BIOLOGICAL ACTIVITY AND METABOLISM OF SELECTED HETEROAROTINOIDS

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BIOLOGICAL ACTIVITY AND METABOLISM

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LIST OF ABBREVIATIONS

ALB	albumin
B/E	magnetic section/electric section geometry
CAD	collisionally activated dissociation
CF	cross-fed
CGC	capillary gas chromatography
CH_3OH	methanol
cm	centimeter
cRABP	cellular retinoic acid-binding protein
cRBP	cellular retinol-binding protein
CSF	cerebrospinal fluid
DHA	diaryl heteroarotinoid
DPM	discentigration
ED_{50}	effective dose at 50% maximum activity level
EGP	epidermal growth factor
ER	endoplasmic reticulum
g	gram
GC/MS	gas chromatography / mass spectrometry
H_2O	water
HAC	acetate
He	helium
HPLC	high performance liquid chromatography
hr	hour(s)

Kg	kilogram
LC	liquid chromatography
LSIMS	liquid secondary ion mass spectroscopy
M	molar
m/z	mass/charge
mAU	milliabsorbance
µci	microcurie
MeOH	methanol
µg	microgram
mg	milligram
MHA	monoaryl heteroarotinoid
min	minute(s)
µl	microliter
ml	milliliter
µm	micrometer
mm	millimeter
mol	mole(s)
MS	mass spectrometry
MS/MS	linked scan mass spectral analysis
MW	molecular weight
N_2	nitrogen
nm	nanometer
nmol	nanomole(s)
NMR	nuclear magnetic resonance
No.	number
^{o} C	degree centigrade
^{o} F	degree Fahrenheit

ODC	ornithine decarboxylase
p.	page(s)
PK-C	protein kinase-C
RA	all-*trans*-retinoic acid
RAR	nuclear retinoic acid receptor
RP	all-*trans*-retinyl palmitate
RXR	nuclear retinoid receptor
RXRE	retinoid receptor response element
TIC	total ion chromatogram
TOC	tracheal organ culture
TPA	12-O-tetradecanoylphorbol-13-acetate
UM	unknown metabolite
UV	ultraviolet
v	volt(s)
VSA	vaginal-smear assay
WHO	world health organization

CHAPTER I

INTRODUCTION

Vitamin A (all-*trans*-retinol) has been shown to be involved in many biological functions, including growth (Moore, 1957; Bieri et al., 1969), vision (Dowling and Wald,1960; Wald, 1968), reproduction (Thompson et al., 1964; Juneja et al., 1964), bone remodeling (Wolbach, 1947; Hayes, 1971), immunocompetence (Dennert, 1984) and normal cellular growth and differentiation (Hixson and Dening, 1978; Goodmann, 1984; McDowell et al., 1987). In large doses, retinoids (vitamin A and its analogs) also demonstrate potent chemopreventive and chemotherapeutic actions against various types of dermatologic disorders (Orfanos et al., 1981), tumors and cancers (Sporn, 1979; Mettlin et al., 1979; Zile and Cullum, 1983). Retinoids are a family of compounds including naturally occurring vitamin A, its natural derivatives (excluding carotenoids), and their synthetic analogs that exhibit some or all of the biological activities of naturally occurring vitamin A. The realization of the necessary function of vitamin A and its derivatives in normal differentiation and maintenance of many epithelial tissues in connection with their effectiveness in the treatment of some cutaneous disorders and cancerous diseases has generated great interest among many biochemists, cell biologists, nutritionists, pharmacologists, pathologists, and dermatologists. A great deal of collective effort has been expended in the exploration of the potential use of retinoids in clinical medicine. Little is known about the mode of retinoid actions, except for the process in vision. It has been proposed that retinoids trigger complex biological activities through cellular signaling systems (Mangelsdorf et al., 1990). The central theme of the proposal involves retinoid binding proteins (cellular retinol binding proteins, cRBPs, and cellular retinoic acid-binding proteins, cRABPs), nuclear retinoic acid receptors (RARs) and retinoid receptors (RXRs).

