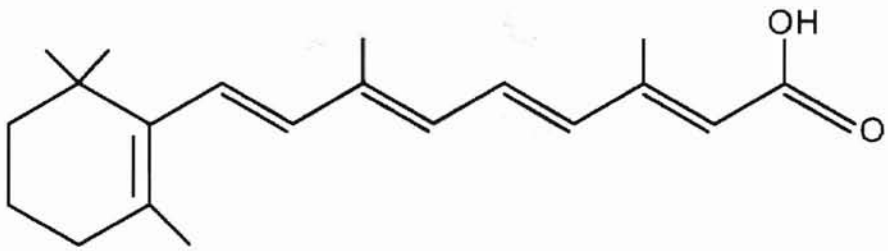
CHAPTER I

LITERATURE REVIEW

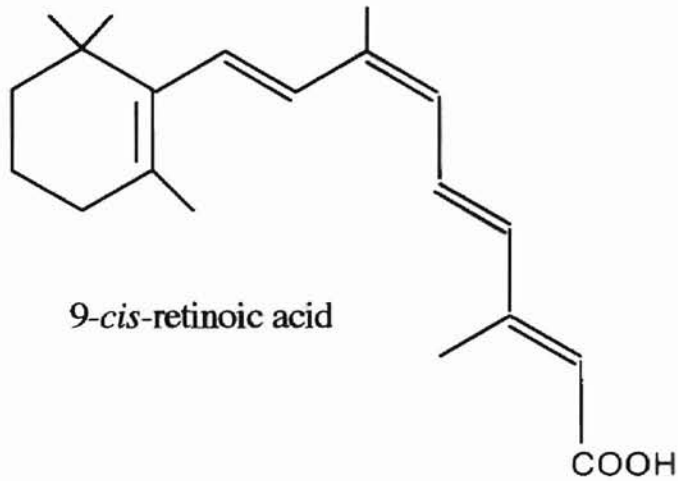
Vitamin A (retinol) and its analogs, retinoids, are essential for many critical life processes including vision, reproduction, cellular differentiation, proliferation, bone development, apoptosis and embryogenesis (Sporn 1994). All-*trans*-retinoic acid (*t*-RA), and its isomer 9-*cis*-retinoic acid (9-*cis*-RA), (Figure 1), are ligands for retinoid receptors which mediate gene expression and control cellular processes throughout an organisms lifetime.

Naturally occurring retinoids, as mediators of cell growth and development, have chemotherapeutic potential in different cancer types but are limited by toxicity (Sporn 1994). Synthetic compounds having good efficacy but lower toxicity are needed. By modifying parts of the retinoid structure, such as the cyclic end group, polyene side chain, or polar end group different therapeutic ratios have been achieved (Bollag 1983 and Lippman et al. 1987). First and second generation retinoids contain modified polar and cyclic end groups, respectively. Third generation arotinoids were created by cyclization of the polyene side chain and are less toxic and more potent than *t*-RA in standard screening tests (Bollag 1983 and Lippman et al. 1987). First, second and third generation retinoids are shown in Figure 2. Heteroarotinoids (Figure 2) are related to arotinoids and contain an aryl group and a heteroatom in the fused ring system (Waugh et al. 1985 and Spruce et al. 1987). Heteroarotinoids exhibit strong activity and lower toxicity when compared to *t*-RA in standard assays and display potential as anticancer agents (Benbrook et al. 1997). Retinoid skeleton modifications have resulted in synthetic retinoids which have an improved therapeutic index over naturally occurring retinoids (Bollag 1983).

In this chapter, various aspects of retinoid biology will be overviewed including retinoid receptors and target genes, metabolism, chemoprevention and toxicity. This

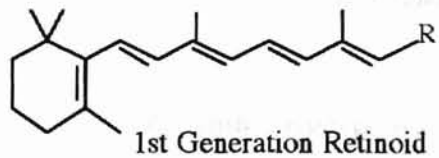


All-*trans*-retinoic acid



9-*cis*-retinoic acid

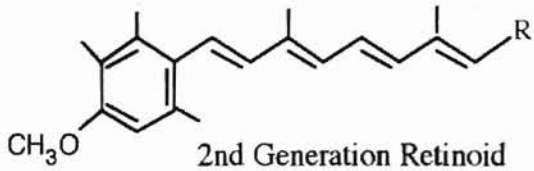
Figure 1: Structures of all-*trans*-Retinoic Acid and 9-*cis*-Retinoic Acid.



1st Generation Retinoid

R= CH_2OH (retinol)

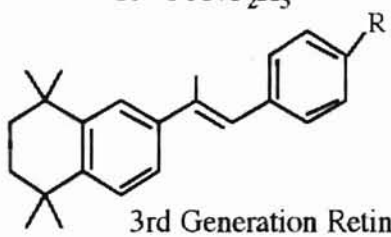
R= CO_2H (retinoic acid)



2nd Generation Retinoid

R= $CO_2C_2H_5$

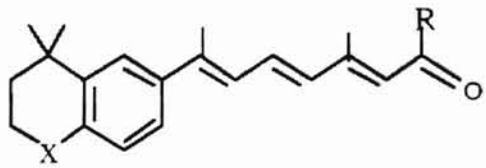
R= $CONC_2H_5$



3rd Generation Retinoid

R= $CO_2C_2H_5$

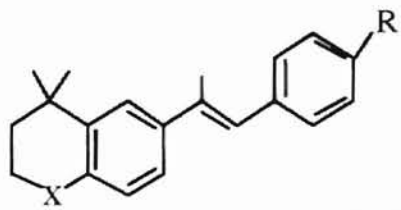
R= CO_2H



Monoaryl Heteroarotinoid

X= O, S

R= OH



Diaryl Heteroarotinoid

X= O, S

R= CO_2CH_3

R= CO_2H

Figure 2: Structures of Retinoids and Heteroarotinoids.

chapter will conclude with an overview of known biological activity of selected heteroarotinoids.

Retinoid Receptors

The understanding of retinoid signaling rapidly advanced with identification of the first retinoic acid receptor (RAR) in the late 1980s (Giguere et al. 1987 and Petkovich et al. 1987). RARα was isolated using sequence homology and identified as a member of the steroid/thyroid hormone nuclear receptor superfamily. With this discovery, vitamin A research rapidly broadened, and the link between retinoids and gene control was established. Retinoid X receptors (RXR) were first identified as "orphan" receptors in which the ligand was unidentified (Brand et al. 1988 and Mangelsdorf et al. 1990). High sequence homology with RAR and the ability to transactivate gene expression confirmed RXRs were related to RARs (Rowe 1997). The RXR ligand was later identified as the isomer of *t*-RA, namely 9-*cis*-RA (Levin et al. 1992).

RARs have three subtypes, α, β and γ, which exist in a variety of vertebrates and are mapped to human chromosomes 17q21.1, 3p24 and 12q13, respectively (Mattei et al. 1991). The RAR family is activated by *t*-RA and 9-*cis*-RA while RXRs are only activated by the later. RARα is widely distributed in both adult and embryo unlike RARβ, which is expressed in adult muscle, prostate and in a spatial and temporal pattern in embryo. RARγ is expressed in liver, skin and kidneys in the adult and spatially in embryos (Mangelsdorf 1994). RXR has three subtypes (α, β and γ) and are mapped to human chromosomes 9q 34.3, 6p21.3 and 1q22.23, respectively (Mangelsdorf 1994). RXRβ is ubiquitous in adult and embryo while RXRα is expressed in adult and embryo liver, skin and kidney. RXRγ is specifically expressed in adult muscle and brain and in the pituitary gland of embryos (Mangelsdorf 1994).

Retinoid receptors can be divided into six domains (A-F) based on homology to members of the steroid/thyroid superfamily (Chambon 1996). Domain A, located near the N-terminus, is not conserved among the RARs or RXRs, while domain B is relatively well

conserved (Chambon 1996). The specific functions of domains A and B are still unclear. The DNA binding domain (DBD), domain C, contains two zinc finger motifs (Chambon 1996). The zinc finger has two α helices. The first fits into the major groove of DNA and makes specific contact with bases in the recognition element half site (Mangelsdorf et al. 1995) while the second is an interface for heterodimerization with RXR (Lee et al. 1993). The RXR DBD has an additional helix which is required for binding of RXR homodimers to the response element (Lee et al. 1993). In a given species, the DBD is highly conserved between RAR and RXR types (Chambon 1996). Domain D serves as a hinge between the DBD and the ligand binding domain (LBD). This domain is conserved between species but interspecies homology decreases for RAR and RXR types, and studies suggest this region may have other functions (Chambon 1996). The ligand binding domain, E, is comprised of 225 amino acids and includes homo- and heterodimerization interfaces and hormone dependent transcriptional activation and repression (Mangelsdorf et al. 1995). The LBD, which has been crystallized for RARγ (Renaud et al. 1995), acts as a molecular switch which shifts the receptor to a transcriptionally active state when the ligand is bound (Mangelsdorf et al. 1995). Currently, a two step model of receptor dimerization has been suggested (Mangelsdorf et al. 1995). First, RXR forms a heterodimeric complex with its partner through the LBD, and then the DBD binds the hormone response element in the target genes promoter region. The function of the carboxy-terminus region, domain F, of RAR and RXR subtypes is still unknown (Chambon 1996).

Other retinoid signaling pathways involve RXR homodimers and other RXR heterodimeric partners including the thyroid receptor and vitamin D receptor. For a review see Sporn 1994.

Target Gene Specificity

Steroid/thyroid superfamily receptors control gene transcription through hormone response elements (HRE) located in the target genes promoter. RAR and RXR response elements (RARE and RXRE, respectively) for many genes have been identified (Sporn

1994). Retinoid response elements consists of a direct repeat (DR) of the half-site consensus sequence AGGTCA separated by a defined number of nucleotides (Sporn 1994). RXRE are DR-1 type where 1 nucleotide separates the direct repeat of the consensus sequence. RARE are DR-5 or DR-2 types elements (Sporn 1994) where 2 or 5 nucleotides separate the direct repeat. RAREs and RXREs are found in many genes directly involved in retinoid biology including mouse and human RARα, β and γ, cellular retinol-binding protein I and II and cellular retinoic acid binding-protein II (Pfhal et al. 1996 and Mangelsdorf et al. 1991 and Husman et al. 1992). Not all retinoid responsive genes contain a RARE or RXRE in their promoter region and many retinoid responsive genes are not associated with retinoid metabolism (for a review, see Sporn 1994).

Zechel et al. (1994) established RXR occupies the 5' half-site and its heterodimeric partner occupies the 3' half site on DR 2 and 5 types. The heterodimer's polarity is reversed in DR 1 types (Kurokawa 1994). The reverse polarity allows repression of RXR-RXR homodimers (Mangelsdorf et al. 1991 and Kurokawa et al. 1995). RAR is close to the TATA box which makes RAR a target for accessory factors that assist in transcription (van der Saag 1996). *In vitro* studies found two proteins, SMRT (silencing mediator for retinoid and thyroid receptor) and N-CoR (nuclear co-repressor), which interact with unliganded receptors and are released upon ligand binding (Chen et al. 1995). The amino termini of both proteins contains a repressor motif while the carboxy termini contains the receptor interaction domain (Chen et al. 1995). In the absence of ligand, the proteins remain bound to the receptor and represses transcription. Upon hormone treatment, a conformation change in the receptor occurs displacing the corepressor and exposing the carboxy terminal activation domain (AF-2) of the RAR (Mangelsdorf 1995). The complex binds a coactivator which results in ligand-dependent induction of transcription (Mangelsdorf 1995). Many proteins interact with the AF-2 domain of RAR and RXRs so that individual receptors may possess multiple pathways for activation (Mangelsdorf 1995). A heterodimer activation model is shown in Figure 3.

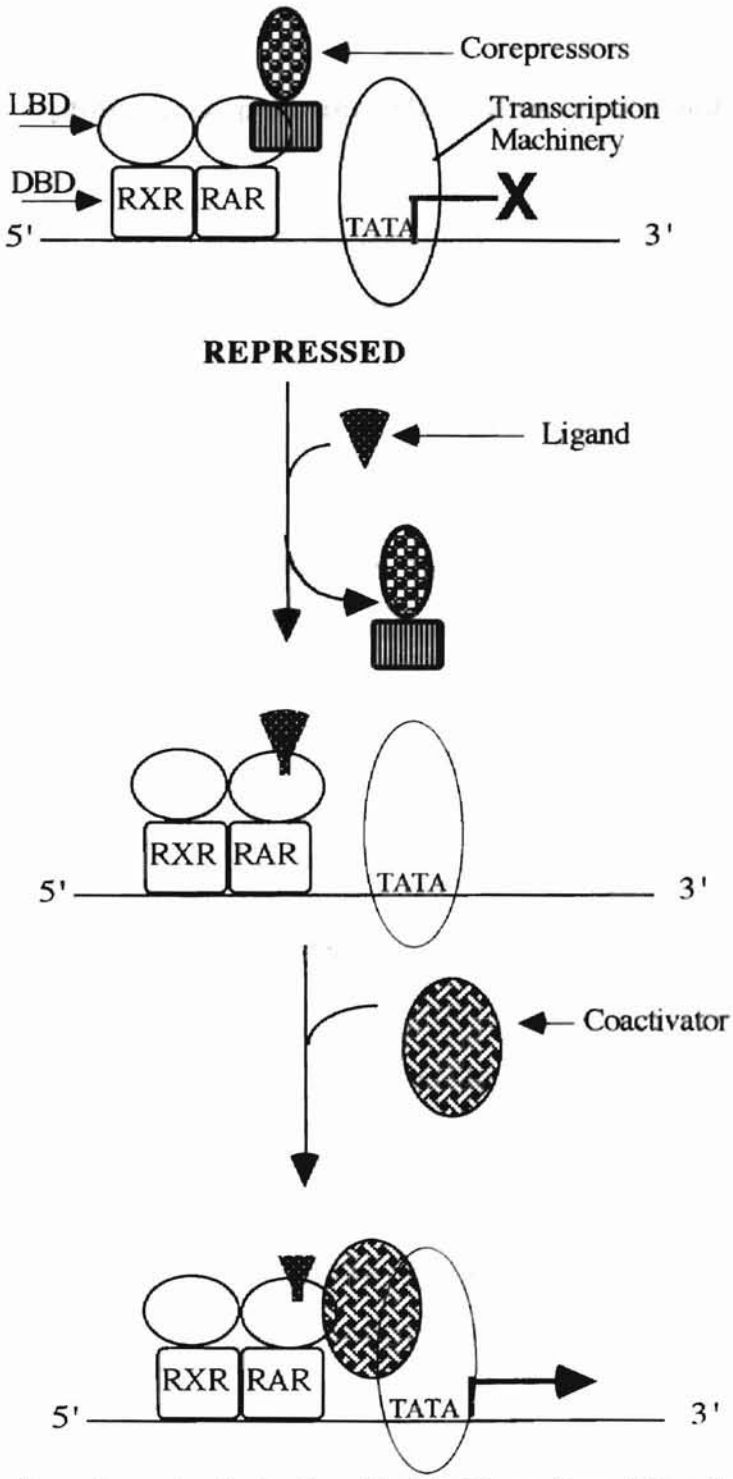


Figure 3: Heterodimer Receptor Activation Model. Figure from Mangelsdorf et al. 1995.

Clearly, the link between retinoids and gene transcription is complex. Whether by transcriptional activation or repression, retinoids play an important role in many cellular processes.

Absorption, Transport and Metabolism of Retinol

Absorption

Carotenoids from fruits and vegetables and retinyl esters from animal sources serve as dietary precursors of retinol. Approximately 600 carotenoids have been identified in nature but only 10% of them are vitamin A precursors, provitamin A (Silveira et al. 1998). Of these, β-carotene is the most biologically active precursor (Napoli 1996). Oxidation of β-carotene occurs by central cleavage to produce retinal in the intestine (Napoli 1996). Cellular retinol binding protein II (CRBP II) binds retinal and protects it from oxidation to retinoic acid. Retinal is reduced to retinol by retinal reductase (Kakkad et al. 1988). Retinyl esters from the diet are hydrolyzed by cholesterol ester hydrolase, and retinol complexes with CRBP II (Silveira et al. 1988 and Napoli 1996).

The retinol-CRBP II complex serves as a substrate for lecithin:retinol acyltransferase (LRAT) which esterifies retinol to retinyl esters (Napoli 1996). LRAT was induced by RAR-selective agonists while RXR-agonists were inactive (Shimada et al. 1997). LRAT uses the acyl group at the sn-1 position of phosphatidylcholine as a fatty acid source unlike acyl:CoA retinol acyltransferase (ARAT) which uses free acyl groups (Silveira et al. 1998). The fatty-acyl moiety is predominantly palmitate (Goodman 1965). Under physiological conditions in the small intestine, the retinol-CRBP II complex is esterified preferentially by LRAT (MacDonald et al. 1988) although at increased concentrations, retinol can be esterified by ARAT (Silveira et al. 1998). LRAT and ARAT esterify retinol although which enzyme is used depends on retinol concentration and whether it is bound by CRBP II (Blomhoff et al. 1991 and Yost et al. 1988).

Transport

Retinyl esters are incorporated into chylomicrons and transferred to the lymph system by exocytosis. After removal of triacylglycerol and apolipoprotein exchange, the retinyl esters remain with the chylomicron remnants and are bound by hepatocytes (parenchymal cells). Extrahepatic uptake of chylomicron remnants occurs primarily in bone marrow, spleen, and to a lesser extent in adipose tissue, skeletal muscle and testes (Blomhoff et al. 1990)

In hepatocytes, retinyl esters undergo hydrolysis, and retinol is bound by the retinol binding protein (RBP) in the endoplasmic reticulum (Napoli 1996). Most retinol does not remain in the hepatocytes but is transferred to liver stellate cells. This transfer mechanism is still unclear. Studies suggest : (1) the holo-RBP mediates intercellular transfer; (2) a carrier protein aids in transfer (Silveira 1998). Retinol reesterification occurs in these cells and retinyl esters are deposited in the cytoplasm as lipid droplets (Napoli 1996). Stellate cells account for 90% of total hepatic retinol which is present as retinyl esters (Napoli 1996).

Retinol mobilization from stellate cells involves hydrolysis to free retinol which is complexed to RBP before secretion into plasma (Napoli 1996). Neither retinol or RBP may depart from the cell in the absence of the other (Napoli 1996). RBP solubulizes and transports retinol through the plasma while protecting it from oxidation and/or isomerization (Napoli 1996). The retinol-RBP complex may associate with the protein transthyretin (TRR, prealbumin) while either still inside the hepatic cell or during systemic circulation (Silveira et al. 1998). TRR is a tetramer of four 55 kDa subunits (Yamamoto et al. 1997) and functions to reduce glomerular filtration of retinol-RBP complex in the kidney.

The mechanism of cellular retinol uptake is still debated. Several reports provide evidence for a specific RBP cell surface receptor in intestinal mucosal epithelium (Rask et al. 1976), human placental brush-border membranes (Sivaprasadarao et al. 1988), cultured

Sertoli cells (Shingleton et al. 1989) and retinal pigment epithelial membrane (Bavik et al. 1991). However, retinol can cross the plasma membrane by a flip-flop mechanism and this occurs with high intracellular apo-CRBP levels (Noy et al 1990 and Noy et al. 1991).

Metabolism

Inside the cell, retinol has different metabolic fates. It may be stored as retinyl esters, metabolized to retinoic acid or returned to systemic circulation. Studies identifying mechanisms regulating intracellular retinoid concentrations became crucial with the discovery of *t*-RA (Emerick et al. 1967) and retinoid receptors. The pathway for conversion of retinol to *t*-RA involves reversible oxidation of retinol to retinal then irreversible oxidation to retinoic acid (Duester 1996). Numerous enzymes involved in retinol and retinal metabolism have been identified and are divided into four distinct families: (Duester 1996).

(1). Alcohol Dehydrogenase (ADH)

Liver ADH catalyzes the oxidation of retinol to retinal in the presence of NAD^+ (Bliss 1951 and Zachman et al. 1961). ADH is a dimeric zinc-dependent enzyme with a subunit molecular weight of 40 kDa (Vallee et al. 1983).

(2). Short Chain Reductases (SDR)

SDRs, which are distinct from ADH, oxidizes retinol to retinal or reduces retinal to retinol in rat liver microsomes (Leo et al. 1987). NADPH dependent SDR is a membrane bound 25-35 kDa protein (Boerman et al. 1995 and Persson et al. 1995). SDR uses all-*trans*-retinol or all-*trans*-retinal either free or bound to CRBP, but can not oxidize free or bound 9-*cis*- or 13-*cis*-retinol (Posch et al. 1991, Boerman et al. 1995 and Duester 1996). Recently, a new member of SDR family was isolated which oxidizes 9-*cis*-retinol to the corresponding aldehyde and is suggested to be a new pathway for generating 9-*cis*-RA (Romert et al. 1998). SDR is expressed primarily in the liver with low levels found in the brain, kidney, testes and lung (Chai et al. 1995 and 1996).

(3). Aldehyde Dehydrogenase (ALDH)

Aldehyde dehydrogenase catalyzes the irreversible oxidation of retinal to retinoic acid (Lee et al. 1991). ALDH metabolizes cis and trans isomers of retinal to the corresponding retinoic acid isomers. ALDH is a cytosolic enzyme with 55 kDa molecular weight (Duester et al. 1996).

(4). Cytochrome P-450 (P-450)

Members of the cytochrome P-450 family oxidize position 4 in the β-ionone ring of retinoic acid, retinal and retinol although generally P-450 is involved in oxidative metabolism of retinoic acid to other polar metabolites (Raner et al. 1996). Figure 4 depicts the absorption, transport and metabolism of retinol.

Retinoic Acid Metabolism

Metabolism of *t*-RA occurs at four major sites: (1) polyene side chain oxidative shortening; (2) oxidation at the C-4 of the cyclohexenyl ring; (3) epoxidation at the 5,6 position of the cyclohexenyl ring; (4) ring and side chain methyl group oxidation (Sporn 1994). One important route of *t*-RA metabolism is hydroxylation at the 4 position of β-ionone ring to form 4-hydroxy-RA which is further oxidized to 4-oxo-RA. A species specific subfamily of cytochrome P450 catalyzes this reaction (Marchetti et al. 1997). 4-oxo-RA is active in the tracheal organ culture assay (TOC), is receptor specific for RARβ and is capable of binding CRABP although with an affinity less than retinoic acid (Frolik et al. 1979, White et al. 1998, Pijnappel et al. 1993 and Fiorella et al. 1993).

Isomers of *t*-RA are metabolized to oxidative products. For example, 9-*cis*-RA is metabolized to 4-hydroxy-9-*cis*-RA and 4-oxo-RA in the rat by cytochrome P450 isozymes yet to be identified (Shirley et al. 1996). Another metabolite of 9-*cis*-RA identified in rat liver is 13,14-dihydro-9-*cis*-RA although it had less activity in transcriptional activation studies than 9-*cis*-RA (Shirley et al. 1996). 13-*cis*-RA is oxidized to 4-hydroxy-13-*cis*-RA and metabolized to more polar metabolites (Marchetti et al. 1997) such as 13-*cis*-4-oxo-retinoic acid (Echoff et al. 1990). 9,13-di-*cis*-RA, a physiologically occurring retinoid, is

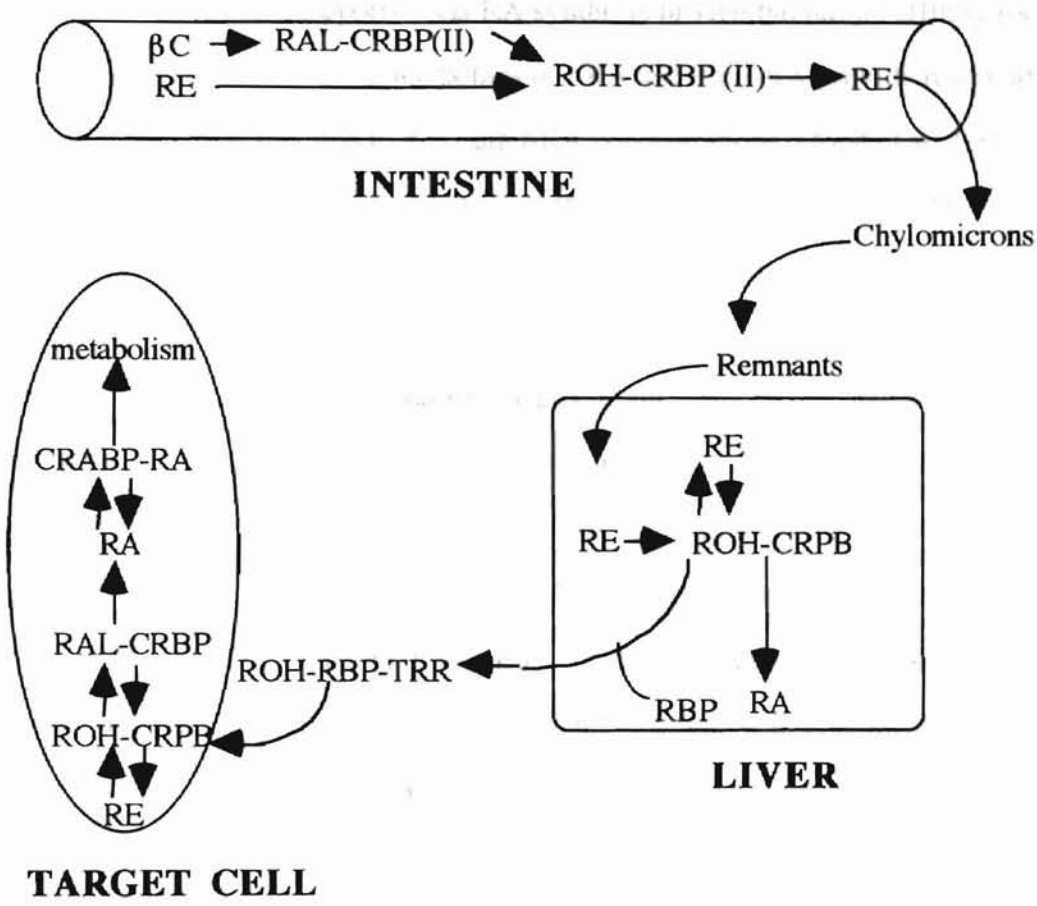


Figure 4: Absorption, Transport and Metabolism of Retinol (Napoli 1996).

produced from 9-*cis*-RA and supports 9-*cis* RA synthesis *in vivo* but has no affinity for CRABP I or II (Horst et al. 1995). Metabolites of *t*-RA and 9-*cis*-RA provide reservoirs of less active retinoids which function to maintain *t*-RA concentrations (Horst et al. 1995).

β-Glucuronides are formed by a family of intracellular UDP-glucuronosyl transferases (UGTs) (Genchi et al. 1996). Of this family, at least two act on *t*-RA (Wang et al. 1996 and Genchi et al. 1996). The glucuronide conjugates of retinol (retinyl-β-glucuronide; ROG) and retinoic acid (retinoyl-β-glucuronide; RAG) promote growth in vitamin A-deficient rats, induce differentiation of HL-60 cells and are less teratogenic than retinol or *t*-RA (Nath et al. 1967 and Gallup et al. 1987 and Zile et al. 1987). ROG and RAG have no significant affinity for CRBP, CRABP or RARs (Mehta et al. 1992 and Sani et al. 1991). RAG is found in human blood (Barua et. al 1986) and is slowly hydrolyzed to RA *in vivo* (Formelli et al. 1996). RAG uptake and metabolism is dependent on animal vitamin A status, when tissues stores are depleted, RAG is readily hydrolyzed to RA (Barua et al. 1998). The route of administration plays a significant role in teratogenicity of RAG. RAG administered subcantenously, but not orally, is teratogenic (Barua et al. 1998). Glucuronidation of xenobiotics and endogenous compounds is usually a detoxification process and allows for excretion of water soluble metabolites which are less biologically active, such is not the case for RAG. RAG regulates the rate of hydrolysis to RA and limits RA concentration to the cells (Formelli et al. 1996).

Clearly, retinoid metabolism is a complex process. Some metabolites are biologically active and have receptor binding affinities while others have no affinity for retinoid binding proteins, but are biologically active. Different metabolic forms may allow for many complex biological processes through receptor interactions or by other mechanisms not identified.

Plasma Retinoid-Binding Protein

RBP was first isolated and identified as the transport protein for vitamin A in plasma in 1968 (Kanai et al.). Since that time, RBP-retinol-TRR interactions have been

extensively characterized. Holo-RBP has been crystallized from human and rabbit serum (Ottonello et al. 1983 and Newcomer et al. 1984). RBP consists of a N-terminal coil, β-barrel core, α-helix and a C-terminal coil (Sporn 1994). The β-barrel contains eight antiparallel β-strands and encapsulates the retinol β-ionone ring while the isoprene tail lies along the barrel axis almost to the protein surface (Sporn 1994).

RBP is synthesized and secreted by the liver although there is a debate as to which liver cell types are responsible for this action. Parenchymal cells do synthesize and secrete RBP but stellate cells are still being analyzed for RBP synthesis and secretion although no clear answer has been obtained. Other tissue which synthesize and secrete RBP include the kidneys, and to a lesser extent the lungs, spleen and testes (Sporn 1994). RBP is synthesized and secreted in the visceral yolk sac and fetal liver (Sporn 1994). RBP secretion is regulated by retinol although other biochemical signals, yet to be identified, can cause cells to retain or secrete RBP (Sporn 1994). RBP regulation in certain cell types is of fundamental importance for understanding the mechanism of retinol storage and delivery.

Cellular Retinoid-Binding Proteins

Two cytoplasmic retinol-binding proteins (CRBP and CRBP II) have been identified (Ong 1984) and share amino acid similarity. These proteins are similar to RBP with a β-barrel motif consisting of 10 anti-parallel β-sheets with a helix-turn-helix between the first and second β-strand (Li et al. 1996). The retinol β-ionone ring lies within the barrel while the isoprene side chain is stretched toward the protein surface with the alcohol close to Gln-108 (Li et al. 1996). Although these proteins are structurally similar, their tissue distribution is different. Levin et al. (1987), using northern analysis, showed CRBP was expressed in liver, kidneys, testes and rat placenta and is the retinoid transporter in these tissues. CRBP II was expressed in the small intestine and is involved in the intestinal uptake of retinol (Levin et al. 1987).

Two cytoplamic retinoic acid-binding proteins have been identified and share amino acid sequence homology. Both proteins have an affinity for *t*-RA but not 9-*cis*-RA

(Donovan et al. 1995). The CRABPs protein structure is similar to CRBPs but the β-ionone ring of *t*-RA is solvent accessible and slightly shifted (Li et al. 1996). The carboxylic acid group interacts with Arg-132 (Li et al. 1996). CRABP is found in seminal vesicles, skin, testes, distal epididymis and kidney with the seminal vesicles having the highest CRABP concentration (Rajan et al. 1991). CRABP II is localized in adult skin and in eight day old mouse embryos (Li et al. 1996). CRABP I and II tissue levels are not effected by retinoid status (Kato et al. 1985 and Blaner et al. 1986 and Madani et al. 1991). The function of CRABP is to transport *t*-RA to the nucleus by passive entry through nuclear pores, while the function of CRABP II is still unknown (Takase et al. 1986 and Donovan et al. 1995).

All four binding proteins transport retinoids through cells although tissue distribution is different. There have been many advances in understanding these proteins but questions still remain on their function in retinoid metabolism and receptor interactions.

Retinoids and Chemoprevention

Retinoids act at the tumor promotion stage rather than the initiation stage in most cancer cases (Lotan 1996). This stage involves cell division of initiated cells to form a preneoplastic lesion followed by conversion to a malignant lesion (Lotan 1996). This process involves unregulated cell proliferation, differentiation and loss of apoptosis (Lotan 1996). Retinoids inhibit cell proliferation, promote differentiation and enhance apoptosis in many cell types. Retinoids suppress invasion and motility of premalignant cells and inhibit angiogensis. Many clinical trials have been conducted with different retinoids (for a review, see Lotan 1996). Although naturally occurring retinoids have been effective, they are limited in use because of toxicity. Other options for using retinoids as chemotheraupetic agents include: (1) a higher initial dose in the initiation stage followed by a lower dose for maintenance; (2) a combination of different retinoids with distinct receptor specificity; (3) synthetic retinoids which are effective and lower in toxicity (Lotan 1996). Much effort has been devoted to the last strategy. In many clinical trials, synthetic retinoids

show promise as chemotherapeutic agents (for review see Hinds et al. 1997). Retinoids have anticancer activity, but when used at chemotherapeutic doses, show teratogenicity, severe hepatotoxicity and anti-keratinizing activity (Holven et al. 1997).

Retinoid Toxicity

Retinoid toxicity was first documented when Arctic explorers became ill after consuming polar bear liver. In 1943, Rodahl et al. found when polar bear liver was fed to rats, the animals had signs of hypervitaminosis A. Since that time, retinoid toxicity has been further investigated, and signs of hypervitaminosis A have been clearly established. Retinoids have many side effects ranging from mild (fatigue, headache or nausea) to severe (teratogenicity and severe liver damage). Retinoids exert their toxic effects through intracellular pathways which these compounds affect (La Vecchia et al. 1996). Retinoid toxicity is observed in several sites in the body and is dependent on age, gender, patient general health and the dose and route of administration (La Vecchia et al. 1996). Table 1 summarizes the side effects of chronic retinoid use.

Vitamin A excess and deficiency leads to teratogenicity. In a recent study, Elmanzar et al. (1996) treated pregnant mice with 37 mg/kg of *t*-RA on day 8 of gestation. Test compounds had the following teratogenic effects: (1) central nervous system (exencephaly, spina bifida); (2) cranofacial (micrognathia, cleft palate and macroglossia); (3) eye (exophthalmus); (4) soft tissue (kidney hypoplasia and undescended and malformed testes); (5) skeletal (unossified bones). In the same study pregnant mice were treated with RARα, RARβ or RARγ agonists. Teratogenicity was observed with all agonists, with the α-ligand being the most potent because of its wide distribution in the embryo (Ellmazar et al. 1996). Receptor selectivity play an important role in retinoid teratogensis.

Heteroarotinoids

Heteroarotinoids are compounds which contain an aryl ring fused to a partially saturated ring and a heteroatom (Waugh et al. 1985 and Spruce et al. 1987).

Table 1: Side Effects of Chronic Retinoid Use[1]

Site	Side Effect
General	Fatigue, headache and nausea
Skin and Mucous Membranes	Dryness of eyes, nose, and oral mucosa, xerosis, alopecia, epitaxsis, pruritis and palmoplantar desquamation
Muscle and Skeletal Systems	Spinal and extraspinal hyperostoses, muscle or joint stiffness and degenerative osteoporosis
Liver	Elevated liver enzymes and severe liver damage
Teratogenicity	Malformation of the cranofacial, cardiac, skeletal and central nervous system

[1] La Vecchia et al. 1996 and Gollinic et al. 1997

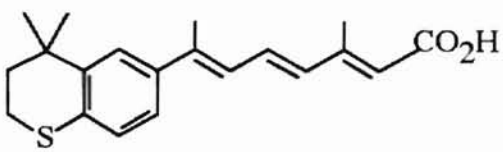
Heteroarotinoids have been tested in several biological assays and show promise as anticancer agents. This section will focus on selected first generation heteroarotinoids which are shown in Figure 5. Compound names are only used to simplify writing and do not represent IUPAC nomenclature.

Heteroarotinoids were first evaluated in the tracheal organ culture bioassay (TOC). Briefly, this procedure measures a compound's ability to reverse keratinization of tracheas from vitamin A deficient hamsters. Tracheas are placed in organ culture, tested with the compound for ten days, fixed and scored for the presence of keratin and keratohyaline granules. If both are observed, the compound is inactive (Newton et al. 1980). HS2 ester had good activity with an ED_{50} of 6×10^{-11} *M* which was comparable to the *t*-RA control. O-DHA acid and ester were the least active in this assay with an ED_{50} of 1×10^{-10} *M* (Waugh et al. 1985).

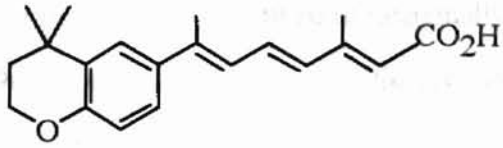
HS, O-MHA and O-DHA acid and ester have been evaluated in the ornithine decarboxylase (ODC) assay. Ornithine decarboxylase catalyzes the reaction of ornithine to putrescine which is decarboxylated to spermidine. These products play an important role in growth and malignant transformation (Verma et al. 1978). Application of 12-O-tetradeconylphorbol-13-acetate (TPA), a tumor promoting agent, increases epidermal ODC activity and is essential for skin tumor promotion (Verma et al. 1978). Systemic and topical applications of *t*-RA inhibit epidermal ODC and formation of skin papillomas (Verma et al. 1977 and Verma et al. 1978). Heteroarotinoids were applied to the backs of mice one hour before TPA application. Mice were sacrificed 5 hours later and assayed for ODC activity (Spruce et al. 1987). HS, O-MHA, O-DHA ester and acid had 89, 74, 43 and 68% ODC inhibition, respectively (Spruce et al. 1987).

Heteroarotinoids were evaluated for their ability to induce differentiation of HL-60 cells. The highest activity was found where sulfur was the heteroatom, although the oxygen counterparts were active in this assay (Spruce et al. 1987).

Monoaryl Heteroarotinoids:

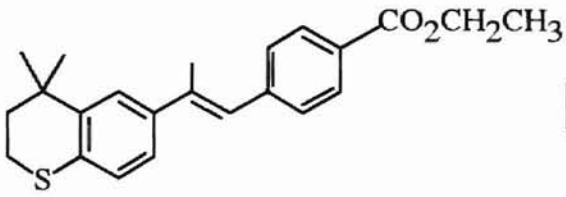


HS

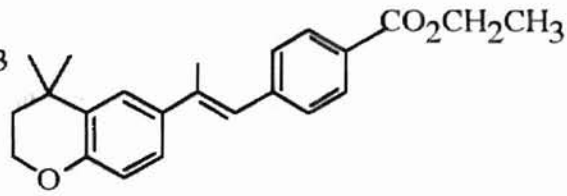


O-MHA

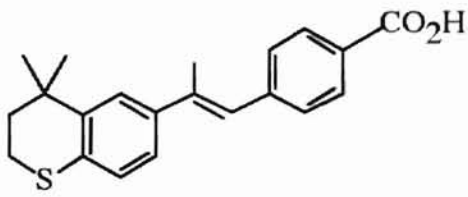
Diaryl Heteroarotinoids:



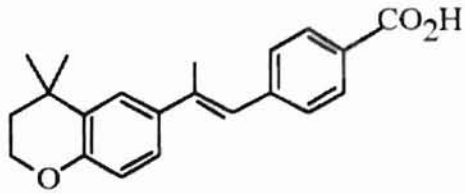
HS2- ester



O-DHA- ester



HS2- acid



O-DHA- acid

Figure 5: Structures of First Generation Heteroarotinoids.

O-DHA ester and O-MHA have been evaluated *in vivo* in the vaginal smear assay. Briefly, the cornification of vaginal epithelium is solely determined by vitamin A status in overiectomized vitamin A-deficient rats. Test compounds were applied intravaginally at dosing levels of $1x10^{-8}$ to $1x10^{-14}$ mol/rat. Disappearance of cornified epithelium and appearance of large number of neutrophils and nucleated epithelium cells were observed after heteroarotinoid applications. O-MHA was 10-fold less active than *t*-RA while O-DHA is comparable or slightly higher than *t*-RA (Thorne 1993).

Selected heteroarotinoids have been evaluated for transactivation using the cervical carcinoma cell line CC-B which contains integrated copies of RARE-CAT reporter gene (Benbrook et al. 1997). Sulfur-containing heteroarotinoids had greater activity in this assay than the oxygen-containing counterparts (Benbrook et al. 1997).

Toxicology data has been determined on HS, 0-MHA and O-DHA acid. Groups of 16 male B6D2F1 mice were dosed with 0.1 (low dose), 0.2, 0.4 (mid-dose 1 and 2, respectively) or 0.8 mg/kg (high dose) of heteroarotinoid or corn oil for 65 total experiment days. Due to lack of toxicity, dose levels were increased 4-fold on day 10, 10-fold on day 29 and 2 fold on day 51 (Benbrook et al. 1997). Animals treated with HS had no significant signs of toxicity until the dose was increased on day 51 and toxicity was limited to the high dose group. Animals had severe skin toxicity and final body weights were reduced by 9.6%. Elevated serum triglycerides were seen in the low and mid-dose groups. Hyperlipidemia is a common side effect of retinoid use because of increases in very low density lipoprotein (Vahlquist et al. 1987 and Gustafson et al. 1990). The maximum tolerated dose (MTD) of HS is 34 mg/kg (Benbrook et al. 1997). O-MHA treated animals had signs of toxicity on day 51 in mid-dose 2 and high dose. Skin toxicity was present in the high dose group and final body weights were reduced from control animals. Bone fractures were observed in mid-dose 2 and high dose groups. Serum triglycerides were elevated and the MTD is 24-32 mg/ kg (Benbrook et al. 1997). Animals treated with O-DHA had no significant signs of toxicity until day 30 with mortality occurring in the mid-

dose 2 and high dose groups. Skin toxicity was present in all groups and weight loss occurred in low dose and mid-dose 1 and 2. Serum triglycerides were elevated and enlarged lymph nodes were present in all groups. The MTD is 6-9 mg/kg for O-MHA and is comparable to *t*-RA (Benbrook et al. 1997). The toxicity of heteroarotinoids is less then naturally occurring retinoids and the same target organs as retinoids are affected (Benbrook et al. 1997 and Lindamood et al. 1987). In a similar study, Lindamood et al. (1990) evaluated a compound which resembles O-DHA acid without the heteroatom. This analog was 100-fold more toxic than *t*-RA (Lindamood et al. 1990). The presence and type of a heteroatom has a profound effect in reducing toxicity with the sulfur containing heteroatom having a higher MTD than its oxygen counterpart (Benbrook et al. 1997).

Receptor specificity was determined for O-MHA, O-DHA ester and acid, HS and HS2 ester and shown in Table 2 (Benbrook et al. 1998). First generation heteroarotinoids have been evaluated in several different biological assays. Table 3 summarizes the assays used to evaluated first generation heteroarotinoids

Summary

Clearly, retinoids participate in a variety of complex cellular processes. Different retinoid receptor subtypes, retinoid responsive genes, retinoid binding proteins and retinoid metabolism all play a critical role in signaling pathways. The role of retinoids in maintaining many epithelium cell types through growth and differentiation is of fundamental importance to chemotherapeutic treatment of many types of cancer. Unfortunately, chemotherapeutic doses of naturally occurring retinoids causes toxicity. In the last few decades, much effort has been devoted to developing synthetic retinoids which have good activity and lower toxicity. Retinoid skeleton modifications have resulted in analogs with a better therapeutic index.

Heteroarotinoids are novel retinoids which have been evaluated in several *in vivo* and *in vitro* assays. They have shown immense potential as anticancer agents and have a much lower toxicity than naturally occurring retinoids and some synthetic analogs.

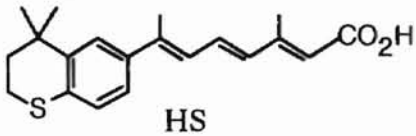
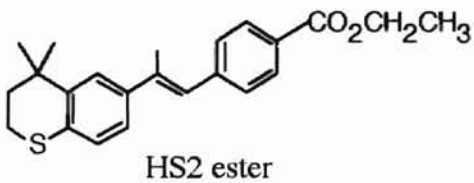
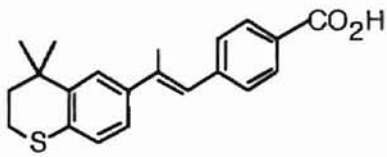
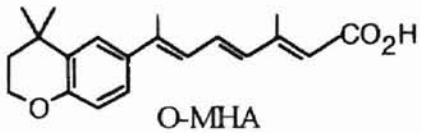
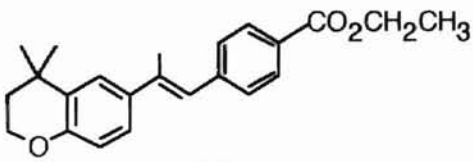
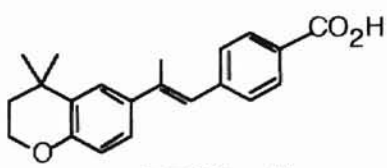
Table 2: Receptor Specificity of Selected Heteroarotinoids[1,2]

Heteroarotinoid	Receptor	Potency (EC_{50} nM)
O-MHA	RARα	740
	RARβ	72
	RARγ	39
	RXRα	2800
O-DHA ester	RARα	1100
	RARβ	140
	RARγ	330
	RXRα	NA
O-DHA acid	RARα	1100
	RARβ	190
	RARγ	200
	RXRα	NA
HS	RARα	1400
	RARβ	43
	RARγ	12
	RXRα	2700
HS2 ester	RARα	620
	RARβ	110
	RARγ	84
	RXRα	NA

1. Table from Benbrook et al. 1998
2. EC_{50} is concentration of retinoid required to produce 50% of the maximal response

NA = non-appreciable

Table 3: Biological Assays Used to Evaluate First Generation Heteroarotinoids

Heteroarotinoid	Biological Assay	Reference
 HS	ODC	Spruce et al. 1987
	Transactivation	Benbrook et al. 1997
	Toxicity	Benbrook et al. 1997
 HS2 ester	TOC	Waugh et al. 1985
	Transactivation	Benbrook et al. 1997
 HS2 acid	Transactivation	Benbrook et al. 1997
 O-MHA	TOC	Waugh et al. 1985
	ODC	Spruce et al. 1987
	Vaginal Smear	Thorne 1993
	Transactivation	Benbrook et al. 1997
	Toxicity	Benbrook et al. 1997
 O-DHA ester	TOC	Waugh et al. 1985
	ODC	Spruce et al. 1987
	Vaginal Smear	Thorne 1993
	Transactivation	Benbrook et al. 1997
 O-DHA acid	TOC	Waugh et al. 1985
	ODC	Spruce et al. 1987
	Transactivation	Benbrook et al. 1997
	Toxicity	Benbrook et al. 1997

The goal of the present study was to address the following aspects: (1) to evaluate four heteroarotinoids for growth promoting activity in vitamin A-deficient rats. Two of the four were related compounds which contained a sulfur as the heteroatom and were monoaryl or diaryl heteroarotinoids; (2) to examine histopathological changes of tissue collected from treated rats; (3) to isolate and identify HS and HS2 metabolites from vitamin A-deficient rat liver and serum; (4) to isolate and identify liver and kidney metabolites from vitamin A-deficient rats injected with radiolabelled O-DHA (O-DHA*).

CHAPTER II

GROWTH PROMOTING ACTIVITY OF SELECT HETEROAROTINOIDS

In 1913, McCollum and Davis first established that a fat-soluble vitamin was required for growth. A rat growth assay was designed as early as 1921 and became a standard approach for evaluating vitamin A activity in diets (Zilva et al.). In 1948 the assay was improved when Johnson and Baumann showed young animals were more easily made vitamin A deficient compared to older animals.