Briefly, retinoids are complexed with retinoid-binding proteins in the cell cytosol, and transported into the nucleus. In the nucleus, retinoids bind to the retinoid receptors. This binding activates the specific transcriptional properties of the receptors, which in turn interact with specific retinoid receptor response elements (RXREs) located in the region of the enhancers of target genes (Mangelsdorf et al., 1990; Blomhoff et al., 1990). The expression of a target gene is regulated by this chain reaction. All the reactions involved in the process are specific. The existence of distinct RAR and RXR subfamilies and of several receptor isomers in each retinoid receptor subfamily (to date, at least three isomers in each subfamily have been discovered) indicates that there are several distinct signaling pathways in response to different retinoids (Mangelsdorf et al., 1990). This notion is consistent with the fact that retinoids exert a broad range of biological effects.

The mechanisms regarding the specific effectors that are regulated down stream in the retinoid-mediated signal transduction pathways are largely unknown. Thus far, protein kinase-C (PK-C) is the most well-studied effector whose critical role in the expression of many oncogenes has been shown to be modulated by retinoids (Cope et al., 1986; Taffet et al., 1983; Cope and Boutwell, 1985). It is suggested that PK-C may be centrally involved in many of retinoids' anticancer actions, such as the effect of retinoids on ornithine decarboxylase, intracellular polyamine levels, and epidermal growth factor receptors (Lippman et al., 1987).

Although the symptoms of acute and chronic hypervitaminosis A (vitamin A toxicity) have been known for over 100 years, nearly a decade of clinical trials using retinoids as preventive and therapeutic anticancer agents has brought the problem of vitamin A toxicity under intense study. To date, the retinoids that have been used clinically, including naturally occurring and synthetic retinoids, are lipophilic in nature. They are poorly distributed in biological tissues and are very slowly eliminated from the body, causing serious prolonged toxicity (Yob and Pochi, 1987). The major toxic effects of clinical retinoid treatment include acute central nervous system damage, teratogenic effects,

mucocutaneous toxicities, major chronic reproductive, skeletal and liver toxicities, and lipid changes (Yob and Pochi, 1987). Therefore, the available retinoids at clinically tolerable doses have only limited activity against human neoplastic processes. These limitations have prompted intense investigations in search of vitamin A derivatives with improved therapeutic values and reduced toxicities.

In the past few decades, extensive chemical modification of the basic retinoid skeleton has led to the discovery of many new types of synthetic retinoids that possess potential therapeutic values in cancer treatment (Bollag, 1979; Bollag and Matter, 1981; Dawson and Okamula, 1990). In addition, synthetic analogs are found to posses marked species- or tissue-specificities (Aszalos, 1981). A group of aromatic retinoids, termed arotinoids, have attracted much attention because of their higher therapeutic potencies when compared to their naturally occurring retinoid counterparts. Unfortunately, they are also much more toxic. "Heteroarotinoids" are a group of compounds modified on the basis of arotinoids. Heteroarotinoids contain aromatic ring(s) and at least one heteroatom within the skeletal framework (Spruce et al., 1987). In several standard evaluation tests, heteroarotinoids have shown marked anticancer activities and much lowered toxicities when compared with some clinically utilized retinoids, such as all-*trans* -retinoic acid, 13-*cis* -retinoic acid, etc. (Dawson et al., 1984; Waugh, et al., 1985; Gale, 1988).

The goal of the present study was to address the following aspects. The first objective was to evaluate the growth-promoting activity of selected heteroarotinoids. The weight-change of vitamin A-deficient rats was monitored for various lengths of time while the animals were fed a vitamin A-deficient diet periodically or continuously supplemented with these compounds. Second, the vitamin A-like activity of these heteroarotinoids was evaluated in terms of maintaining normal differentiation of epithelial cells. An *in vivo* vaginal-smear assay (VSA) was developed to quantitatively measure and compare the decornification activity of the selected heteroarotinoids with ovariectomized vitamin A-deficient rats. A further investigation on the histopathological effect of the heteroarotinoids

was performed on various epithelial tissues from rats supplemented with these heteroarotinoids. Third, methodologies were developed to isolate and identify major biliary metabolites formed *in vivo* after a pharmacological dose of a selected heteroarotinoid was administered.