Vitamin A deficiency results in many physiological changes including ocular manifestations ranging from night blindness to corneal keratomalacia (Somner 1998). Early physiological signs of chronic vitamin A deficiency include an increase in cerebrospinal fluid pressure (Nelson et al. 1962 and 1964) followed by loss of appetite and a decrease in weight gain. A higher incidence of mortality is observed in children who are mildly deficient and the risk is increased as the deficiency becomes more severe (Somner 1998). Diarrhea and aggravated anemia are also severe signs in vitamin A-deficient animals (Somner 1998).

Many clinical and physiological stresses can influence vitamin A utilization. For example, immobilized rats had decreased serum, liver, testes and kidney vitamin A concentrations (Morita et al. 1982). Artificially induced fever decreases serum vitamin A levels (Mendez et al. 1959). Zinc maintains normal plasma vitamin A concentrations and is important for alcohol dehydrogenase and RBP synthesis and function (Smith et al. 1973 and Solomons et al. 1980). In a recent study, animals fed a vitamin A-deficient diet had decreased zinc concentrations in the urine (Grases et al. 1998). Vitamin A deficiency and zinc loss alters the papillar urothelium and these animals have a higher risk of renal uroliths (Grases et al 1998). Vitamin A deficiency increases copper and iron concentrations in rat

testes (Rahman et al. 1995). Other dietary components such as vitamin E, protein and fat must be adequate to provide full utilization of vitamin A.

Signs of vitamin A deficiency are difficult to characterize since most animals succumb to infection before signs of vitamin A deficiency appear (Beaver 1961). Vitamin A-deficient animals have impaired blood clearance of *Escherichia coli* and are more susceptible to *Staphylococcus aureus* infection (Ongsakul et al. 1985 and Wiedermann et al. 1996). Young vitamin A-deficient rats have a decreased ability to recover from the rodent malarial parasite *Plasmodium berghei* (Stoltzfus et al. 1989). Furthermore, deficient animals are more susceptible to viral infections including herpes simplex virus, swine influenza virus and rotavirus (Underhall et al. 1956, Nauss et al. 1985 and for review see Ross et al. 1996).

Clearly, immunity is compromised in vitamin A-deficient animals. The mechanisms underlying this response are unclear although many observations have been reported: (1) vitamin A-deficiency alters the mucosal epithelium integrity in the intestine and leads to an increased risk in intestinal bacteria translocation and compromised mucosal immunity (Semba 1998 and Wiedermann et al. 1995); (2) bacteria adhesion, phagocytosis and generation of active oxidative molecules were lower in neutrophils taken from vitamin A-deficient animals (Twining et al. 1997); (3) inflammation in animals causes a redistribution of vitamin A and RBP reduction in liver and plasma (Rosales et al. 1998); (4) a decrease in natural killer (NK) cell activity is observed in vitamin A-deficient animals (Bowman et al. 1990); (5) antibody production is decreased in vitamin A-deficient rats after immunization with antigens from *Pseudomonas aeruginosa* and *Serratia marcesens* (Pasatiempo et al. 1990).

In the present study, four heteroarotinoids were assayed for their ability to support growth in vitamin A-deficient rats. Two feeding trials were conducted in different years and are designated feeding trial one and two, respectively. All-*trans*-retinoic acid, a known growth-supporting compound, was included as a positive control. Synchronous vitamin A

deficiency was introduced in all animals prior to testing any compounds (Lamb et al. 1974). The structures for compounds tested are shown in Figure 6. Compound names are used to simplify writing and do not represent IUPAC nomenclature.

Materials and Methods

In the text the designation FT1 and FT2 are defined as feeding trial one and feeding trial two, respectively. These abbreviations are used because the trials were slightly different from each other.

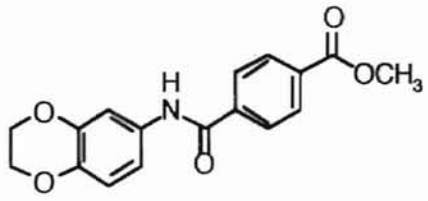
Animals and Animal Care

Forty-eight (FT1) or thirty-six (FT2) F-344 male rats (Charles Rivers Laboratories, Wilmington, MA) were obtained at seventeen days of age with dams (10 pups per dam). Immediately upon arrival, dams were placed on the vitamin A-deficient diet listed in Table 4 (Harlan Teklad, Madison, WI). At the age of twenty-one (FT1) or twenty (FT2) days pups were weaned and maintained on the same vitamin A-deficient diet. Animals were weighed every two days to monitor their vitamin A-deficiency status. Weighing was done at approximately the same time of day to avoid diurnal variations. Rats were placed in individual polycarbonate cages. Cages were placed side-by-side for visual and odor socialization and were switched frequently in a random manner to avoid geographical variations. All procedures were approved by the Laboratory Animal Care Committee at Oklahoma State University and conformed to NIH guidelines. Fresh diet and water were given daily and available *ad libitum*. The room was cleaned daily and cages were changed twice weekly.

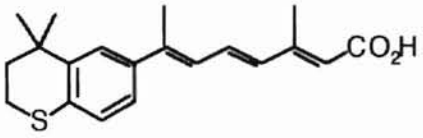
Chemicals

Heteroarotinoids HN, HN2, HS and HS2 were synthesized and crystallized by Dr. K.D. Berlin's research group at Oklahoma State University. The compounds were tightly capped under nitrogen and stored in the dark at -20 °C. Retinoic acid was in the form of a stabilized gelatin beadlet containing 10.3% retinoic acid and was provided by Hoffmann-La Roche, Inc.

Feeding Trial One Structures

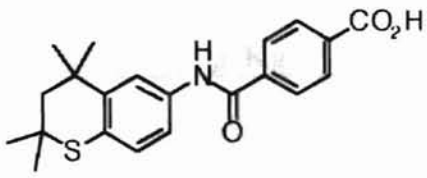


HN

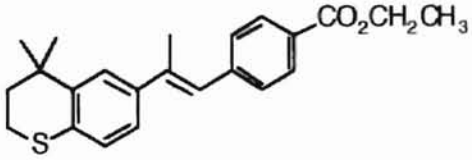


HS

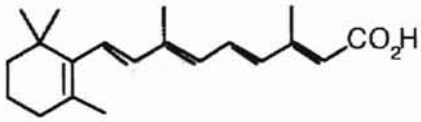
Feeding Trial Two Structures



HN 2



HS 2



All-*trans* -retinoic acid
Positive control for feeding trials 1 and 2

Figure 6: Structures of Compounds Tested in Vitamin A-Deficient Rats in Growth Promoting Assay.

Table 4: Vitamin A-Deficient Diet[1]

COMPONENTS	g/Kg
Casein, "Vitamin-Free" Test	193.0
DL-Methionine	3.0
Corn Starch	661.1343
Cottonseed Oil	50.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (TD 94046)	35.0
Calcium Phosphate, dibasic $CaHPO_4$	4.0
Biotin	0.0004
Vitamin B_{12} (0.1% in mannitol)	0.0297
Calcium Pantothenate	0.0661
Choline Dihydrogen Citrate	3.4969
Menadione Sodium Bisulfate Complex	0.05
Niacin	0.0991
Pyridoxine HCl	0.022
Riboflavin	0.022
Thiamin HCl	0.022
DL-Alpha Tocopheryl Acetate (1000 U/g)	0.05
Vitamin D_2 in corn oil (400,000 U/g)	0.0055

1. Teklad Custom Diet TD 97146

Introduction of Synchronous Vitamin A Deficiency

The method of Lamb et al. (1974) was used to introduce synchronous vitamin A deficiency in forty-eight (FT1) and thirty -six (FT2) F-344 rats. Diets were supplemented with 45 μg *t*-RA/rat/day in the form of retinoic acid beadlets for two consecutive weight gain and weight loss cycles. Retinoic acid beadlets were premixed with the vitamin A-deficient diet at a level of 25 mg retinoic acid beadlets per Kg diet. Before the feeding experiment, a six (FT1) or seven (FT2) day retinoic acid supplementation period was implemented to negate the signs of vitamin A-deficiency from the preceding deprivation phase.

Stock Solutions and Feeding Procedures

Stock solutions were prepared in aldehyde free-ethanol at a concentration of 500 μg/ml. A two-day supply was made each time, wrapped in aluminum foil and stored at -20 °C under N_2 until diet preparation. Each rat was given 20 g of diet. For retinoic acid control rats, the beadlets were premixed with the diet to give the appropriate doses of 10, 100, or 200 μg/rat/day. For test compounds HN and HS, diets were prepared by diluting the appropriate stock solution to achieve a final dose of 10, 100 or 200 μg/rat/day. This dilution was placed directly on the feed and the ethanol was allowed to evaporate in the dark for one hour. For test compounds HN2 and HS2, diets were prepared by diluting the appropriate stock solution to achieve a final dose of 10, 100 or 200 μg/rat/day. This dilution was placed directly on 5 g of feed and the ethanol was allowed to evaporate in the dark for one hour. The 5 g treatment was placed directly on 15 g of the vitamin A-deficient diet. The feeding procedures are shown in Figure 7 and 8 for FT1 and FT2, respectively.

In Figure 7 and 8 the letters represent the compound which was fed and the number denotes the amount fed per rat. For example, rats fed retinoic acid at a level of 10 μg/day are designated as RA-10.

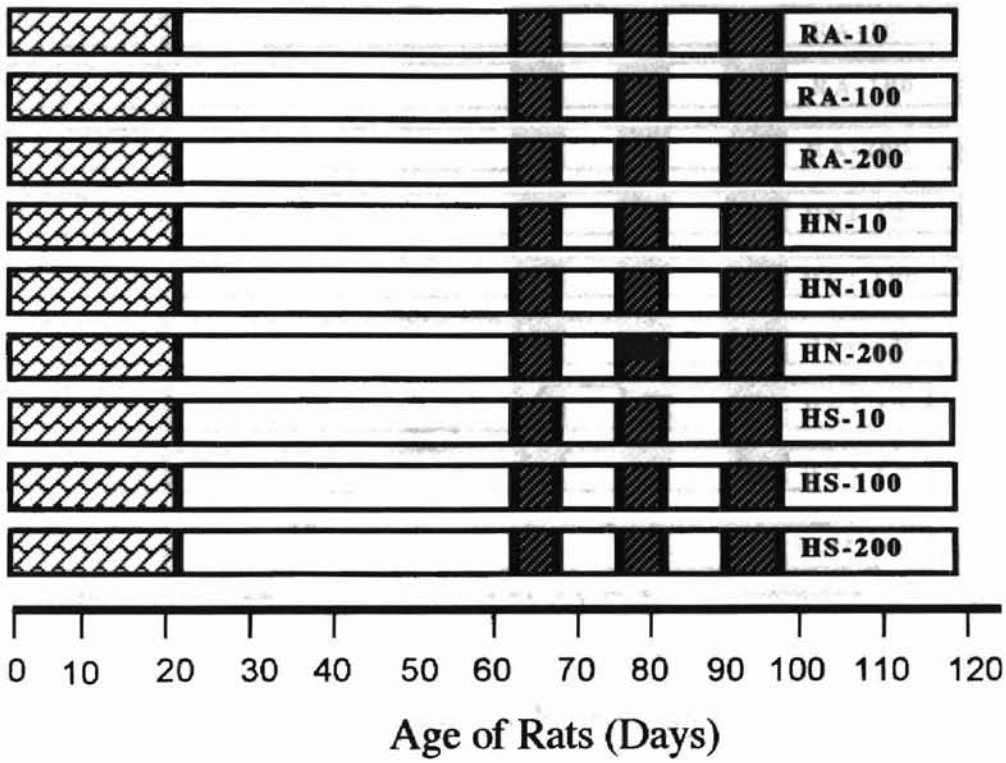


Figure 7: Experimental Feeding Procedure for Feeding Trial One.

=Nursing = RA supplement 45 µg/rat/day

= Vitamin A-deficient diet

n = 4 rats per group

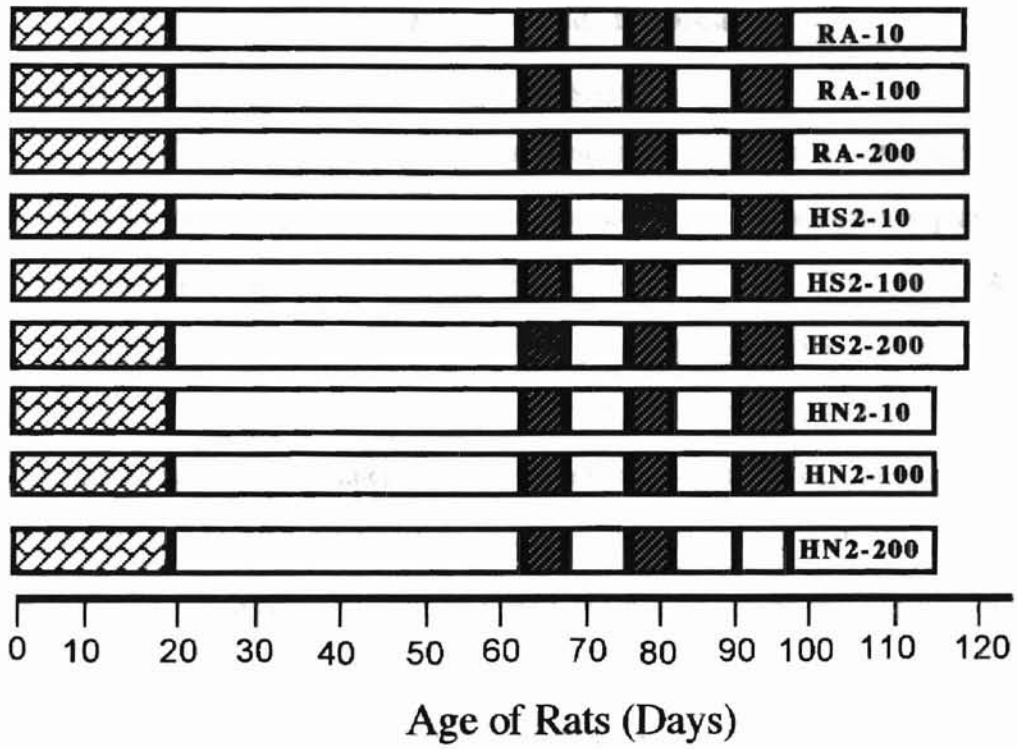


Figure 8: Experimental Feeding Procedure for Feeding Trial Two.
= Nursing = RA supplement 45 µg/rat/day
= Vitamin A-deficient diet
n = 4 rats per group

Results and Discussion

Introduction of Synchronous Vitamin A Deficiency

Figure 9 and 10 represent the synchronous vitamin A-deficiency growth curve for rats used in feeding trial one and two, respectively. The first growth plateau was reached at 62 days with an average weight of 174 g (FT1) and 175 g (FT2). Animals fed 45 μg/rat/day of *t*-RA for five days had an average weight of 194 g (FT1) and 192 g (FT2) at the end of supplementation period. A second growth plateau was achieved approximately two weeks later when rats were 76 days of age with an average weight of 212 g (FT1) and 204 g (FT2). Rats were again supplemented with 45 μg of *t*-RA/rat/day for five days and averaged 222 g (FT1) and 213 g (FT2) at the end of supplementation. At the end of the last cycle, all animals were synchronously vitamin A-deficient. A final 45 μg/rat/day retinoic acid supplement was given prior to feeding the test compounds to negate signs of vitamin A deficiency. Animals were 91 and 90 days of age, averaged 232 g and 215 g and were supplemented for six or seven days with a final average weight of 243 g and 224 g, for feeding trials one and two, respectively.

Rats in the first feeding trial have a sharp decline in weight on day 38. This decrease in weight gain is not due to vitamin A deficiency but results from animals not being fed over a weekend by laboratory animal resource personnel. In some groups animals lost up to 25% of their body weight and had diarrhea. Rats were immediately fed and watered. Animals were weighed the next day and had compensatory weight gain. Animals were maintained on the same vitamin A-deficient diet until the first growth plateau was reached. Dr. E.C. Nelson's research group assumed all responsibility for the feeding and watering of rats for the duration of feeding trial one and two.

Growth Patterns of Rats Fed 10, 100 or 200 μg/rat/day of *t*-RA, HN or HS

Figure 11 shows the growth curves for rats fed 10 μg/day of *t*-RA, HN or HS for 21 days. At 10 μg/day, *t*-RA fed rats maintained growth with a final average weight of 265 g. At 10 μg/day of HN or HS, rats had a marked decline in growth and average final

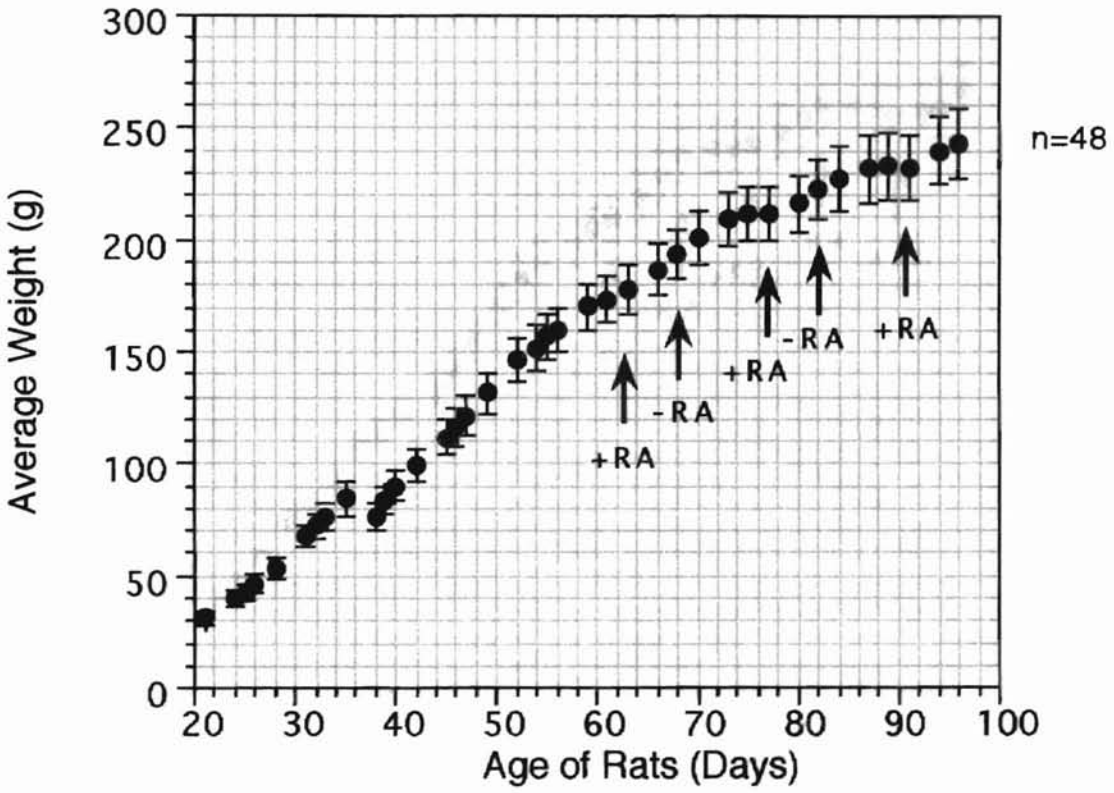


Figure 9: Synchronous Vitamin A-Deficient Growth Curve for F-344 Male Rats in Feeding Trial One. Mean weight ± SD.

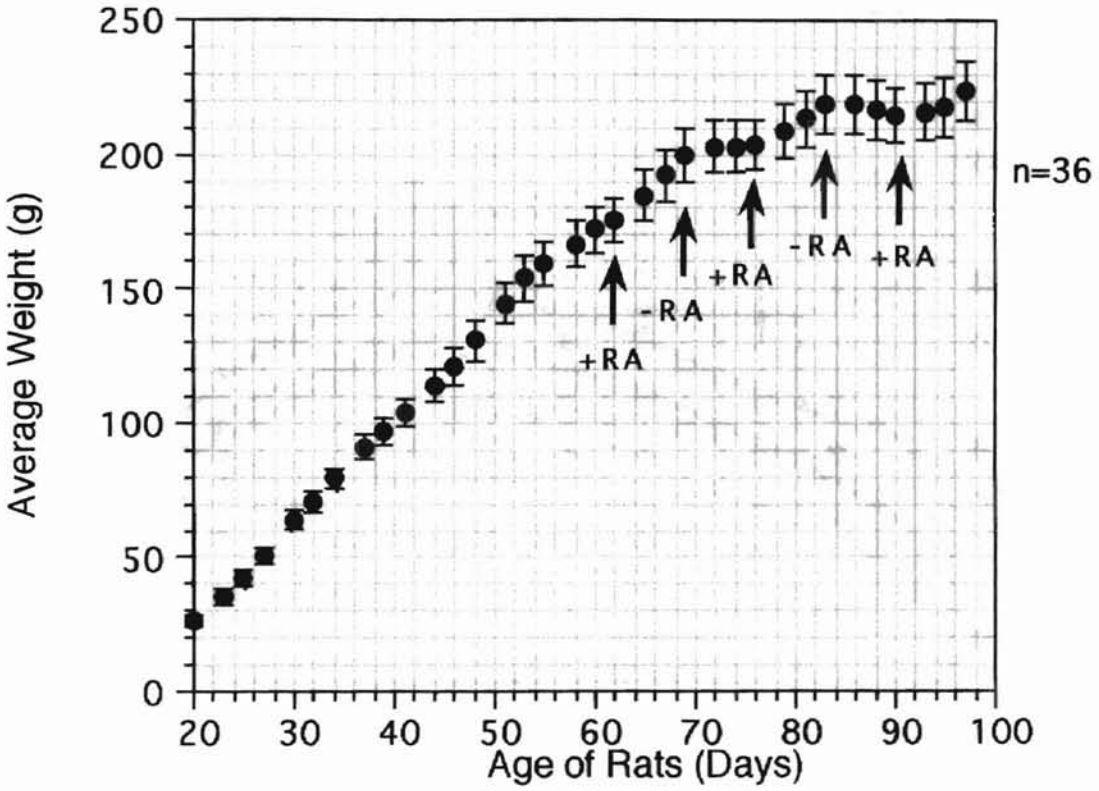


Figure 10: Synchronous Vitamin A-Deficient Growth Curve for F-344 Male Rats in Feeding Trial Two. Mean weight ± SD.

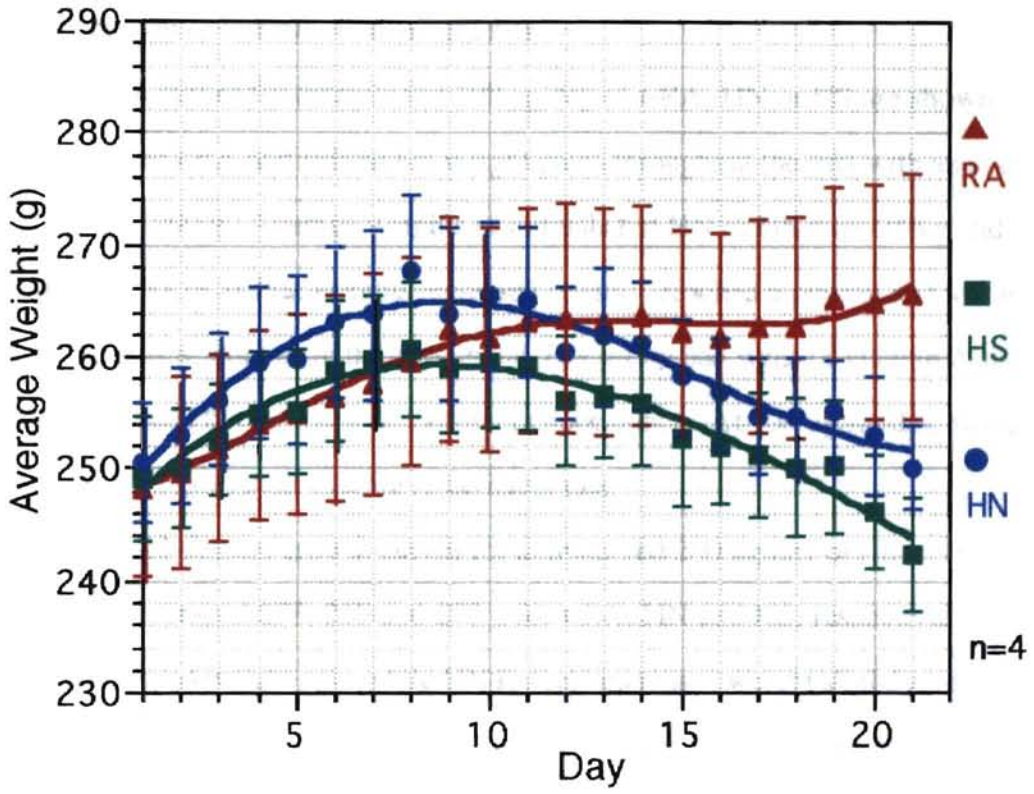


Figure 11: Average Weights ± SD of FT 1Rats Fed a Vitamin A-Deficient Diet Supplemented with 10 μg/day of *t*-RA, HN or HS.

weights of 250 and 242 g, respectively. HN and HS fed rats at this dose did show signs of vitamin A deficiency (humped posture, encrusted eyes and rough haircoat) while *t*-RA fed control animals showed no physical changes. At 10 μg/rat/day neither HN or HS supported growth of vitamin A -deficient rats.

Growth curves for animals fed 100 μg/day of *t*-RA, HN or HS are shown in Figure 12. Animals fed *t*-RA had substantial weight gains throughout the trial with an average final weight of 290 g. Average weights of animals fed HN or HS were 255 g and 252 g, respectively. HN or HS rat weights increased slightly, reached a plateau and lost weight by 21 days. A few animals in the 100 μg/day HN group had signs of vitamin A-deficiency while animals fed HS or *t*-RA were normal in appearance. At 100 μg/rat/day, neither HN or HS supported growth in vitamin A-deficient rats.

Figure 13 depicts growth curves for animals fed 200 μg/day of *t*-RA, HN or HS. Final weights averaged 278, 250 and 270 g for *t*-RA, HN and HS, respectively. Rats fed *t*-RA had increased weight gain while HN fed rats increased weight slightly, reached a plateau and lost weight by the end of 21 days and had marked signs of vitamin A deficiency. HS fed rats had weight gains comparable to *t*-RA throughout the trial and final average weights were comparable. HS treated animals had no signs of vitamin A-deficiency. These results indicate that HS at 200 μg/day was capable of supporting growth in vitamin A-deficient rats as compared to the 200 μg/rat/day *t*-RA control.

Growth Patterns of Rats Fed 10, 100 or 200 μg/rat/day of *t*-RA, HN2 or HS2

Figure 14 represents the growth curves for rats fed 10 μg/day of *t*-RA, HN2 or HS2. At 10 μg/day, *t*-RA fed rats maintained growth with a final average weight of 261 g. HN2 rats fed 10 μg/day had a marked decline in growth, showed signs of vitamin A-deficiency and were terminated on day 18 with an average final weight of 215 g. HS2 rats fed 10 μg/day maintained growth and had an average final weight of 250 g.

Growth curves for animals fed 100 μg/day of *t*-RA, HN2 or HS2 are shown in Figure 15. Animals fed *t*-RA had substantial weight gains throughout the trial with an

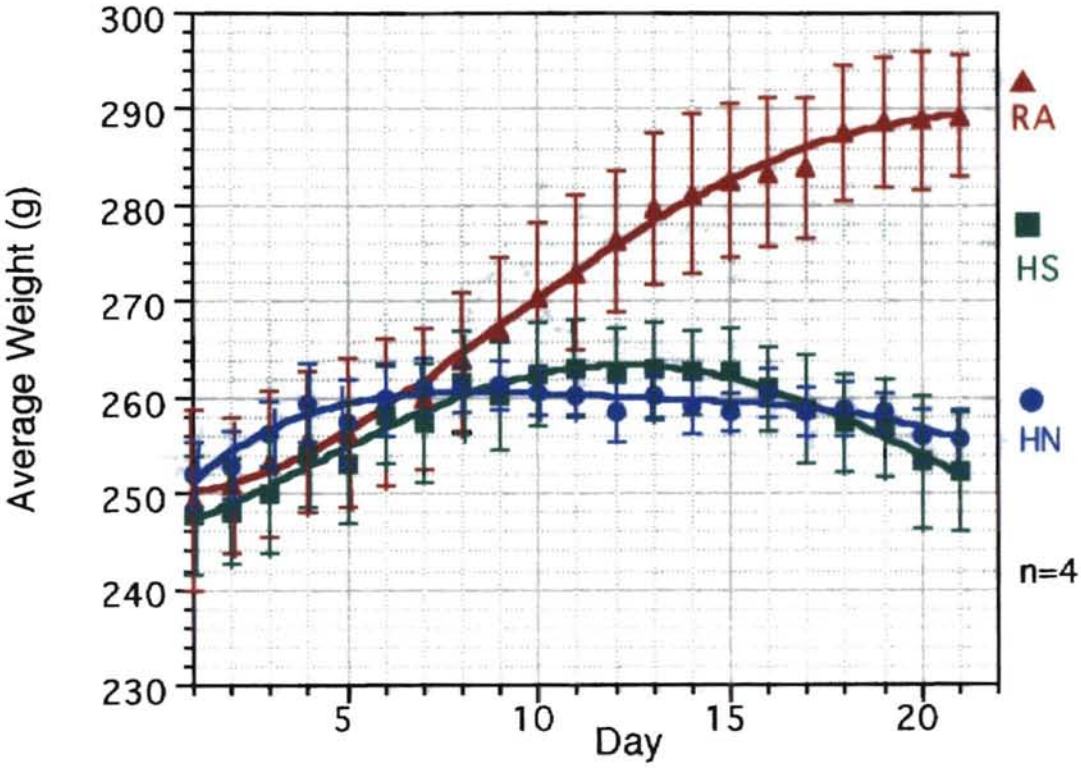


Figure 12: Average Weights ± SD of FT1 Rats Fed a Vitamin A-Deficient Diet Supplemented with 100 μg/day of *t*-RA, HN or HS.

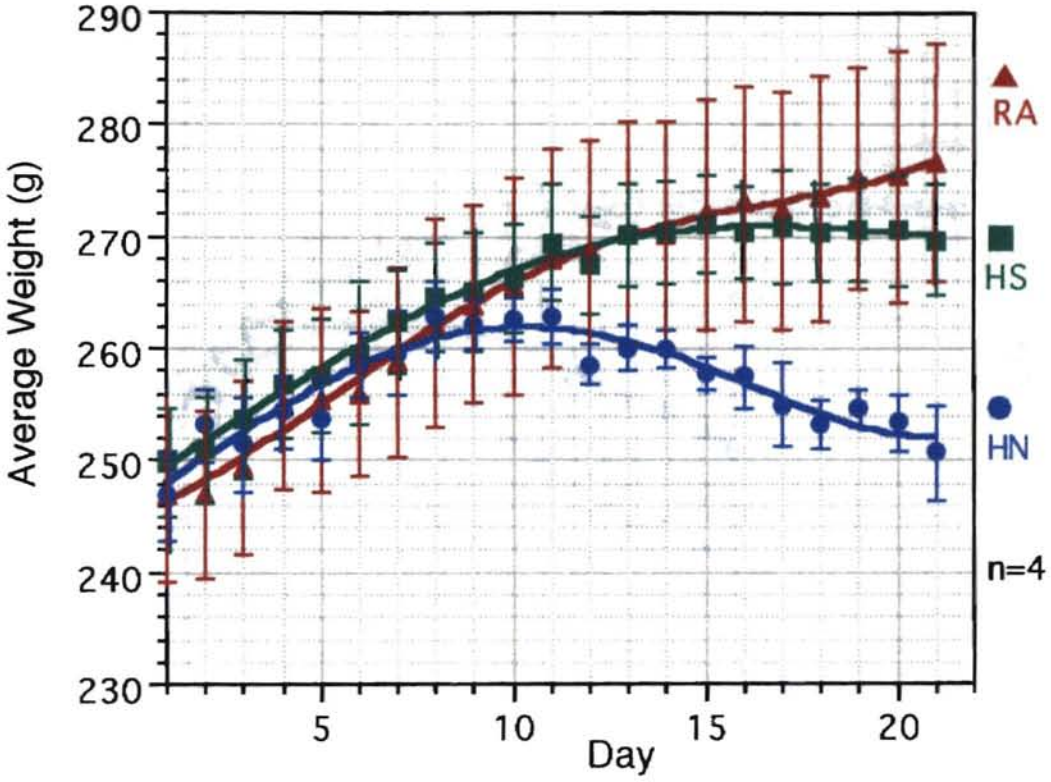


Figure 13: Average Weights ± SD of FT 1 Rats Fed a Vitamin A-Deficient Diet Supplemented with 200 μg/day of *t*-RA, HN or HS.

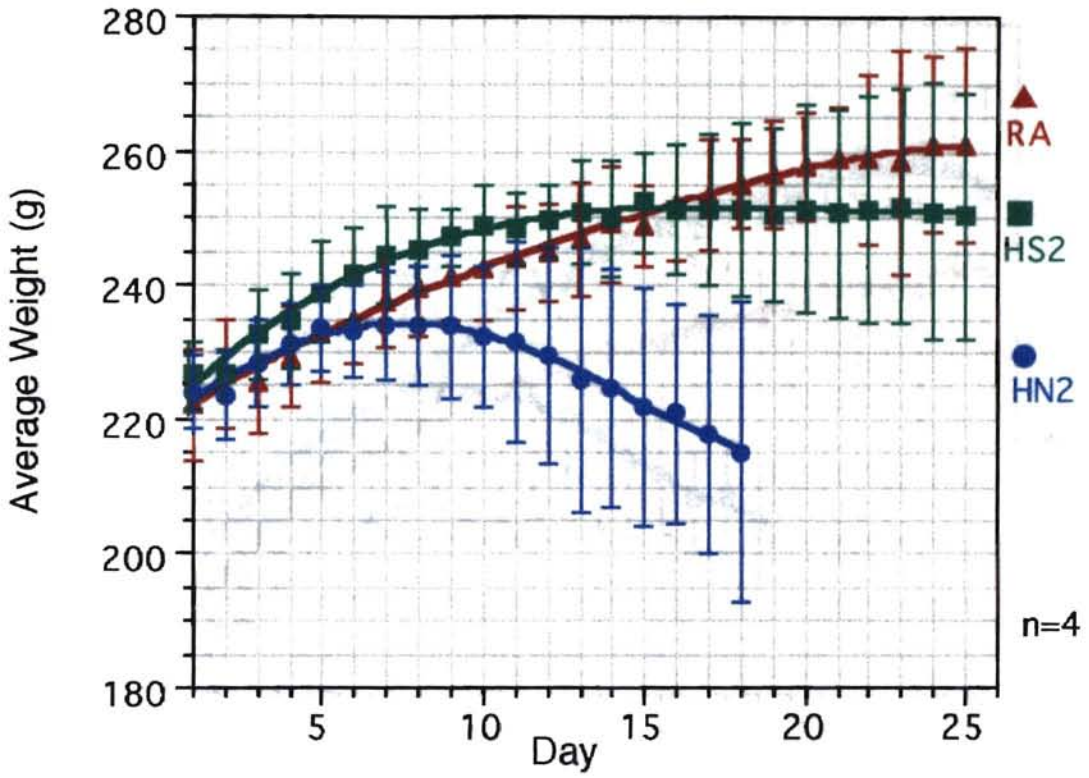


Figure 14: Average Weights ± SD of FT2 Rats Fed a Vitamin A-Deficient Diet Supplemented with 10 μg/day of *t*-RA, HN2 or HS2.

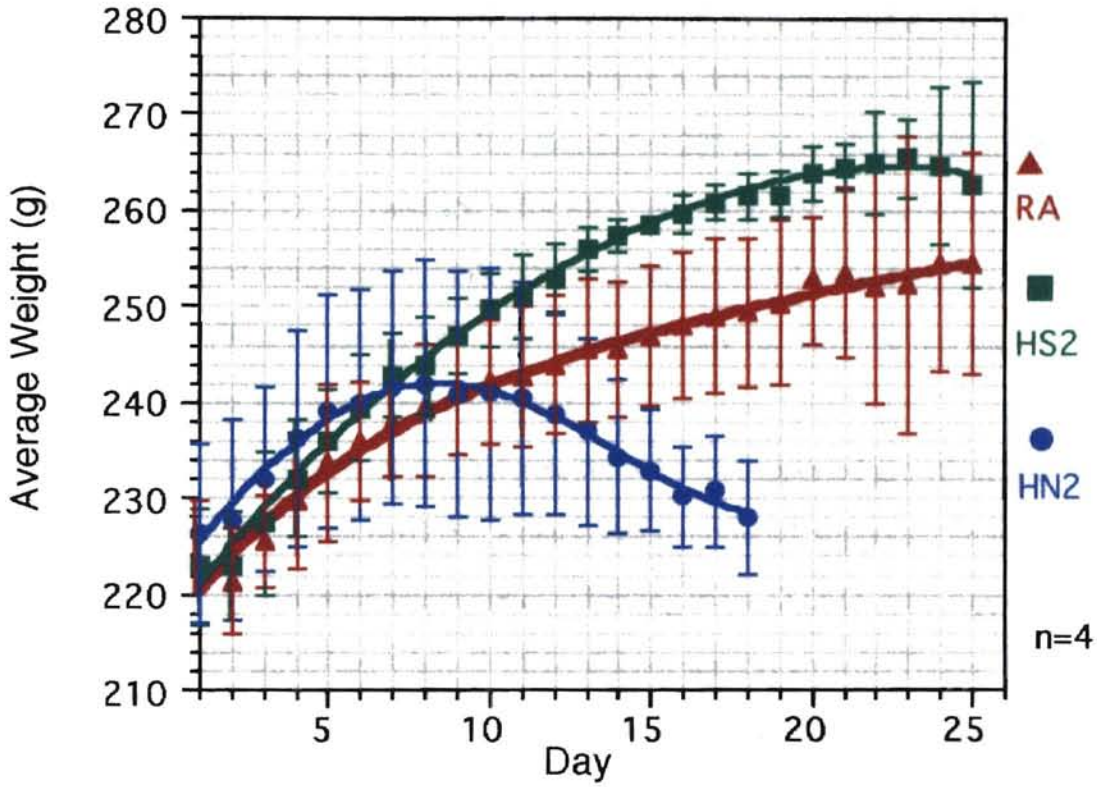


Figure 15: Average Weights ± SD of FT2 Rats Fed a Vitamin A-Deficient Diet Supplemented with 100 μg/day of *t*-RA, HN2 or HS2.

average final weight of 255 g. At 100 μg/day HN2 rats increased weight slightly, reached a plateau and lost weight by 18 days and were terminated with an average final weight of 228 g. Rats fed 100 μg/day HS2 had increasing weight gain with HS2 fed rats gaining more than *t*-RA fed rats. HS2 fed rats had an average final weight of 263 g. At 100 μg/day HS2 is capable of supporting growth in vitamin A-deficient rats.

Figure 16 depicts growth curves for animals fed 200 μg/day *t*-RA, HN2 or HS2. Final weights averaged 272, 235 and 257 g for *t*-RA, HN2 and HS2, respectively. Rats fed *t*-RA had increased weight gains while HN2 fed rats increased weight slightly, reached a plateau and rapidly lost weight and were terminated on day 18. HS2 fed rats had increased weight gains and were comparable to *t*-RA.

In feeding trial one, a monoaryl heteroarotinoid, HS, supported growth at 200 μg/rat/day while in feeding trial two HS2, a diaryl heteroarotinoid, supported growth at 10, 100 and 200 μg/rat/day. In other heteroarotinoid feeding trials, O-DHA ester supported growth at 100 and 200 μg/rat/day while O-MHA did not support growth (Thorne 1993). The differences between monoaryl and diaryl heteroarotinoids and the heteroatom they contain plays an important role in growth promoting activity. Look et al. (1995) observed RARγ mutant mice weighed 40-90% less than heterozygous or wild type siblings and they suggest RARγ is important in growth. As shown in Table 2, sulfur-containing heteroarotinoids have a greater specificity for RARγ than their oxygen counterparts. Although the sulfur containing heteroarotinoids are more specific for RARγ and better at promoting growth when compared against there oxygen counterparts, this does not imply that RARγ is the only receptor responsible for this activity. Moreover, it does not imply a given RARγ specific heteroarotinoid will support growth in this assay. It may be a combination of all retinoid receptors which play a role in this activity. Furthermore, compounds which support growth may have activity or be metabolized to other compounds with higher activities.

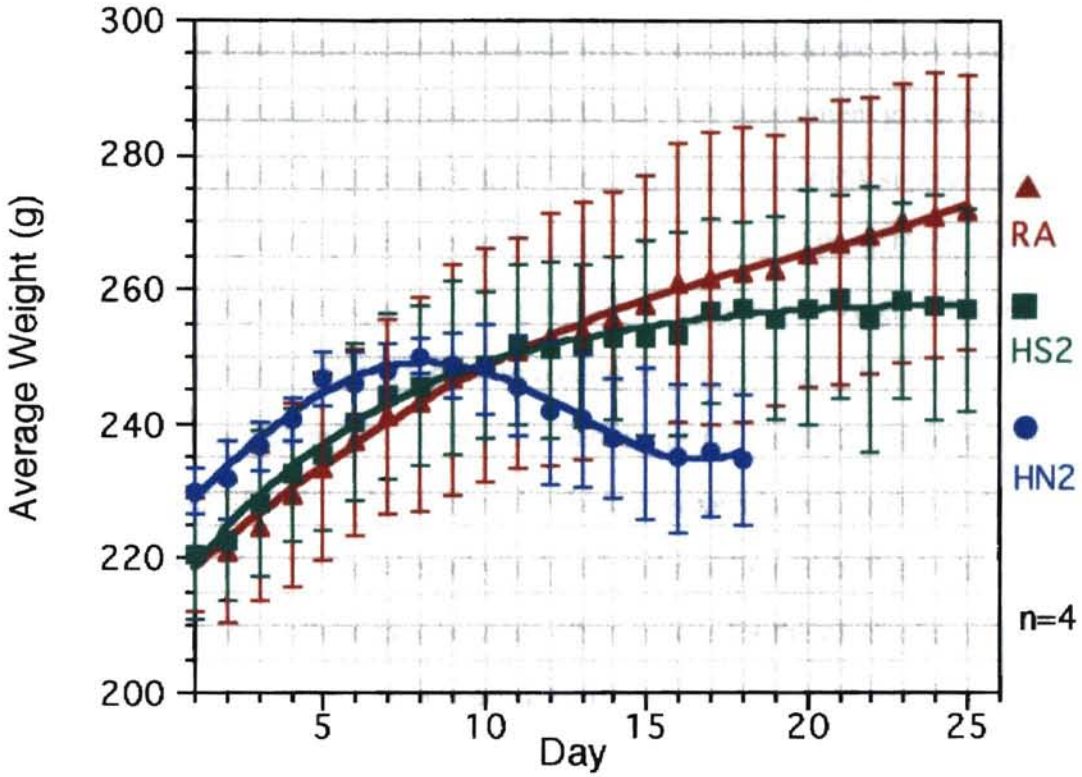


Figure 16: Average Weights ± SD of FT2 Rats Fed a Vitamin A-Deficient Diet Supplemented with 200 μg/day of *t*-RA, HN2 or HS2.

HN and HN2 did not support growth in this assay when fed at any level although initially in the feeding trials these heteroarotinoids were comparable to the *t*-RA control. The explanation for this initial activity may be due to the *t*-RA supplement given prior to feeding the test compounds. In general, it takes nine days from one *t*-RA supplementation period to another for a growth plateau to be reached. Animals, fed HN or HN2, sustained growth for approximately nine days and then gradually started to lose weight. These animals were supplemented with *t*-RA prior to feeding HN or HN2 and the initial effects are due to this supplementation and not from the activity of HN or HN2. Since growth is a complex process, it is difficult to determine why these compounds are inactive in this assay. These compounds may have been hydrolyzed and rapidly metabolized which would effect their bioavailability or there may be many other reasons for their lack of activity.

Conclusions

Results from rat feeding trial one and two clearly demonstrate that HS and HS2 are potent growth promoters in vitamin A-deficient rats. The activity of HS was comparable to *t*-RA at 200 μg/rat/day while the activity of HS2 was comparable to *t*-RA at 10 and 200 μg/rat/day and higher than *t*-RA at 100 μg/rat/day. Secondly, there is a clear change in activity when the oxygen is replaced with sulfur in the monoaryl heteroarotinoid. Finally, this data agrees with other heteroarotinoid data which suggests sulfur containing heteroarotinoids have better activity than their oxygen containing counterparts in a variety of assays.

CHAPTER III

HISTOPATHOLOGICAL EXAMINATION OF THE EFFECTS OF SELECT HETEROAROTINOIDS IN VITAMIN A-DEFICIENT RATS

Wolbach and Howe first reported physiological and histopathological changes in vitamin A-deficient rats (1925). They observed atrophy of the submaxillary, parotid, lacrimal, thyroid, pituitary and Haderian glands. There was a decrease in adipose tissue, liver and spleen and almost a complete disappearance of thymus and enlarged lymph nodes. Squamous metaplasia, a building of multiple layers of generally keratinized epithelial cells, was present in all animals and in some cases was so severe it resulted in either the inability to swallow or ureter blockage which caused death. Histopathological changes in vitamin A-deficient animals included normal epithelia being replaced by stratified keratinizing epithelium in the following locations: (1) respiratory tract-larynx, trachea and bronchi; (2) alimentary tract-submaxillary and parotid glands; (3) genitourinary tract-bladder, ureter, epididymis, prostate and seminal vesicles; (4) eyes and glands-cornea and lacrimal and Hardian gland; (5) ductless glands-thymus. Keratinization of the trachea and bronchi was usually the first tissues effected followed by urinary tract mucosa with the pancreas being the last tissue to show keratinization (Wolbach et al 1925). Atrophy was also observed in the testes although squamous metaplasia was not seen.

Lack of spermatogenesis is characteristic of vitamin A deficiency in laboratory animals. Spermatogenesis takes place in the seminiferous tubules. Within the tubules, Sertoli cells create two regions, the basal and adluminal compartments. The basal compartment contains early germ cells (spermatogonia and early spermatocytes) and open directly to the blood vessels while the adluminal compartment contains more advanced germ cells (advanced spermatocytes, spermatids and spermatozoa) and is separated from the blood vessels by a permeable barrier (Poccia 1994). Primordial germ cells move from

the yolk sac endoderm to the gonadal ridge and differentiate to spermatogonia. Spermatogonia contain different subtypes, A_0 cells which are non cycling, and A_1-A_4 which divide into differentiated type B spermatogonia (Poccia 1994). Tight junctional complexes between adjacent Sertoli cells form a barrier which type B spermatogonia cross and are enveloped within the Sertoli cells. Type B spermatogonia differentiate to a primary spermatocyte which divides to produce two secondary spermatocytes (Guyton 1996). A second meiotic division occurs to produce two spermatids. Cytodifferentiation of spermatids converts them through nineteen different stages to spermatozoa. These stages can be differentiated by acrosome presence and placement, flagella position and length and the nuclear chromatin condensation (for review of the stages see Lebond et al. 1952). During spermatid development, the seminiferous tubule contains an older spermatid generation which are released as spermatozoon when the younger generation reaches stage VIII. From stages VIII-XV the younger spermatid generation are the only ones present in the tubule, when they reach stage XV a new spermatid generation appear in the same area of the tubule. Once the sperm is released it enters the lumen of seminiferous tubules, migrates to the ducts of the testes and then to the ductus of the epididymis. A photomicrograph of seminiferous tubules is shown in Figure 17.