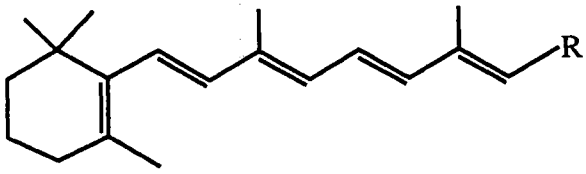
The vital role of retinoids in growth and differentiation of many types of epithelial cells indicates their fundamental importance in chemoprevention and chemotherapeutic treatment of various cancerous diseases. The activity of an individual retinoid depends on its biological and pharmacokinetic properties, including metabolism. Investigation of the biological activities and metabolisms of these selected heteroarotinoids will provide important information for their pharmacological evaluation and potential clinical utilizations in the future.

CHAPTER II

LITERATURE REVIEW

Historical Perspective

The term "retinoids" was coined to refer to a family of structurally-similar compounds comprising naturally occurring retinoids, including all-*trans*-retinol (vitamin A), retinal, retinoic acid (Figure 1), and their synthetic analogs. This definition emphasizes the structural similarities regardless of their differences in biological activities. As revised from the original description of "fat-soluble A" and "vitamin A", which strictly referred to all-*trans*-retinol, the IUPAC-IUB Joint Commission on Biochemical Nomenclature in 1982 recommended that, "The term vitamin A should be used as the generic descriptor for retinoids exhibiting qualitatively the biological activity of retinol. This term should be used in derived terms such as vitamin A activity, vitamin A deficiency, vitamin A antagonist." (1982). Chytil (1984) argued, however, that the term "vitamin A" should be strictly reserved for retinol because only this compound, when taken in sufficient quantities, satisfies all the vitamin A requirement for proper development and growth of an animal (Chytil, 1984). Some retinol metabolites, such as retinoic acid and retinal, are undoubtedly involved in many processes of vitamin A activity. Their functions, however, are not identical and frequently overlap. The author of this presentation tends to agree with Dr. Chytil's point of view and refers to all-*trans*-retinol only wherever "vitamin A" is used in this writing. Animals are not capable of *de novo* synthesis of vitamin A. The major sources of naturally-occurring retinoids are fish liver oils, visceral parts of fish, eggs, and animal kidneys, lungs, eyes, and intestinal mucosa (Underwood, 1984). Carotenoids are the precursors of vitamin A. They are synthesized exclusively by photosynthetic



R	$= CH_2OH$	Retinol
	$= CHO$	Retinal
	$= CO_2H$	Retinoic Acid

Figure 1. The Naturally-occurring Retinoids.

microorganisms and plants (Underwood, 1984). Among carotenoids found in the most important mammalian food sources, β-carotene has by far the most provitamin A activity (Bauernfeind, 1972). Vitamin A compounds are derived enzymatically from carotenoids in the intestinal mucosa and in the liver of animals (Goodman and Olson, 1969).

Although the existence of an essential nutritional factor, later to be termed vitamin A, was recognized and its physiological effects were described more than 3500 years ago (Moore, 1957), systematic studies on the characterization of its chemical and physical properties did not begin until early this century (Hopkins, 1906; Stepp, 1909; McCullum and Davis, 1913; 1915; Osborne and Mendel, 1913). In 1920, Drummond named this essential factor "vitamin A" (Drummond, 1920).

In the second quarter of this century, progress was made in the chemical isolation, characterization and synthesis of vitamin A, its natural derivatives, and their biologically active precursors (Aszalos, 1981; Frickel, 1984; Frolik, 1984; Sporn, et al., 1984; Pawson, et al., 1982). In the 1960s, the elucidation of the biochemical process of vision revealed, for the first time, the specific role of 11-*cis* -retinal in the visual cycle (Wald and Habbard, 1970). With the introduction and development of high-performance liquid chromatography (HPLC) in early 1970s, a great deal of information on the metabolism of natural retinoids was obtained (Frolik, 1984). At the same time, problems in human vitamin A deficiency (hypovitaminosis A) and vitamin A toxicity (hypervitaminosis A) had gained worldwide recognition as a series of activities in controlling these problems were initiated (WHO, 1976).