Mason (1933) first demonstrated that vitamin A-deficient rat seminiferous tubules were greatly reduced in size and the tubules only contained spermatogonia, Sertoli cells and a few spermatocytes. Vitamin A-deficient animals supplemented with retinoic acid have normal growth but are unable to maintain or reinitiate spermatogenesis unless they are supplemented with retinol (Howell et al. 1963). When vitamin A-deficient rats are supplemented with retinol, normal spermatogenesis is reinitiated (Huang et al. 1979). In vitamin A-deficient rats, the Sertoli cells-spermatid association is disrupted resulting in sloughing of germ cells from germinal epithelium (Unni et al. 1983). Retinoic acid fed rats have disrupted Sertoli cell junctions and when animals were supplemented with retinol there was reestablishment of Sertoli cell junctions and spermatocytes reappeared (Unni et

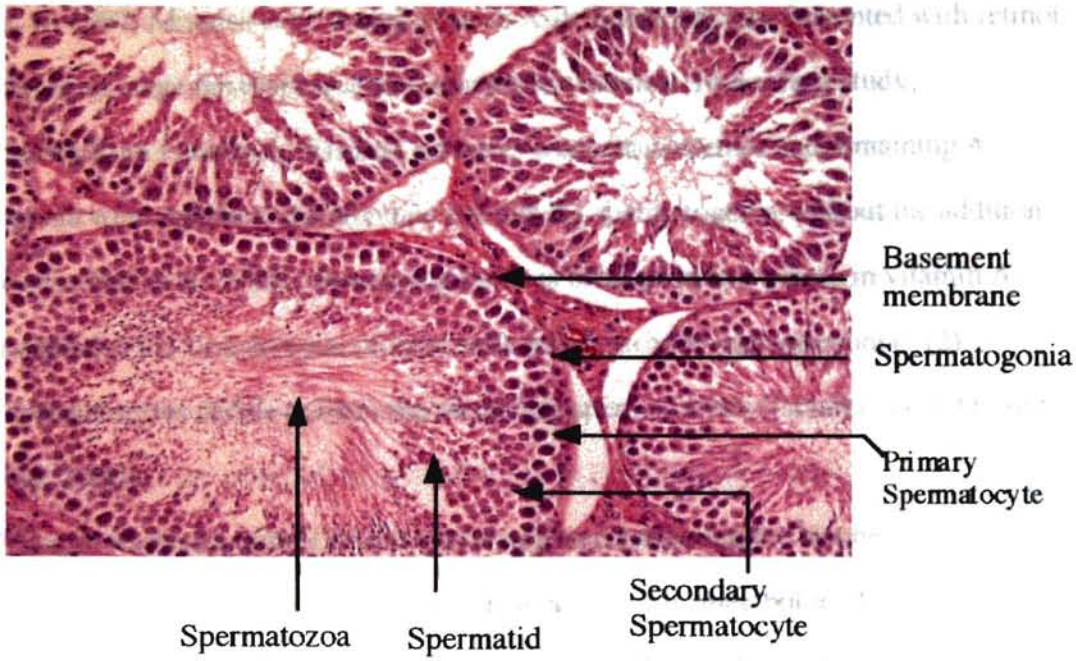


Figure 17: Photomicrograph of Cross Section of Rat Seminiferous Tubule (200x). F-344 rats were 118 days of age. Samples were trimmed, placed in plastic cassette and processed by dehydration in ethanol. Samples were embedded in paraffin wax, microtome sectioned and stained with hematoxylin and eosin.

al. 1983). In 1987, Morales et al. found vitamin A-deficient rats supplemented with retinol had synchronous stage development of seminiferous tubules. In a recent study, spermatogonia from vitamin A-deficient rat testes were characterized and remaining A spermatogonia were unable to differentiate into the A_1 spermatogonia without the addition of retinol (van Pelt et al. 1995). Spermatogenesis is blocked in two places in vitamin A-deficient animals: (1) first division of stem cells which provide spermatogonia; (2) primary spermatocytes divide to form the secondary spermatocytes (Ismail et al. 1990 and Zheng et al. 1996).

Vitamin A plays a critical role in spermatogenesis although the biochemical mechanisms for this process are unclear. Retinoic acid and its metabolite, 4-oxo-retinoic acid, reinitiate spermatogenesis when given to vitamin A-deficient rats at increased doses (van Pelt et al. 1991 and Gaemers et al. 1996). Retinoid receptors play an important role in spermatogenesis. RARα mRNA is regulated during the spermatogenic cycle with a seven fold increase at the stage where spermatocytes are moved from the basal to adluminal compartment. RARα is probably required for movement across the tight junctional complexes (Kim et al. 1990). Using normal adult rat testes, RARα was highest in round spermatids at stage VII and then decreased dramatically at stage IX as spermatids differentiated into elongated spermatids. RARα was found in primary spermatocytes and is important for differentiation of Sertoli cells (Akmal et al. 1997). RARα knockout mice were observed to have a testicular phenotype similar to vitamin A-deficient rats (Lufkin et al. 1993). RARα in vitamin A-deficient rat testes is decreased in early germ cells or inactivated in advanced germ cells and may lead to cell apoptosis (Akmal et al. 1998). Testicular gene expression of RARα, RARβ and RARγ in normal and deficient rats was examined after injection of retinoic acid. In normal rats, there was no significant increase in RARα, RARβ or RARγ after treatment with retinoic acid . In vitamin A-deficient rat testes, RARβ was induced after treatment with retinoic acid with no change in RARα or RARγ (Gaemers et al. 1997). RXRα, RXRβ and RXRγ were found in normal and

vitamin A-deficient rat testes but there was no significant increase in induction upon treatment with retinoic acid (Gaemer et al. 1997). Other retinoid binding proteins have been examined in rat testes. RBP mRNA is only expressed in Sertoli cells and is not detected in other testicular cells while CRABP mRNA was only detected in germ cells (Eskild et al. 1991). Clearly, spermatogenesis is a complex process in which vitamin A plays a critical part although the mechanisms underlying this process are still unclear.

Retinoids are important for maintaining normal epithelium in many tissues. The present study reports the histopathological changes in vitamin A-deficient rats fed either all-*trans*-retinoic acid or one of the four heteroarotinoids. Test compounds were evaluated for their ability or inability to replace vitamin A in the tissues and/or toxicity. The effects of different doses of the same compound or different compounds were compared.

Materials and Methods

Animals

The animals from the growth promoting assay (Chapter II) were used in the histopathology examinations. Description of the rats, animal care and feeding procedures are given in the Materials and Methods in Chapter II. Two feeding trials were conducted in separate years and are designated as feeding trial one (FT1) or feeding trial two (FT2), respectively. Briefly, F-344 male rats (Charles Rivers Laboratory, Wilmington, MA) were obtained at seventeen days, weaned at approximately 20 days and maintained on the vitamin A-deficient diet shown in Table 4 until they were approximately 9 weeks of age and had reached a weight plateau. Animals were supplemented with 45 μg/day of all-*trans*-retinoic acid for two consecutive weight gain-weight loss periods. Rats were approximately 13 weeks old and were synchronously vitamin A-deficient. After 6-7 days of *t*-RA supplementation, rats were randomly divided into 4 rats per group. Each group contained rats classified as heavy, light or intermediate based on body weight. Each group contained one heavy, one light and two intermediate rats. Four heteroarotinoids (HS, HS2, HN and HN2) and *t*-RA (see Figure 6 for structures) were fed at a level of 10, 100

or 200 μg/rat/day for approximately 22 days. Each group of rats was fed one compound at a given dose. For example, HS-10 rats were fed HS at a level of 10 μg/rat/day for 22 days. At the end of the supplementation period, rats were approximately four months of age and were killed by an overdose of CO_2 (rats in the HN2-10, 100 and 200 groups were terminated on day 18 because they were moribund). F-344 control rats (4 animals for FT1 and 5 animals for FT2) were age matched and were maintained on Purina 5001 laboratory chow (Ralston Purina, St. Louis, MO).

Rat Necropsy Procedure

In the text formalin will refer to 10% neutral buffered formalin which was supplied at 10 times the tissue volume in order to achieve adequate tissue fixation.

Rats were pinned to the necropsy board and the fur wetted. All animals were examined for exudate from the eyes, mouth and anal-genital region. Animals were checked for physical signs of abnormal growth. Skin over the inguinal canal region was cut with scissors and reflected cranially over the abdominal and thoracic walls. The abdominal wall musculature was cut and removed. Ribs were cut on both sides with scissors and removed. The stomach and intestinal tract was infused with formalin using a 23 gauge needle and 10 ml syringe. The esophagus was transected immediately caudal to the diaphragm and the gastrointestinal tract was removed and placed in formalin. The spleen and liver were removed and the liver was sliced into 3mm sections and both organs were placed in formalin. The pelvis was split with small bone cutters and the testes were pushed up through the inguinal canal and into the abdominal cavity. Testes, accessory sex glands, penis, urinary bladder and remaining colon were removed through the split pelvis. The tunica albuginea was punctured with a scalpel to allow for better formalin penetration of the testes and all tissues were placed in the formalin. Kidneys were removed, sliced in half lengthwise and placed in formalin. Adrenals glands, located at the cranial of the kidney in the perirenal fat, were removed and placed in formalin. The right jaw was cut with bone cutters and the skin reflected over the chest and neck, and the salivary glands and lymph

nodes were removed and placed in formalin. The heart was removed, cut in half lengthwise and placed in formalin. Grasping the tongue, the entire pluck (tongue, trachea and lungs) was reflected caudally and removed. Trachea and lungs were infused with formalin. The carcass was placed in sternal recumbancy and the skin was incised over the neck where the head attaches. The remaining skin was reflected posteriorly and removed. Using scissors, a 1 cm-strip of skin was cut from over the dorsolateral aspect of the shoulder and placed in formalin. The head was removed using bone cutters and remaining skin reflected anteriorly. The calvarium was cut open using scissors at a 90° angle to the bone and the brain was exposed. The skull with brain *in situ* was placed in formalin. Both rear legs were removed at the coxofemoral joint and the muscle was trimmed away from the bone and the rear limb was placed in formalin. The remaining skinned carcass was placed in formalin.

Rat Serology

Serology profiles were done on four randomly selected FT1 and FT2 rats. Briefly, rats were lightly anaesthetized with CO_2. Blood was collected using a 20 gauge needle inserted into the heart. Approximately, 1 ml of blood was collected, serum was removed and sent to the University of Missouri for a basic serology profile designed to test for exposure to the following: rat corona virus, reovirus 3, sendaivirus, lymphocytic choriomeningitis, pneumonia virus of mice, parvovirus, *Mycoplasma pulmonis* and Theiler's murine encephalomyelitis virus.

Tissue Preparation

After necropsy, 0.5 cm thick or smaller tissue sections were fixed in 10% neutral buffered formalin for 24 to 48 hours. Selected tissues (Table 5) were trimmed to approximately 3 mm thick, placed in plastic tissue cassettes and processed by dehydration in sequentially increasing concentrations of ethanol (from 70% to 100%) and embedded in paraffin wax at 60 °C using a Leica TP 1050 automated vacuum tissue processor. Samples were microtome sectioned at 5 mm and stained with hematoxylin and eosin using an

Table 5: Selected Tissues Examined in the Histopathology Study

Tissue	Tissue
Brain	Liver
Cerebrum	Spleen
Cerebellum	Kidneys
Eye	Urogenital Tract
Lymph Nodes	Bladder
Mandibular	Seminal Vesicles
Heart	Vas deferens
Thymus	Epididymis
Respiratory Tract	Prostate
Trachea	Testes
Lungs	Glands
Gastrointestinal Tract	Hardian
Tongue	Salivary
Stomach	Submandilar
Pancreas	Sublingual
Duodenum	Parotid
Jejunum	Thyroid
Cecum	Parathyroid
Ileum	Coagulation
Skeletal Muscle	Skin
Bones	Brown Fat
Tibia	
Femur	
Stifle Joint	

automated Fisher Histomatic Slide Stainer. After the slides were stained, each tissue sample was examined in a blind fashion for evidence of vitamin A deficiency, or toxicity, inflammation or other abnormal condition.

Results and Discussion

Gross Pathology

Rats in the HN-10, 100 and 200 and HS-10 groups had crusting around the eyes before necropsy. Animals fed HN2-10, 100 and 200 had weight loss, decreased abdominal fat, eye crusting and humped posture. Seminal vesicles were grossly smaller in vitamin A-deficient animals versus control animals but the size variation was not group or dose specific. Animals in both trials had liver and kidneys with normal coloration and texture although livers were slightly smaller in vitamin A-deficient animals versus control animals. Deficient animals had a rougher haircoat then normal animals. Abnormal growth was not observed in any animal during the necropsy procedure. Animals in both trials exhibited signs of vitamin A deficiency but did not have evidence of toxicity.

Histopathology for Animals in Feeding Trial One and Two

The following tissues had a normal histopathological appearance when examined in all FT1 and FT2 animals: brain, lymph nodes, thymus, skeletal muscle, skin, brown fat, liver, spleen, kidneys, respiratory tract, gastrointestinal tract, femur and tibia.

Inflammation

Rats in the following dosing groups HS-10, 100 and 200 and HN-200 had suppurative to less frequent pyogranulomatous inflammation (pus filled nodules containing mononuclear phagocytes) in the accessory sex glands (seminal vesicles, prostate, bulbourethral and urethral glands) while HN-10 and HS-100 had inflammation of the Hardian gland. Lesions were not seen at necropsy and a bacterial culture was performed. Gram stains of the affected tissue did not reveal an etiological agent. Lesions found in the accessory sex glands may have been due to infection by one or more generally facultative pathogens commonly found in the urogenital tract of laboratory rats, such as *Escherichia*

coli, *Proteus vulgaris* or a staphococcus or streptococcus organism (Benirschke et al. 1978). Squamous metaplasia (normal epithelium transformed into stratified epithelium) of the urinary bladder was observed in one HN-200 rat and is believed to be associated with the coagulation gland adenititis. There was no gross or histologic evidence in this rat of squamous metaplasia in the other tissues examined.

Rats in feeding trial two fed RA or HS2 at 10, 100 or 200 μg/day demonstrated suppurative inflammation involving accessory sex glands, and/or heart and/or eyes. All rats in each of the HN2 dosing groups had suppurative inflammation of the accessory sex glands, and/or heart and/or eye. Lesions were not observed at the time of necropsy and no attempt to culture bacteria was made. Etiological agents were not identified but it is believed that inflammation foci developed in response to pathogens such as *E.coli*, *P. vulgaris*, streptococcus or staphylococcus organism isolated in the urogenital tract of laboratory rats (Benirschke et al. 1978). As discussed in chapter two, deficient animals succumb to infections before many signs of vitamin A deficiency appear (Beaver 1961). Vitamin A-deficient animals have a decreased number of neutrophils (Twining et al. 1997), NK cell activity (Bowman et al. 1990), antibody production (Pasatiempo et al. 1990) and compromised mucosal immunity (Semba 1998 and Wiedermann et al. 1995). In FT1 and FT2, only vitamin A-deficient rats developed non-specific inflammatory foci regardless of treatment group or dose while the control group did not have evidence of non-specific infection suggesting vitamin A supplemented rats had a better functioning immune system. The histopathology results for rats in FT1 and FT2 are summarized in Table 6.

Other signs of vitamin A deficiency (inflammation of the eye, retinal atrophy, optic nerve degeneration, squamous metaplasia of mucus-secreting surfaces or defective bone reabsorption) were not observed. Vitamin A toxicity such as excessive soft tissue calcification irregular closure or degeneration of growth plates or perisinusoidal hepatic fibrosis was also not seen in any animals (Jones et al. 1997).

Table 6: Histopathology Results for F-344 Rats in FT1 and FT2[1]

Group	Conjuctivitis	Myocarditis	Prostatitis	Coagulation Gland or Seminal Vesicle Adenititis	Salivary Gland Aden-ititis
FT1:					
RA-10	0	0	0	0	0
RA-100	0	0	0	0	0
RA-200	0	0	0	0	0
HN-10	1	0	0	0	0
HN-100	0	0	0	0	0
HN-200	0	0	1	1	0
HS-10	0	0	1	1	0
HS-100	1	0	0	1	0
HS-200	0	0	0	1	0
FT2:					
RA-10	0	1	2	0	0
RA-100	0	2	1	1	0
RA-200	0	1	2	2	0
HN2-10	2	0	0	2	3
HN2-100	3	1	2	0	3
HN2-200	2	0	1	2	3
HS2-10	0	2	3	0	1
HS2-100	0	0	2	2	0
HS2-200	0	0	3	2	0

1. Numbers in the table represent the number of rats with that condition. All treatments groups had 4 animals per group (n = 4).

Conjuctivitis: inflammation of the mucous membrane of the eye

Myocarditis: inflammation of the muscle walls around the heart

Prostatitis: inflammation of the prostate

Adentitis: inflammation of the lymph nodes or glands

Spermatogenesis

A common finding in all FT1 treated groups is a lack of spermatogenesis while age-paired control animals had active spermatogenesis. Photomicrographs of the seminiferous tubules of control, RA-200, HN-200 and HS-200 animals are shown in Figures 18, 19, 20 and 21, respectively. The seminiferous tubules of rats fed 10 or 100 μg/day of RA, HN or HS are similar to those shown in Figures 19, 20 and 21, respectively. The seminiferous tubules of vitamin A-deficient rats are smaller than control rat seminiferous tubules and frequently lack basement membrane contact between adjacent tubules. These findings are observed in all treated groups and are not compound or dose specific. Rats in the treatment groups lack spermatids in the seminiferous tubules. This is a result of germ cell depletion, because vitamin A deficiency was imposed on the animals prior to feeding the test compounds. The prepubertal phenotype is consistent in all treatment groups and is not compound or dose specific.

A typical finding in treated FT2 rats is lack of active spermatogenesis in the seminiferous tubules of the testes. Vitamin A-deficient rats also had smaller seminiferous tubules when compared to control rat tissue and had lost basement membrane contact between adjacent seminiferous tubules. This was observed for all vitamin A-deficient animals and was not group or dose specific. Treated animals have a prepubertal phenotype with a lack of spermatids in the lumen of the seminiferous tubules. This phenotype results from germ cell depletion due to vitamin A-deficiency and is not specific to treatment group or dose. The photomicrographs of seminiferous tubules of rats fed Purina 5001 laboratory chow, RA-200, HN2-200 or HS2-200 are shown in Figures 22, 23, 24 and 25, respectively. Animals fed 10 or 100 μg/day of RA, HN2 or HS2 had seminiferous tubules resembling those shown in Figures 23, 24 and 25, respectively. The seminiferous tubules of vitamin A-deficient rats resemble the histological changes observed by Wolbach and Howe in 1925: "The liquid lies outside the basement membrane, the connective tissue between the tubules does not seem to be permeated by liquid, so that the appearance in

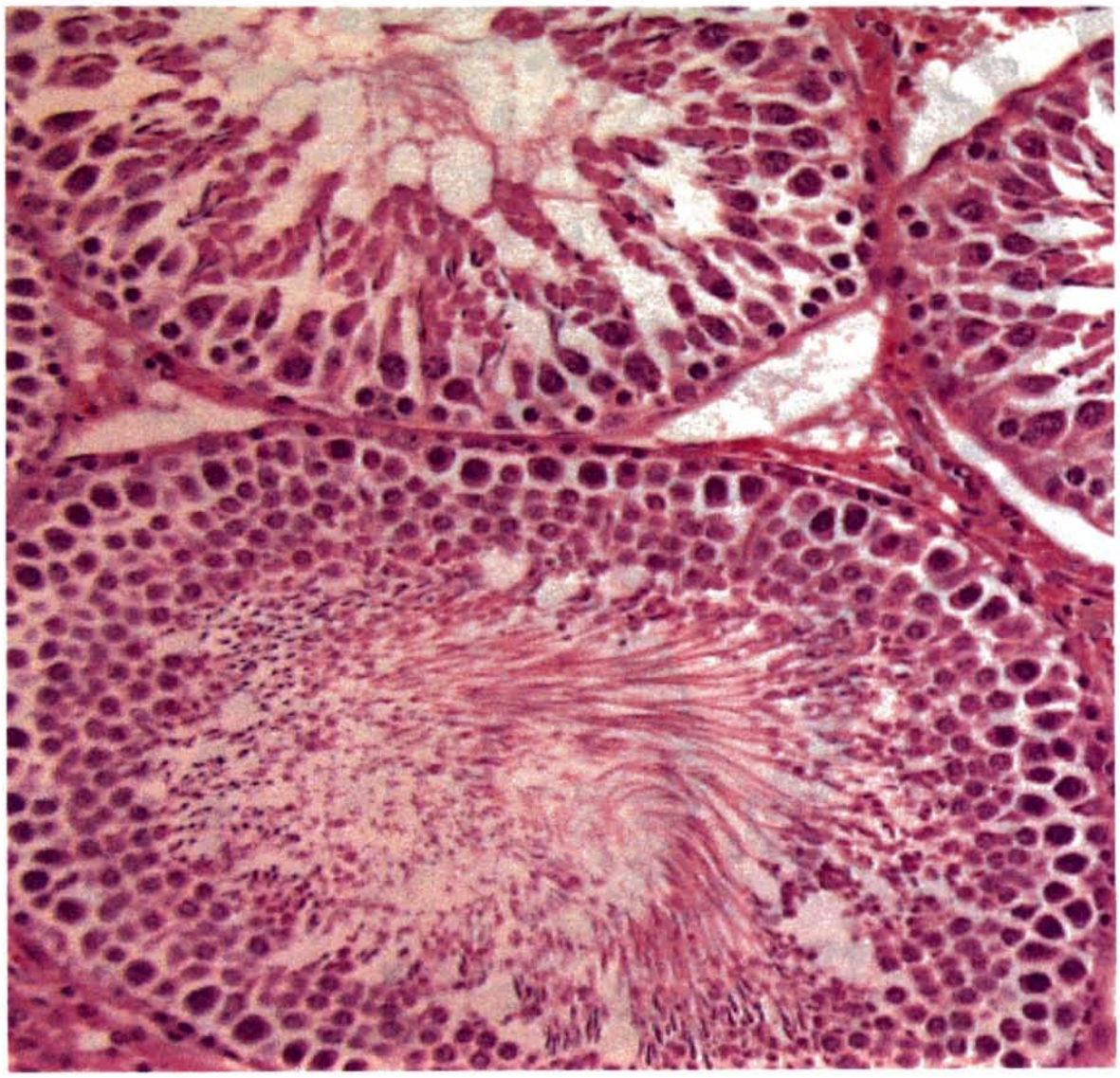


Figure 18: Photomicrograph of FT1 Rat Seminiferous Tubules. Control rats were fed Purina 5001 Laboratory Chow. Magnification 320x.

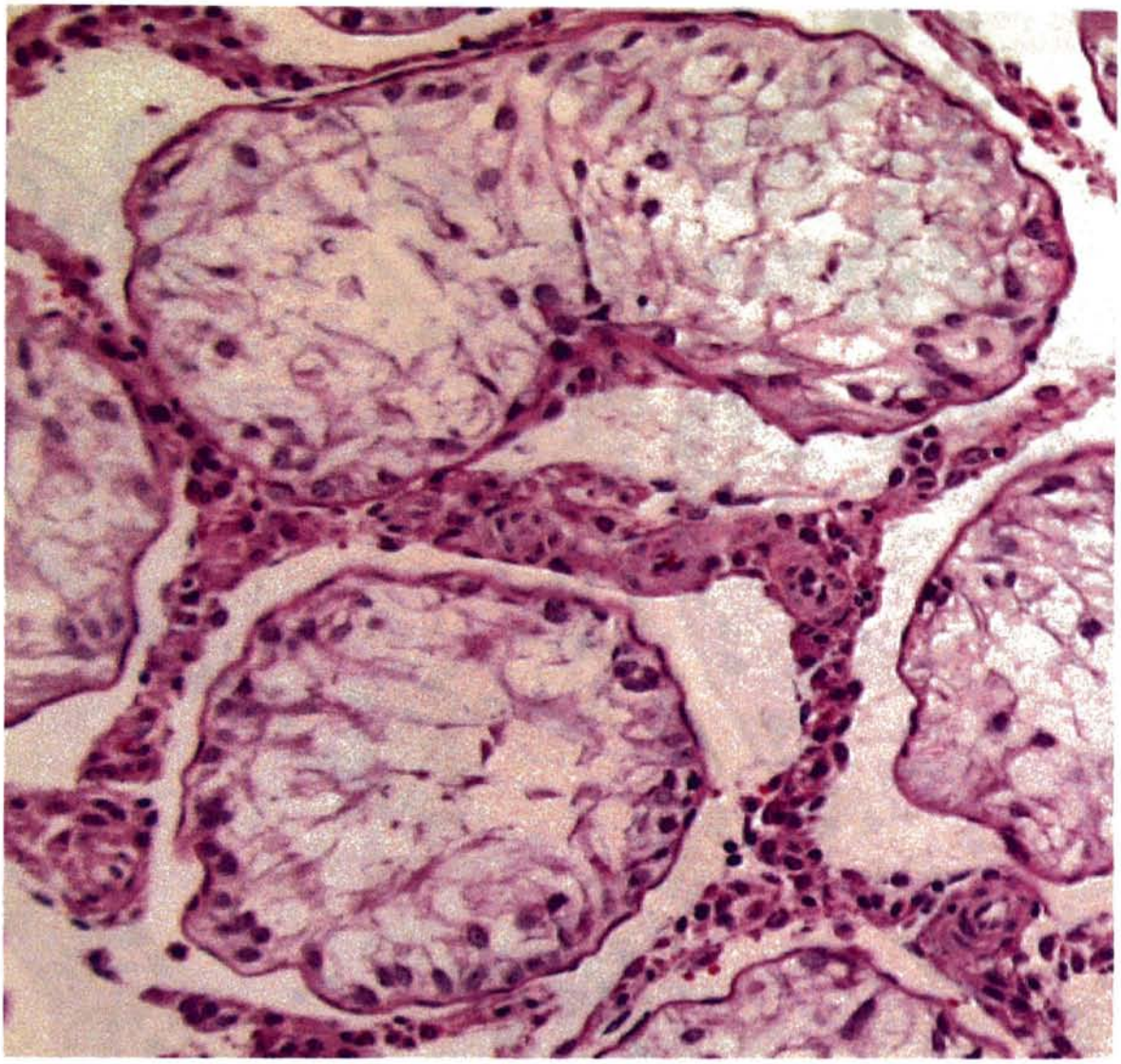


Figure 19: Photomicrograph of FT1 Rat Seminiferous Tubules. Rats were fed 200 μg/day of RA for 21 days. Magnification 320x.

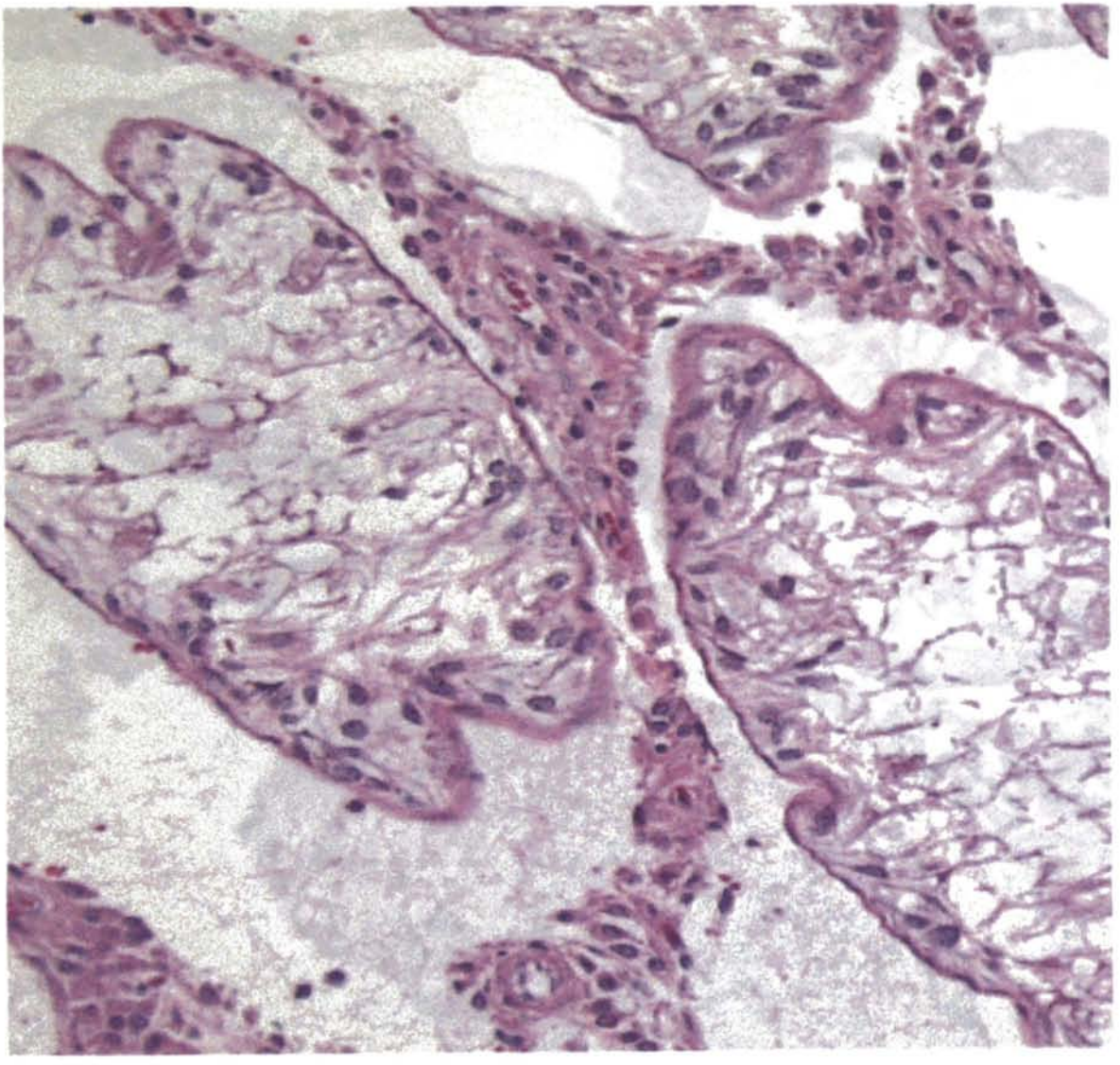


Figure 20: Photomicrograph of FT1 Rat Seminiferous Tubules. Rats were fed 200 µg/day of HN for 21 days. Magnification 320x.

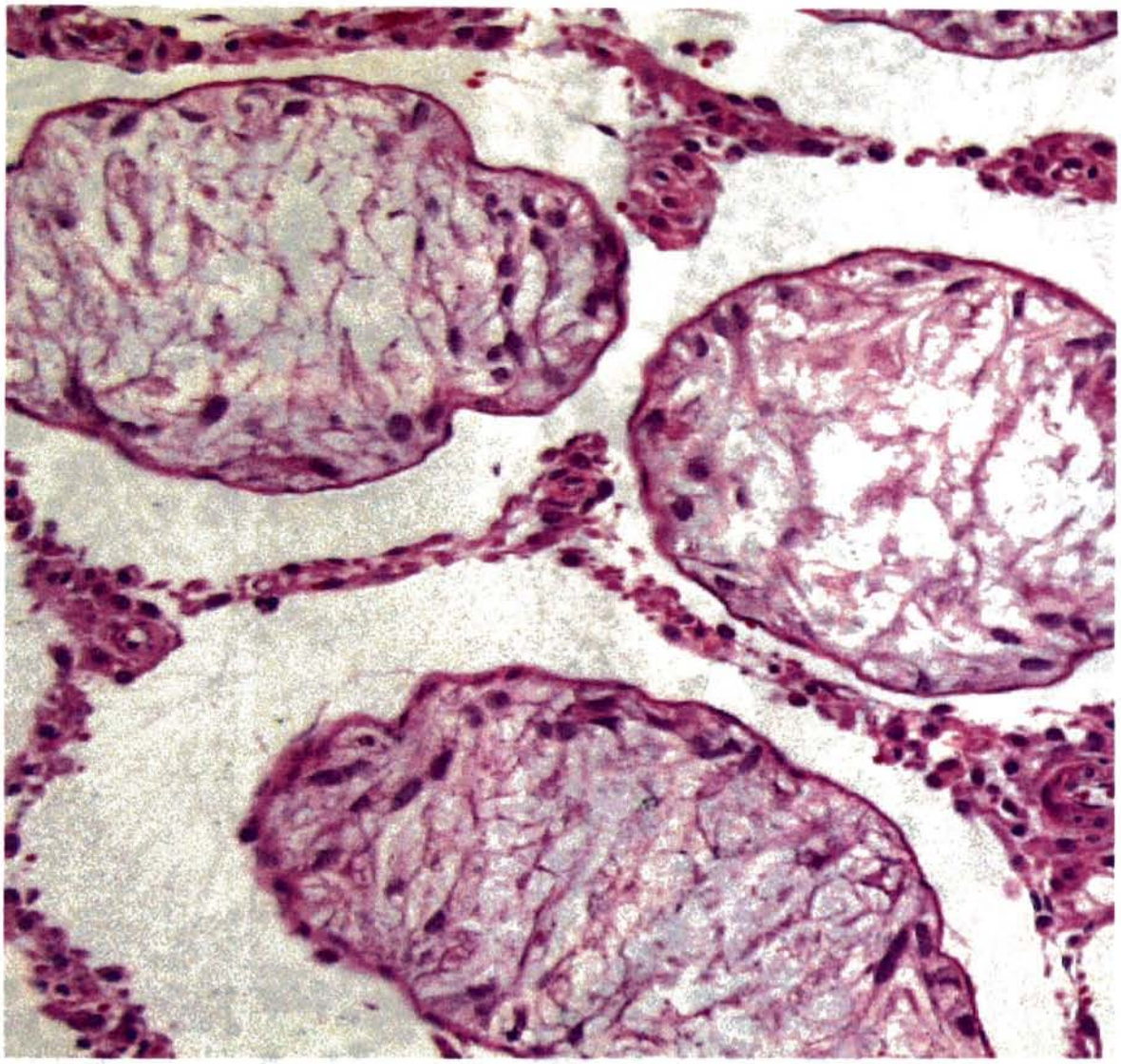


Figure 21: Photomicrographs of FT1 Rat Seminiferous Tubules. Rats were fed 200 µg/day of HS for 21 days. Magnification 320x.

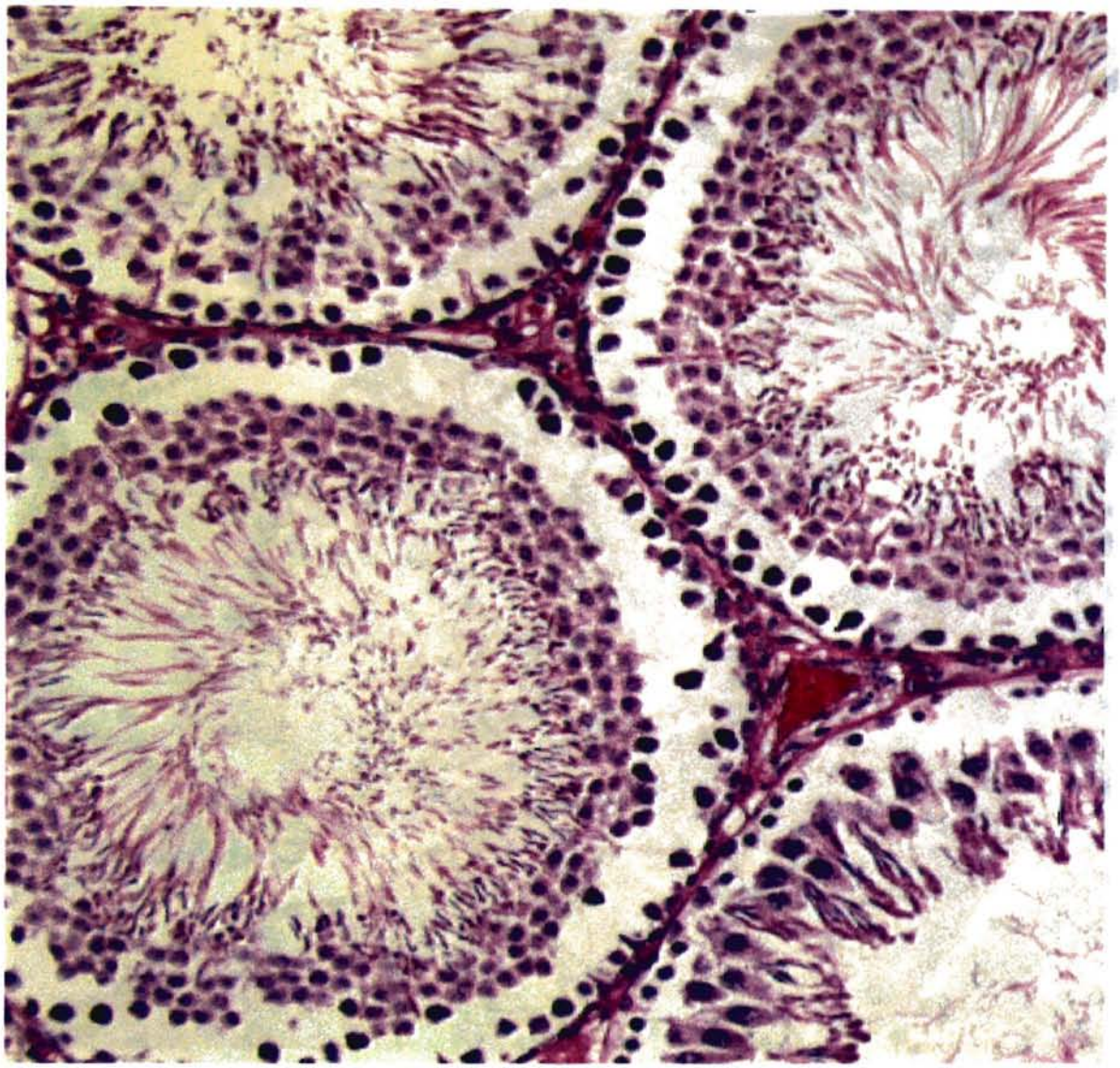


Figure 22: Photomicrograph of FT2 Rat Seminiferous Tubules. Control rats were fed Purina 5001 Laboratory Chow. Magnification 320x.

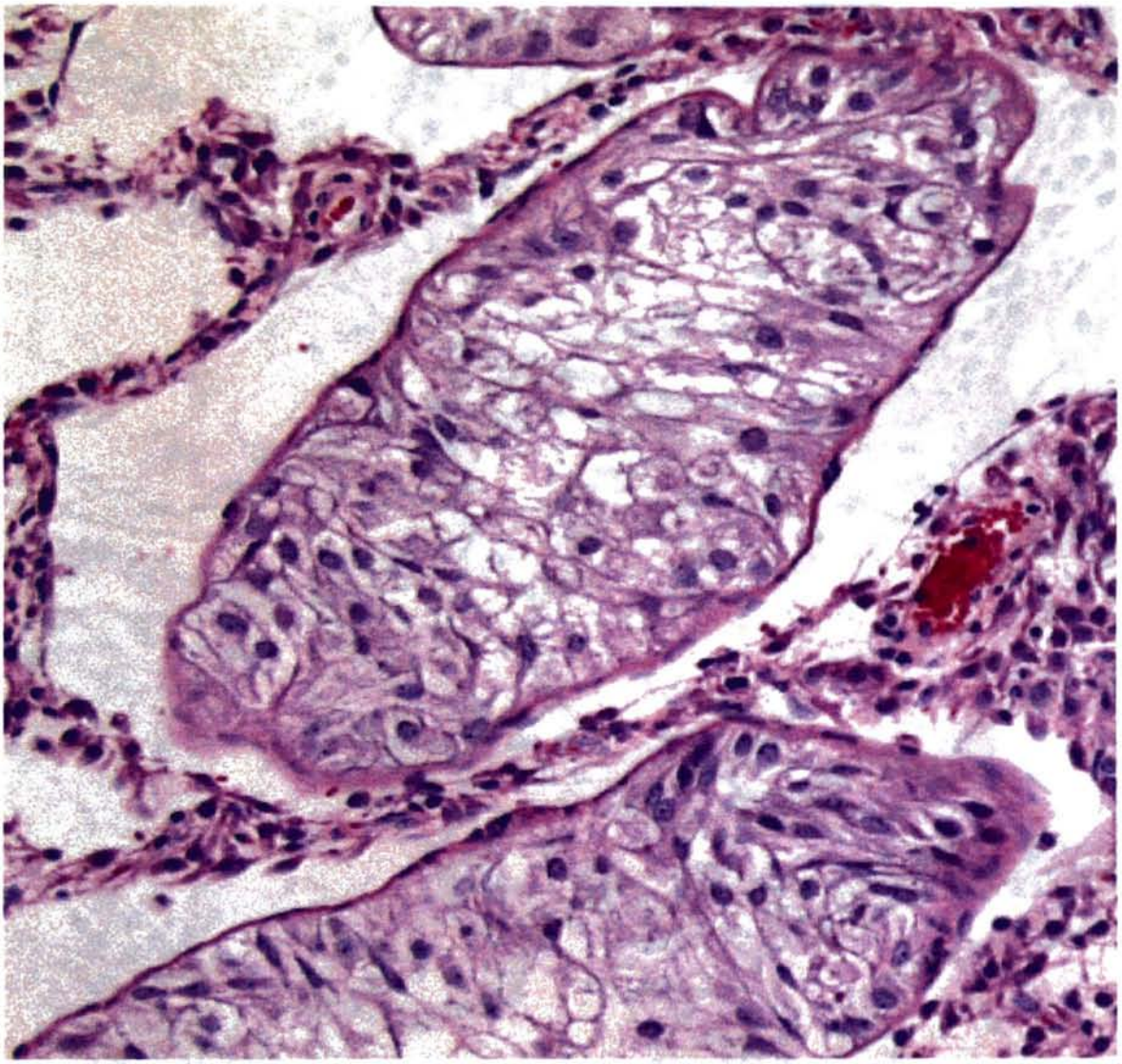


Figure 23: Photomicrograph of FT2 Rat Seminiferous Tubules. Rats were fed 200 µg/day of RA for 22 days. Magnification 320x.

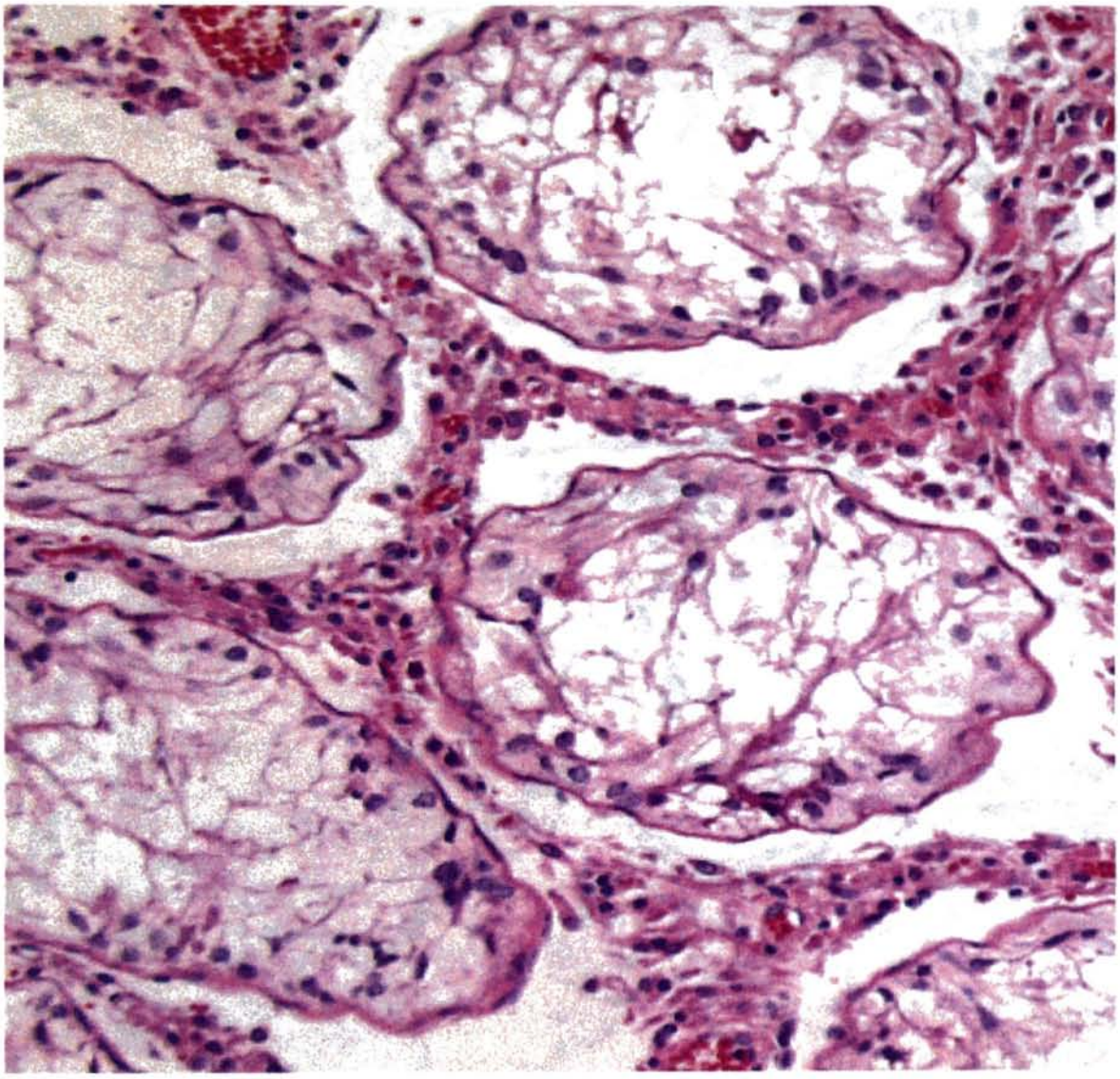


Figure 24: Photomicrograph of FT2 Rat Seminiferous Tubules. Rats were fed 200 μg/day of HN2 for 18 days. Magnification 320x.

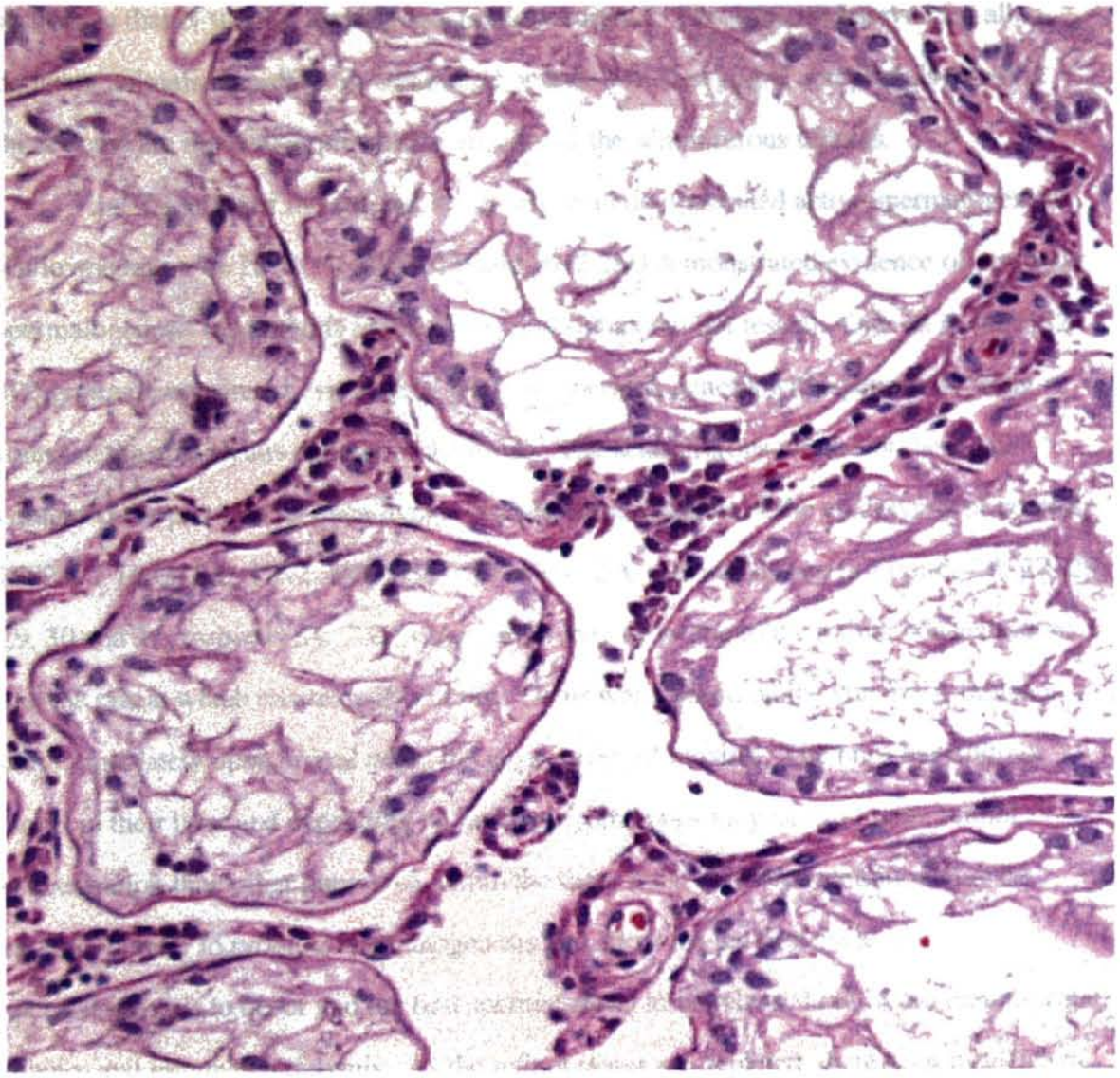


Figure 25: Photomicrograph of FT2 Rat Seminiferous Tubules. Rats were fed 200 μg/day of HS2 for 22 days. Magnification 320x.

sections is that of tubules floating in a liquid medium." The same is observed for all deficient animals regardless of compound or dose which suggests compounds tested at these doses are inactive in preventing atrophy of the seminiferous tubules.

In FT2, none of the rats fed RA at any level demonstrated active spermatogenesis while one rat in the HN2-10, HN2-100 and HN2-200 demonstrated evidence of previous spermatogenesis (spermatids found in the epididymis but not testes). One rat in the HS2-100 group demonstrated evidence of previous spermatogenesis with spermatids being found in the epididymis and not in the testes. The seminiferous tubules of these rats contained the prepubertal phenotype. The photomicrographs of the epididymis of control, HN2-10, HN2-100, HN2-200, HS2-100 or RA-200 rats are shown in Figures 26, 27, 28, 29, 30 and 31, respectively. This observation may be to due to the retinol concentrations and the rate at which rats sexually mature. Dams were placed on the vitamin A-deficient diet immediately upon arrival while the pups were still nursing. The pups have retinol stores in their liver and depending on the dams some pups may have a higher retinol concentration than other pups even within the same litter. This concentration may be enough initially to promote spermatogenesis and allow for the first cycle of spermatogenesis to occur and the first spermatozoon to be released out of the seminiferous tubules and into the epididymis. As the retinol stores are depleted, there is an insufficient retinol concentration to promote spermatogenesis as evidenced by lack of spermatids in the seminiferous tubules. Furthermore, rats in these groups might have reached sexual maturity earlier when the retinol stores were not yet totally depleted. Other explanations for this observation may be that HN2 or HS2 promotes spermatogenesis in vitamin A-deficient rats. Although if this was the case, all rats fed HS2-100 or HN2 at any level would show evidence of active spermatogenesis in the epididymis and the seminiferous tubules.

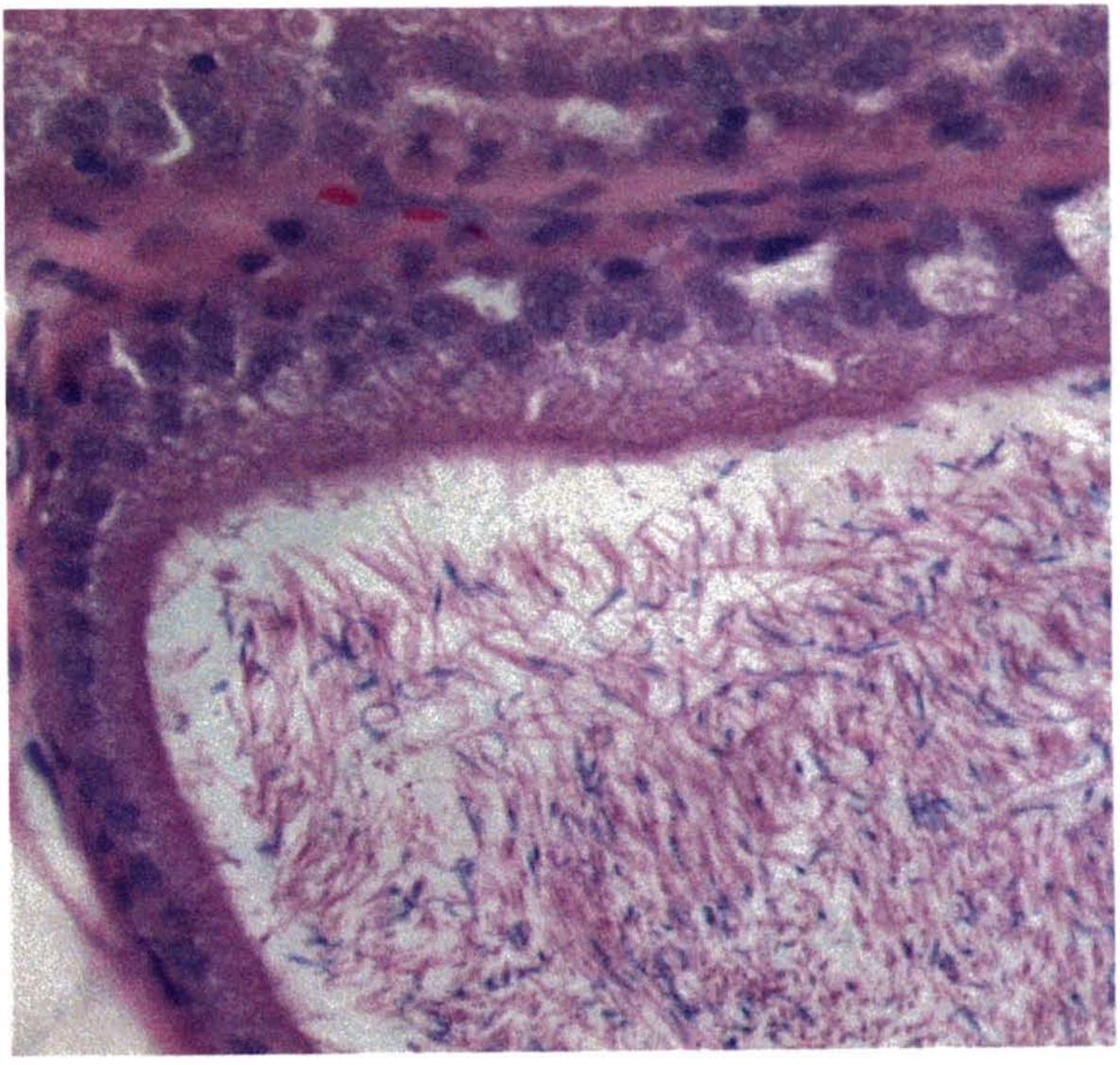


Figure 26: Photomicrographs of Rat Epididymis. Rats were fed Purina 5001 Laboratory Chow for 22 days. Magnification 640x.

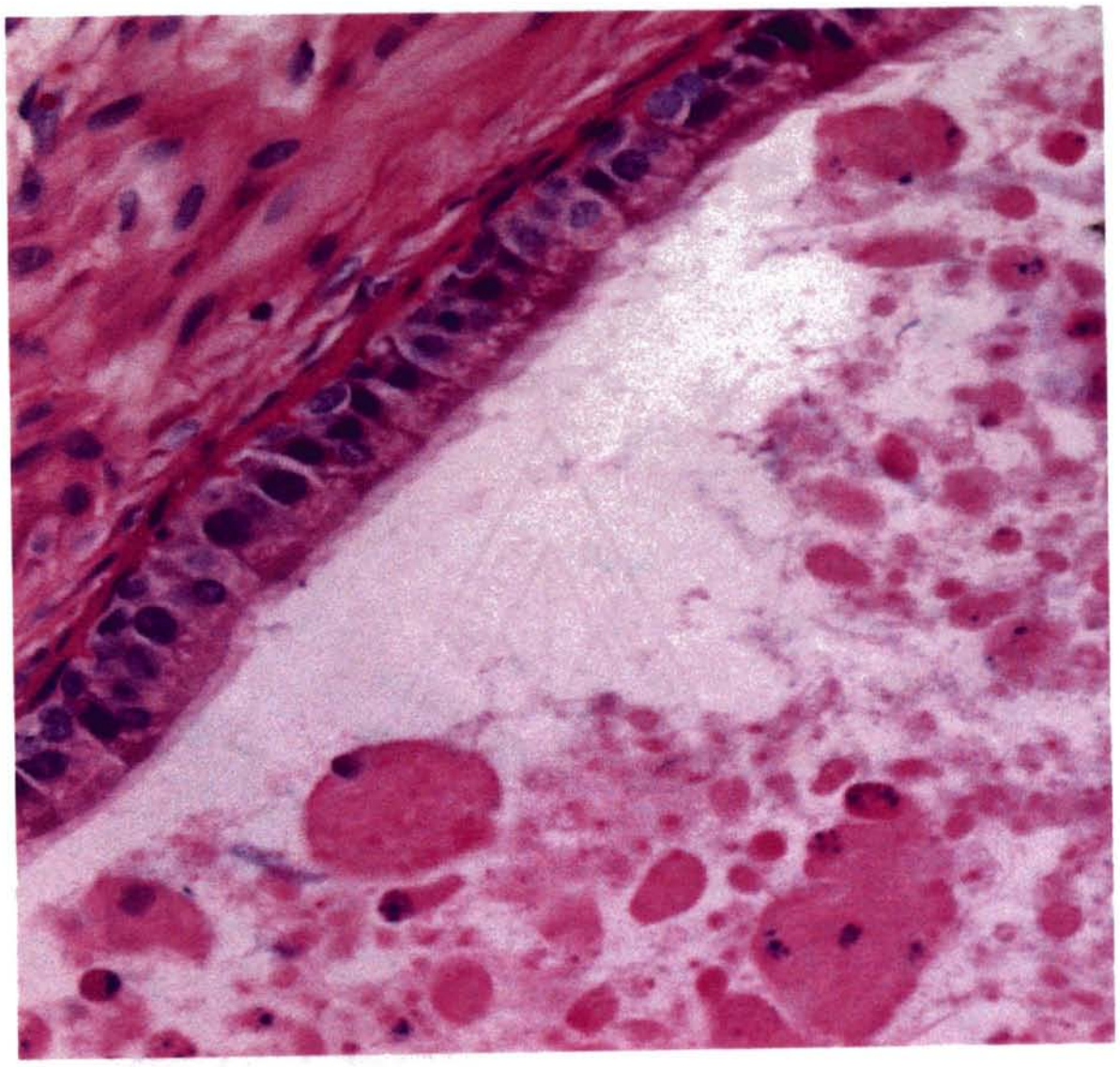


Figure 27: Photomicrograph of Rat Epididymis. Rats were fed 10 μg/day of HN2 for 18 days. Magnification 640x.

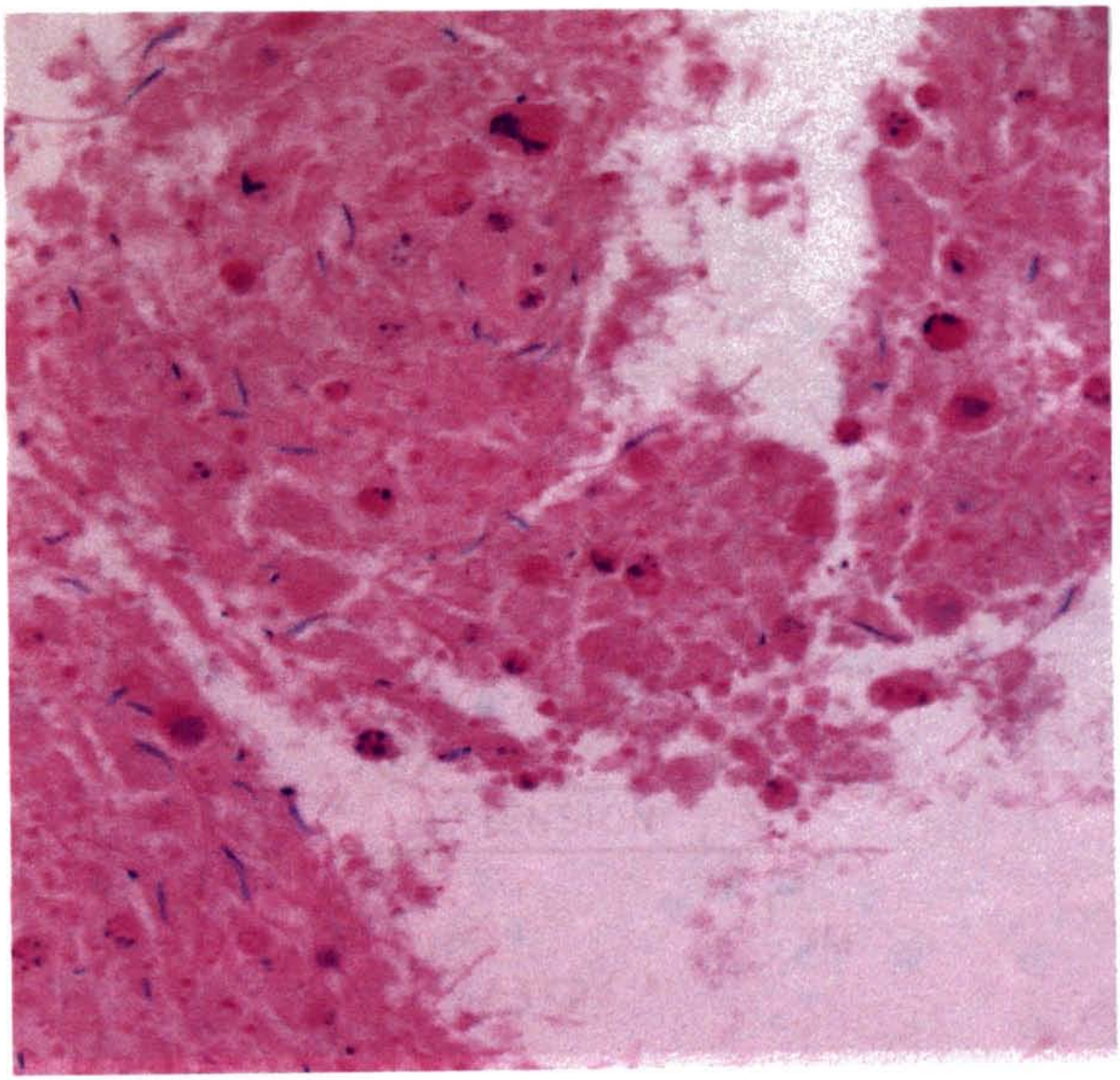


Figure 28: Photomicrograph of Rat Epididymis. Rats were fed 100 μg/day of HN2 for 18 days. Magnification 640x.

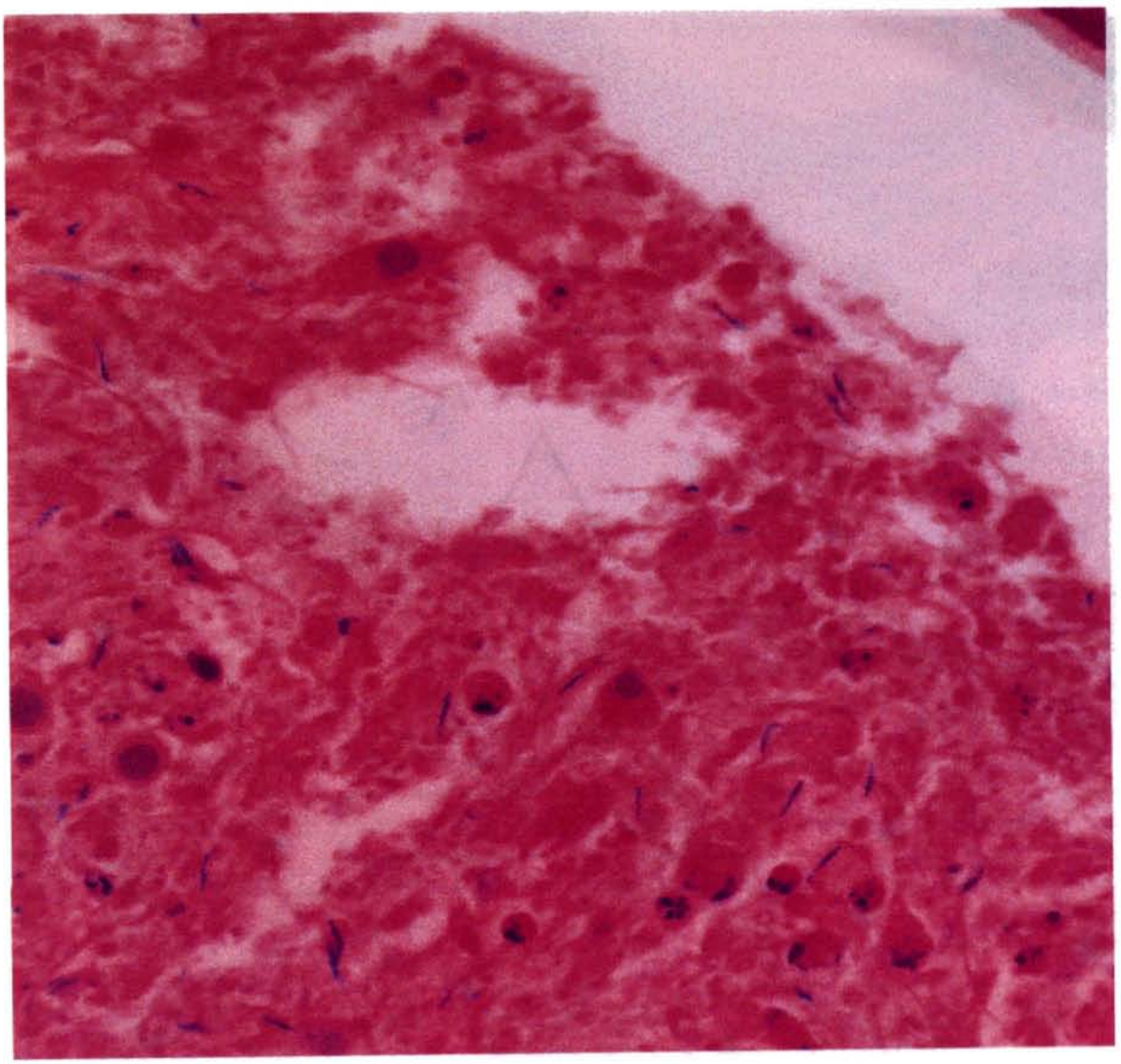


Figure 29: Photomicrograph of Rat Epididymis. Rats were fed 200 µg/day of HN2 for 18 days. Magnification 640x.

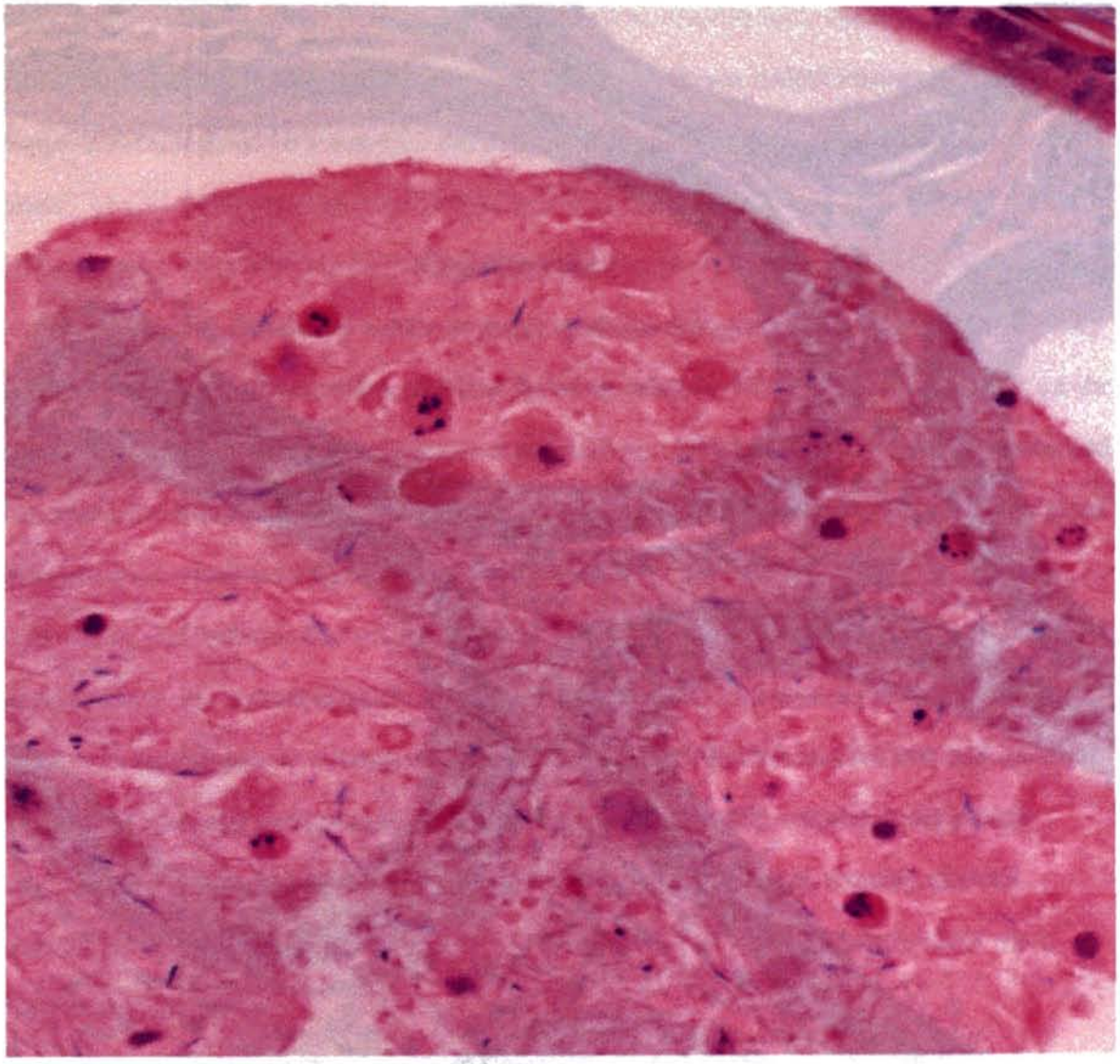


Figure 30: Photomicrograph of Rat Epididymis. Rats were fed 100 μg/day of HS2 for 22 days. Magnification 640x.

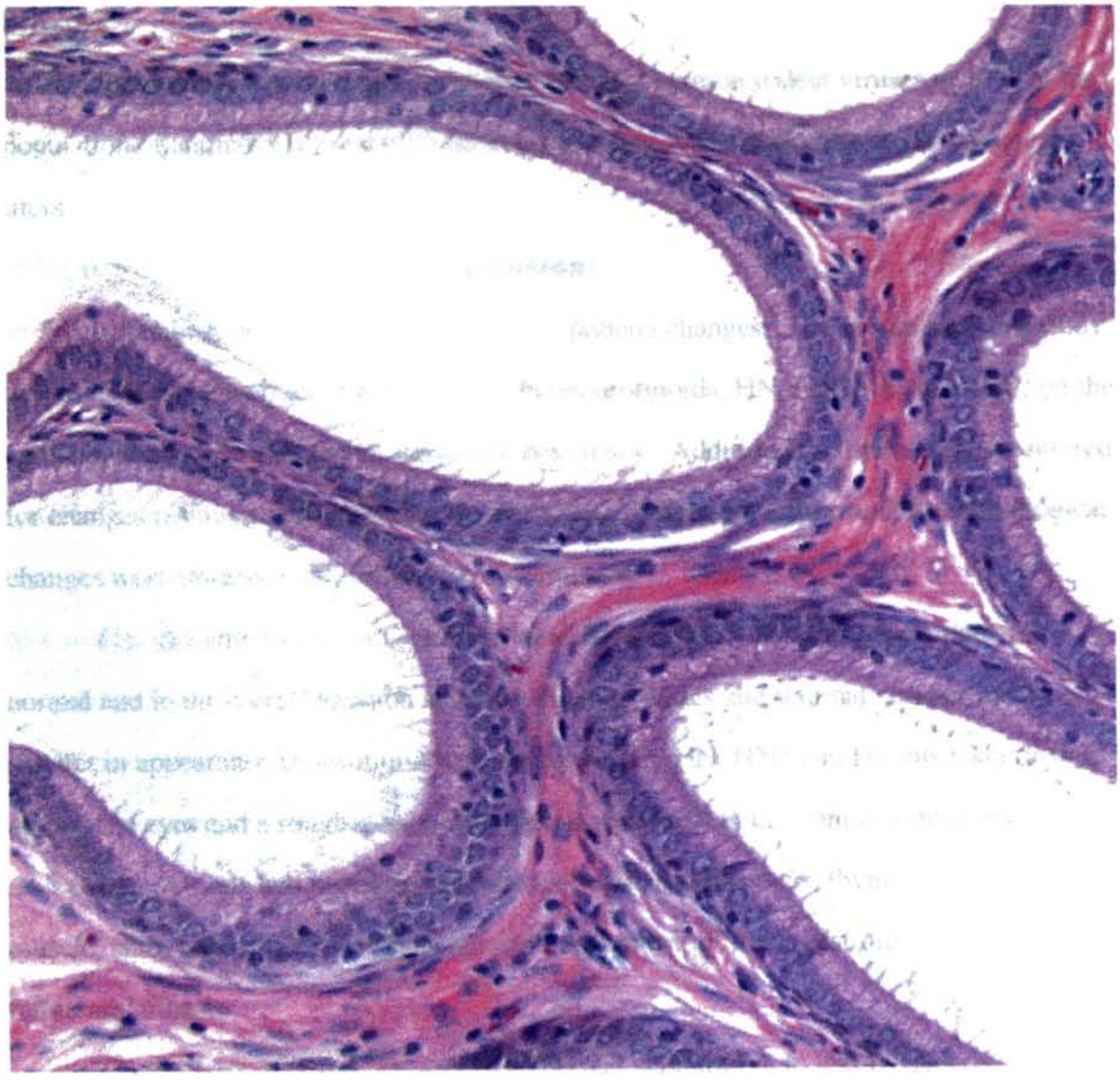


Figure 31: Photomicrograph of Rat Epididymis. Rats were fed 200 μg/day of RA for 22 days. Magnification 320x.

Serology

Random testing for exposure or infection by common rodent viruses in 10% of the population of animals FT1 and FT2 did not reveal any significantly elevated serological titers.

Conclusions

Vitamin A deficiency results in many epithelia changes. The objective of this study was to evaluate the effect of RA and four heteroarotinoids, HN, HN2, HS and HS2, on the epithelia commonly affected by vitamin A deficiency. Additionally, tissues were monitored for changes resulting from heteroarotinoid toxicity. Gross pathology and histopathological changes were observed and summarized as follows:

(1). All animals had no abnormal conditions or growth. All organs appeared normal and in the correct location although the liver, testes and seminal vesicles were smaller in appearance in vitamin A-deficient animals. HN, HN2 and HS rats had crusting around the eyes and a rougher haircoat which is consistent with vitamin A deficiency.

(2). All rats had histologically normal brain, lymph nodes, thymus, skeletal muscle, skin, brown fat, liver, spleen, kidneys, respiratory tract, gastrointestinal tract, femur and tibia.

(3). HS-10, 100 and 200 and HN-200 had suppurative to less frequently pyogranulomatous inflammation in the accessory sex glands. HN-10 and HS-100 had inflammation of the Hardian gland. Squamous metaplasia of the bladder of one HN-200 rat was observed.

(4). Rats in feeding trial two fed RA or HS2 demonstrated suppurative inflammation involving accessory sex glands and/or heart and/or eyes.

(5). Animals fed a vitamin A-deficient diet lacked active spermatogenesis regardless of the treatment or dose although a few animals in the HN2 and HS2 treatment groups had evidence of previous spermatogenesis.

(6). Animals in all treatment groups lacked spermatids as a result of germ cell depletion due to vitamin A deficiency

(7). Toxicity was not observed in any treatment group or dose.

(8). Random testing for exposure or infection by common rodent viruses in 10% of the population of animals FT1 and FT2 did not reveal any significantly elevated serological titers.

The changes observed in this study, such as the reduced size of the seminiferous tubules, lack of spermatids, epithelium changes of the salivary and Hardian glands are characteristic of vitamin A deficiency. Comparisons between compounds and doses of the same compound did not show any obvious differences. Tissues examined had the same histopathology as the tissue from rats treated with RA with a few exceptions such as the epididymis from HN2 and HS2 treated rats. In the eye and urogenital tract, neither RA or the heteroarotinoids tested supported epithelial integrity. These studies show that heteroarotinoids have a similar activity as all-*trans*-retinoic acid in maintaining rat epithelium.

CHAPTER IV

METABOLISM OF SELECT HETEROAROTINOIDS IN VITAMIN A-DEFICIENT RATS

Identification of drug metabolites has become important in clinical toxicology, pharmacology and pharmacokinetics. Reverse-phase HPLC has become a dominant technique for the analysis of metabolites in biological media (Blake et.al. 1989). Retinoids have strong UV absorption due to their conjugated system of double bonds and coupling reverse-phase HPLC with a photodiode array (PDA) detector provides a good system to separate metabolites (Wingerath et al. 1997). However, this technique does not allow for structure elucidation. Mass spectrometry is a powerful technique which gives molecular weight and characteristic fragment ion information which is helpful for structure elucidation.

Vecchi et al. (1967) was the first to obtain mass spectra for vitamin A and some of its isomers. Mass spectra of retinol, retinyl acetate, retinal, retinoic acid, methyl retinoate, anhydroretinol and 9-*cis* -retinal have been obtained (Lin et. al 1970). Mass spectra of vitamin A analogs have also been obtained (Reid et al. 1973). Although GC/MS (gas chromatography/ mass spectrometry) has proved to be a powerful technique in identifying metabolites, retinoids are not well suited for GC/MS. Vitamin A compounds are heat labile and dehydration of retinoids occurs on GC columns (Sporn 1994). Retinoids have been isolated and identified using LC/MS (liquid chromatography/mass spectrometry). Etretinate, a vitamin A analog useful in the treatment of psoriasis, is metabolized to acitretin and isoacitretin. These metabolites were quantitated and identified using LC/MS (Fayer et al. 1991). LC/MS was used to identify isoacitretin and the glucuronide of isoacitretin in prefused rat liver (Cotler et al. 1992). Ranalder et al. (1993) quantitated all-*trans* -retinoic

acid and 4-oxo-retinoic acid in human plasma using LC/MS. Retinoyl glucuronide was identified by LC/MS from intestinal microsomes (Salyers et al. 1993).

Reverse-phase HPLC and mass spectrometry have been great aids in isolating and identifying heteroarotinoid metabolites. Based on retinoid metabolism, potential metabolites of O-DHA ester were synthesized and mass spectra were obtained (Sunthanker et al. 1993 and Thorne 1993). Three bilary metabolites of O-DHA ester were identified in rats. Bile cannulated rats were injected interpertionally with O-DHA ester and bile was collected for six hours. Metabolites were isolated using reverse-phase HPLC, compared to standards and tentatively identified. Mass spectra of the metabolites were obtained and compared to corresponding standards and identified (Thorne 1993). Bilary metabolites are shown in Figure 32.

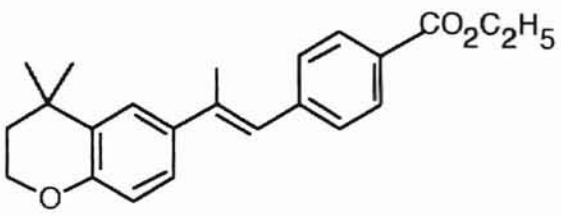
Isolation and identification of new heteroarotinoid metabolites may yield new compounds which have lower toxicity and are more effective than the parent compound. In the present study, metabolites of HS, HS2-ester and C-14 radiolabelled O-DHA* (O-DHA*) were isolated and identified in vitamin A-deficient rat liver, kidneys and/or serum. These compounds were chosen because of their biological activity in the growth assays (Chapter 2). HS, HS2 ester and O-DHA* are shown in Figure 33.

Materials and Methods

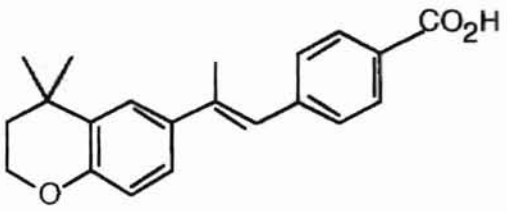
HS metabolism studies were conducted in the same year as feeding trial one (FT1). HS2-ester and O-DHA* studies were conducted in the same year as feeding trial two (FT2)

Animals and Animal Care

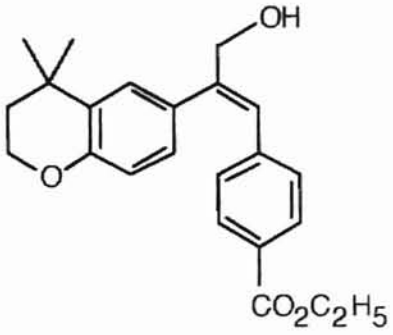
The description's of animals and animal care is given in Chapter II. Briefly, F-344 male rats (Charles Rivers Laboratories, Wilmington, MA) were obtained at seventeen days of age with dams (10 pups per dam) and dams were placed on the vitamin A-deficient diet listed in Table 4 (Harlan Teklad, Madison, WI). At the age of twenty-one (FT1) or twenty (FT2) days pups were weaned and maintained on the same vitamin A-deficient diet. Animals were weighed every two days to monitor their vitamin A-deficiency status. Rats



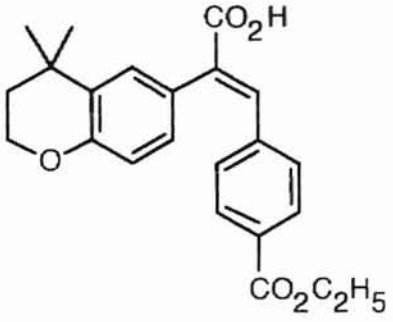
O-DHA ester



Bile Metabolite 1

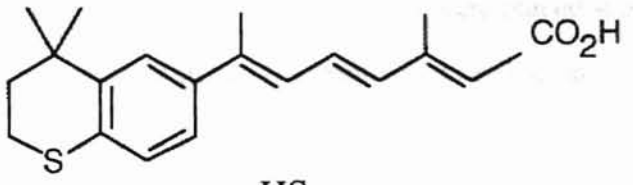


Bile Metabolite 2

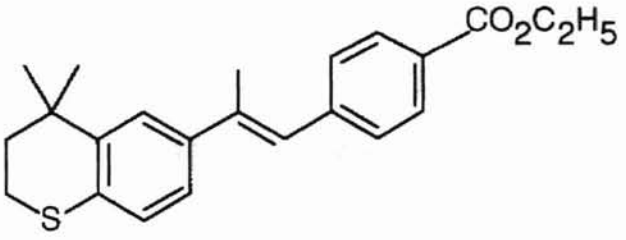


Bile Metabolite 3

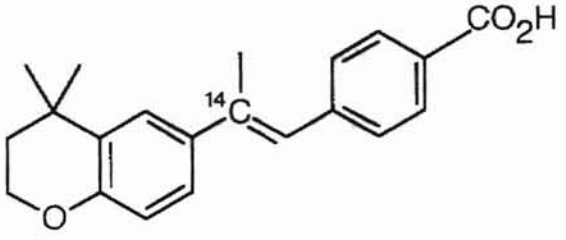
Figure 32: Bilary Metabolites of O-DHA-ester (Thorne 1993).



HS



HS2-ester



O-DHA*

Figure 33: Heteroarotinoids Used in Metabolism Studies.

were placed in individual polycarbonate cages. Cages were placed side-by-side for visual and odor socialization and were switched frequently in a random manner to avoid geographical variations. All procedures were approved by the Laboratory Animal Care Committee at Oklahoma State University and conformed to NIH guidelines. Fresh diet and water were given daily and available *ad libitum*. The room was cleaned daily and cages were changed twice weekly. Animals were maintained on 45 μg/rat/day of *t*-RA until the metabolism study was initiated. All rats were starved overnight prior to the initiation of the metabolism study.

Chemicals

HS, HS2-ester, O-DHA* and heteroarotinoid standards were synthesized by Dr. K.D. Berlin's group in the Department of Chemistry at Oklahoma State University (Waugh et al. 1985, Spruce et al. 1987, Sunthankar et al. 1993 and Liu et al 1999) . All heteroarotinoids received from Dr. Berlin were sealed and tightly capped under nitrogen and were stored in the dark at -20 °C. Other reagents used in the extraction process, analysis, or standard preparation are Optima grade hexane (Fisher Scientific, Pittsburgh, PA), Optima grade methanol (Fisher Scientific, Pittsburgh, PA), HPLC grade water (Fisher Scientific, Pittsburgh, PA), Tracemetal grade acetic acid (Fisher Scientific, Pittsburgh, PA) EcoLume (ICN Biomedical Inc., Aurora, OH), and compressed nitrogen gas (Airgas, Stillwater, OK)

HPLC Gradients and Instrumentation

Two types of HPLC gradients were used to separate heteroarotinoid metabolites and are listed in Table 7. The HPLC gradients consists initially of 75 or 50% methanol/ 25 or 50% 0.01 *M* acetic acid which is employed for 10 minutes. This is followed by a linear gradient up to 100% methanol which is then used to elute for 20 minutes at a flow rate of 1 ml/min. Fractions were collected using a Model 1850 Fraction Collector (ISCO, Lincoln, NE).

Table 7: Gradient Used in Heteroarotinoid Metabolite Isolation and Separation

Gradient #	Gradient	Sample
1	50% CH_3OH:50% 0.1M HAc-Hold 10 min. ↓ 100% CH_3OH- Linear 60 min ↓ 100% CH_3OH-Rinse 20 min	HS 0-DHA*-acid
2	75% CH_3OH:25% 0.1M HAc-Hold 10 min. ↓ 100% CH_3OH- Linear 60 min ↓ 100% CH_3OH-Rinse 20 min	HS2-ester O-DHA*-acid purity check and syringe wash

HPLC analysis was performed with a Waters Model 6000A solvent delivery system, a Whatman Partisil 5 ODS-3, 0.47 x 23.5 cm, 5 μm particle size reverse-phase column (Whatman Inc., Clifton, NJ), a 6 port sample injector (Valco Instruments Co., Houston, TX) and a Waters Millennium Chromatography Manager. The latter consists of a pump control module, a 996 photodiode array detector (PDA), and the Millennium chromatography manager software version 2.1 (Waters Inc., Milford, MA).

Synthesis of HS2-acid

The HS2-acid was prepared from HS2-ester and the protocol is shown in Figure 34. HS2-ester stock solution was prepared in aldehyde free-ethanol at a concentration of 500 μg/ml. Five milliliters of a 1:1 10% KOH/ 95% ethanol solution (v:v) was added to 15 μg of HS2-ester. The sample was incubated in a 45-50 °C water bath for 15 minutes and acidified with 4 *N* HCl until the sample was pH 2. Ten milliliters of hexane was added, the sample was mixed for 30 seconds and placed on ice for 20 minutes. The upper layer was removed and the sample was evaporated to dryness under nitrogen. The residual sample was stored at -20 °C until time of analysis. The sample was analyzed by HPLC using gradient 2.

Administration of HS

For the HS metabolism study, the two rats used had an average weight of 315 g and were 165 days of age. The HS stock solution was prepared in aldehyde free-ethanol at a concentration of 500 μg/ml. Each rat was given 3 g of vitamin A-deficient diet treated with aldehyde free-ethanol (n=1) or 200 μg of HS (n=1). Diets were allowed to evaporate in the dark for 1 hour prior to feeding. Animals were allowed to feed for 2 hours and then were killed with an overdose of CO_2. Liver, kidneys and testes were removed, washed with ice cold distilled water to remove blood and frozen immediately in dry-ice. Samples were stored at -20 °C until time of analysis.

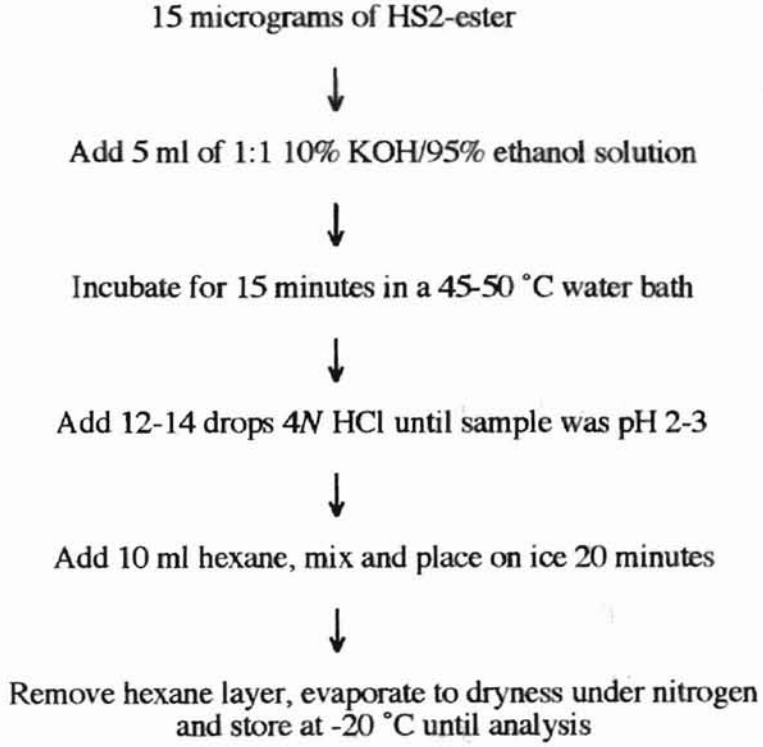


Figure 34: Procedure for HS2-acid Synthesis.

Administration of HS2-ester

For the HS2-ester metabolism study, 10 vitamin A-deficient rats with an average weight of 277 g and 123 days of age were used. The HS2-ester stock solution was prepared in aldehyde free-ethanol at a concentration of 500 μg/ml. Animals were given 5 g of diet which was treated with aldehyde free-ethanol (n = 5) or 200 μg HS2-ester (n = 5). Diets were allowed to evaporate in the dark for 1 hour prior to feeding. Animals were allowed to feed for 4 hours.

Serum was collected on all animals by terminal bleeding. Briefly, rats were lightly anaesthetized, a 1.5 inch 20 gauge needle was inserted into the heart and 1.5-2 ml of blood per rat was collected and immediately stored on ice. Blood was centrifuged at 750 x g using a KOMPspin Carbon Fiber Rotor Model KAD-21.50 (Composite Rotors, Mountain View, CA), and serum was collected and stored at -20 °C. Animals were killed by overdose of CO_2 and livers were removed, washed with ice cold distilled water, immediately frozen in dry-ice and stored at -20 °C until analysis.

Extraction Procedures

The procedure for extracting and isolating metabolites from vitamin A-deficient rat serum is shown in Figure 35. Control (4.5 ml) and treated (5 ml) serum were thawed at room temperature for 30 minutes. Ten milliliters of 95% ethanol was added, and the solution was mixed. The sample was acidified using 12-14 drops of 4*N* HCl and 10 ml of hexane was immediately added. The sample was mixed for thirty seconds and stored on ice for 20 minutes. The upper layer was removed and hexane was added and removed twice. The hexane fractions were combined and evaporated to dryness under nitrogen. The residual sample was stored at -20 °C until HPLC analysis. The sample was analyzed by HPLC using gradient 2.

The extraction method of Chaudhary et al. (1985) was used with a slight modification and is shown in Figure 36. Tissue samples were thawed, weighed and cut with scissors into 3-5 mm slices. Thirty milliliters of methanol was added and the sample

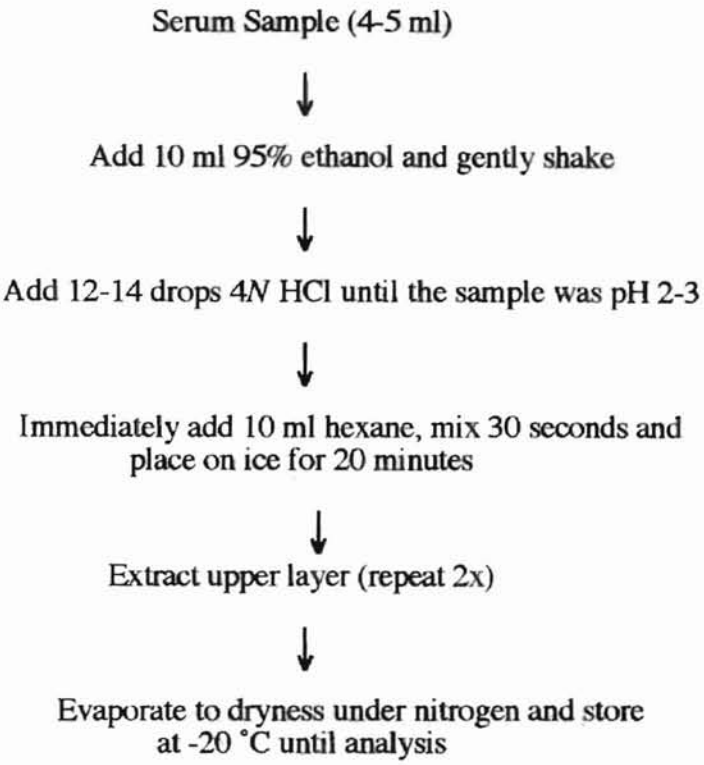


Figure 35: Extraction Procedure for Vitamin A-Deficient Rat Serum Metabolite Isolation.