Detailed descriptions regarding the classical discoveries in vitamin A research, including the original description of vitamin A substances and their carotenoid precursors; the development of quantitative chemical methods for the analysis and assay of vitamin A compounds; the elucidation of the chemical structure and the total synthesis of vitamin A; the pathology of hypovataminosis A and hypervitaminosis A in experimental animals and man; and the nutritional needs for vitamin A in humans and animals, can be found in

several references (Karrer and Jucker, 1950; Moore, 1957; Wald, 1968; Pommer, 1977; Wolf, 1980).

In the late 1970s, the recognition of the variety of vitamin A activities in maintaining the normal physiology of the body, such as growth promotion, reproduction, bone remodeling, and the differentiation and integrity of epithelial tissues, led to rapid progress in the chemistry and biology of retinoids. The connection between vitamin A and cancer became increasingly clear as squamous metaplastic changes in epithelial tissues due to vitamin A deficiency were recognized as morphologically similar to the changes found in certain precancerous lesions caused by carcinogen exposures (Harris et al., 1972). Studies to reveal the molecular basis of the actions of retinoids have become the subject of much of the active research. As retinoids proved to be highly potent in controlling cell differentiation and proliferation, they have become valuable tools in studies dealing with the most fundamental problems in the field of biological research. By the same token, they have been enthusiastically trailed in clinical practices in the treatment of severe cystic acnes, psoriasis, and other dermatological disorders. Furthermore, potential uses of retinoids were explored in the prevention and treatment of various cancers, and the outcomes were promising (Dawson and Okamura, 1990). Investigation into the toxic effects of retinoids, although recognized long ago, has intensified since increased clinical utilizations of retinoids were inevitably accompanied by the problems of toxicity.

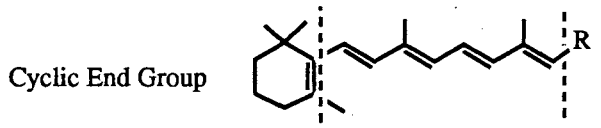
In the last decade, great interest has been generated in the development of synthetic retinoids in a search for new drugs with greater tissue specificity, greater therapeutic efficacy, and lower toxicities. Major advances in synthetic organic chemistry have been made. As a result, over a thousand new retinoids have been synthesized (Dawson and Okamura, 1990). A series of *in vitro* and *in vivo* bioassay systems have been developed to screen the newly synthesized retinoids for their biological activities (Sporn, 1984). There has been a striking thrust in the development of the newly synthesized retinoids for prevention and/or treatment of diseases, particularly in the areas of oncogene and

dermatology. There are many similarities in the general effects that have been experienced with different systemic retinoids, although in varying degrees (Dicken, 1984). Like those that have been observed for natural retinoids, the effects that are most notable with synthetic retinoids are in the areas of cellular differentiation, inflammation, immunomodulation, keratinization, cell membrane stability and organization, posttranslational glycosylation, and anticancer activity (Yob and Pochi, 1987). Structural modifications of the ring, the side chain, or the polar terminal group of the retinoid skeleton resulted in synthetic retinoids with improved therapeutic index over the two original retinoid drugs, all-*trans*- and 13-*cis*-retinoic acid (Pawson et al., 1982). The fact that some synthetic retinoids bear little structural resemblance to retinol or retinoic acid has led to a new definition of retinoids, namely, "a retinoid is a substance that can elicit biological responses by binding to and activating a specific receptor or set of receptors" (Sporn et al., 1986). This new definition emphasizes the biological responses and the mode of action rather than the structure of a compound when it is considered to be a member of the retinoid family.

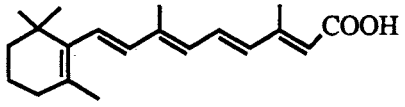
This review will be focused on synthetic retinoids. The development of this contemporary domain of retinoid research will be discussed in conjunction with reviews of various aspects of the natural retinoids including the metabolism, biological activities, toxic effects, and mechanisms of actions.