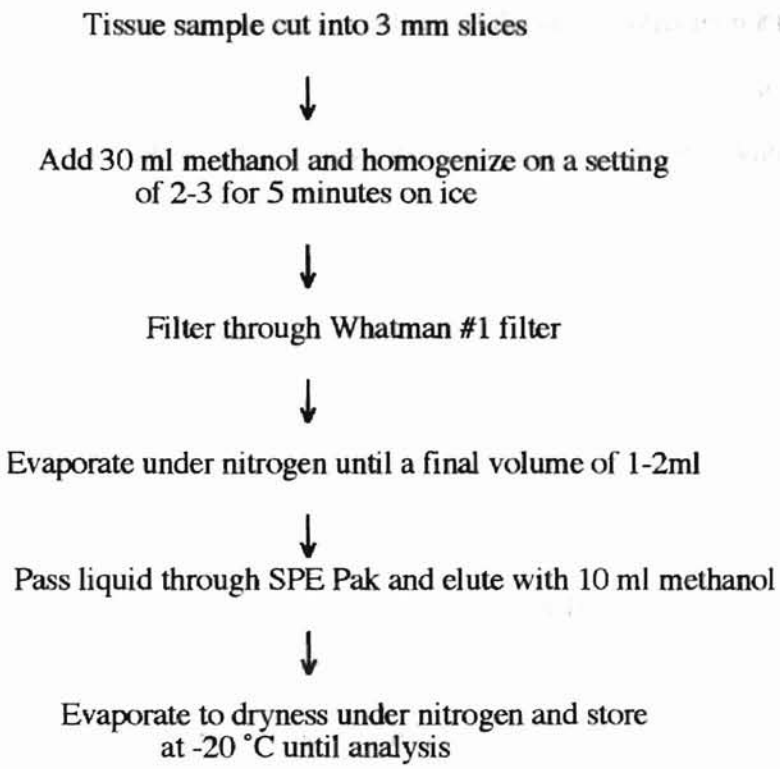


Figure 36: Extraction Procedure for Vitamin A-Deficient Rat Liver, Kidney or Testis Metabolite Isolation.

was homogenized on a setting of 2-3 for 5 minutes on ice using a Sorvall Omni-Mixer (Sorvall Inc., Norwalk, CT). The homogenate was filtered through a Whatman #1 filter and rinsed with 5 ml of methanol. The filtrate was evaporated under nitrogen in a 32 °C heating block to a final volume of 1- 2 ml. The sample was passed through a Waters C-18 solid phase extraction (SPE) cartridge (Waters Corp., Milford, MA) and was then eluted with 10 ml methanol. The eluent was evaporated to dryness under nitrogen and the residual sample was stored at -20 °C until HPLC analysis. The sample was analyzed by HPLC using gradient 1 and 2 for HS and HS2-ester samples, respectively.

O-DHA* Purity

The specific activity of O-DHA* was determined to be 57.2 μCi/mg (Liu et al 1999). O-DHA* purity was checked prior to administration (see Figure 55). A 5 μg/ml O-DHA* stock solution was prepared in aldehyde free-ethanol, and 0.20 μg was injected into the HPLC and analyzed using gradient 2. One milliliter fractions were collected, and 5 ml of EcoLume scintillation cocktail was added. Radioactivity was assayed using a 1900CA Tri-carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL).

Administration of O-DHA*

For O-DHA* metabolism studies, four vitamin A-deficient rats had an average weight of 256 g and were 142 days of age. Animals were starved overnight prior to the O-DHA* metabolism study. Animals were lightly anesthetized with CO_2 and injected intraperitoneally (i.p.) with either 700 μl of dosing vehicle (n = 2) (50% normal saline: 50% absolute ethanol) or O-DHA* (n = 2) (10 μCi/rat) using a 1.5 inch 23 gauge needle. The syringe was washed with 300 μl of methanol, and the sample was injected into the HPLC and analyzed using gradient 2. One milliliter fractions were collected, and the radioactivity was assayed.

Animals were given 5 g of vitamin A-deficient diet treated with absolute ethanol or O-DHA (170 μg/rat). The diet was allowed to evaporate in the dark one hour before the

metabolism study was initiated. Animals were placed in metabolism cages and given tap water and 5 g of treated diet. Animals were killed after 4 hours by an overdose of CO_2 and liver and kidneys were removed, washed with ice cold distilled water, frozen immediately in dry-ice and stored at -20 °C until time of analysis.

All glassware used in the extraction process was swiped and radioactivity was assayed prior to the extraction process. Liver or kidney samples (1-2 g) were cut into 3-5 mm slices, 15 ml of methanol was added and samples were homogenized on a setting of 2-3 for 5 minutes using a Sorvall Omni-Mixer as previously described. The homogenate was passed through a Whatman #1 filter, rinsed with 5 ml of methanol and filtrate volume was determined. A 50 µl sample was removed and assayed for radioactivity. The filtrate was evaporated under nitrogen until a final volume of 1-2 ml. The sample was passed through a SPE cartridge and eluted with 5 ml, 2 ml (repeated twice) and 1 ml (repeated twice) of methanol. The final volume was determined, 10 µl was removed and radioactivity was assayed for each rinse. The sample was evaporated to dryness under nitrogen.

Isomerization

Retinoid isomers are very easily produced in response to light. Precautions were taken to reduce light-isomerization of compounds. All extractions were done in a dark room or under gold lighting using GE F20T12/GO gold bulbs. When samples were exposed, they were wrapped in aluminum foil. Samples were stored dry at -20 °C until time of analysis. Freezing and re-thawing tissue samples was avoided as much as possible.

Results and Discussion

Metabolites were named according to the parent compound which was administered, biological media extracted and polarity of the compound. For example, HS-LM1, HS was the compound administered, L is for liver and M1 is metabolite 1 and it is the most polar metabolite in the chromatogram.

Artifacts Produced from the Extraction Procedure

Metabolites can be artifacts of the extraction procedure. Although the extraction procedures used in this chapter are mild and samples were kept cold and under dim lighting, artifacts may be produced because of interactions between the tissue sample and the parent compound. Moreover, artifacts may be produced in response to heat or natural/-fluorescent lighting since retinoids are sensitive to oxidation and/or isomerization. For all metabolism experiments, a small amount of parent compound was added to the control serum or tissues and the sample was processed in the same manner as the metabolite sample. These experiments were designed to serve as controls for oxidation and/or isomerization processes which may occur in the extraction process.

Artifacts may be produced from an interaction of a metabolite with the biological media. In cases where the metabolites were tentatively identified with a standard, the standard was added to the control biological media and processed in the same manner as the metabolite sample. These experiments served as additional controls for the metabolism studies.

Repurification of Samples

In cases where the entire extract was injected into the HPLC, the quality of the UV spectra may be poor and the retention time may vary. This observation is probably due the extract having many unidentified compounds which interact with the column and this does not allow for good metabolite separation. This problem was circumvented by re-analyzing fractions which only contained the metabolite of interest. Fractions were pooled, dried under nitrogen and reanalyzed. This repurification usually gave an improved UV spectra and more reliable retention times of the metabolites.

Use of Vitamin A-Deficient Animals

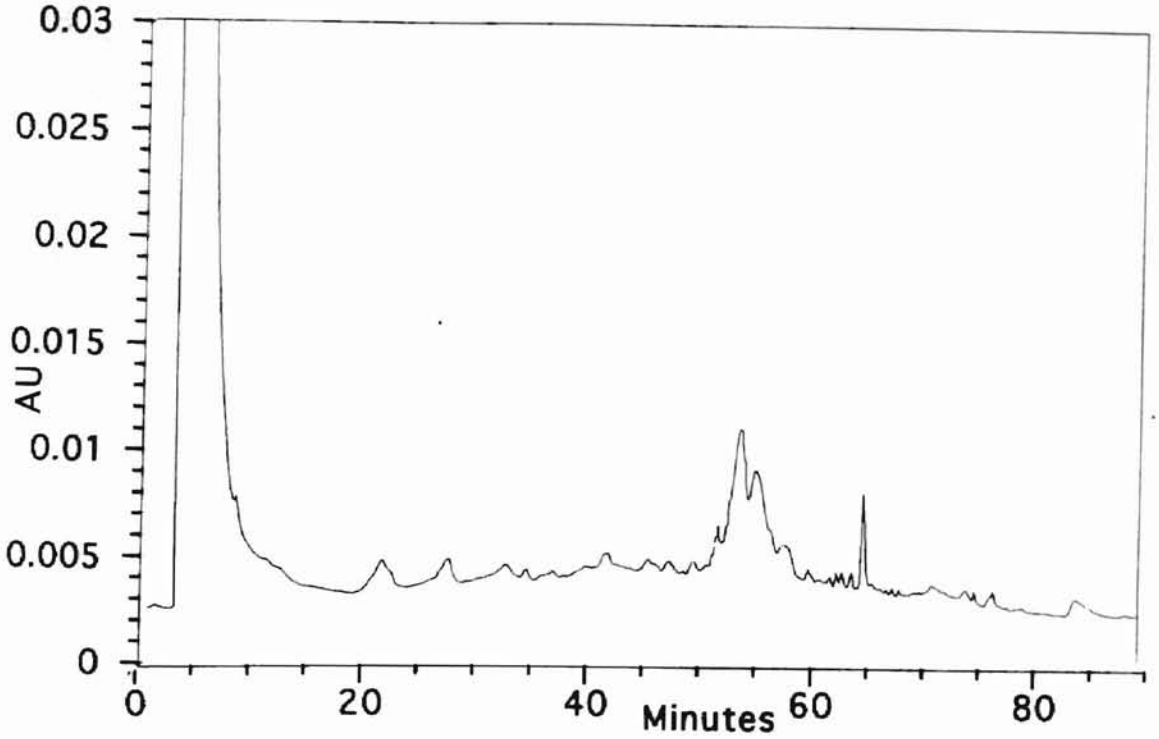
Animals which were not vitamin A deficient were initially used in HS studies (data not shown). This proved to be very difficult when livers from different animals were removed and metabolites isolated since different animals have different retinoid stores. It

became difficult to identify metabolites of the parent compound when rats were not deficient. Control and treated chromatograms from animals were impossible to compare since peaks which are present in the treated chromatogram may or may not be metabolites of the parent compound. For example, the heteroarotinoid extract may have a peak in the chromatogram which may be a heteroarotinoid metabolite or it may be a metabolite from stored retinoids, and this peak may or may not be present in the control chromatogram since the retinoid stores between the two animals being compared may be different.

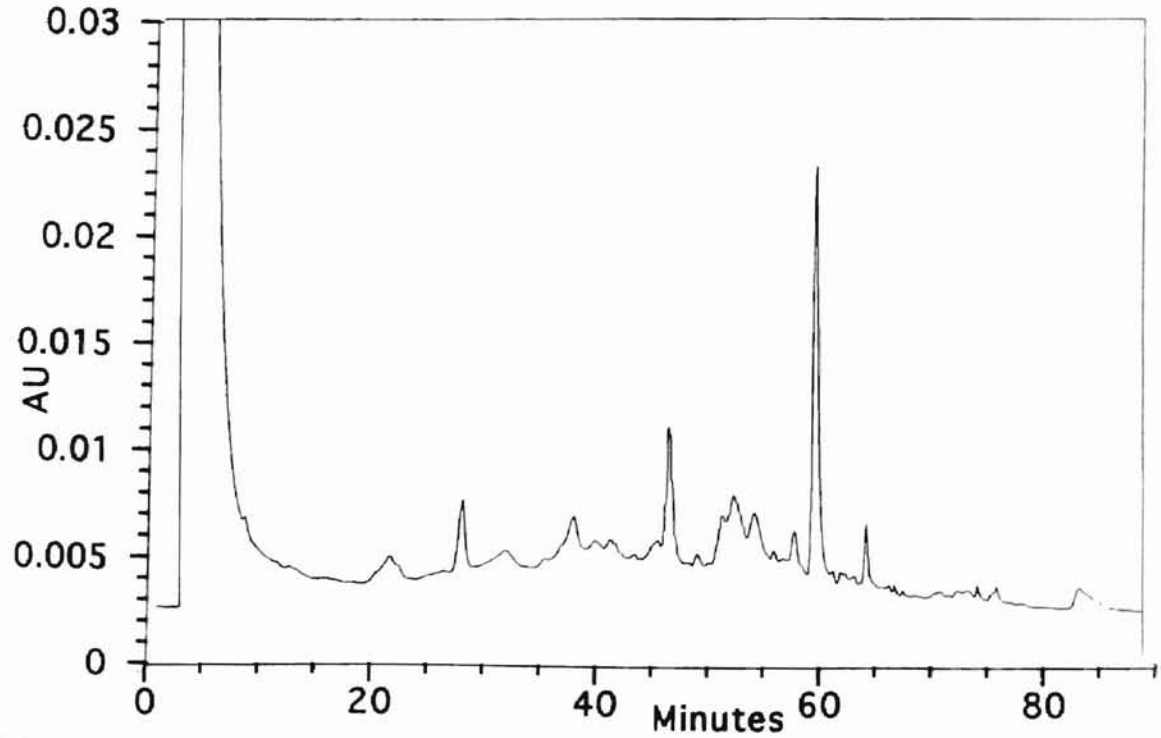
This problem could be circumvented by using radiolabelled compounds which are much easier to trace although extremely expensive to synthesize. Another alternative is to use a biological sample which can be collected from the same animal before and after the parent compound administration. Isolating metabolites from rats which are deficient gives an extract which contains only the parent compound and its metabolites since the retinol stores are depleted from the tissues. Furthermore, it is much easier to compare control and treated liver chromatograms and identify potential metabolites.

HS Metabolism Studies

Liver weights were comparable with the control and HS treated rat livers weighing 11.9 and 10.9 g, respectively. Control and HS samples were chromatographed the same day. The HPLC profiles of control and HS liver extracts are shown in Figure 37 . Two major HS liver metabolites, HS-LM1 and HS-LM2, were isolated. The first metabolite, HS-LM1, had a retention time of 38.1 minutes and a maximum UV absorbance at 342.3 nm and a second-to-maximum absorbance at 224.1 nm. A second metabolite HS-LM2 was detected at 46.9 minutes and a maximum UV absorbance at 374.2 nm. The parent compound, HS, was recovered in the extract with a retention time of 60.2 minutes and a maximum UV absorbance of 360.7 nm. A peak with a retention time of 60.1 minutes was observed in the control chromatogram with a maximum UV absorbance of 252.3 nm. This peak is an unidentified component in both the control and HS liver. Chromatograms of HS-LM1, HS-LM2 and HS are shown in Figures 38, 39 and 40, respectively.

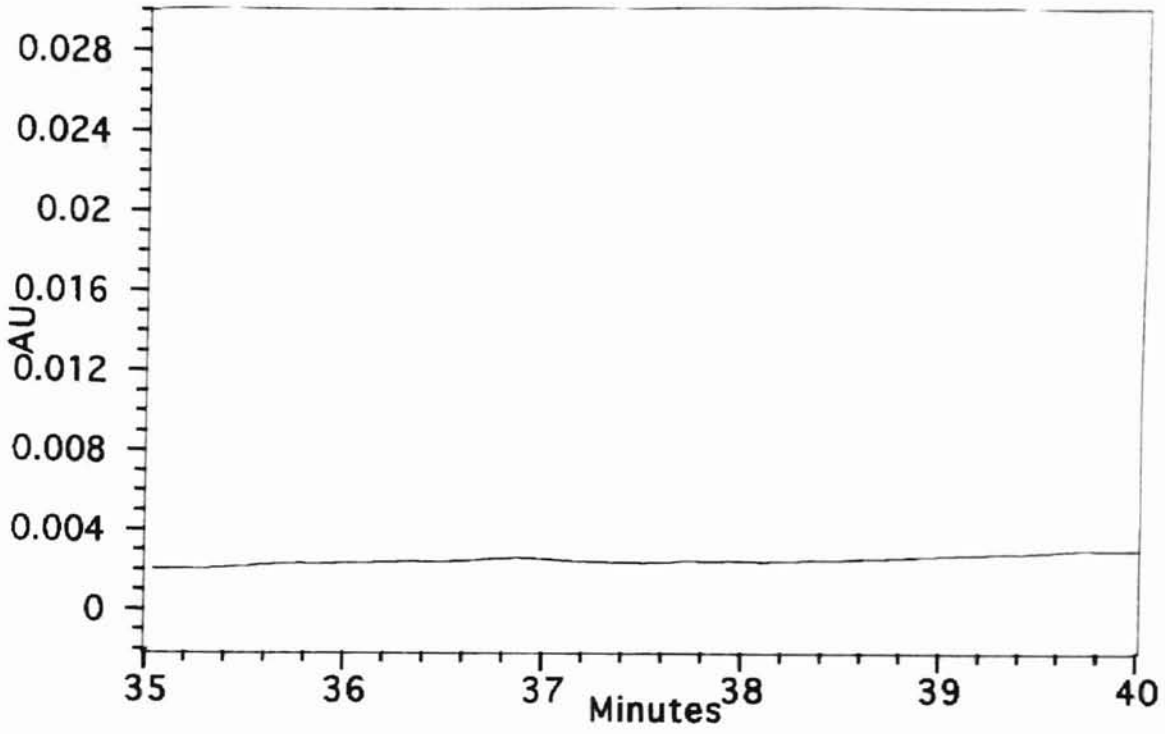


(a)

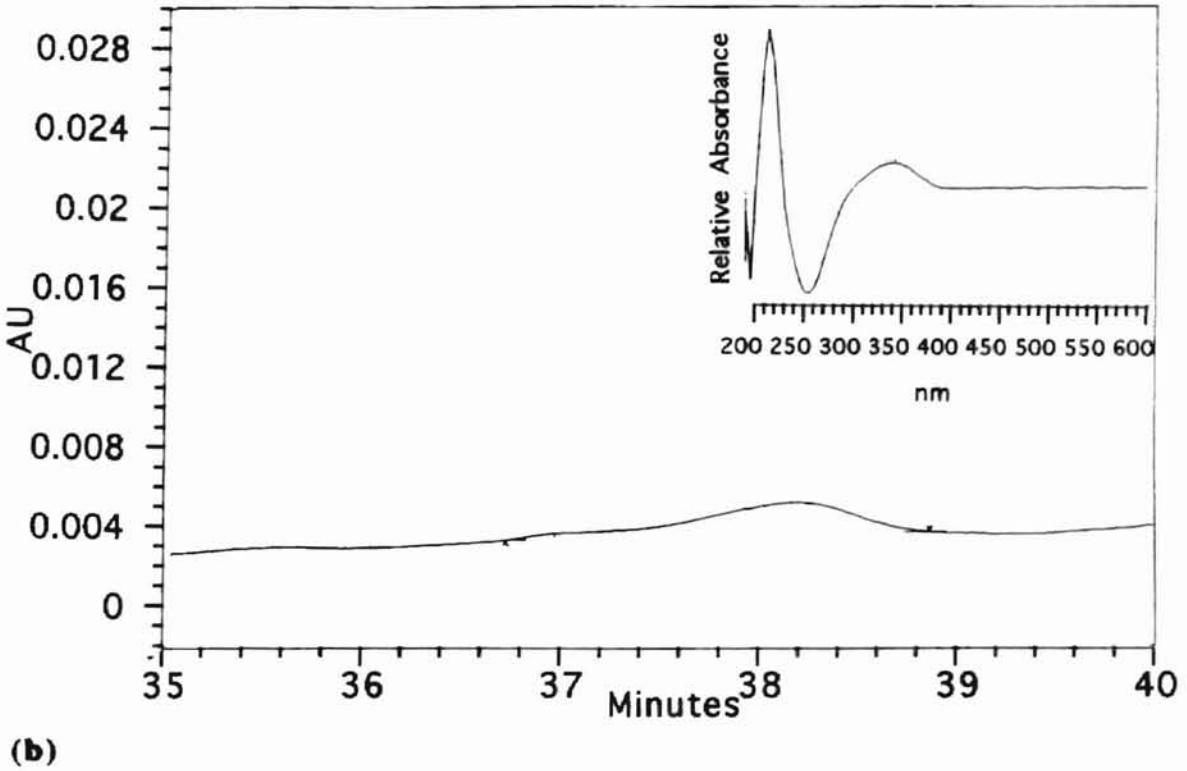


(b)

Figure 37: HPLC Profiles of Control (a) and HS (b) Liver Extracts.

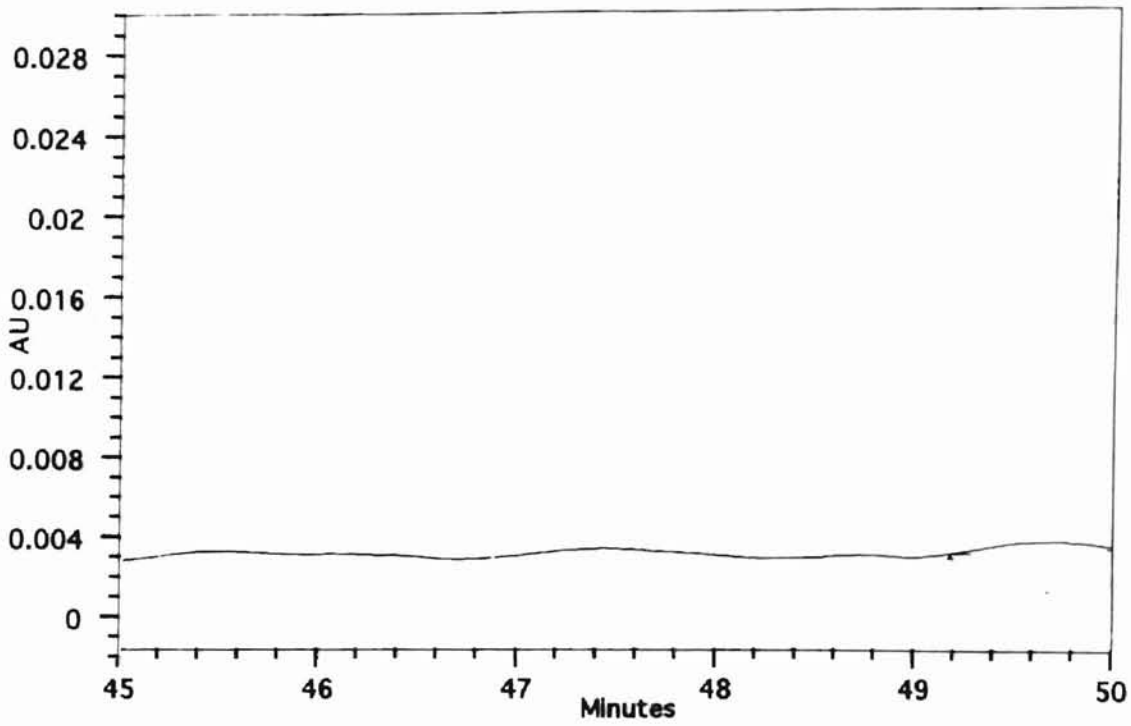


(a)

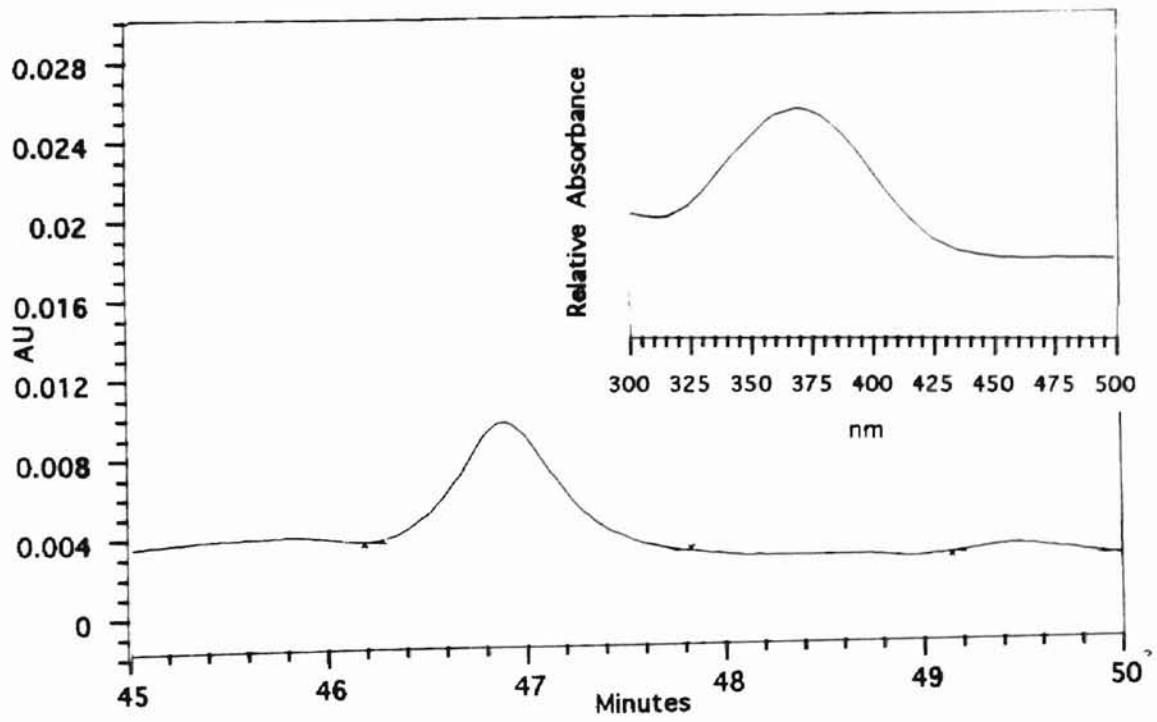


(b)

Figure 38: HPLC Profiles of Control Liver Extract (a) and HS-LM1 (b). UV Spectrum of HS-LM1 is shown in the insert of (b)

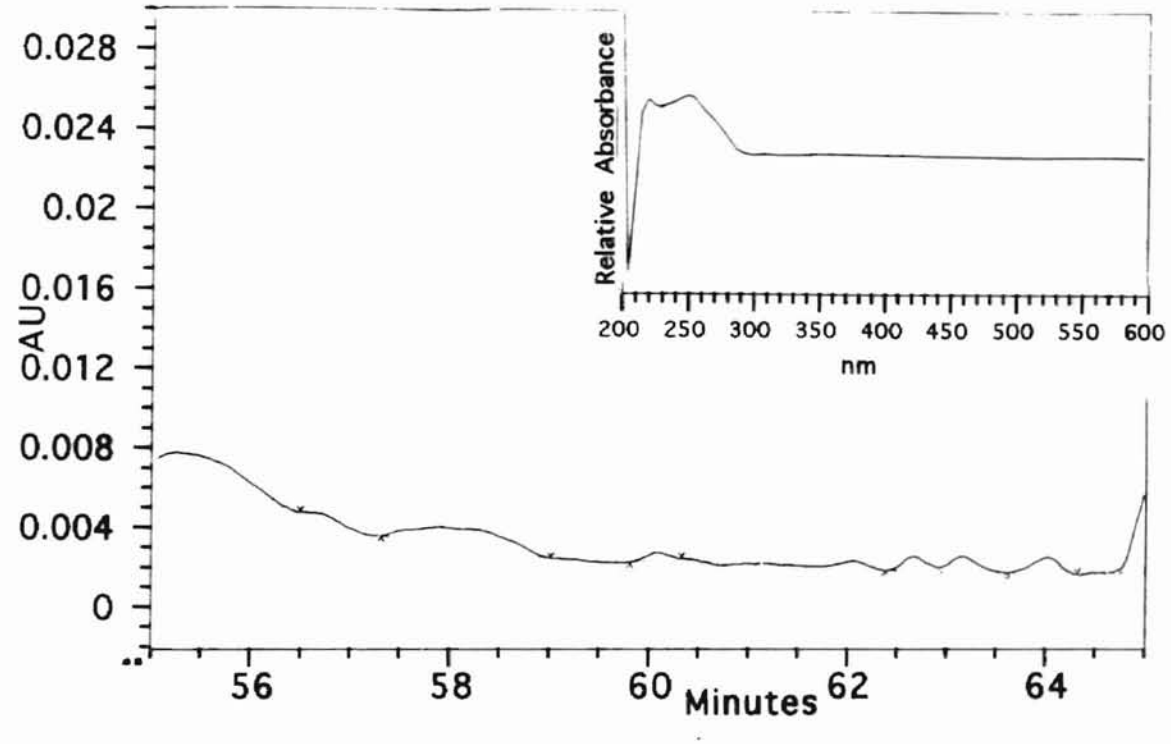


(a)

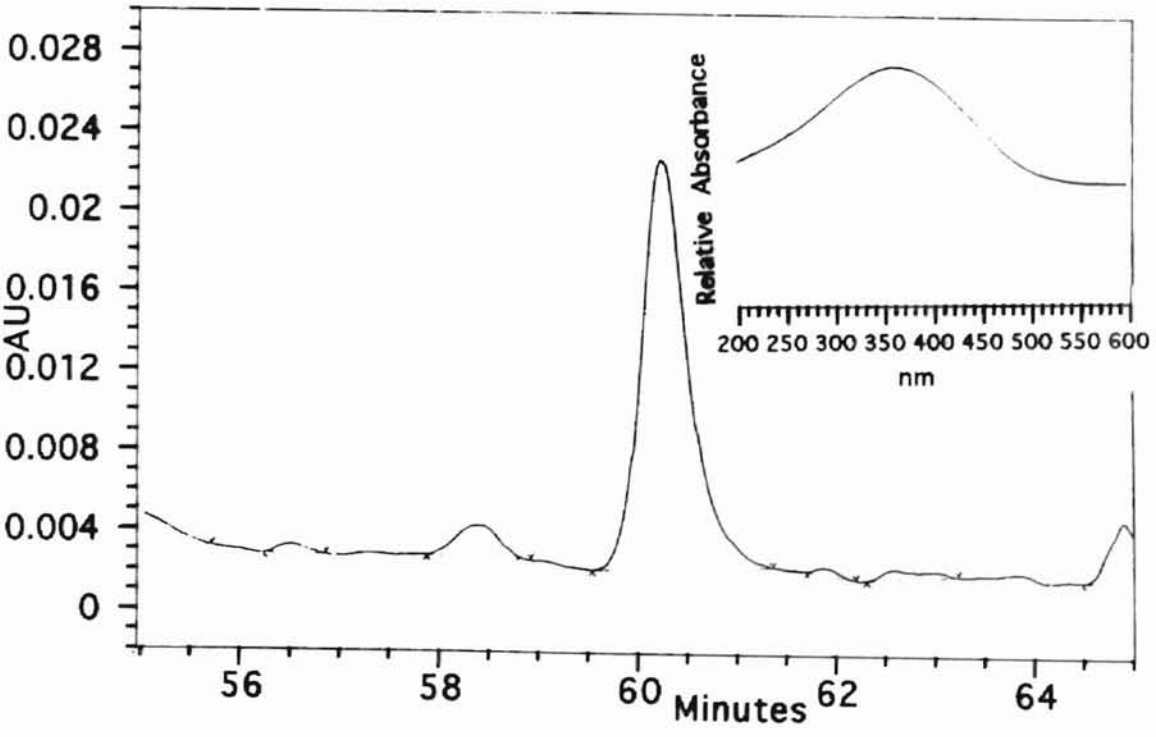


(b)

Figure 39: HPLC Profiles of Control Liver Extract (a) and HS-LM2 (b). The UV Spectrum of HS-LM2 is shown in the insert of (b)



(a)



(b)

Figure 40: HPLC Profiles of Control (a) and HS (b) Liver Extracts. UV Spectra of Control and HS are shown in the insert of (a) and (b), respectively.

To ensure that HS metabolites were not artifacts produced from the extraction process, HS was added to 0.5 g of control liver and the sample was processed in the same manner as liver metabolite samples. The HPLC profile is shown in Figure 41. HS has a retention time of 59.9 minutes and a maximum UV absorbance of 360.7 nm. Another peak was observed in the spiked chromatogram with a retention time of 38.8 minutes and maximum UV absorbance at 342.3 nm, and the HPLC profile is shown in Figure 42. The retention times and the maximum UV absorbance are similar to HS-LM1 which suggests HS-LM1 is an artifact of the extraction procedure and may be an unidentified oxidation product of HS. No peaks were detected in the 46 minute range suggesting HS2-LM2 is not an artifact of the extraction process and is a metabolite of HS. The retention times are slightly different between the metabolite and spiked samples but, this could be explained by variations in column temperature or electronic drift.

HS metabolites were not detected in the kidney or testes. The control extract was the same as the heteroarotinoid extract for both tissues (data not shown). Two hours may be an insufficient amount of time to produce metabolites in these tissues. The metabolism of HS may depend on the individual animal's metabolism, route of administration and bioavailability of HS. As seen with later metabolism studies, four hours is a sufficient time period to detect liver and kidney metabolites although this may be compound dependent.

HS2-ester Rat Serum Metabolites

Serum was collected and pooled on vehicle control (n = 5) and HS2-ester treated animals (n = 5). Serum volumes for control and HS2-ester treated animals were 4.5 and 5 milliliters, respectively. Samples were chromatographed on the same day and 1 milliliter fractions were collected and stored at -20 °C until further analysis. HPLC profiles of control and HS2-ester serum extracts are shown in Figure 43.

Two rat serum metabolites, HS2-SM1 and HS2-SM2, were tentatively identified. The first metabolite, HS2-SM1, had a retention time of 27.2 minutes and a maximum UV absorbance at 318.5 nm. The second metabolite, HS-SM2, was detected at 30.5 minutes

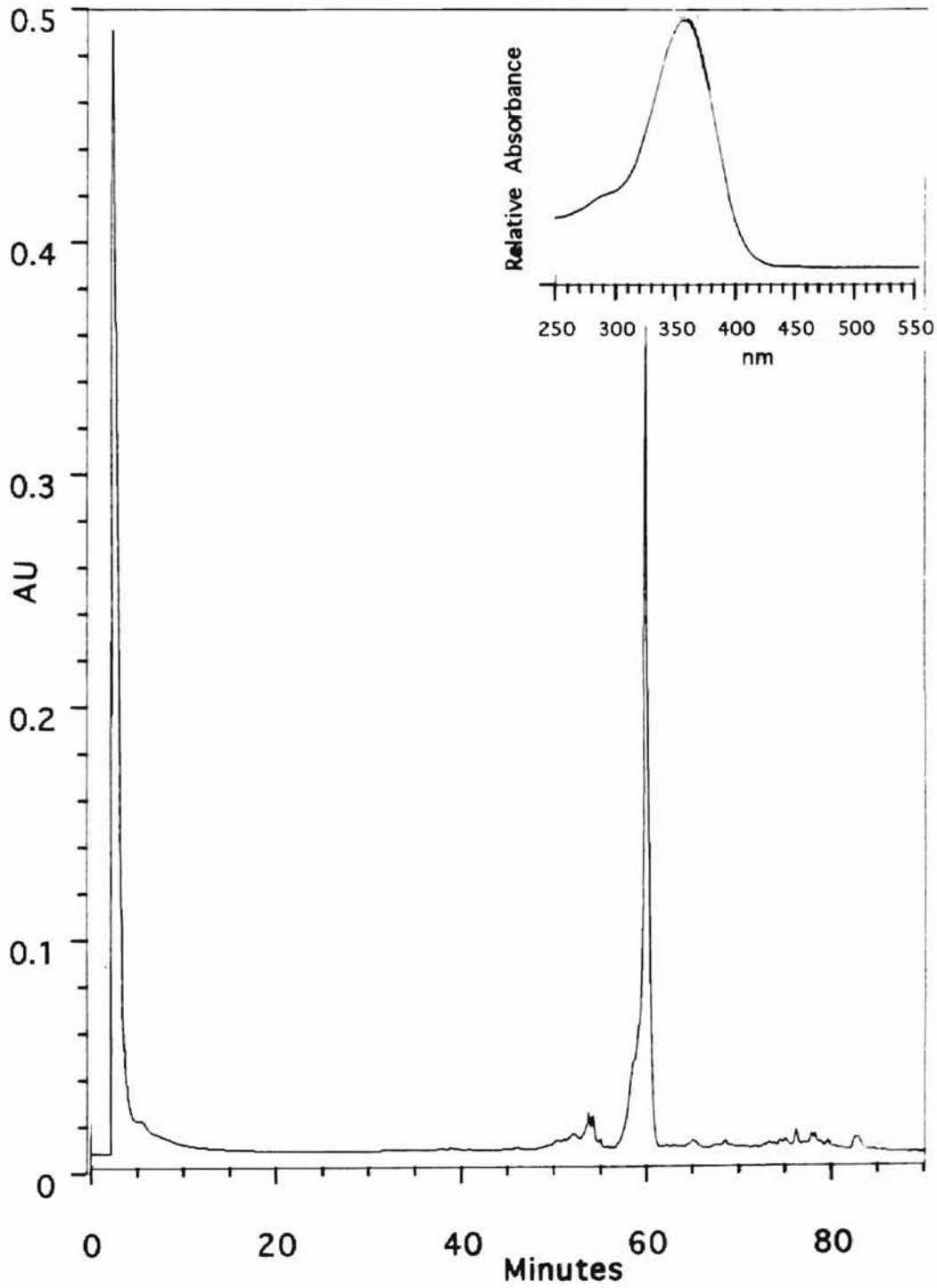


Figure 41: HPLC Profile of HS Spiked Control Liver. UV Spectrum of HS is shown in the insert.

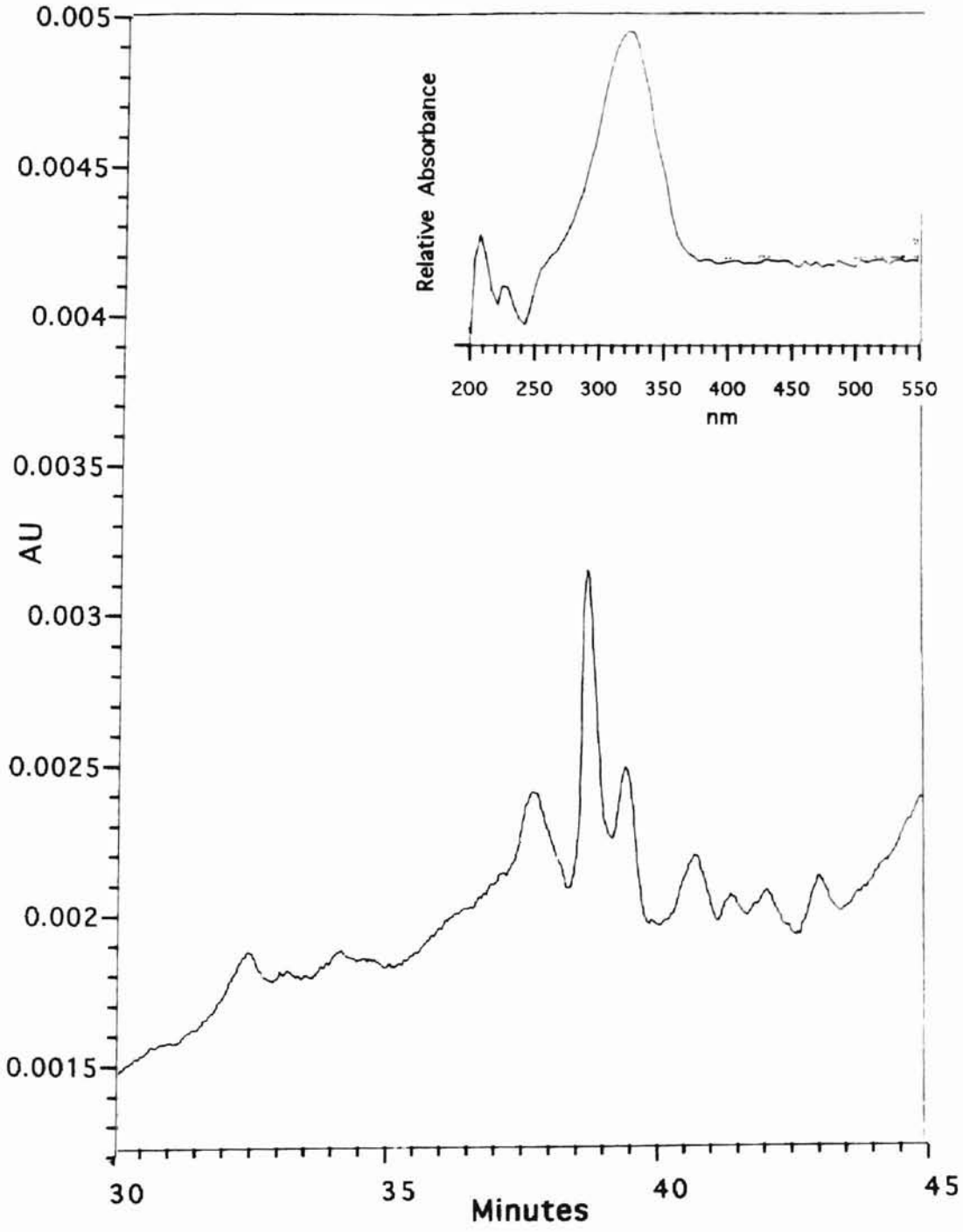
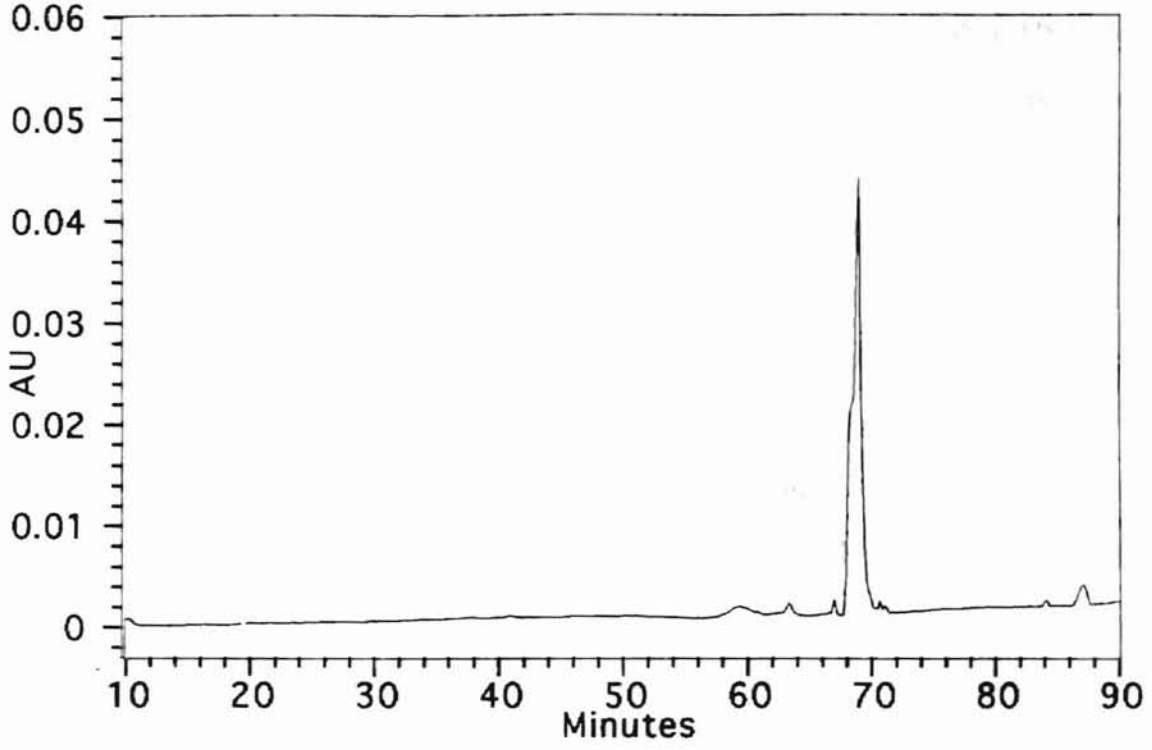
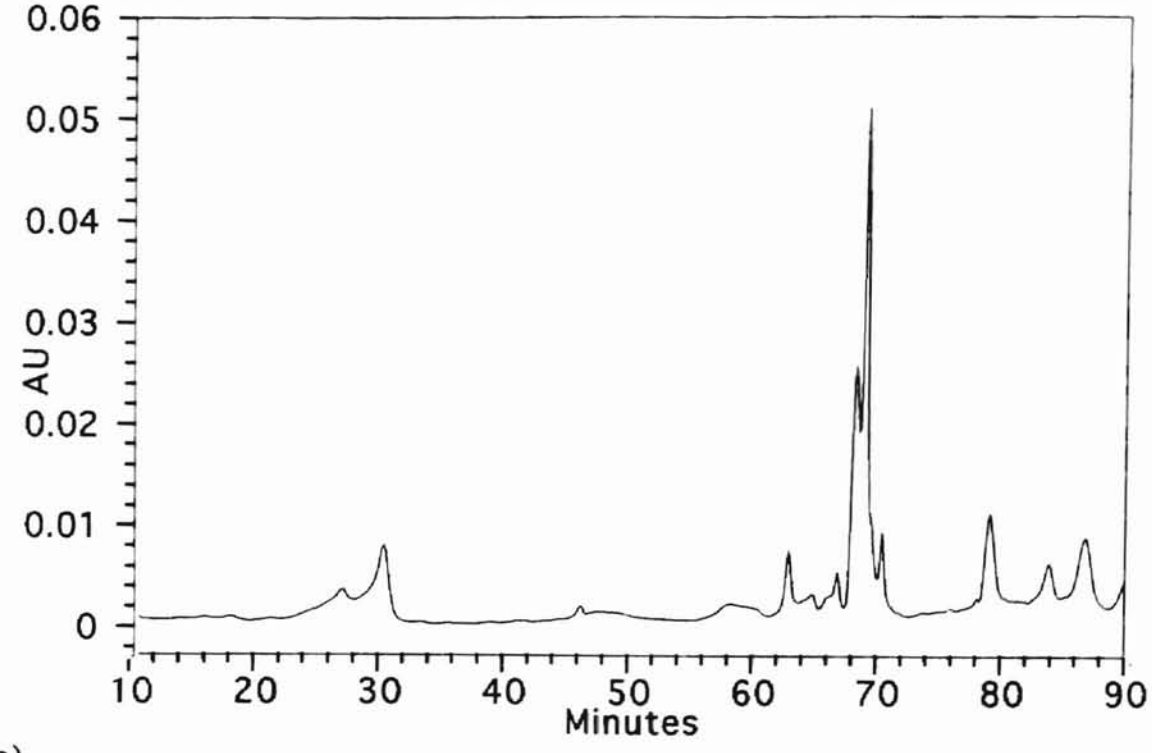


Figure 42: HPLC Profile of the HS Artifact Produced From the Extraction Process. UV Spectrum of the artifact is shown in the insert.



(a)



(b)

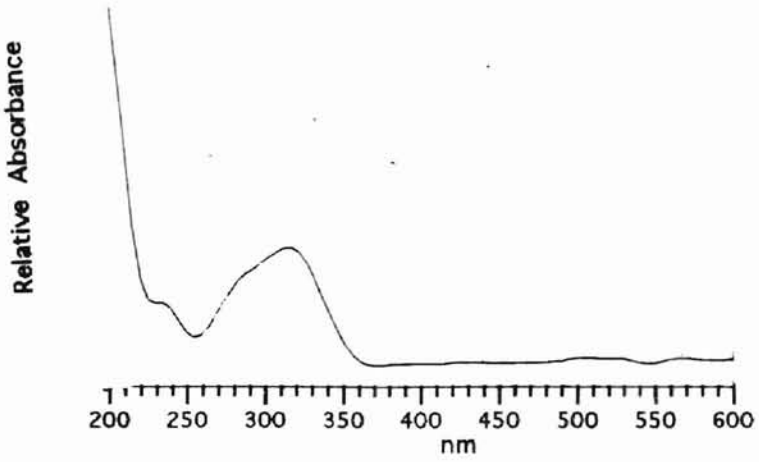
Figure 43: HPLC Profiles of Control (a) and HS2 (b) Serum Extracts.

with a maximum UV absorbance of 323.3 nm. The UV spectra of HS2-SM1, HS2-SM2, and HS2-ester are shown in Figure 44. The parent compound, HS2-ester was recovered with a retention time of 46.3 minutes and a maximum UV absorbance of 323 nm. The parent compound and HS2-SM2 had the same maximum UV absorbance but different retention times suggesting these compounds are related.

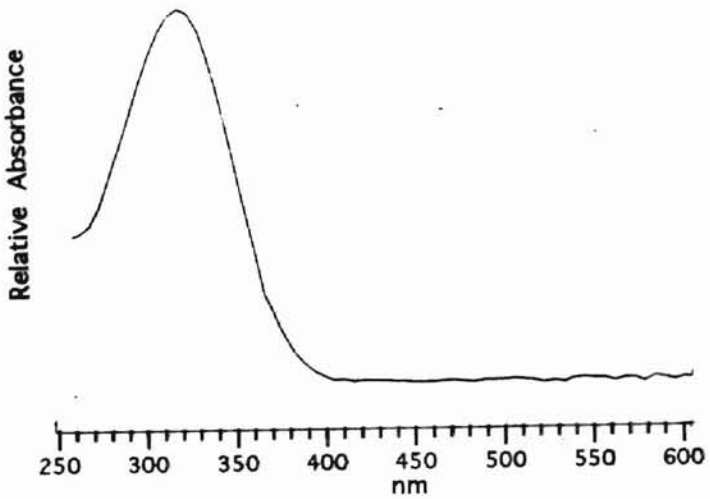
HS2-acid was synthesized from HS2-ester and allowed to isomerize in the light for 24 hours to give a mixture of HS2-acid and the corresponding E-isomer. The HPLC profile is shown in Figure 45. The E-isomer had a retention time of 24.4 minutes and a maximum UV absorbance of 318.5 nm and a second maximum absorbance at 266.5 nm. The UV spectra and retention times of HS2-SM1 and the E-isomer match suggesting these compounds are the same structure. The UV spectrum of HS2-acid and HS2-SM2 match and both compounds have a similar retention time which suggests they are the same structure.

The same serum sample was spiked with a small amount of HS2-acid and its E-isomer and analyzed using the same chromatographic conditions. The peak heights of HS2-SM 1 and 2 increased in response to the addition of HS2-acid and the E-isomer (see Figure 46), suggesting HS2-SM2 and 1 are the same as HS2-acid and its E-isomer, respectively.

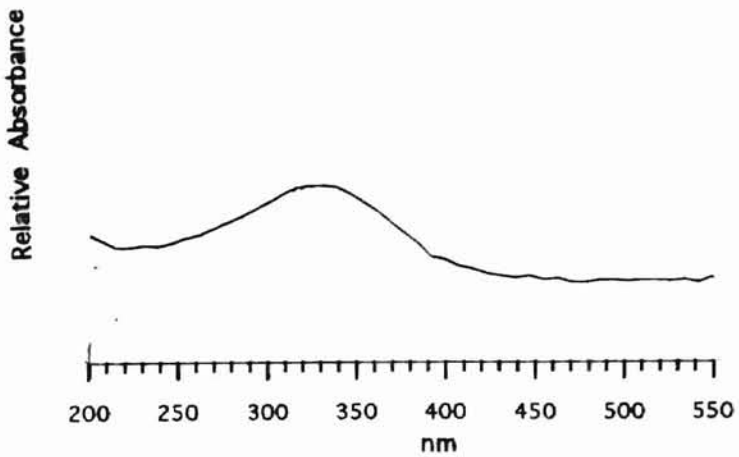
To ensure HS2 serum metabolites were not artifacts of the extraction process, HS2-ester was added to control serum and the sample was processed in the same manner as metabolite samples. The HPLC profile of the HS2-ester spiked serum is shown in Figure 47. HS2-ester has a retention time of 46 minutes and a maximum UV absorbance of 323.3 nm. Two additional peaks are associated with HS2-ester standard. These peaks have a retention time of 40 and 54 minutes, respectively and a maximum UV absorbance of 323.3 nm. These peaks are not produced from the extraction procedure and are present in the HS2-ester standard. Other peaks were not detected in the 20-35 minute range suggesting the metabolites are not artifacts produced from the extraction process.



(a)



(b)



(c)

Figure 44: UV Spectra of HS2-SM1 (a), HS2-SM2 (b) and HS2 (c).

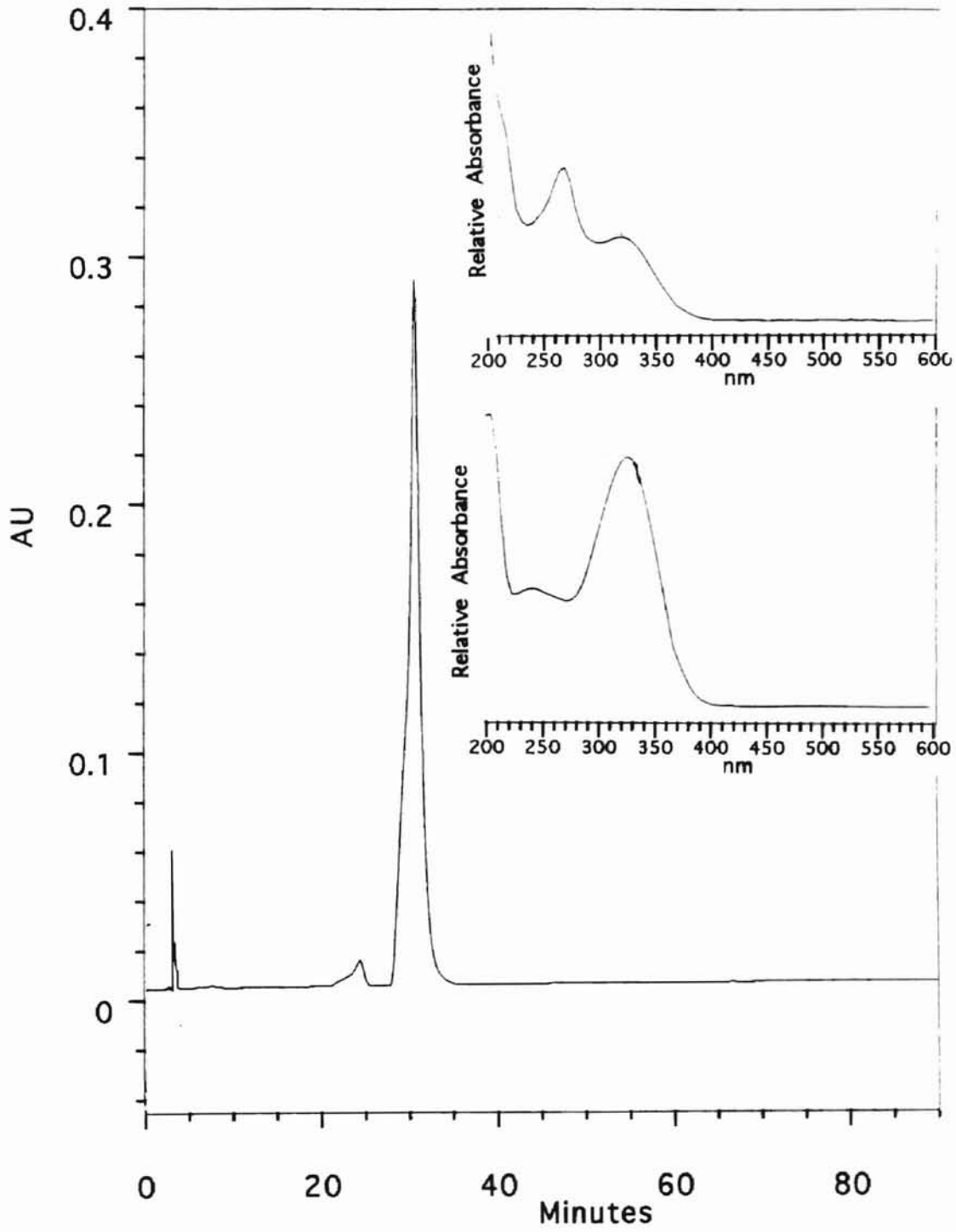
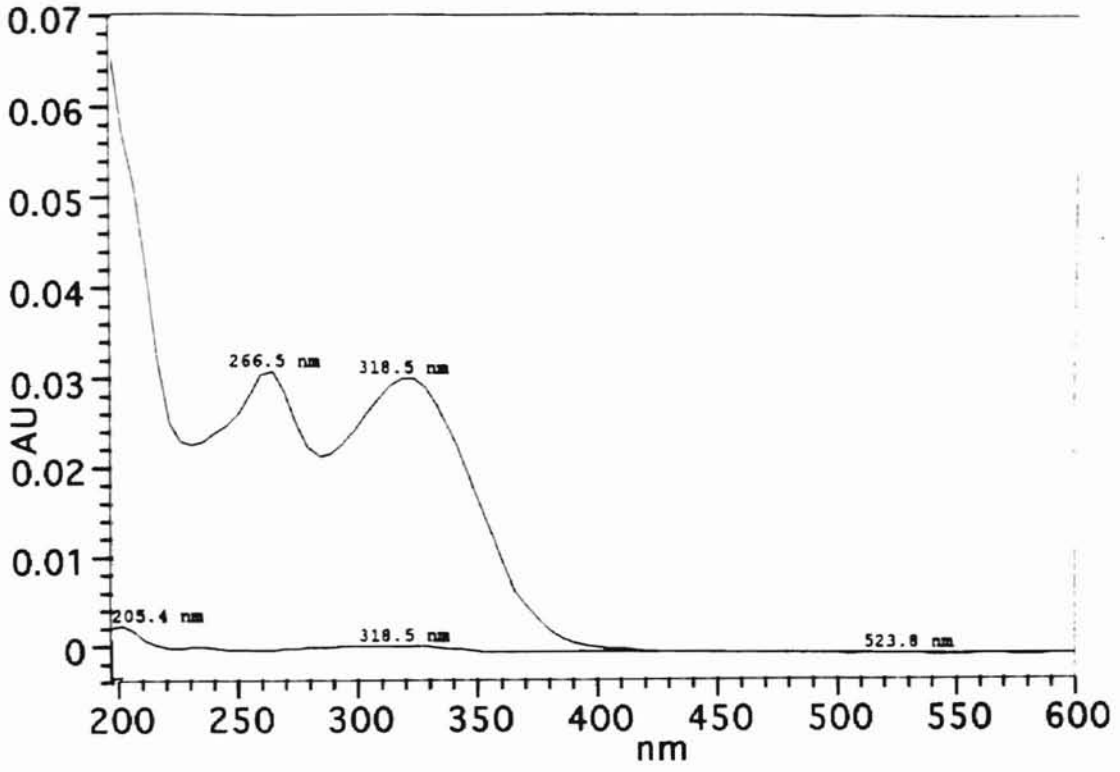
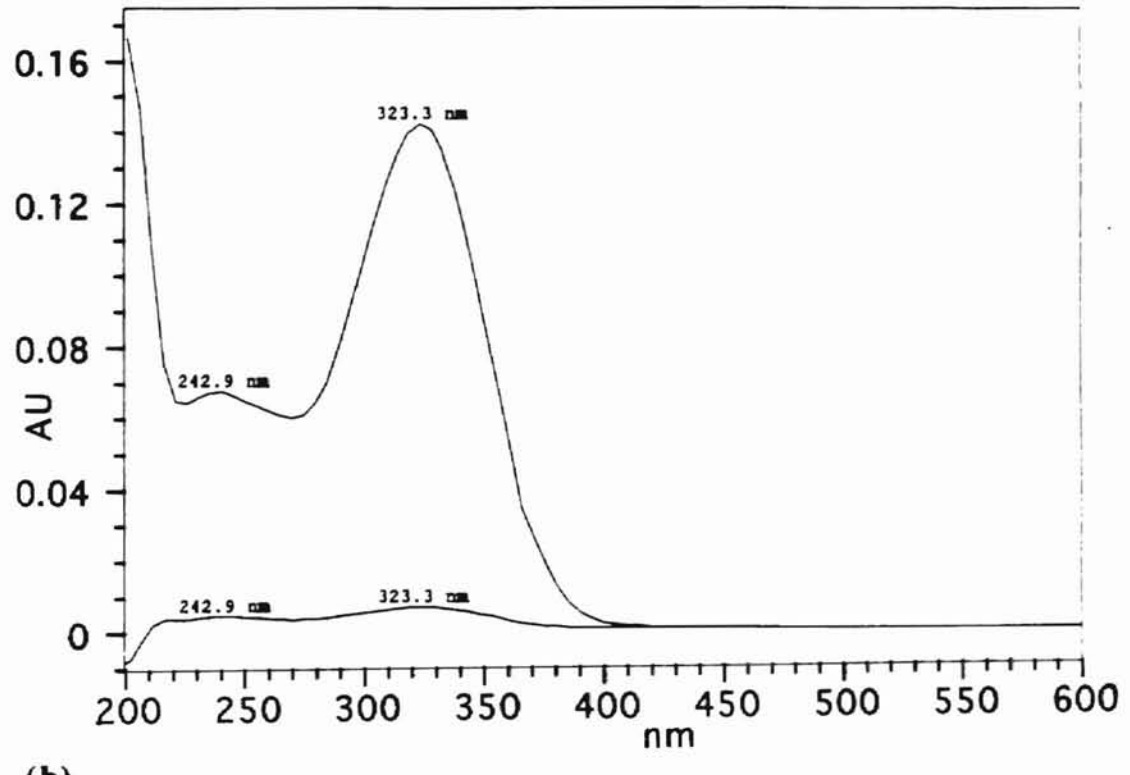


Figure 45: HPLC Profiles of HS2-acid and the Corresponding E-isomer. UV Spectra of HS2-acid-E-isomer and HS2-acid are shown in the insert.



(a)



(b)

Figure 46: UV Spectra of HS2-SM1 Spiked with HS2-acid E-isomer (a) and HS2-SM2 Spiked with HS2-acid (b).

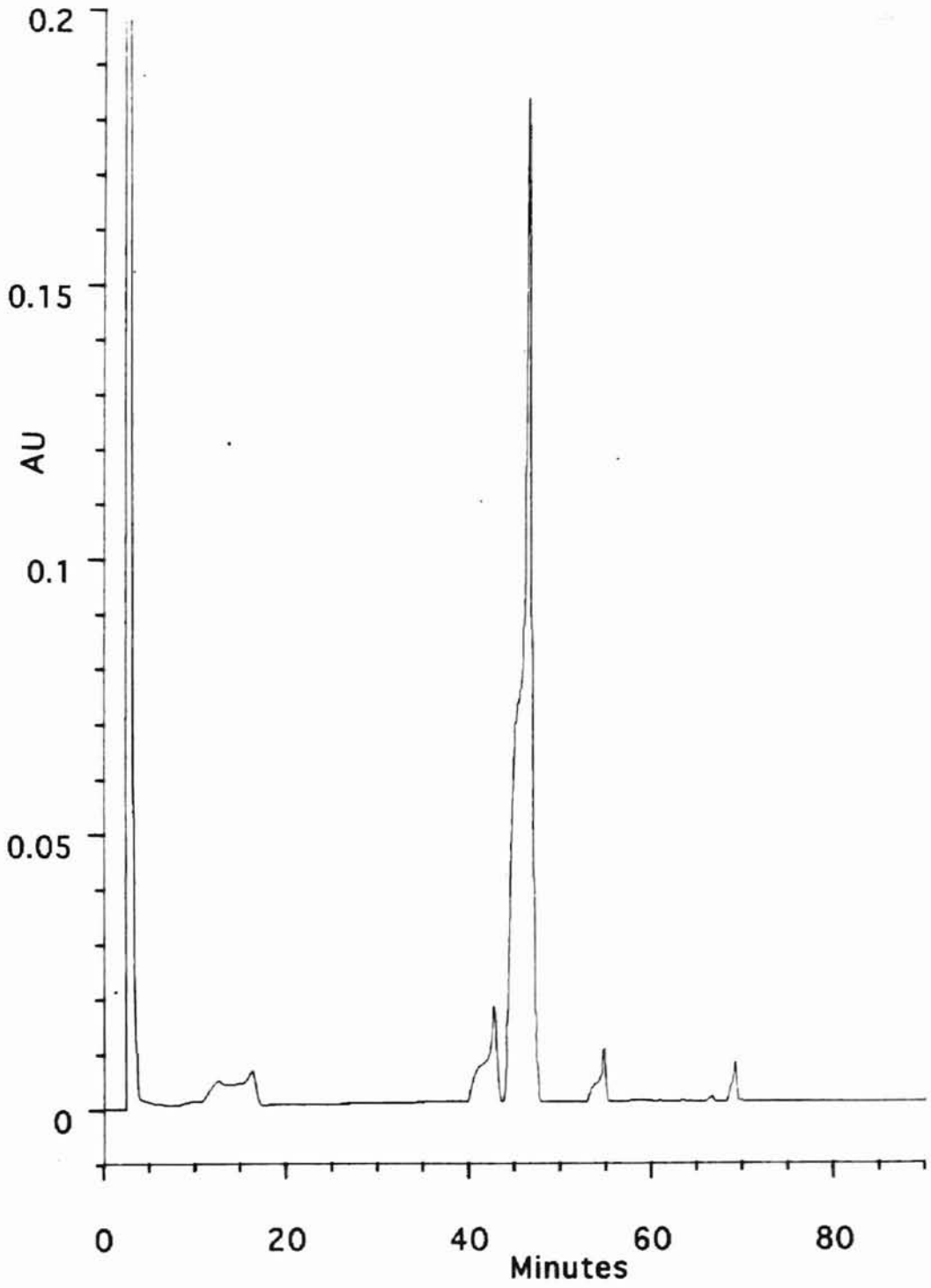


Figure 47: HPLC Profile of HS2-ester Serum Spike.

As an additional confirmatory measure, HS2-acid was added to control serum and extracted in the same manner as the metabolite samples. The HPLC profile of the HS2-acid spiked serum is shown in Figure 48. HS2-acid has a retention time of 28 minutes and a maximum UV absorbance of 323.3 nm. A shoulder was observed on the HS2-acid peak which suggests the E-isomer may be produced in the extraction process. To evaluate if the isomer was present, one minute increments (slices) were taken across the peak from 24-31 minutes. The UV spectra are shown in Figure 49. All slices showed a maximum UV absorbance at 323.3 nm which is consistent with the HS2-acid maximum UV absorbance. None of the slices had a maximum UV absorbance of 318.5 nm which suggests the metabolite is not an artifact of the extraction procedure. Although every precaution was taken to ensure the extractions were consistent, slight variations between samples do exist which could cause isomerization of the metabolites. It becomes very difficult to determine if isomers are true metabolites although several have been reported in the literature (see Literature Review, Chapter I). For example, 13-*cis*-RA is found in the blood after administration of *t*-RA (Eckoff et al. 1990). Additional extractions of control serum spiked with HS2-acid would aid in determining if the isomer is an artifact although the amount of control serum available was limited. The same extraction was done several times using fetal bovine serum (FBS) spiked with a small amount of HS2-acid and the isomer was not detected in the HPLC profile (data not shown) suggesting HS2-SM1 may be a true metabolite of HS2-ester in the serum.

HS2-ester Rat Liver Metabolism Studies

A total of 41 (control) or 45 g (HS2-ester) of liver was extracted for the metabolism studies. Each control or HS2-ester (n = 5/group) treated liver was extracted separately and samples were pooled prior to HPLC analysis. Control and HS2-ester liver extracts were chromatographed the same day and fractions were collected. HPLC profiles are shown in Figure 50. Four peaks were identified in the HPLC profile of the HS2-ester extract and had retention times of 23.1, 31.5, 32.5 and 52.9 minutes (parent compound, HS2-ester).

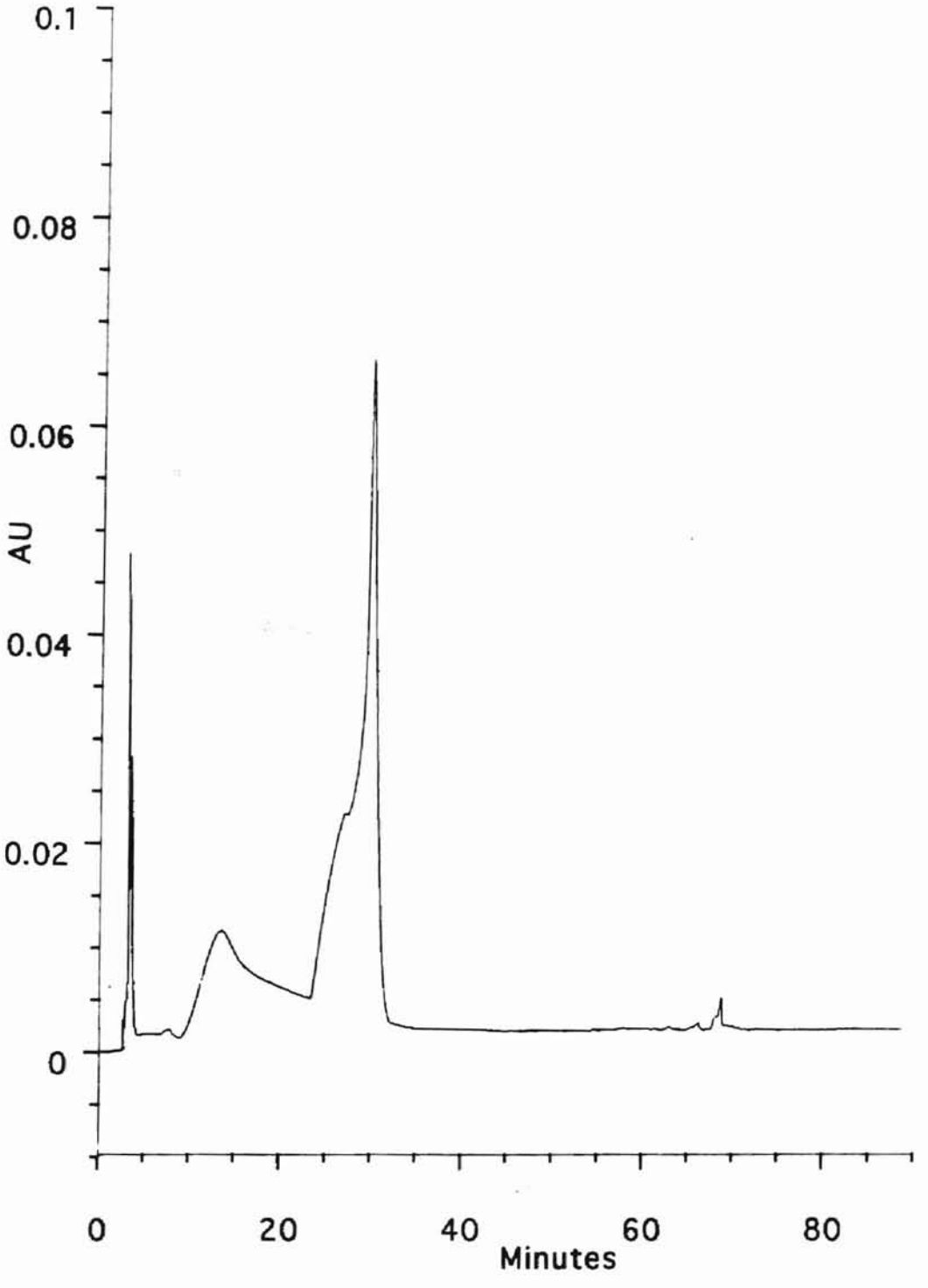


Figure 48: HPLC Profile of HS2-acid Serum Spike.

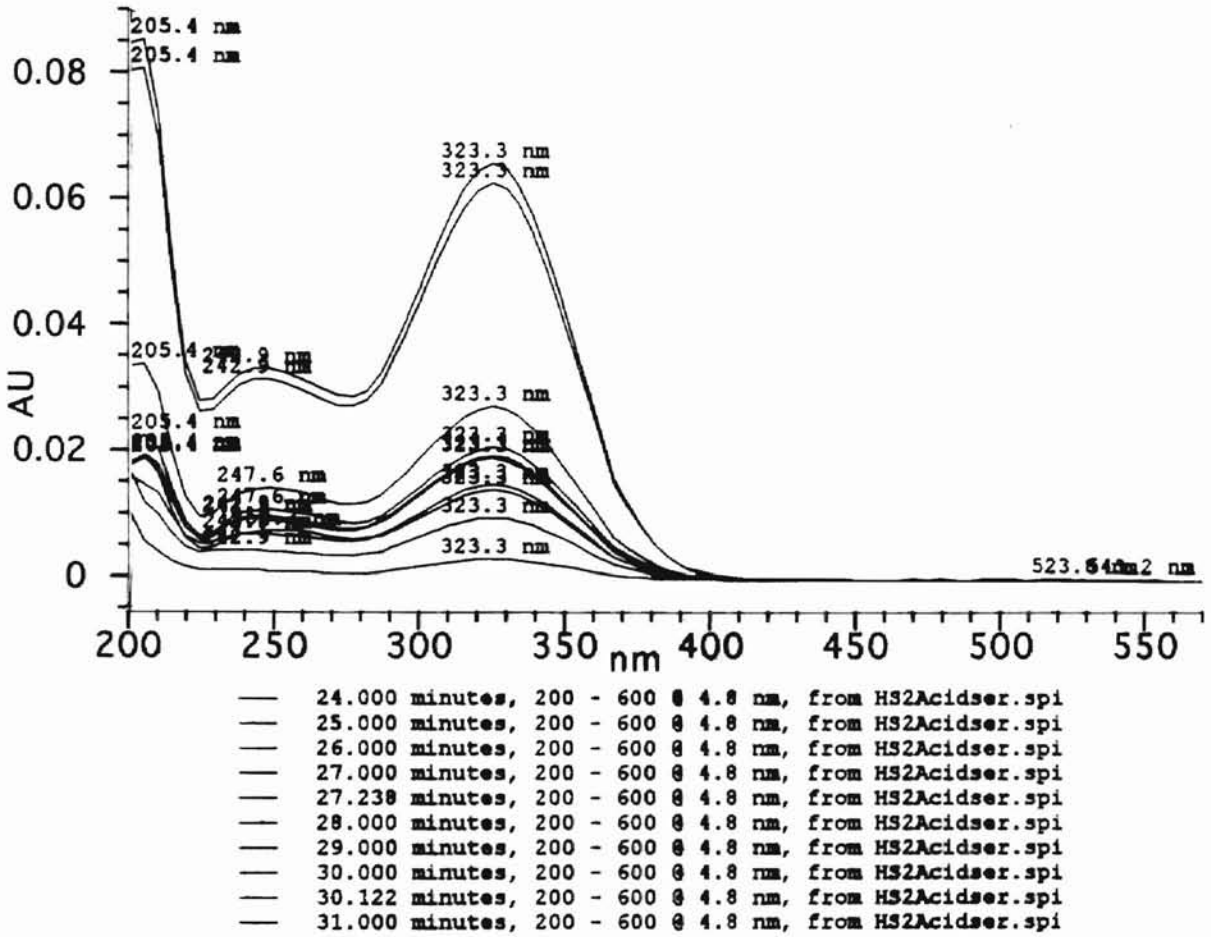
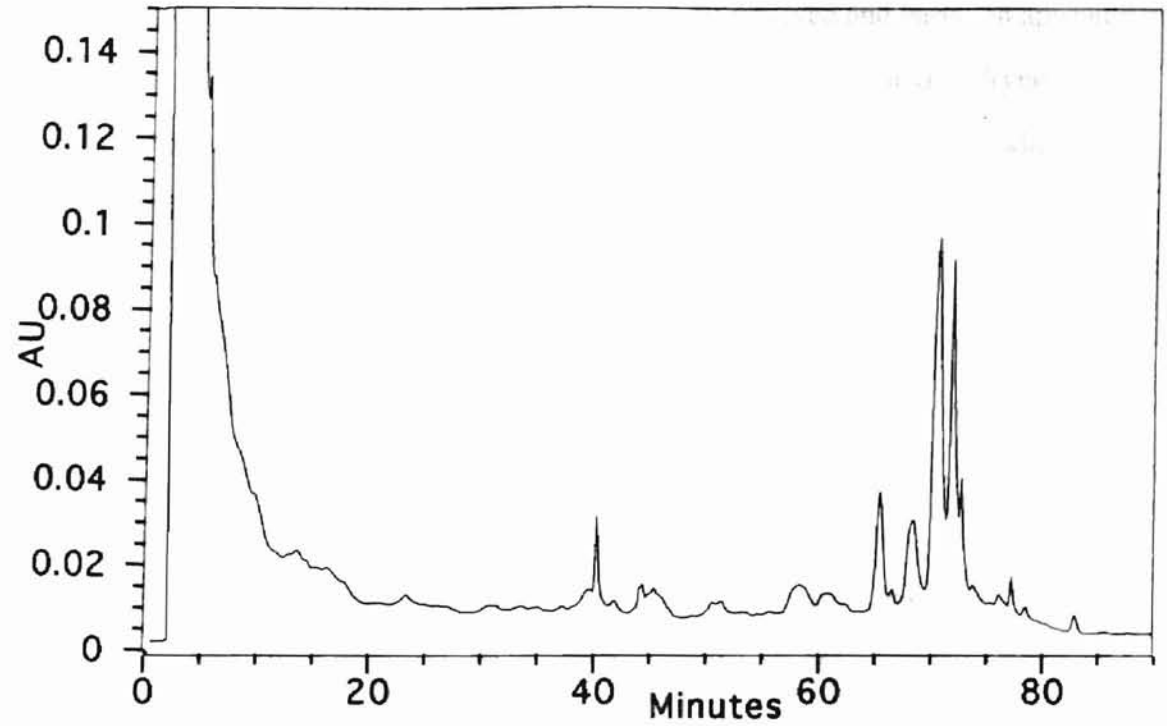
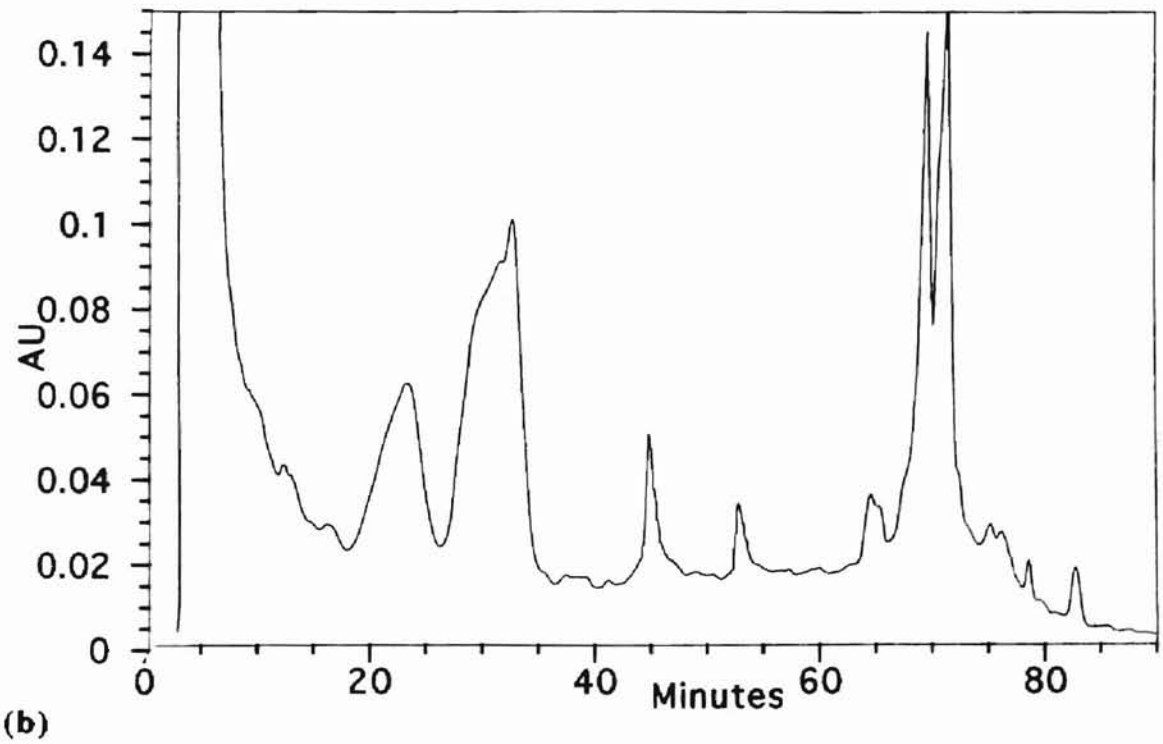


Figure 49: UV Spectra of 24-31 Minute Slices of HS2-Acid Spike.



(a)



(b)

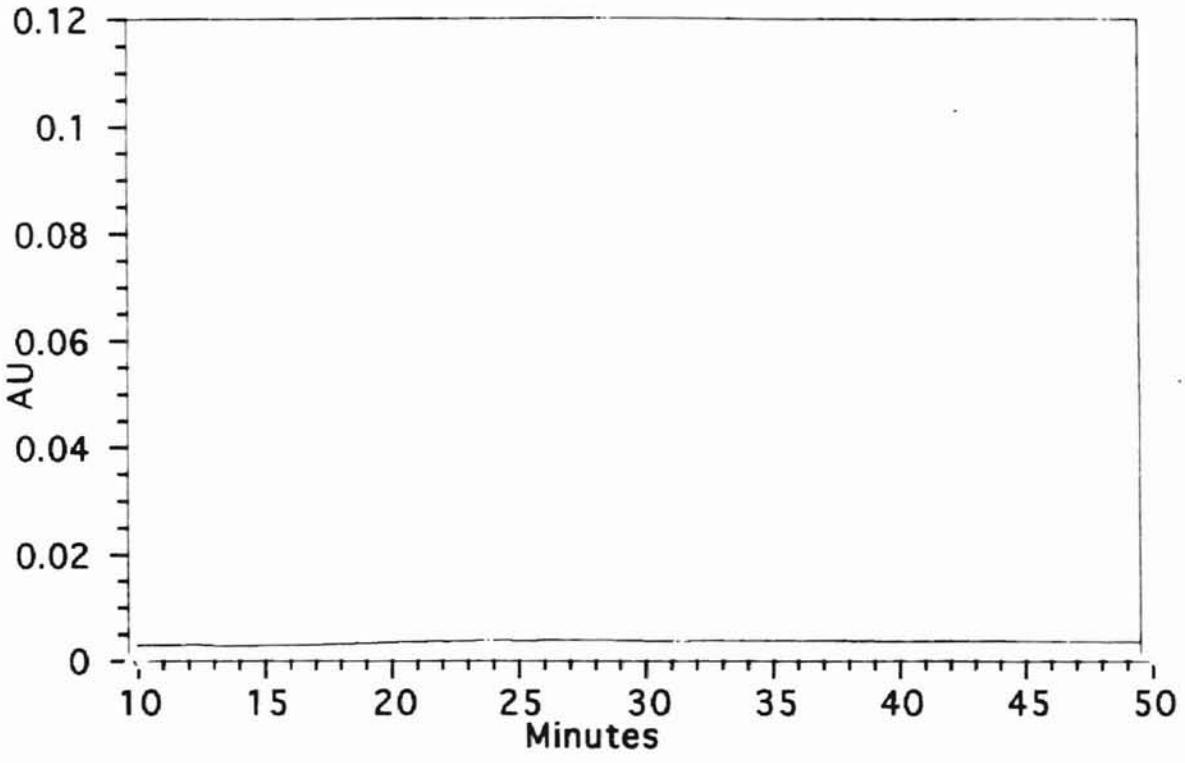
Figure 50: HPLC Profiles of Control (a) and HS2-ester (b) Liver Extracts.

The peaks in the region of 20-35 minutes were not well resolved and based on appearance were not pure. The 15-35 minute fractions were pooled and evaporated to dryness under nitrogen. The sample was injected and analyzed by HPLC using the same gradient. The regional HPLC profiles of the 15-35 minute control and HS2-ester fractions are shown in Figure 51.

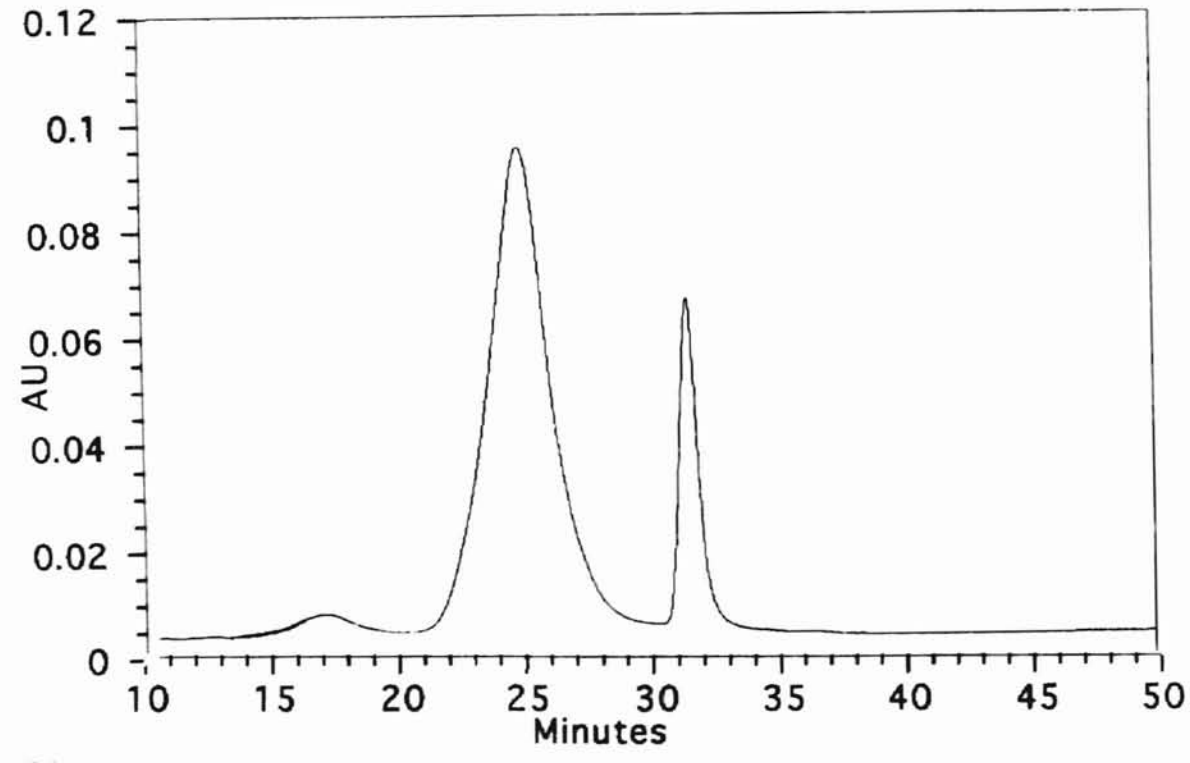
The repurification greatly improved peak resolution and three metabolites were identified in the 15-35 minute fraction. The first metabolite, HS2-LM1, had a retention time of 17 minutes and a maximum UV absorbance at 313.8 nm and a second maximum absorbance at 266.5 nm. A second metabolite, HS2-LM2, had a retention time of 24.8 minutes and a maximum UV absorbance of 323.3 nm. The third metabolite, HS2-LM3, had a maximum UV absorbance of 323.3 nm and a retention time of 31.5 minutes. The UV spectra of HS2-LM1, 2 and 3 are shown in Figure 52. The UV spectrum and retention time of HS2-LM3 match well with the HS2-acid standard (see Figure 53). Repurification of the 15-35 minute fraction greatly increased sample purity and allowed the metabolites to be separated and collected as pure peaks.

A small amount of control liver was spiked with HS2-ester to confirm metabolites were not artificially produced due to the interaction between the parent compound and liver. Samples were processed in the same manner as the metabolite samples. The spiked sample, HS2-ester liver extract, control liver extract and HS2-ester standard profiles were compared. Peaks were detected which corresponded to the peaks present in the HS2-ester standard or control extract. In the region where metabolites elute (15-30 minutes), no peaks were detected suggesting metabolites are not produced due to the extraction process.

As an additional confirmatory measure, control liver was spiked with a small amount of purified HS2-acid to ensure metabolites were not produced due to metabolite interaction with the liver sample. Samples were processed in the same manner as the metabolite samples. The HS2-acid spiked control sample, HS2-ester liver extract, control liver extract and HS2-acid standard HPLC profiles were compared. Peaks were detected

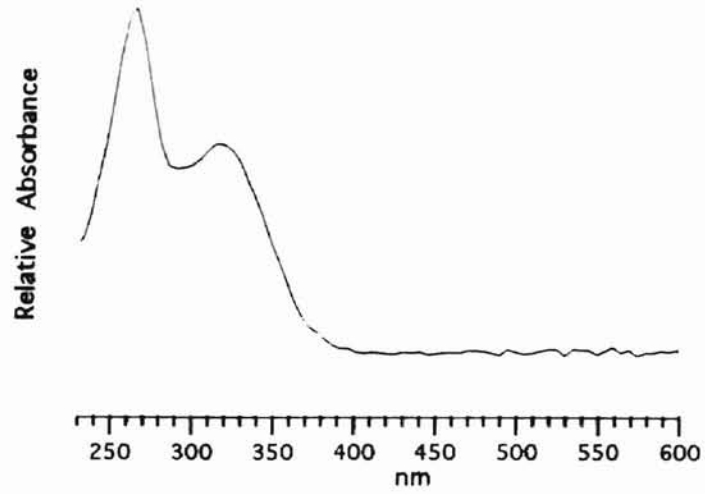


(a)

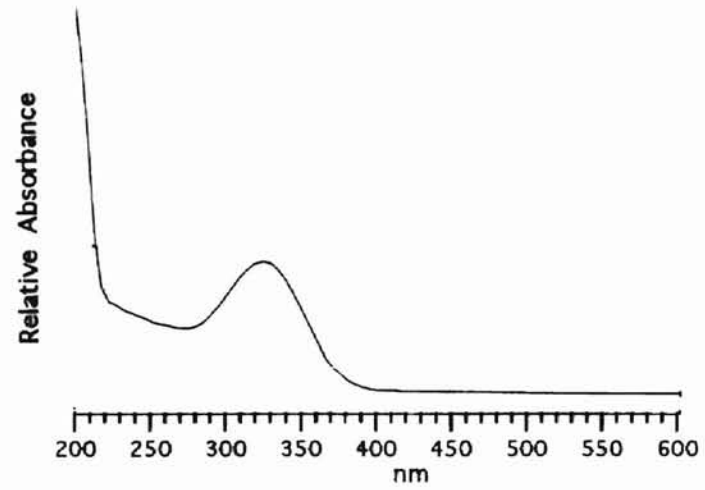


(b)

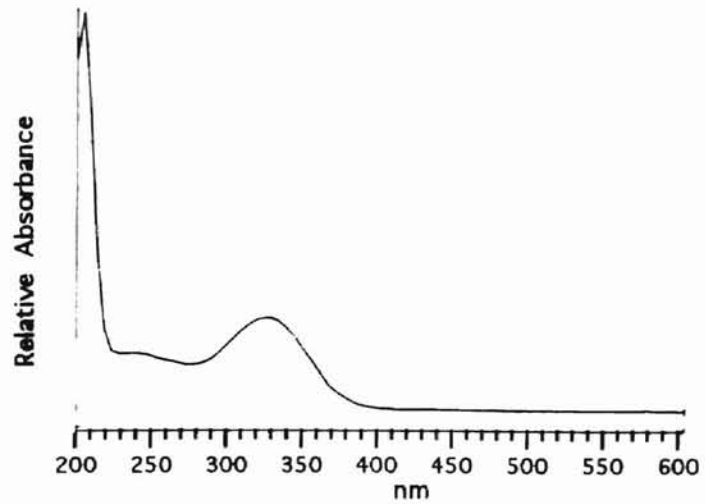
Figure 51: HPLC Profiles of Control (a) and HS2-ester (b) 15-30 Minute Fractions.



(a)

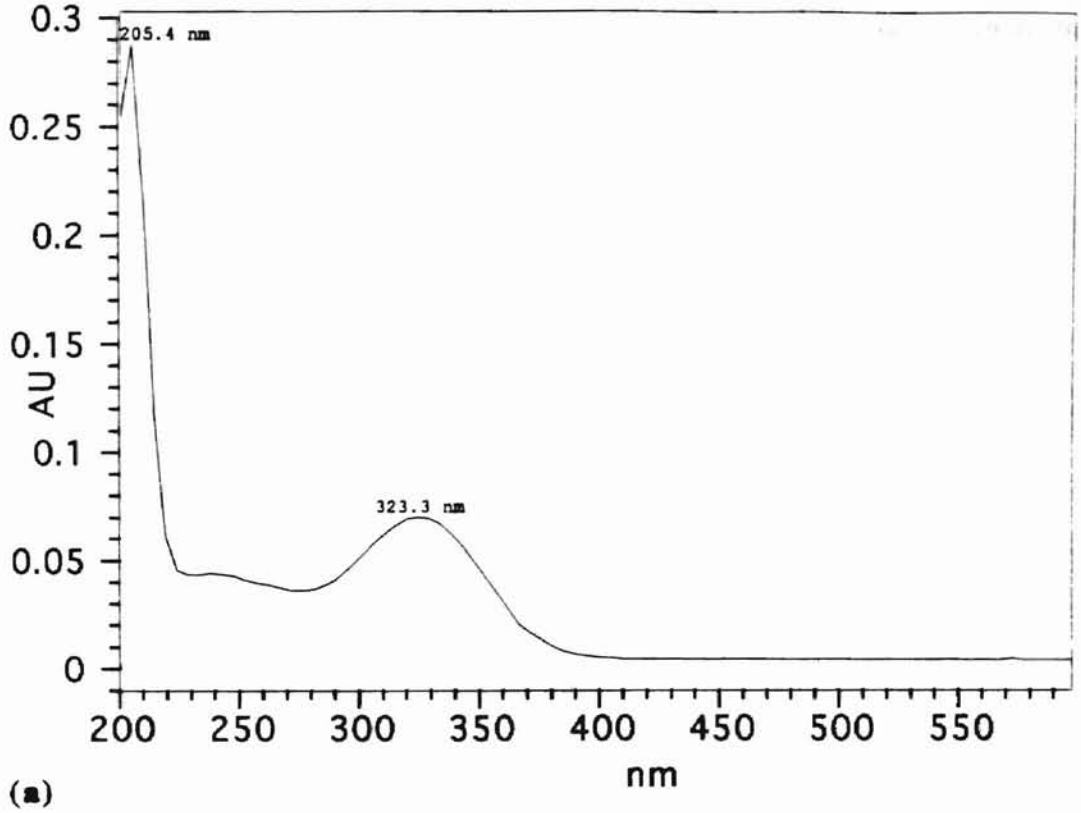


(b)

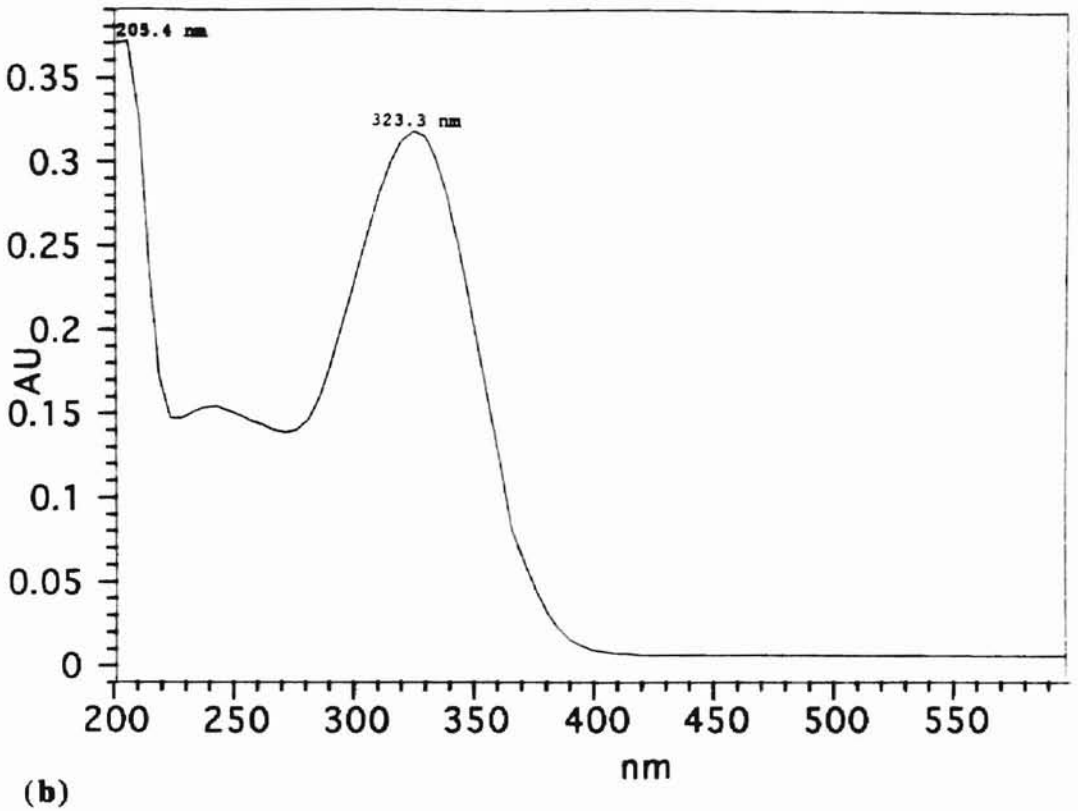


(c)

Figure 52: UV Spectra of HS2-LM1 (a), HS2-LM2 (b) and HS2-LM3 (c).