Synthetic Retinoids

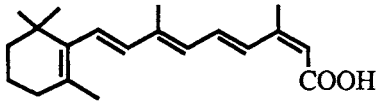
The basic structure of retinoids consists of three portions: a cyclic end group, a polyene side chain, and a polar end group (Figure 2). Synthetic retinoids have been prepared by making alterations in one or more parts of the basic retinoid skeleton (Lippman and Meyskens Jr., 1988). The first-generation synthetic retinoids were produced by alterations made at the polar end group and the polyene side chain. The most important first-generation retinoids are Tretinoin (all-*trans*-retinoic acid) and Isotretinoin (13-*cis*-



Basic Structure

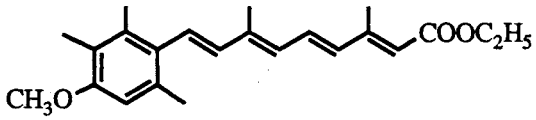


Tretinoin



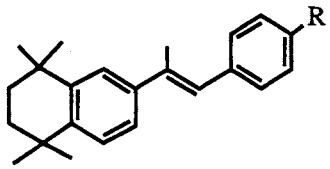
Isotretinoin

First Generation



Etretinate

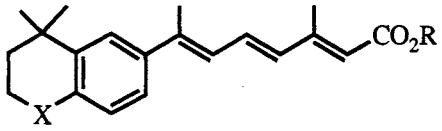
Second Generation



Arotinoids

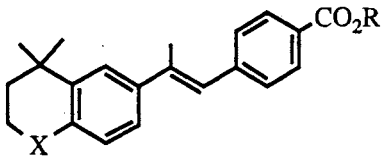
TTNPB (R-COOH)

Third Generation



X = O, S
R = H, Et, etc.

Monoaryl Heteroarotinoids



X = O, S, NH, NMe, etc.
R = H, Me, Et, etc.

Diaryl Heteroarotinoids

Fourth Generation

Figure 2. Classification of the Synthetic Retinoids.

retinoic acid). Some retinoamides and series of chlorinated and fluorinated vitamin A analogs were also synthesized. Some of them, such as fluorinated retinoids, have shown enhanced anti-tumer activity (Lovely and Pawson, 1982). The second-generation retinoids were developed by aromatizing the cyclohexenyl ring. A representative second-generation molecule is Etretinate, which is widely used in clinics. Cyclization of the polyene side chain produced retinoids of the third generation, the retinoidal benzoic acid derivatives, also known as arotinoids. Arotinoids are structural systems that contain an aryl ring within the side chain or fused to the cyclohexyl ring of the basic retinoid skeleton. By condensing the polyene side chain onto one or more aromatic ring structures, the conformations of the molecules become more rigid and planer. The most extensively studied arotinoids in this category is (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-hephthalenyl)-1-propenyl] benzoic acid (Ro 13-7410), also known as TTNPB, and its ethyl ester (Ro 13-6298). These compounds were more potent than the first- or the second-generation retinoids in several standard screening tests and in the treatment of keratinizing and hyperproliferative disorders including psoriasis (Tsambaos and Orfanos, 1983). The newest class of synthetic retinoids are the heteroarotinoids developed on the the base of arotinoids by Berlin and co-workers (Waugh et al., 1985; Spruce et al., 1987; Spruce et al., 1991) and Dawson and co-workers (Dawson et al., 1984; Metra et al., 1986). Heteroarotinoids consist of at least one aryl ring and a heteroatom within the retinoid skeletal framework (Spruce et al., 1987). They are categorized as the fourth generation of synthetic retinoids in this writing.

Metabolism of Retinoids

Naturally Occurring Retinoids

Various aspects of the metabolism of the naturally-occurring retinoids have been reviewed (Olson, 1969; Roberts and Frolik, 1979; Frolik, 1984). The major nonoxidative

metabolism of vitamin A and its derivatives, namely esterification, isomerization and conjugation, mainly occur during the absorption and transportation processes. The utilization of vitamin A by the body starts with absorption in the intestine, which is followed by transportation to and storage in the liver. Dietary retinol is quickly esterified in the intestinal mucosa with long-chain fatty acids (Goodman et al., 1966). β-Carotene, the main natural dietary precursor of vitamin A for man, is converted to retinol by two enzymatic steps. First, it is cleaved by β-carotene 15, 15'-oxygenase found in the intestine and liver to two molecules of retinaldehyde (Goodmann and Huang, 1965), which is then reduced to retinol by retinaldehyde reductase in the intestinal mucosa (Huang and Goodmann, 1965).