(a)



(b)

Figure 53: UV Spectra of HS2-LM3 (a) and HS2-acid (b).

which match the peaks present in the control extract or HS2-acid standard. In the region where metabolites were detected (15-30 minutes), only one peak was detected which matched the HS2-acid standard peak suggesting other metabolites are not produced from interaction between the liver sample and metabolite. HPLC profiles of HS2-ester and HS2-acid spiked control liver extracts are shown in Figure 54a and 54b, respectively.

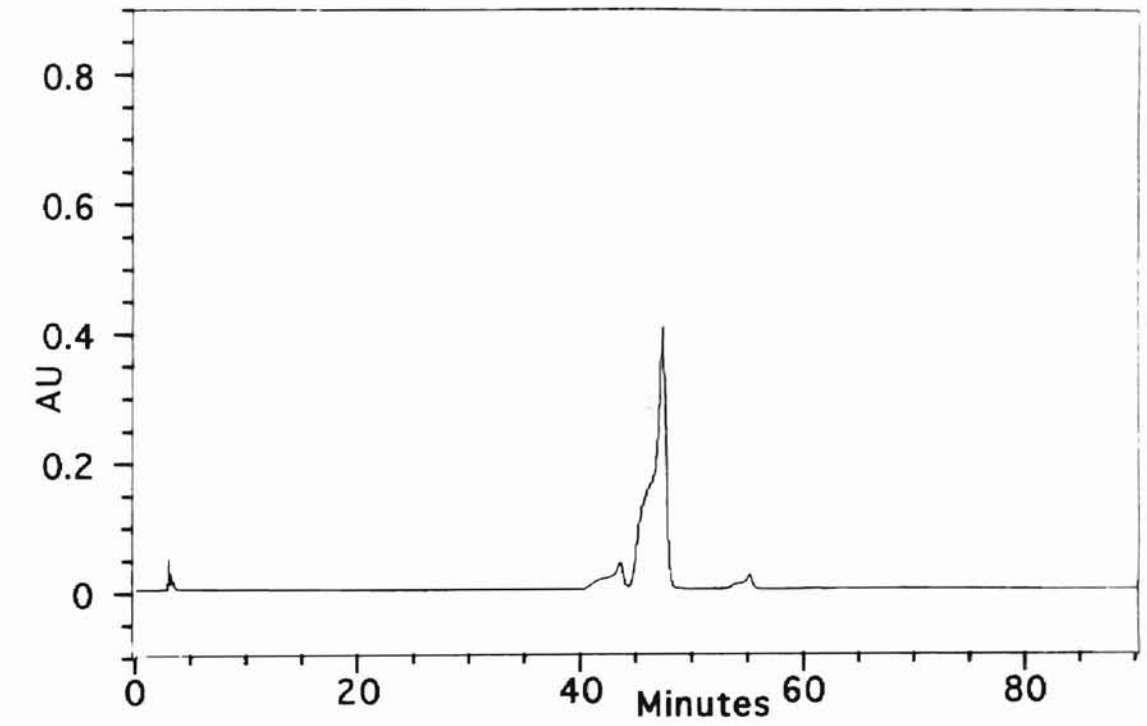
O-DHA* Purity Check

Prior to checking the purity of O-DHA*, radioactive contamination of the HPLC system was tested by injecting 300 μl of methanol and running the same gradient as was used to check O-DHA* purity (gradient 2-Table 7). One milliliter samples were collected and assayed for radioactivity. Only background levels of radioactivity were detected in the HPLC system. This same test was conducted prior to any radiolabelled extract being injected. In all cases, only background levels were detected in the system (data not shown).

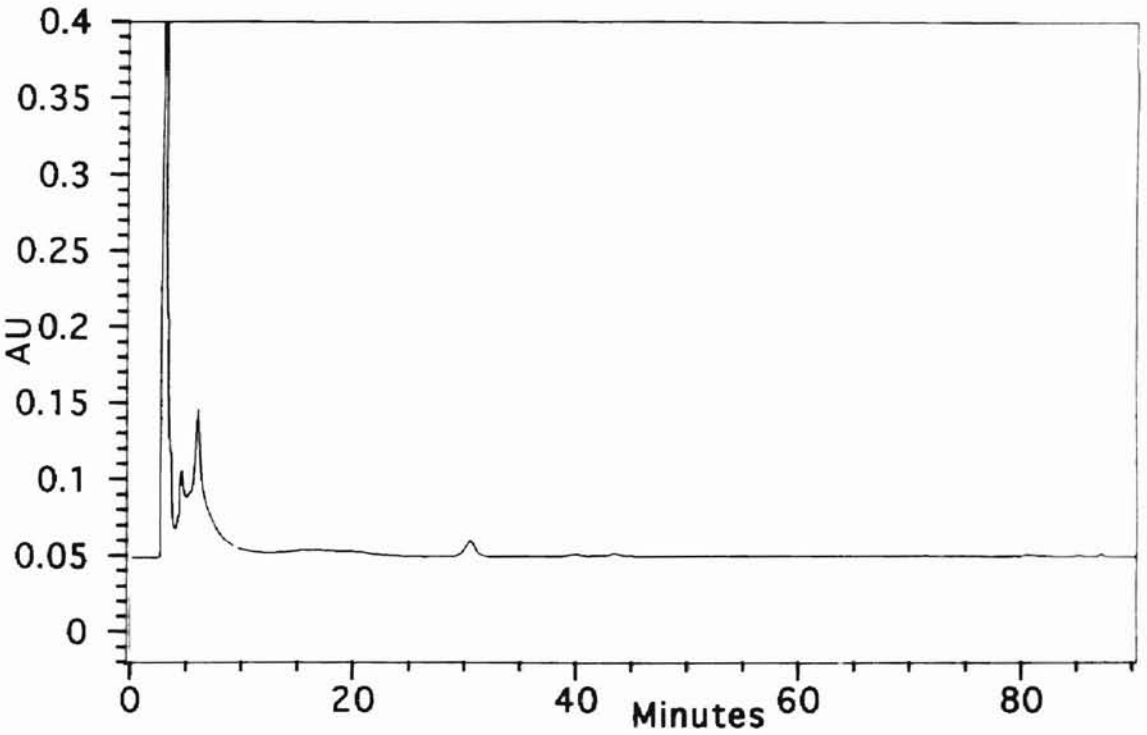
The purity of O-DHA* was tested by injecting 0.2 μg of O-DHA* and running gradient 2. Ninety 1-ml samples were collected and assayed for radioactivity. The HPLC profile and ^{14}C radioactive trace are shown in Figure 55. O-DHA* has a retention time of 24.5 minutes and an UV maximum absorbance of 313.8 nm. The ^{14}C radioactive trace shows one peak containing radioactivity. Other impurities were not detected in the chromatogram or the ^{14}C radioactive trace. Hence, O-DHA* was a pure compound and additional purification was not needed prior to administration.

O-DHA* Liver Metabolism Studies

Although the O-DHA* purity was tested prior to administration, it is important to check that only pure O-DHA* was injected into the animals since the parent compound could isomerize and/or oxidize prior to administration. After administration of O-DHA*, the syringe was rinsed with methanol, and the sample was injected and analyzed by HPLC using gradient 2. One milliliter fractions were collected and assayed for radioactivity. Only one peak containing radioactivity was detected in the ^{14}C radioactive trace and the HPLC



(a)



(b)

Figure 54: HPLC Profiles of HS2-ester (a) and acid (b) Spiked Control Liver Extracts.

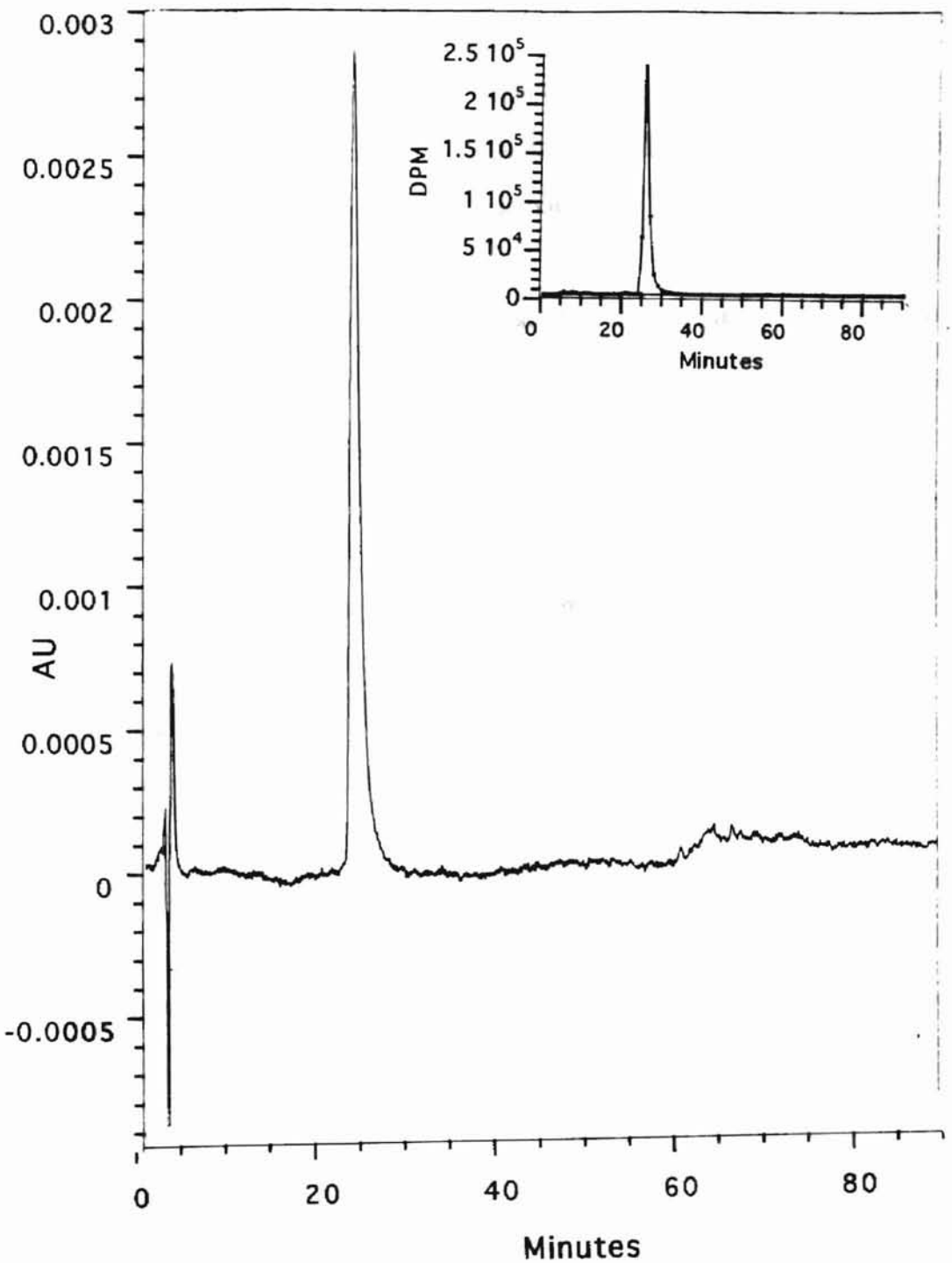


Figure 55: HPLC Profile of O-DHA*. The ^{14}C Radioactive Trace is shown in the insert.

profile shows one peak eluting at 24 minutes with an UV maximum absorbance at 313.8 nm (see Figure 56). This matches well with the O-DHA purity results. Rats received only the parent compound, and isomerization and/or other oxidation process commonly associated with retinoids did not occur.

Since metabolites are in low concentration in biological media, it is difficult to isolate large quantities for identification and maintain the radiation safety office standards which stipulate the maximum amount of radioactivity given to a rat which is to be incinerated is 10 μCi. This problem was circumvented by feeding rats an equal amount of O-DHA which increases metabolite concentration while allowing the metabolites to be easily traced with the ^{14}C radiolabel.

The control and O-DHA* liver samples were processed and analyzed by HPLC using gradient 1. The HPLC profile of control and O-DHA* liver extracts and the ^{14}C radioactive trace shown in Figure 57. The chromatogram showed a number of polar metabolites of O-DHA*. A peak was detected at 4 minutes but the UV spectrum of this peak was difficult to obtain since the solvent front overwhelms the UV spectrum. Several metabolites were detected in the 35-50 minute range but were poorly resolved. O-DHA* and its E-isomer were recovered in the liver extract and were detected at 55.6 and 54.7 minutes with a maximum UV absorbances of 313.8 nm and 318.5 nm, respectively. Although the parent compound was pure when administered, the E-isomer was detected and is an artifact of the extraction procedure which will be discussed later.

Since the metabolite resolution was poor in the 35-50 minute range, these fractions were pooled and re-analyzed. The samples were dried under nitrogen and analyzed by HPLC using gradient 1. One milliliter fractions were collected, and 10 μl was assayed for radioactivity. The resolution was improved and three metabolites were identified, O-DHA*-LM1, O-DHA*-LM2 and O-DHA*-LM3. The first metabolite, O-DHA*-LM1, had a retention time of 39.5 minutes and a maximum UV absorbance of 323.3 nm. A second metabolite, O-DHA*-LM2, was detected at 41 minutes with an UV maximum absorbance

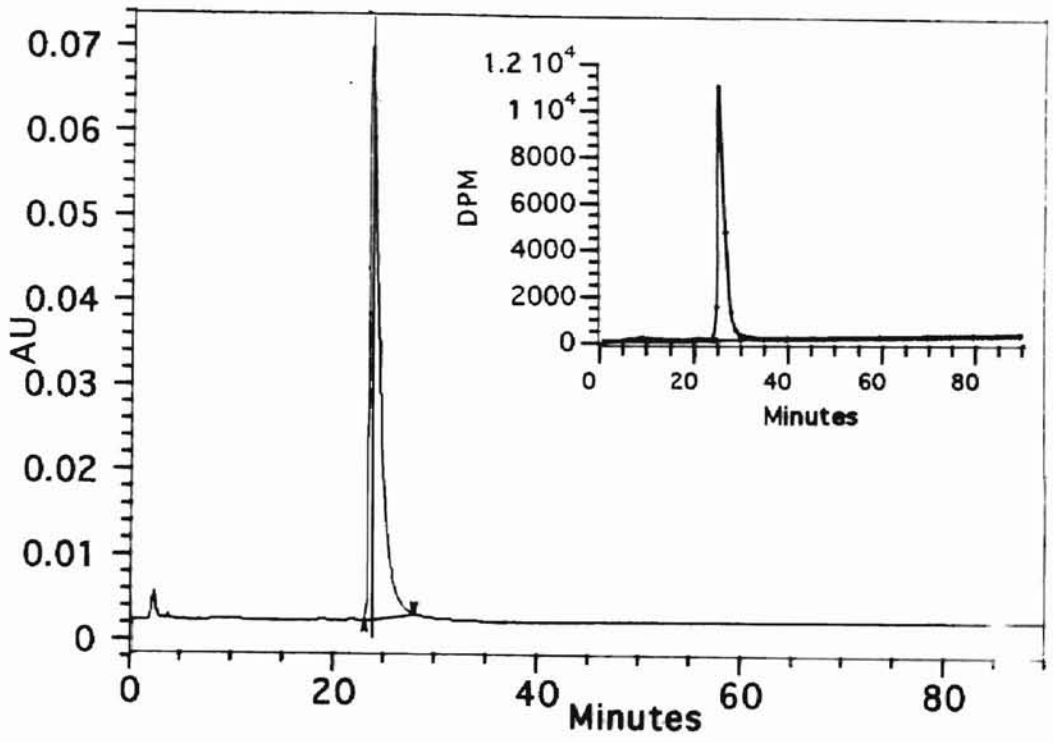
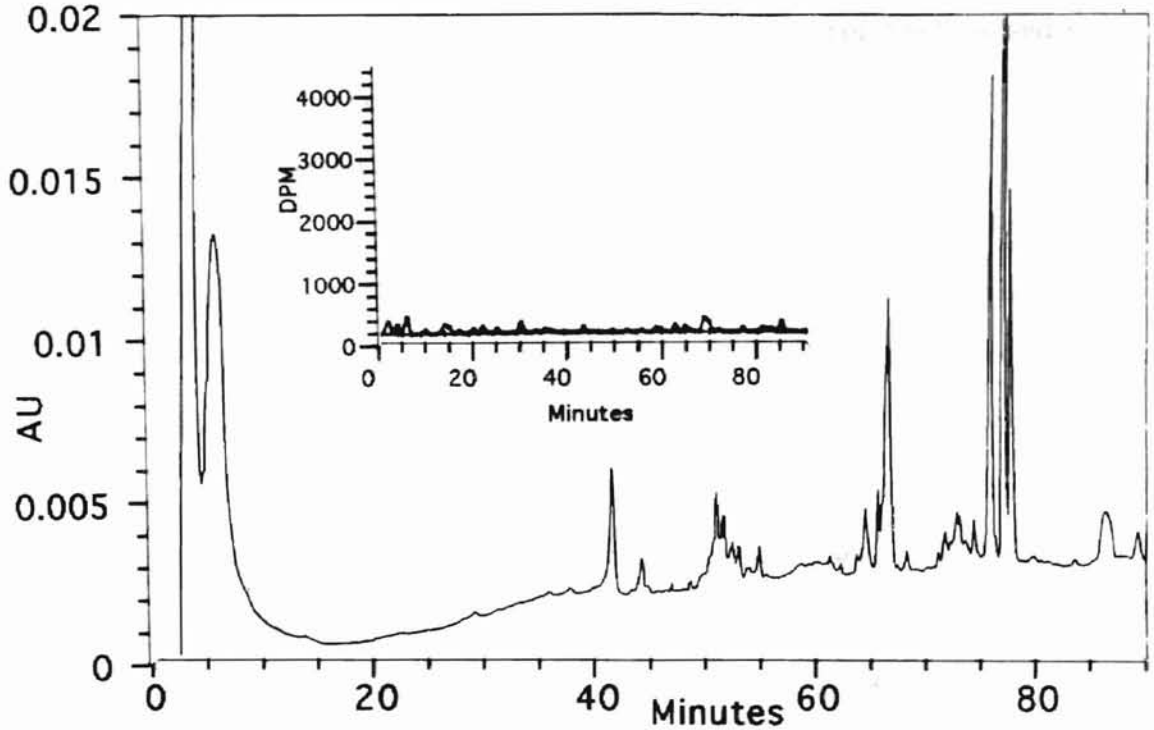
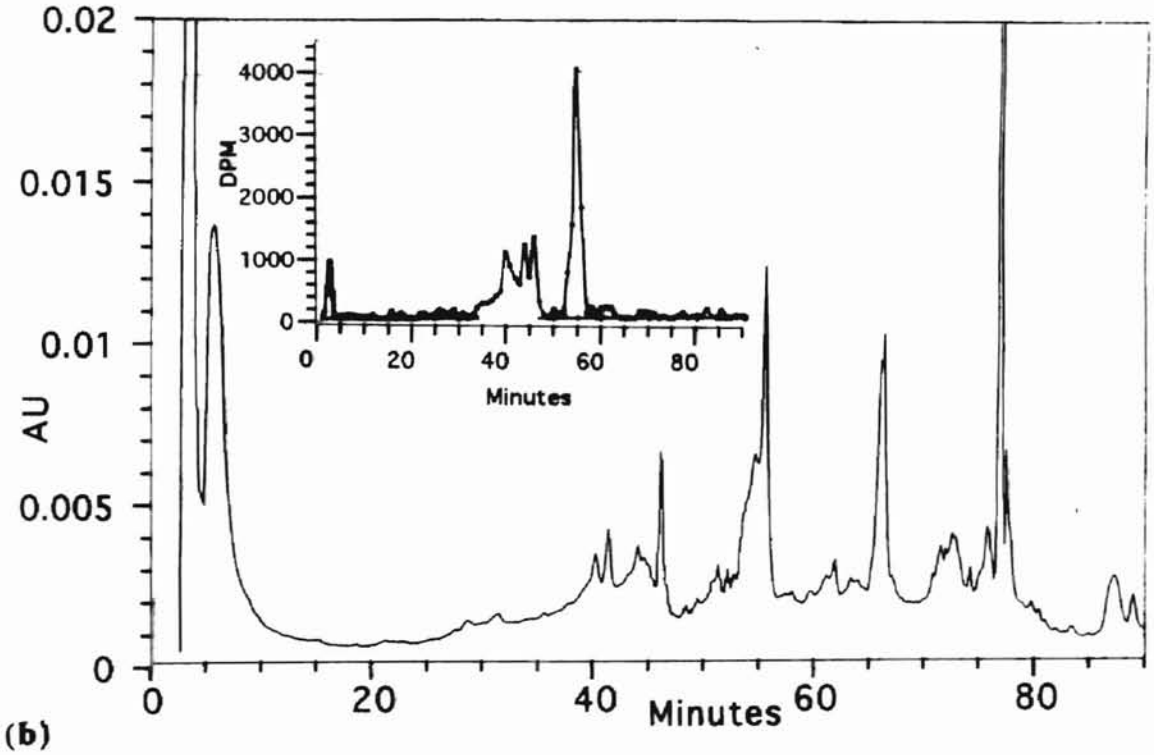


Figure 56: HPLC Profile of O-DHA* Syringe Wash. The ^{14}C Radioactive Trace is shown in the insert. The line in the chromatogram represents the peak apex. The smaller lines on the bottom indicate the presence of a peak.



(a)



(b)

Figure 57: HPLC profiles of Control (a) and O-DHA* (b) Liver extracts. The ^{14}C Radioactive Trace is shown in the insert.

at 304.3 nm. A third metabolite had a maximum UV absorbance of 299.5 nm and a retention time of 45.2 minutes. The ^{14}C radioactive trace and UV spectra are shown in Figures 58 and 59, respectively.

Heteroarotinoids standards were synthesized based on known retinoid metabolism in which there is oxidative cleavage of the polyene side chain or cyclic ring oxidation (Sunthankar et al. 1993). These standards were designed to aid in metabolite identification. Various standards were analyzed by HPLC using the same gradient which was used for metabolite isolation (gradient 1). Standards are listed in Table 8. Although the standards had similar retention times as the metabolites, the UV spectra of the standards did not overlay the metabolites. The metabolites and the standards did have similar maximum UV absorbances suggesting the metabolites are similar in structure to the standards. Since metabolite concentration was low in many cases, reliable UV spectra were difficult to obtain.

As a confirmatory measure, a small amount of O-DHA* was added to 1 g of control liver and processed in the same manner as the metabolite samples. This was to ensure the metabolites produced were not artifacts of the extraction procedure. One milliliter samples were collected and assayed for radioactivity. The HPLC profile and ^{14}C radioactive trace are shown in Figure 60. The parent compound was recovered at 55 minutes with a maximum UV absorbance of 313. 8 nm. An additional peak was detected at 54 minutes with a maximum UV absorbance of 318.5 nm which is characteristic of the E-isomer of O-DHA*. The isomer was not administered to the animals (see Figure 56) and is a product of the extraction procedure. Every precaution was taken to ensure that isomerization and/or oxidation did not occur since retinoids are extremely sensitive to low levels of light and heat. This experiment was designed to serve as a control for isomerization and/or oxidation processes which may occur during the extraction process.

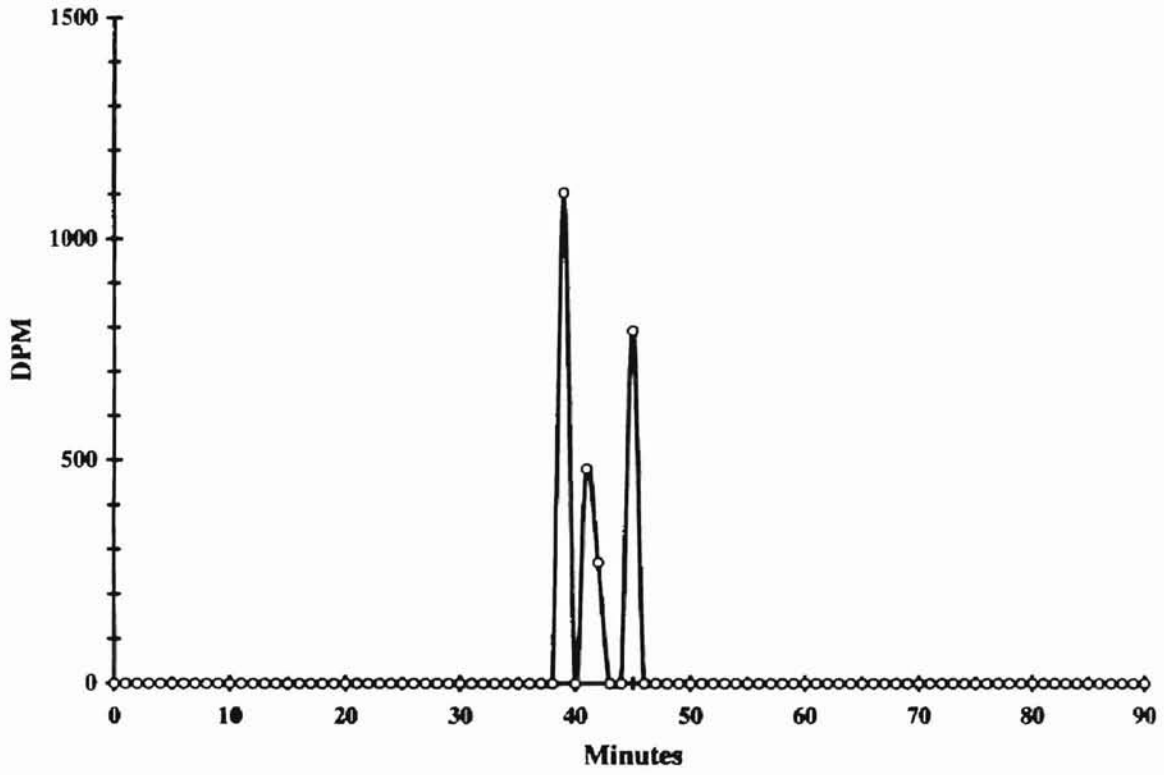
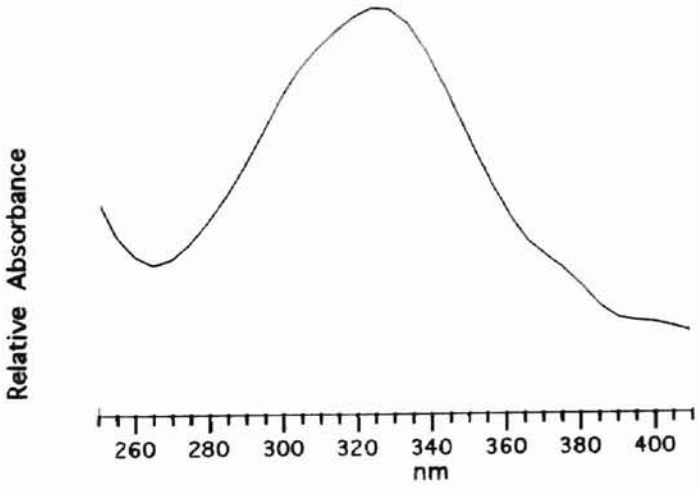
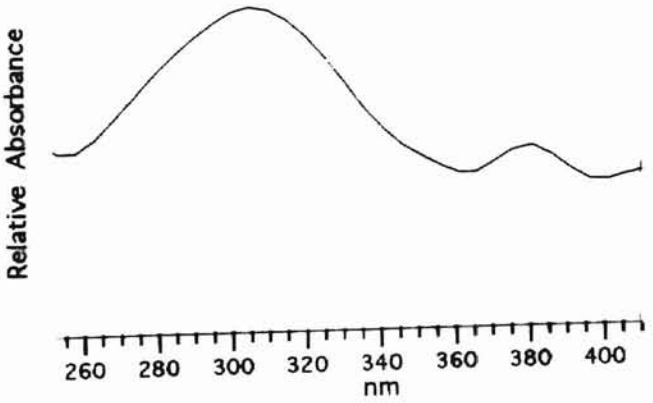


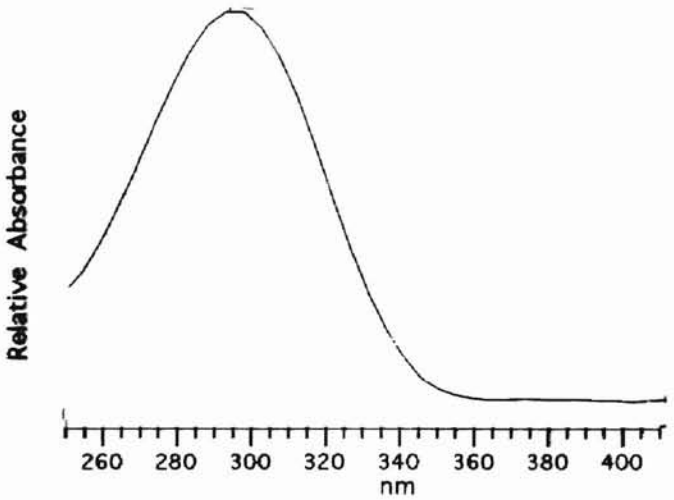
Figure 58: The ^{14}C Radioactive Trace of the 35-50 Minute Fraction of O-DHA* Extract.



(a)



(b)



(c)

Figure 59: UV Spectra of O-DHA*-LM1 (a), O-DHA*-LM2 (b) and O-DHA*-LM3 (c).

Table 8: List of Heteroarotinoid Standards

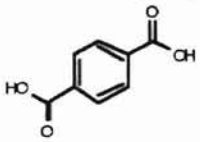
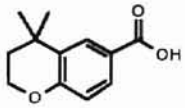
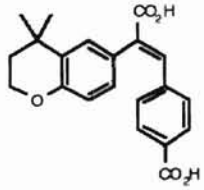
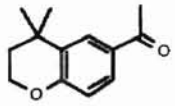
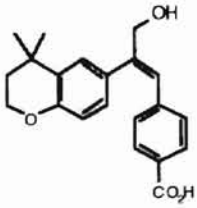
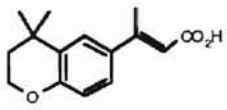
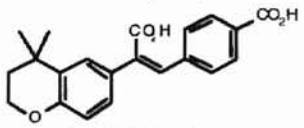
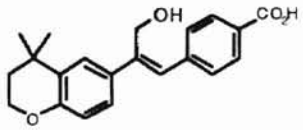
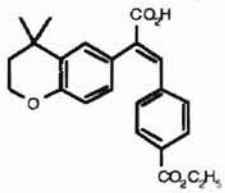
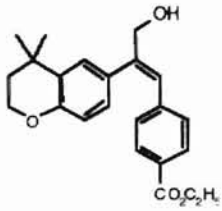
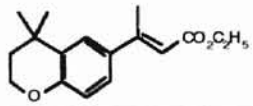
Name/Structure	Retention Time (minutes)	Maximum UV Absorbance (nm)
Standard 1(S-1) 	5.8	242.9
Standard 2 (S-2) 	25.2	261.7
Standard 3 (S-3) 	27.5	294.8
Standard 4 (S-4) 	29.1	280.6
Standard 5 (S-5) 	33.3	285.3
Standard 6 (S-6) 	37.9	294.8

Table 8: List of Heteroarotinoid Standards Cont.

Name/Structure	Retention Time (minutes)	Maximum UV Absorbance (nm)
Standard 7 (S-7) 	38.0	285.3
Standard 8 (S-8) 	42.5	313.8
Standard 9 (S-9) 	43.5	285.3
Standard 10 (S-10) 	47.3	295.3
Standard 11 (S-11) 	59.0	299.5

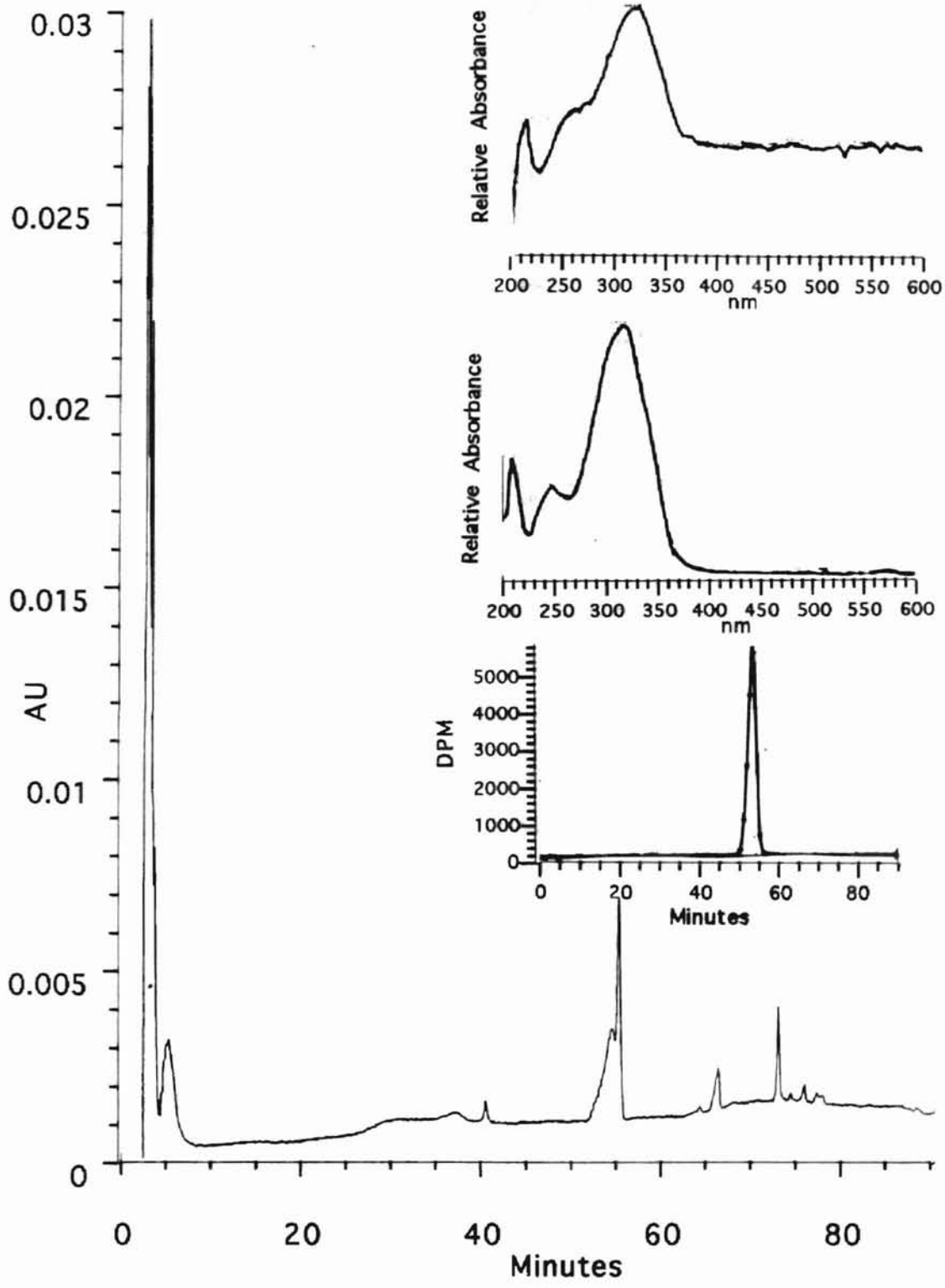


Figure 60: HPLC Profile of the O-DHA* Spiked Control Liver Extract. The ^{14}C Radioactive Trace and UV Spectrum are shown in the insert.

O-DHA* Kidney Metabolism Studies

The control and O-DHA* kidney extracts were analyzed on the same day by HPLC using gradient 1. A number of polar metabolites were detected and the HPLC profiles of the control and O-DHA*extracts are shown in Figure 61. Two metabolites, O-DHA*-KM1 and 2, eluted very early with retention times of 4 and 12 minutes, respectively. The UV spectra were not obtainable for either since the solvent front and other polar material from the extract overwhelmed the spectrum. This is especially true for the 4 minute peak while the 12 minute peak is small and difficult to detect among all the other polar components in this range. A peak was detected at 20 minutes in the O-DHA* extract. This peak was not detected in the control extract and was not present in the radioactive trace, suggesting this peak is an unidentified compound in the kidney and is not a metabolite of O-DHA*.

Four metabolites were detected in the 30-50 minute range. O-DHA*-KM3 eluted at 29.2 minutes with a maximum UV absorbance of 285.4 nm while O-DHA*-KM4 was detected at 37.2 minutes with a maximum UV absorbance of 299.5 nm. O-DHA*-KM5 had a maximum UV absorbance of 294.8 nm and a retention time of 44.1 minutes while O-DHA*-KM6 had a maximum UV absorbance of 299.5 nm and eluted at 47.8 minutes. The parent compound was recovered from the liver and detected at 54 minutes with a maximum UV absorbance of 313.8 nm. The UV spectra of O-DHA*-KM3, 4, 5, 6 and the parent compound are shown in Figure 62 and 63.

In cases where the entire extract was injected into the HPLC, the quality of the UV spectra may be poor and the retention time may vary. This observation is probably due the extract having many unidentified compounds which interact with the column and this does not allow for good separation of the metabolites. This problem was circumvented by re-analyzing fractions which only contained the metabolite of interest. This usually gave an improved UV spectra and more reliable retention times.

The 25-32 and 35-40 minute fractions of the O-DHA* extract were re-analyzed by HPLC using gradient 1. One milliliter fractions were collected and 10 μl were assayed for

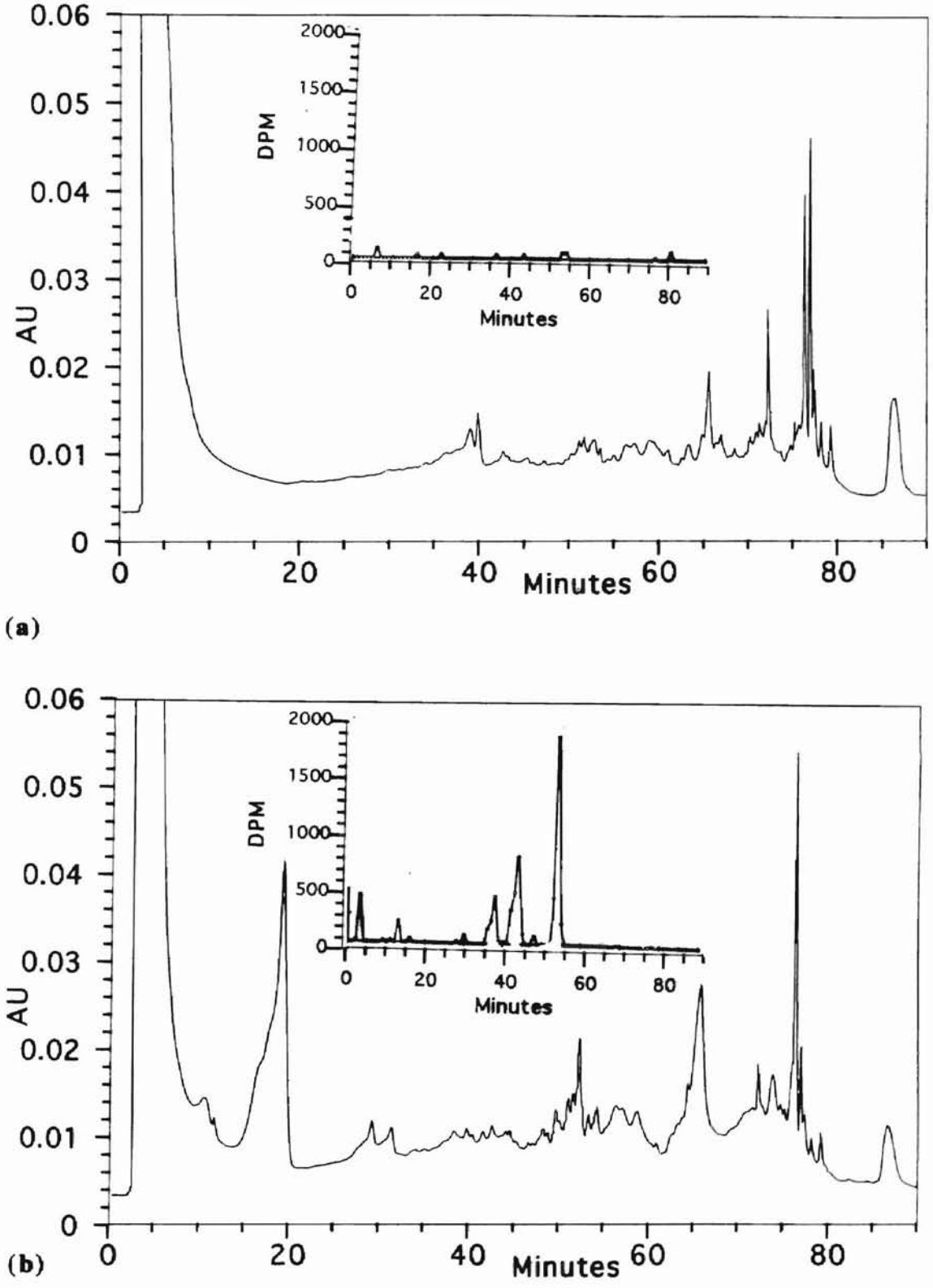


Figure 61: HPLC Profiles of Control (a) and O-DHA* (b) Kidney Extracts. The ^{14}C Radioactive Trace is shown in the insert.