The esterified retinol is incorporated into chylomicra and transported via the lymph to the liver. The retinyl esters are then hydrolyzed and reesterified in the liver for storage (Lawrence et al., 1966; also see the Mode of Action for more detailed discussion on the transport and storage of retinoids). It was reported that retinyl palmitate is the major ester in both the lymph (Huang and Goodman, 1965) and liver (Mahadevan and Ganguly, 1961). Another important ester of retinol formed in the liver is retinyl phosphate, which is further conjugated with mannose to form mannosyl retinyl phosphates (Masushige et al., 1978). It has been postulated that retinoids control the glycoprotein synthesis through the formation of these glycosyl phosphoryl retinoid intermediates (De Luca et al., 1979; Wolf et al., 1979). Recently, Rimoldi et al. (1990) demonstrated that the incorporation of mannose into lipidic intermediates and glycoproteins was greatly reduced during the progression of vitamin A deficiency. Their study indicated that vitamin A deficiency caused major impairment in the GDP-mannose biosynthesis.

Conjugation of retinol with glucoronic acid to form β-glucuronide was first found in bile (Dunagin et al., 1965), but later was detected in the urine (Nath and Olson, 1967) and intestinal mucosa as well (Zile et al., 1982). Glucuronides of retinoids are water soluble agents and possess some biological activities of vitamin A. Stimulatory effects of

glucoronides of retinoids in the growth of vitamin A deficient rats (Chytil, 1985), and in the differentiation of HL60 cells and vaginal epithelial cells have been reported (Wong and Bucl, 1971; Stahlmann et al., 1988). Marked inhibitory effect on hormone-induced differentiation of mammary glands in organ culture was also found (McDowell et al., 1984). Recently, Mehta and co-workers (1992) demonstrated that the effects of retinoid glucuronides are independent of cRBP or cRABP in mammary gland organ culture. They speculated that retinoid glucuronides may either function through a mechanism different from that of retinoids, or they may be metabolized to active components of retinoids, which may in turn bind to retinoid binding proteins for the glucuronide action (Mehta et al., 1992).

Isomerization of retinoids occur in many physiological processes. For example, retinaldehyde functions in the visual cycle by isomerization of all-*trans*-retinaldehyde to 11-*cis*-retinaldehyde (Wald, 1968). 13-*cis*-Retinoic acid was identified in tissue extract of rats given all-*trans*-retinoic acid (Zile et al., 1967; Lippel and Olson, 1968). In spite of the fact that isomerization of all-*trans*-retinoic acid to its 13-*cis* isomer occurred artificially during the extraction and chromatographic procedures, repeated detection of 13-*cis* isomers in the metabolism of all-*trans*-retinoic acid led many to speculate its occurrence as a normal physiological event and of possible physiological importance (see review in Frolik, 1984).

Major oxidative pathways of natural retinoids have been extensively investigated in many *in vitro* and *in vivo* studies (see review by Gale, 1988). The major oxidation sites are: (1) at C(4) of the cyclohexenyl ring; (2) at C(5,6) double bond in the cyclohexenyl ring through epoxidation; (3) at the methyl groups on the ring and on the side chain; and (4) on the side polyene chain by oxidative shortening. The metabolism of retinoic acid *in vivo* is a complex process that gives rise to many metabolites, the relative quantities of which vary in different tissues (Chytil, 1984). This phenomenon may imply its diversified activities in different tissues.