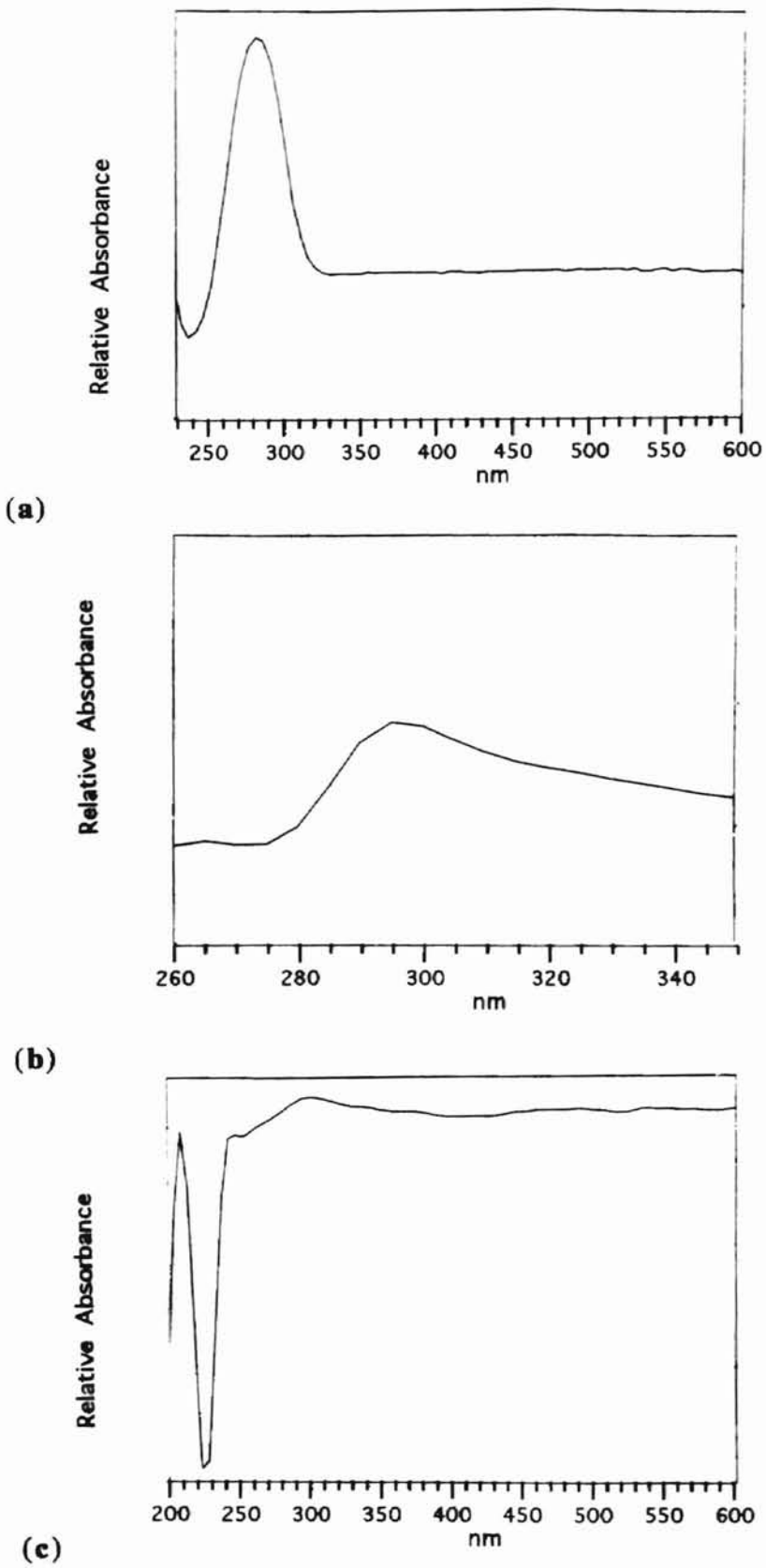
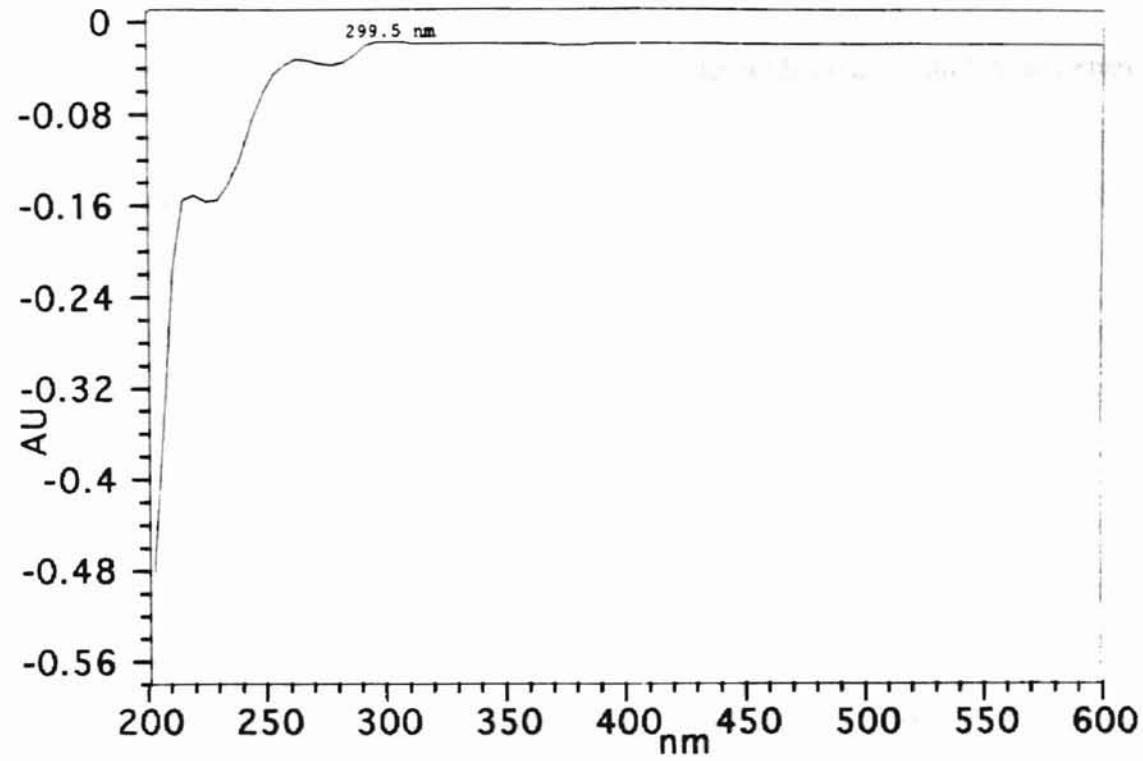
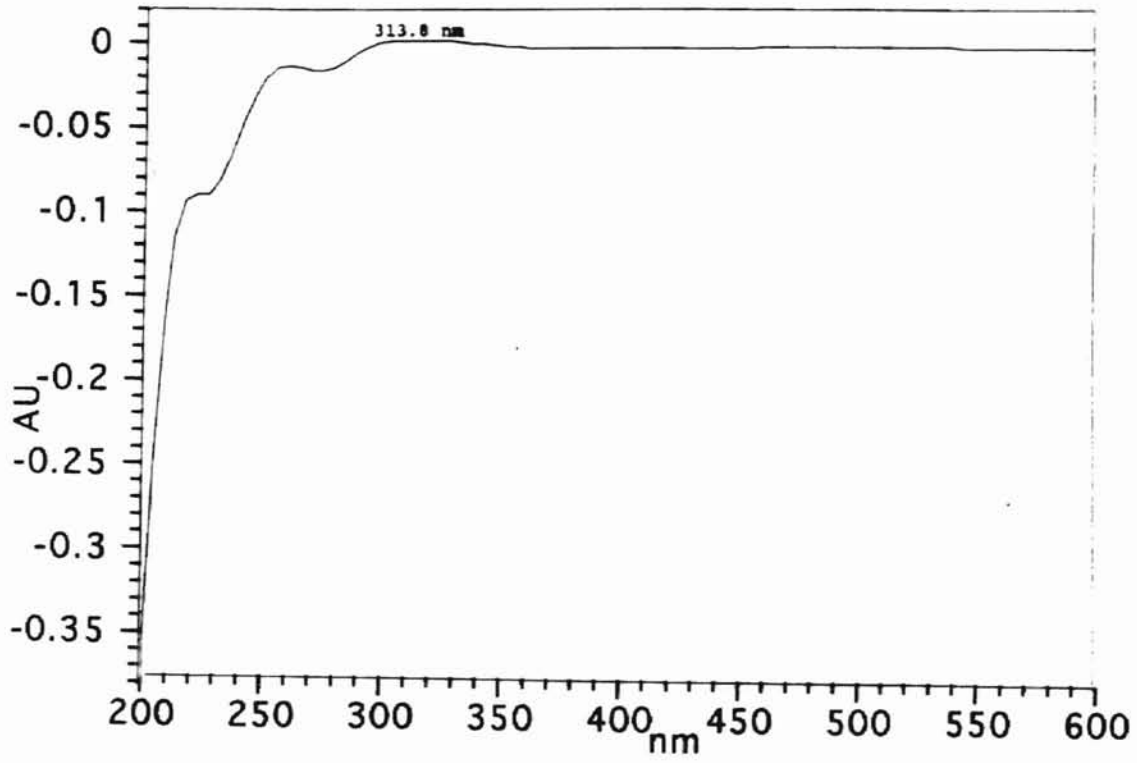


Figure 62: UV Spectra of O-DHA*-KM3 (a), O-DHA*-KM4 (b) and O-DHA*-KM5 (c).





(b)

Figure 63: UV Spectra of O-DHA*-KM6 (a) and O-DHA* (b).

radioactivity. O-DHA*-KM3 was detected at 32 minutes with a maximum UV absorbance of 280.6 nm. The retention time and maximum UV absorbance match well with S-4 (see Table 8). As a confirmatory measure, a small amount of S-4 was added to the O-DHA*-KM3 sample and re-analyzed by HPLC using gradient 1. The peak height of O-DHA*-KM3 did increase in response to the addition of S-4 (see Figure 64). These results support an initial identification of O-DHA*-KM3. O-DHA*-KM4 was detected at 39 minutes with a maximum UV absorbance of 294.8 nm and a second peak was detected with a maximum UV absorbance at 266.5 nm. A peak was detected in the control 35-40 minute fraction with a retention time of 39 minutes and a UV maximum absorbance of 266.5 nm. This suggests the peak at 266.5 nm is an unidentified compound in the control and O-DHA* extracts. The spectrum and retention time of O-DHA*-KM4 match with S-6 providing an initial identification of O-DHA*-KM4. The UV spectra for O-DHA*-KM4, the control fraction and S-6 are shown in Figure 65.

The 40-45 minute fraction was re-analyzed by HPLC using gradient 1 and one milliliter fractions were collected and assayed for radioactivity. O-DHA*-KM5 eluted at 45.5 minutes with a maximum UV absorbance of 304.3 nm. No other peaks in the corresponding control fraction were detected. The UV spectrum is shown in Figure 66. The UV spectrum did not match well with the standards having similar retention times. The metabolite is probably structurally related to some of the standards but is unidentified. Moreover, the UV spectrum of O-DHA*-KM6 did not match well with the standards having similar retention times and it is probably structurally related to the standards but is unidentified.

A small amount of O-DHA* was added to the control kidney and processed in the same manner as the metabolite sample to ensure metabolites were not artifacts produced from the extraction process. One milliliter fractions were collected and assayed for radioactivity. O-DHA* was recovered from the kidney at 54.6 minutes and a maximum UV absorbance of 313.8 nm. Other peaks were not detected in the HPLC profile or the

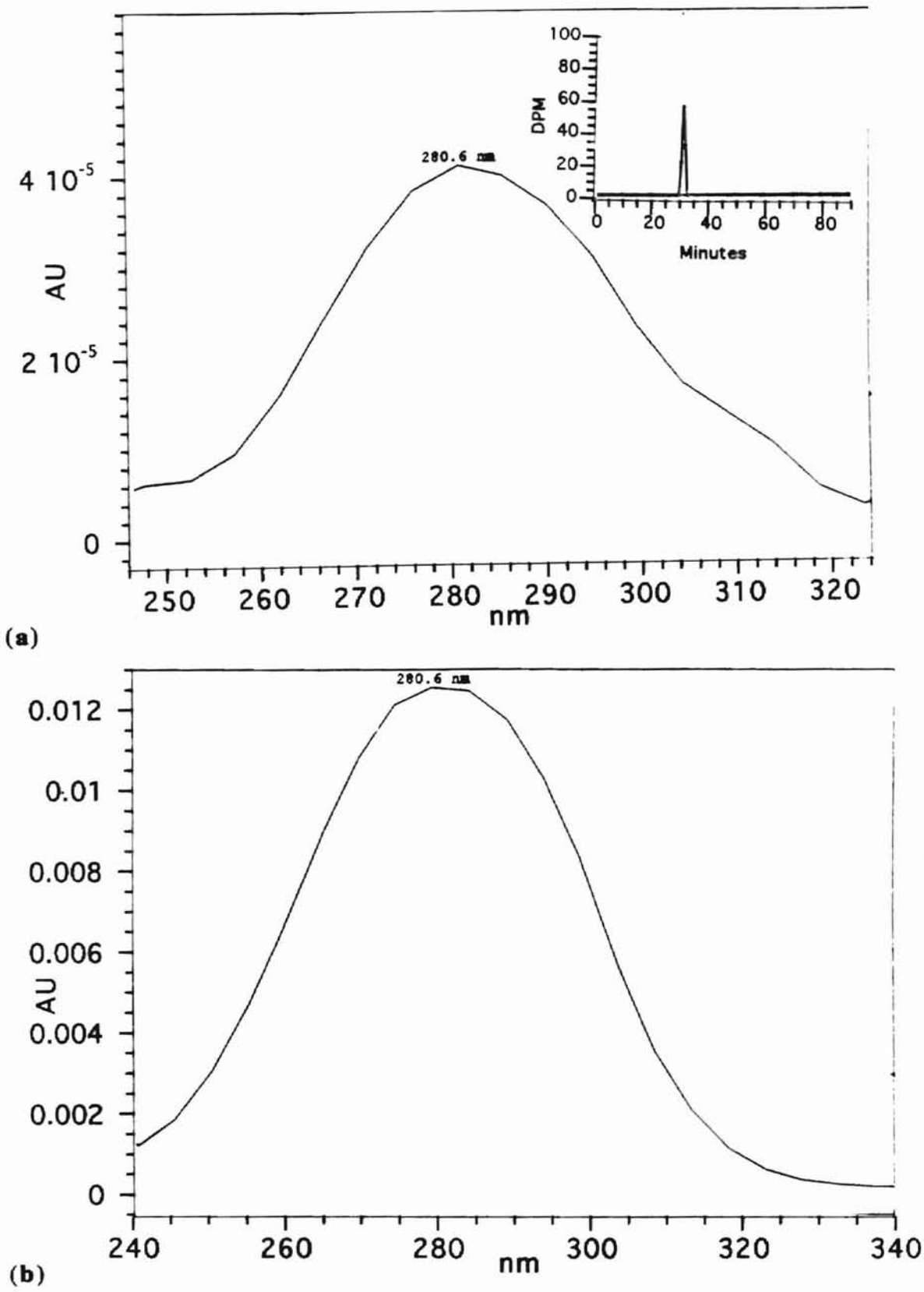


Figure 64: UV Spectra of O-DHA*-KM3 (a) and O-DHA*-KM3 Spiked with S-4 (b). The ^{14}C Radiolabeled Trace of O-DHA*-KM3 is shown in the insert of (a).

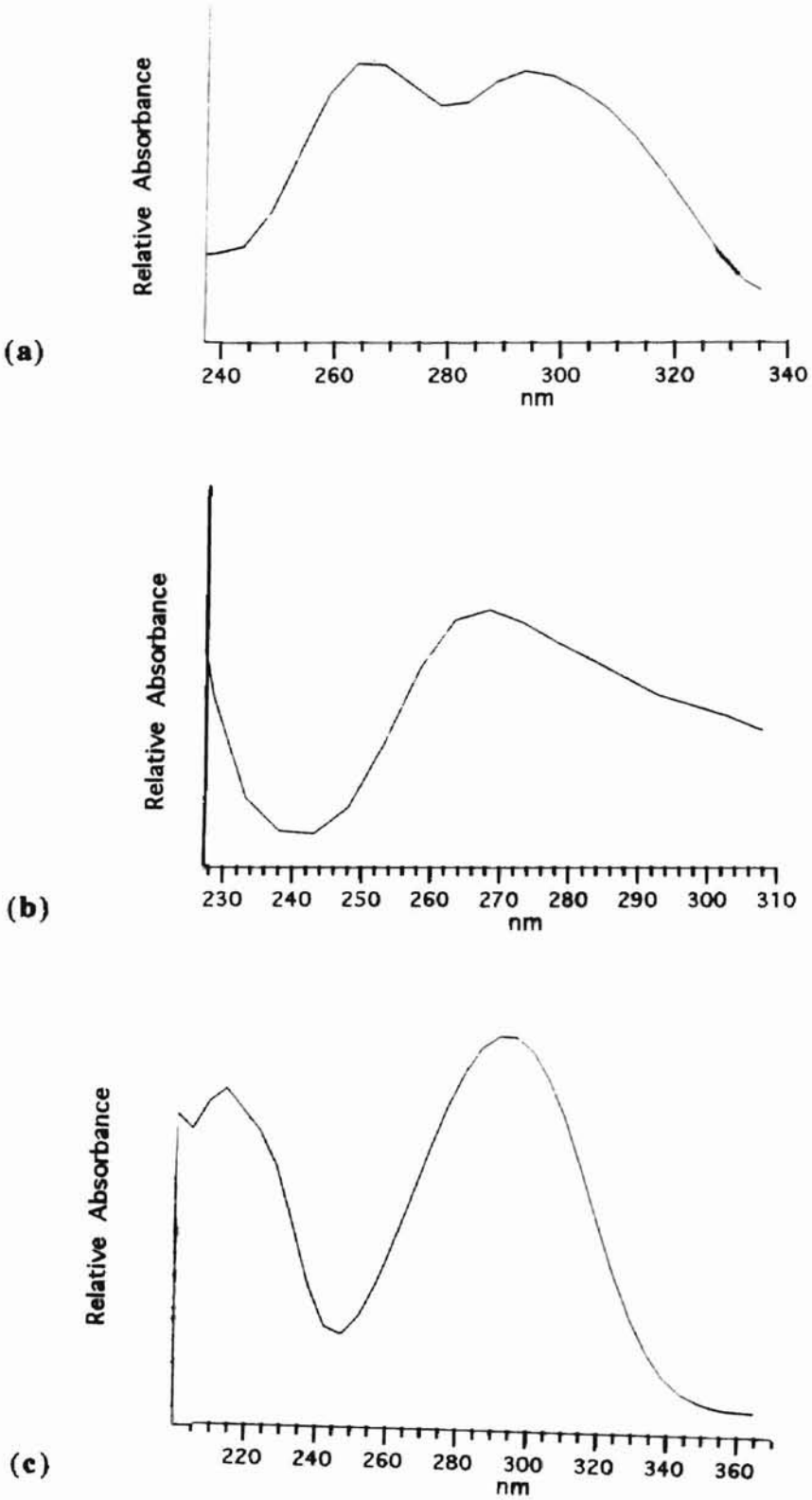
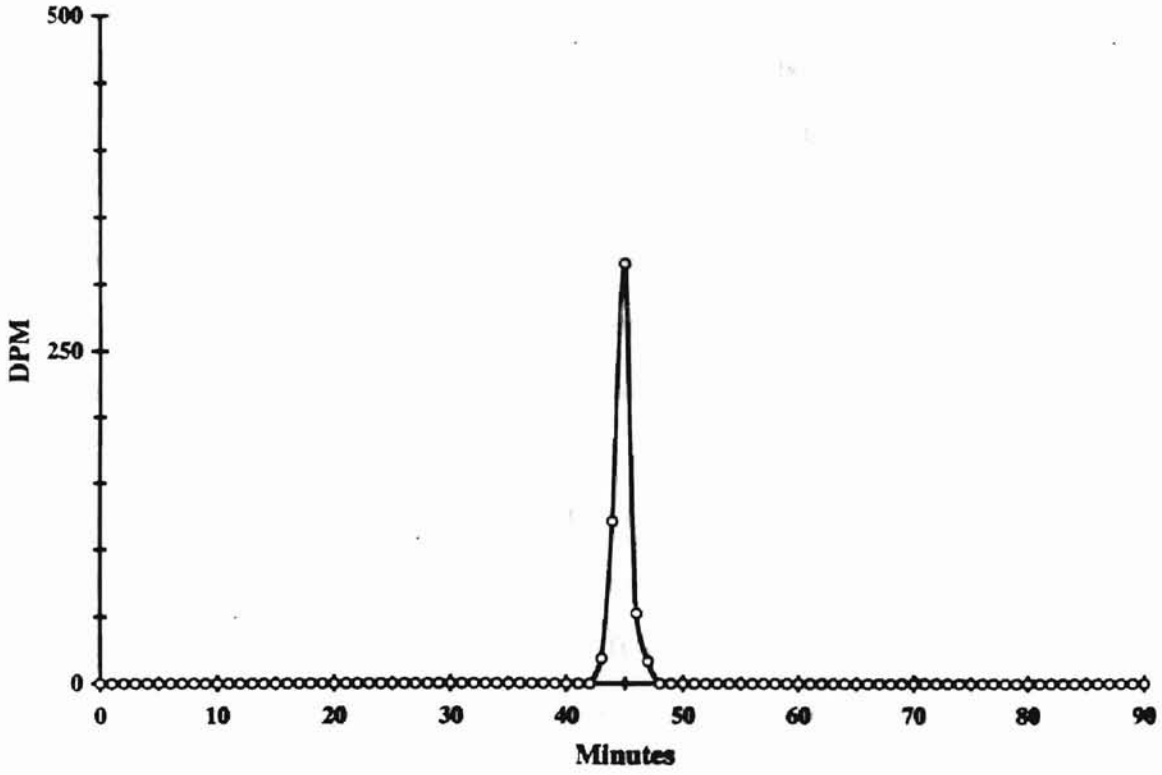
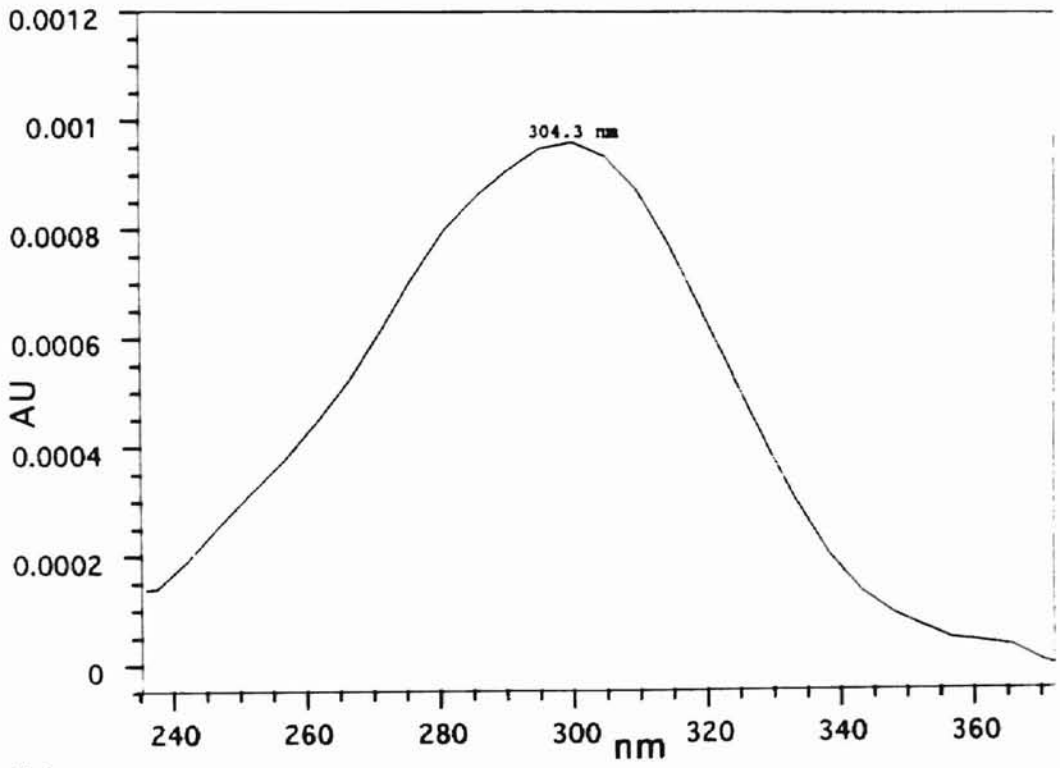


Figure 65: UV Spectra of O-DHA*-KM4 (a), Control Fraction (b) and S-6 (c).



(a)



(b)

Figure 66: The ^{14}C Radiolabeled Trace of O-DHA*-KM5 (a) and the UV Spectra of O-DHA*-KM5 (b).

^{14}C radioactive trace (Figure 67). These results suggest metabolites are not produced from the extraction process and are metabolites of O-DHA* in the kidney.

As an additional confirmation, a small amount of S-4 or S-6 was added to control kidney and processed in the same manner as the metabolite samples. The S-4 and S-6 spiked kidney HPLC profiles are shown in Figures 68 and 69, respectively. The S-4 standard was detected at 34.8 minutes with a maximum UV absorbance of 280.6 nm. The S-6 standard was recovered at 41.4 minutes with a maximum UV absorbance of 294.8 nm (see Figure 69). Other peaks matching O-DHA* kidney metabolites were not detected when the spike S-4 and S-6 samples were analyzed suggesting other metabolites are not artifacts produced by the extraction procedure. Both S-4 and S-6 spiked samples had retention times which ran two minutes later than standards ran previously. This observation may be variations in column temperature or electronic drift.

Different metabolites were found in the kidney than from the liver although a few had similar retention times and maximum UV absorbances particularly in the 41-48 minute range of liver and kidney extracts (see Table 9). These metabolites may be the same in both tissues although the retention times and the maximum UV absorbance are slightly different between the samples. This observation may be due to differences between HPLC runs and low metabolite concentration which does not give high quality UV spectra. More polar compounds were identified in the kidney than in the liver suggesting that these compounds may have been already metabolized by the liver and then excreted by the kidney or these metabolites could have been produced by metabolism of the parent compound in the kidney. However, it is clear that four hour time period is sufficient for metabolites of O-DHA* to be produced in the liver and kidney. In HS metabolism studies, rats were allowed to feed for two hours and then tissue was collected. Metabolites were found in the liver but were not detected in the kidneys or testes suggesting a two hour time period may be insufficient amount of time for metabolites to be metabolized in other tissues. This may depend on a number of factors including individual animal metabolism and heteroarotinoid

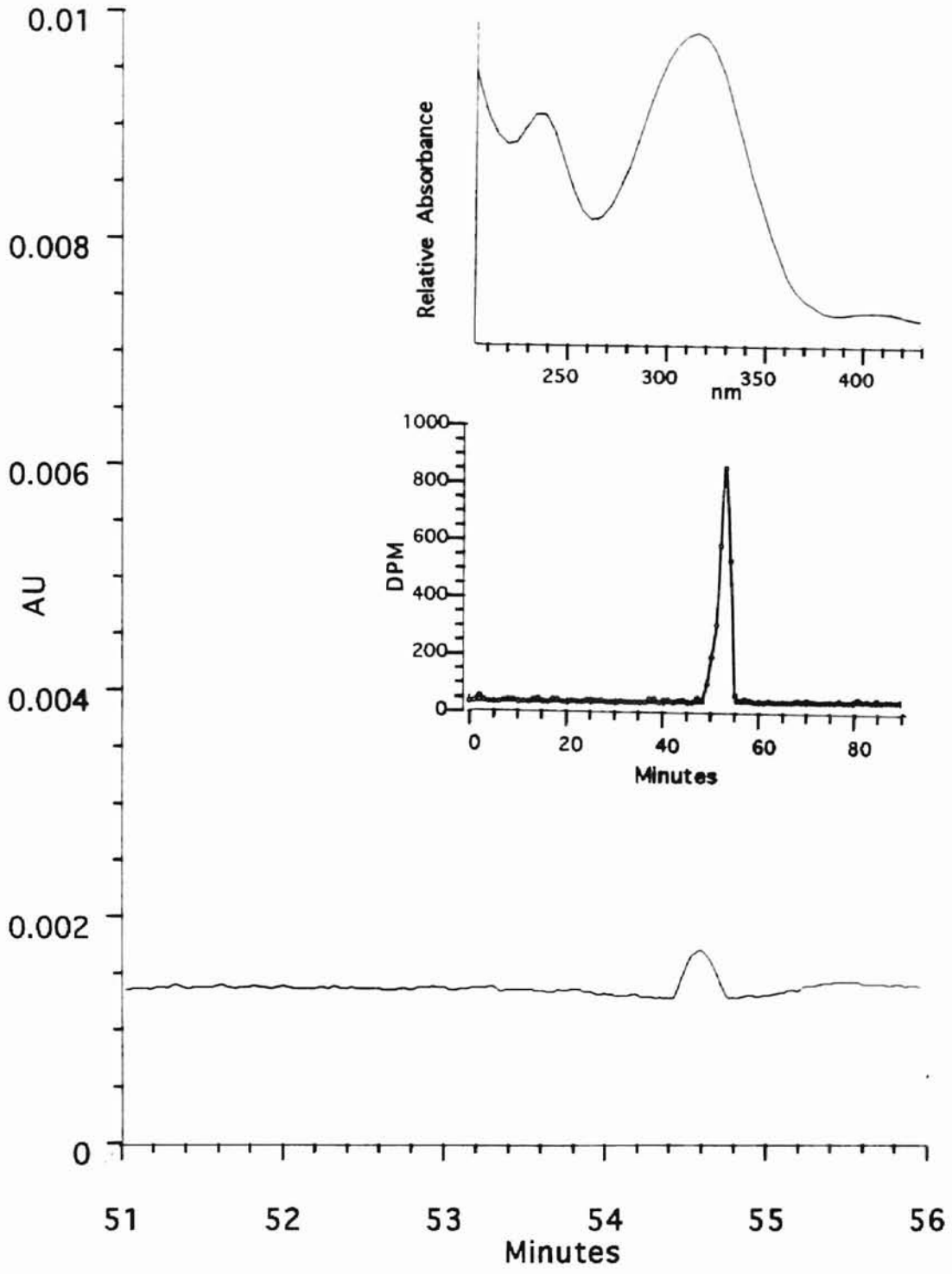


Figure 67: HPLC Profile of O-DHA* Spiked Liver. The UV Spectrum of O-DHA* and the ^{14}C Radioactive Trace of O-DHA* Spiked Liver is shown in the inserts.

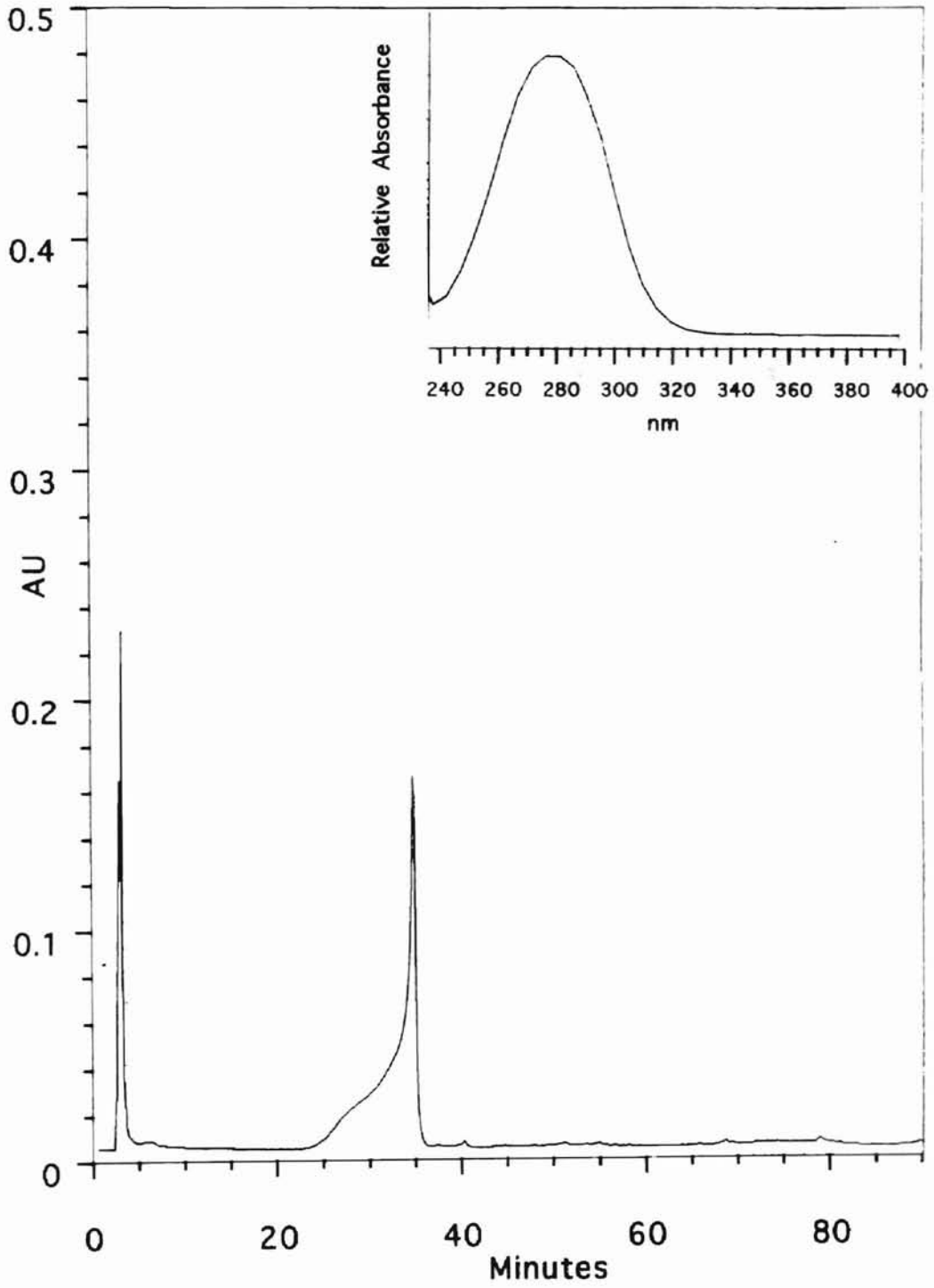


Figure 68: HPLC Profile of S-4 Spiked Kidney Extract. The UV Spectrum of S-4 is shown in the insert.

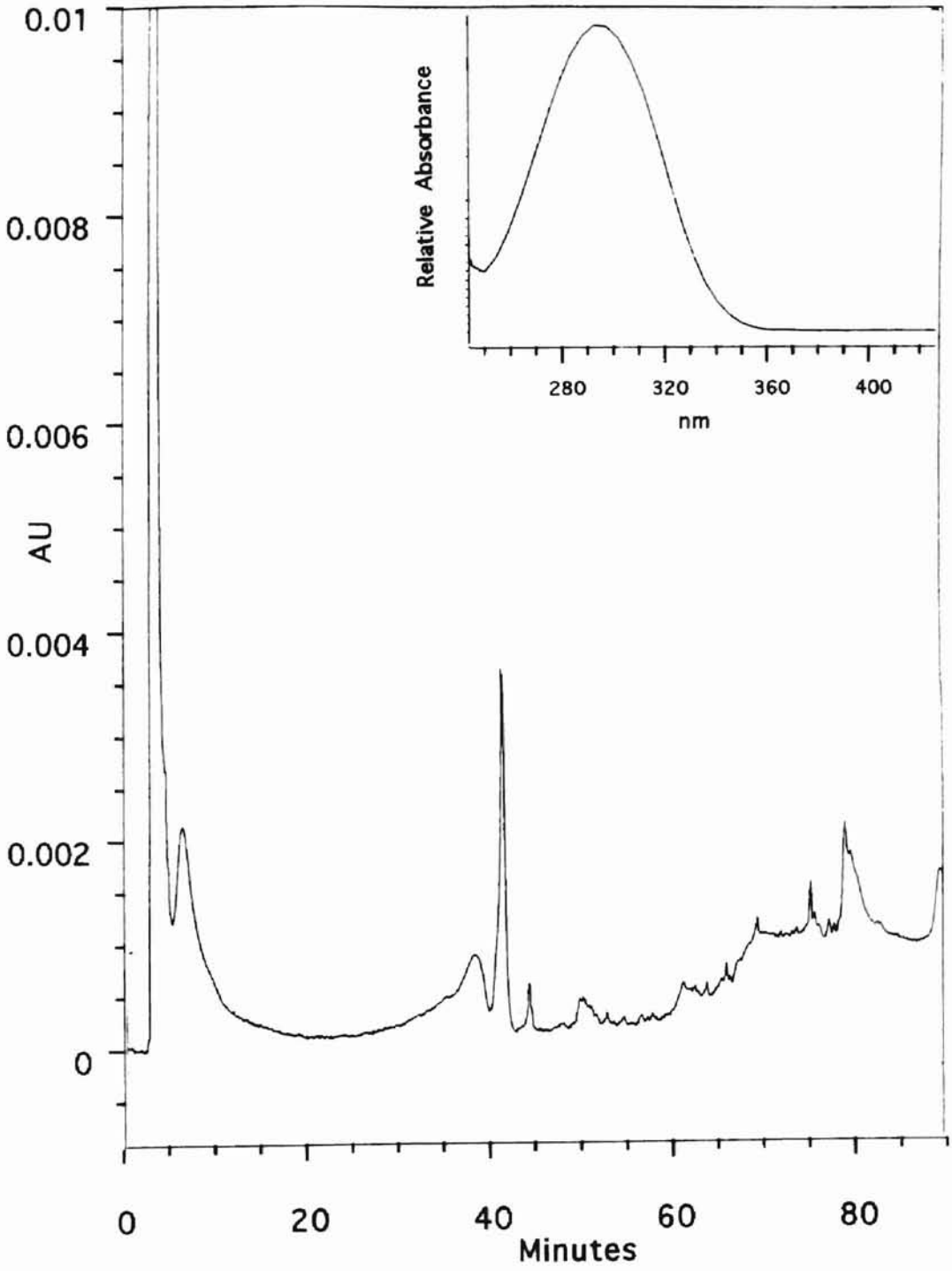


Figure 69: HPLC Profile of S-6 Spiked Kidney Extract. The UV Spectrum of S-6 is shown in the insert.

Table 9: Comparsions of Liver and Kidney Metabolites in the 41-48 Minute Range

Metabolite	Retention Time (minutes)	Maximum UV Absorbance (nm)
O-DHA*-LM2	41.0	304.3
O-DHA*-KM5	44.1	294.8
O-DHA*-LM3	45.2	299.5
O-DHA*-KM6	47.9	299.5

bioavailability. The HS2-ester and O-DHA* metabolism studies suggests four hours is a sufficient time period for the parent compound to be metabolized in liver, kidney or serum.

Problems with Data Recovery From Floppy Disks

In running long gradients to get metabolite separation, a large amount of data is stored on the computers hard drive. It often becomes necessary to store data (i.e. chromatograms) on disks so that there is room available on the hard drive to collect additional data. In the current system, to save data to a floppy disk the program Norton Backup for Windows (version 3.1) was used. This allows the user to go from the Millennium Chromatography Manager software, where the data is collected, analyzed and stored, to a DOS shell which is imported into the Norton program. Once in the Norton program, the data can be compressed and stored on a floppy disk and is cataloged by date. This data can be deleted from the hard drive and can be restored from the floppy disk using Norton. This is done by choosing the correct catalog (date that the data was stored on the floppy) and then restoring the data through Norton. This will then allow the data to be imported through a DOS shell into the Millennium Chromatography Manager. Before the year 2000, this system worked well. Frequently, data was moved from floppy disk to the hard drive at regular intervals so that data could be reviewed.

Shortly after the first part of this year, a problem was discovered with copying and restoring data from the floppy disks to the hard drive. An error would occur in which the computer could not import the data from the DOS shell so that no data was loaded on to the hard drive. The same error occurred when data was to be copied to a floppy disk or to another project on the hard drive. Waters Technical Service was called and our group was told that we had a Y2K problem and Waters no longer supported this version of Millennium. The only solution was to update the Millennium software to the newest date on the computer, but this did not correct the problem since the computer had trouble recognizing the dates which were cataloged. Numerous attempts to restore the data were tried including defragging the hard drive, reloading Norton, restoring the catalog, copying

data from Norton to Millennium, copying into another Millennium project, copying into a different directory and ensuring the floppy disk drive was recognized by Norton, but the data was not recovered and the DOS shell was never able to import or export data to or from Norton. During this time, however, the Millennium Chromatography manager was able to control the HPLC system and data could be collected, analyzed and stored. The system works well as long as no data is to be copied to or restored from floppy disks.

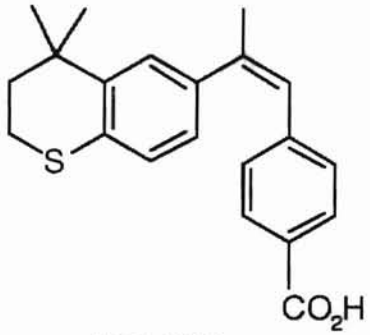
Currently, we are in the process of updating our system and are trying to recover data from the older version. The majority of the data for this chapter was saved on the hard drive but a few chromatograms were saved on disk. All of the data are shown and none of the data was lost since a paper copy did exist in laboratory notebooks although it may not be in as nice of a format as other chromatograms which could be re-analyzed and printed.

Conclusions

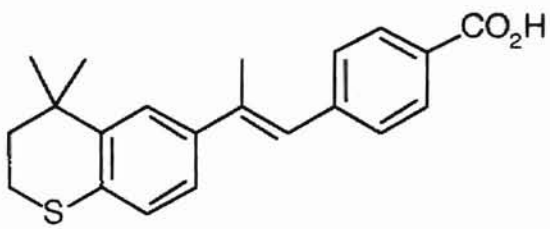
In this chapter, metabolites of selected heteroarotinoids were isolated and in some cases initially identified. Methods were developed to extract and isolate metabolites from serum and tissue. Vitamin A-deficient animals were used for metabolism studies. This system allowed for metabolite detection without the interference from stored retinoids.

HS metabolism studies were conducted and two metabolites were isolated from the liver, HS-LM1 and 2, respectively. HS-LM1 is an artifact produced from the extraction procedure while HS-LM2 is a metabolite of HS. HS metabolites were not detected in kidneys or testes after two hours.

Methods were developed to synthesize HS2-acid and its E-isomer from HS2-ester These compounds were used as standards for the HS2-ester metabolism studies. Two HS2-ester serum metabolites, HS2-SM1 and 2, were initially identified as the HS2-acid and its E-isomer based on HPLC. The structures of tentatively identified HS2-ester metabolites are shown in Figure 70. Three HS2-ester liver metabolites were isolated which were more polar than the parent compound. One metabolite, HS2-LM3, was initially identified by HPLC as HS2-acid (see Figure 70).



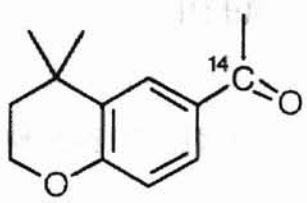
HS2-SM1



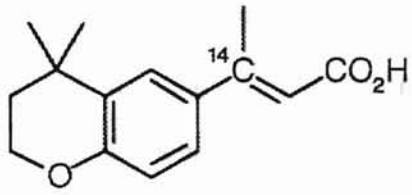
HS2-SM2 and HS2-LM3

Figure 70: Tentatively Identified HS2-ester Metabolites.

A radiolabelled heteroarotinoid, O-DHA*, was synthesized and had high specific activity which allowed the liver and kidney metabolites to be easily traced. Four polar O-DHA* liver metabolites were detected with three in the 35-50 minute range. Although the UV spectra of the standards in this range did not overlay the liver metabolites, the spectra had similar maximum UV absorbances. Several O-DHA* kidney metabolites were isolated and two, O-DHA*-KM3 and O-DHA*-KM4 were initially identified as S-4 and S-6 by HPLC. The structures of the tentatively identified O-DHA* kidney metabolites are shown in Figure 71. Other kidney metabolites had similar retention times and maximum UV absorbances as liver metabolites although none of the metabolites were identical to the standards in this range. It was observed that fours hours after administering O-DHA*, metabolites were detected in the liver and kidneys of vitamin A-deficient rats.



O-DHA*-KM3



O-DHA*-KM4

Figure 71: Tentatively Identified O-DHA* Kidney Metabolites.

CHAPTER V

SUMMARY AND CONCLUSIONS

Heteroarotinoids are a class of retinoids which contain an aryl ring fused to a partially saturated ring and a heteroatom. Heteroarotinoids inhibit the induction of ornithine decarboxylase, induce differentiation in HL-60 cells, reverse trachea keratinization and in some examples transactivate retinoid receptors. By adding a heteroatom in the cyclic ring, the toxicity of these compounds is far less than naturally occurring and some synthetic retinoids and heteroarotinoids show significant promise as anticancer agents. Important information can be provided by studying their biological activity and metabolism. This information can direct heteroarotinoid synthesis and provide pharmacological data which will aid in clinical applications.

Vitamin A was first recognized as a requirement for growth in 1913. Since this time, a growth assay was developed to test compounds for growth-promoting activity. In the current study, four heteroarotinoids, HN, HN2, HS and HS2, were tested for their ability to support growth in vitamin A-deficient rats when fed at levels of 10, 100 or 200 μg/rat/day. All-*trans*-retinoic acid, a known growth-supporting compound, was used as a positive control. Synchronous vitamin A-deficiency was introduced in all animals prior to testing. Feeding trials 1 and 2 were conducted in different years and designated FT1 and FT2, respectively. In feeding trial one, HS, a sulfur containing monoaryl heteroarotinoid, supported growth at 200 μg/rat/day, while HS2, a sulfur containing diaryl heteroarotinoid, supported growth at 10, 100 and 200 μg/rat/day. In other heteroarotinoid feeding trials where the sulfur was replaced with an oxygen, the monoaryl heteroarotinoid failed to support growth at any level while the diaryl supported growth at 100 and 200 μg/rat/day. The differences between monoaryl and diaryl heteroarotinoids and the heteroatom they contain plays an important role in growth promoting activity. Similar observations have

been seen in other bioassays where the sulfur containing heteroarotinoids have higher activity than their oxygen containing counterparts (Benbrook et al. 1998).

Physiological and histopathological changes were first observed in vitamin A-deficient rats by Wolbach and Howe in 1925. The same classic changes are observed today where normal epithelium is replaced by stratified keratinized epithelium and there is atrophy of the adipose tissue, liver, spleen and testes. Lack of spermatogenesis is also characteristic of vitamin A deficiency in laboratory animals. Animals in FT 1 and 2 were subjected to post mortem examination and histopathological changes of rats fed all-*trans*-retinoic acid or 4 heteroarotinoids for 18-22 days were observed and compared against age matched vitamin A supplemented controls. The effects of different doses of the same compound or different compounds were compared. Animals were found to have no abnormal conditions or growth. Gross pathology of HN, HN2 and HS animals revealed a rougher haircoat and crusting around the eyes which is consistent with signs of vitamin A deficiency. Rats fed HN at a level of 10 or 100 μg/day had Hardian gland inflammation while a rat fed 200 μg/day of HN had squamous metaplasia of the bladder. Rats fed 10, 100 and 200 μg/day HS or 200 μg/day HN had suppurative to less frequently pyogranulomatous inflammation of the accessory sex gland. Rats in feeding trial two fed HS2 or *t*-RA demonstrated suppurative inflammation involving accessory sex glands, and/or heart and/or eyes. Animals fed a vitamin A-deficient diet lacked active spermatogenesis regardless of the treatment or the dose although a few animals fed HN2 or HS2 had evidence of previous spermatogenesis. Animals in all treatment groups lacked spermatids as a result of germ cell depletion due to vitamin A deficiency. Heteroarotinoid toxicity was not observed in any treatment group or dose. Testing for exposure or infection to common rodent virus in animals in FT1 or FT2 did not reveal any significant elevated serological titers. The changes observed in this study are characteristic of vitamin A deficiency. It is likely that heteroarotinoids have the same activity as all-*trans*-retinoic

acid in maintaining epithelium. In the eye or urogenital tract, neither *t*-RA or the heteroarotinoids tested maintained epithelial integrity.

Metabolism studies were conducted on HS and HS2 compounds since these compounds showed biological activity in the growth-assays. Non-radiolabelled metabolite studies were initially performed since radiolabelled heteroarotinoids with high specific activity are time consuming, difficult and expensive to synthesize. Furthermore, it is difficult to predict which heteroarotinoid may be active *in vivo*. Vitamin A-deficient rats were used to study metabolism of select heteroarotinoids. This system allowed for metabolite isolation without the interference from endogenous retinoids. Studying the metabolism of heteroarotinoids in serum or tissue is a labor intensive process although with the use of vitamin A-deficient rats this process was made easier. Although no two animal's metabolism was the same and variations in metabolism may exist in vitamin A-deficient versus supplemented animals, this system provides a good technique to initially isolate metabolites. Two HS metabolites were isolated from the liver, HS-LM1 and 2, respectively. HS-LM1 was an artifact of HS produced from the extraction process. Metabolites of HS were not detected in the kidneys or testes two hours after administration. HS2-acid and its E-isomer were synthesized and used as standards for HS2-ester metabolism studies. HS2-ester serum metabolites were initially identified as HS2-acid and the E-isomer based on HPLC analysis. Three HS2-ester liver metabolites were isolated and HS2-LM3 was initially identified as HS2-acid based on HPLC analysis.

A radiolabelled heteroarotinoid, O-DHA*, with high specific activity, was synthesized. Vitamin A-deficient rats were injected with O-DHA* and fed O-DHA. Four liver metabolites were detected and elute in the 35-50 minute range. Several O-DHA* kidney metabolites were detected and two were tentatively identified based on HPLC analysis and comparisons to O-DHA standards. Metabolites of O-DHA* were detected in the liver and kidney four hours after administration. Metabolites were easily identified using a radioactive tracer.

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VITA

Melissa Simms-Kelley

Candidate for the Degree of

Doctor of Philosophy

Thesis: GROWTH PROMOTING ACTIVITY AND METABOLISM OF SELECT HETEROAROTINOIDS IN VITAMIN A DEFICIENT RATS

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Columbus, Kansas, on December 31, 1971, the daughter of Chris and Ann Simms. Married Jason Patrick Kelley on June 4, 1994, in Columbus, Kansas.

Education: Graduated from Columbus Unified High School, Columbus, Kansas in May, 1990; received a Bachelor of Science degree in Biochemistry from Kansas State University in 1994. Completed the requirements for the Doctor of Philosophy Degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in May, 2000.

Experience: Employed by Kansas State University, Department of Biochemistry as an undergraduate research assistant; employed by Oklahoma State University as a graduate assistant researcher, 1994 to present.

Professional Memberships: Phi Lambda Upsilon and American Chemical Society.