To date, much of the research in the metabolism of the natural retinoids has focused upon a search for biological metabolites of retinol or retinoic acid that are more active than the parent compounds in biological functions. Although further investigation is still required, results from many *in vitro* and *in vivo* assay systems that are used to test various vitamin A activities indicated that retinoic acid is the most active form of vitamin A in maintaining normal cell differentiation (Lotan, 1980) and in reversing malignant tissue changes induced by exposure to carcinogens (see review by Lotan, 1986). It has also been shown that retinoic acid was just as active as retinol in supporting growth (Nelson et al., 1964; DeMan et al., 1964; Zile and DeLuca, 1968). In addition, retinoic acid was detected as normal metabolite of retinol and retinaldehyde (see review by Frolik, 1984). For over three decades, it was widely accepted that retinoic acid was unable to satisfy the visual and reproductive requirements for retinal and retinol (Dowling and Wald, 1960; Thompson et al., 1964) because it could not be converted back to either retinaldehyde or retinol in the body (Dowling, 1961). More recently, however, Van Pelt and co-investigators reported that retinoic acid, like retinol, could support spermatogenesis when a large dose was administered repeatedly (Van Pelt and De Rooij, 1991). They concluded that all-*trans*-retinoic acid (RA) was able to induce synchronous proliferation and differentiation of A-spermatogonia, and support the full development of spermatogenic cells into elongated spermatids.

Synthetic Retinoids

As mentioned earlier, the use of natural retinoids in dermatology and in the prevention of epithelial cancer has generated great interest in the fields of clinical medicine and basic research. Because of the unacceptable toxic effect of the natural retinoids at pharmacological levels, seeking less toxic analogs has become the focus of much research in this area. Compared to the number of retinoid analogs that have been synthesized and screened for biological activity, studies on the metabolism of synthetic retinoids has lagged

behind. Only a few synthetic retinoids have been studied extensively (Frolik, 1984). The major route of the metabolism of 13-cis-retinoic acid (considered a synthetic retinoid although evidence has indicated that it is a natural retinoid and a physiological metabolite of all-*trans*-retinoic acid) in hamsters, rats, and humans was found to be the oxidation at C(4) of the cyclohexenyl ring (Frolik, 1984). Like all-*trans*-retinoic acid, 13-*cis*-retinoic acid and its metabolites were rapidly excreted from the body via bile.

The metabolism of Etretinate (Ro 10-9359), the TMMP analog of ethyl retinoate and a drug widely used in the clinics, was also reported. Hanni and co-workers (1977, 1979) were able to identify a number of metabolites in human plasma, urine and feces after an oral dose of Etretinate. The types of metabolic pathways can be generalized as follows: (1) conjugation with glucuronic acid, sulfuric acid, or glycine; (2) initial hydrolysis of the ethyl ester to free acid; (3) hydrolysis of the methoxy group, or the 3-methyl group in the aromatic ring (see the structure of Etretinate in Figure 2); and (4) oxidative cleavage of the tetraene side chain. The major metabolic product of Etretinate was found to be its corresponding acid Ro 10-1670 in the bile of rats (Hanni et al., 1977) and in human epidermal keratinocytes in an *in vitro* study (Kitano et al., 1982). This hydrolysis was believed to be the activation step in the therapeutic activity of Etretinate (Hanni et al., 1977), and the responsible esterase was found to be widely distributed in many tissues (Wang and Hill, 1977).

Studies on the metabolism of various analogs of retinamide have also been reported (Kalin et al., 1982; Wang et al., 1980; Swanson et al., 1980, 1981). A few glucoronide conjugates and fatty acid esters were identified. Biliary polar metabolites were generally observed, but none of the structures were identified.

The metabolism of difluoro analogs was found to be very similar to that of vitamin A (Barua and Olson, 1984). Esters, 4-oxidative products, and polar conjugated derivatives were the major metabolites identified in the intestine, liver, kidney and plasma.

The pharmacokinetic tissue distribution and placental permeability of a representative arotinoid, TTNPB (Ro 13-7410), was studied after a single oral bolus was administered to pregnant hamsters (Howard et al., 1989). The absorption of the retinoid as determined in the blood plasma level was fast (t 1/2 = 1.2 h), with a peak plasma level at 2.4 h. The tissue distribution following an 100 μg/kg [^{3}H]-TTNPB administration was in the order of liver > fetus > adrenal > lung ≈ kidney > plasma. An unidentified polar metabolite was detected at 0.5 h in the blood plasma, and constituted > 90% of the total circulating radioactivity by 12 h. TTNPB was cleared more slowly, and concentrated in the conceptus to a higher degree. These findings were consistent with the markedly high teratogenic activity of TTNPB as compared to the natural retinoids.

The Role of Retinoids in the Control and Maintenance of Epithelial Tissue Differentiation

The main theme regarding the biological activity of vitamin A is its activity in maintaining normal differentiation and growth of epithelial cells and cell-surface structures (Pawson et al., 1982). The processes of cell differentiation and growth are regulated by vitamin A compounds (Floyd and Jetten, 1988). In the reproductive system, vitamin A is required for maintaining a normal epithelium in processes such as spermatogenesis, gestation, organogenesis, and embryonic development (Thompson et al., 1964; Juneja et al., 1964; Takahashi et al., 1975). Vitamin A is important in the bone structuring because it regulates the normal differentiation of mesenchymal "undifferentiated" cells (Hayes, 1971), and the balance between the number and the activity of osteoblasts and osteoclast cells (Gallina et al., 1970).

The hypothesis that retinoids control epithelial differentiation was initiated in the early 1920s. In 1922, Mori (1922) conducted the first detailed study of epithelial tissue changes in rat eyes resulting from vitamin A deficiency. He reported the loss of secretions by the lacrimal gland and marked keratinization of the rat cornea and conjunctiva due to

vitamin A deficiency. As an ultimate result, the cornea lost its normally ordered architecture, and developed an ulcer. This symptom was later termed as keratomalacia (Wolf, 1980). The first sign of the pathological process was xerophthalmia, or dry eye, as a result of abnormal tear production due to vitamin A deficiency (Moore, 1957b). Wolbach and Howe (1925) showed that epithelial tissues in general were affected by vitamin A deficiency. They demonstrated that the columnar and transitional epithelium of the respiratory tract, alimentary tract, genitourinary tract, eyes, and glands were all replaced by stratified squamous keratinized cells in vitamin A-deficient animals. The generalization was further supported by the correlation between low vitamin A status and high incidence of tumors as observed by many other investigators (Fujimaki, 1926; 1929; Burk and Winzler, 1944). Tumor development was the result of abnormal differentiation of epithelial tissues. An important nature of the vitamin A-deficiency-induced epithelial changes is that the conditions are reversible as repletion of vitamin A results in the reappearance of ciliated and mucus-secreting columnar cells replacing the squamous cells of the deficient state (Wolbach and Howe, 1933). The role of retinoids in the modulation of epithelial differentiation was firmly established in some more recent studies (Olson et al., 1983; Ong and Chytil, 1983; De Luca and Shapiro, 1981).

Epithelial cells in different tissues interact differently with retinoids (Olson, 1972). The morphological appearances of different epithelial cells as the result of vitamin A deficiency are therefore different. Epidermis, a stratified squamous epithelium, is on one extreme of the spectrum. The proliferating basal cells of the epidermis are the lowest layer of cells. Studies showed that cells superior to the basal cells became more stratified and further differentiated as they moved up and ultimately differentiated into keratinized cells (Matoltsy, 1976). Retinoids inhibited keratinization and induced mucus metaplasia (Yaar et al., 1981; Fuchs and Green, 1980; 1981). Intestinal cells are at the other extreme of the spectrum. The major goblet cells of the intestine normally synthesize and secret mucus materials. It was found that the number of the mucus-secreting goblet cells was greatly

reduced in vitamin A deficiency (Manville, 1937; De Luca and Wolf, 1969). Later, it was determined that the rate of differentiation of a particular subclass of goblet cells from precursor cells was decreased in vitamin A deficiency (De Luca et al., 1971; Olson et al., 1981). Other epithelial tissues, such as the cornea, the respiratory tract, and the genital tract, contain both goblet and keratinized cells in normal situations. Nevertheless, they respond to vitamin A in the same manner, i.e., in the absence of vitamin A, tissues are transferred into more keratinized status (see review by Shapiro, 1986). In addition to the loss of goblet cells, the normally columnar-like goblet cells appeared flattened (Wong and Bulk, 1971), and the overall surface integrity of the epithelium was affected because of the loss of protective mucoid secretions (De Luca and Wolf, 1969).

Toxicity and Pharmacology

The development and clinical utilization of retinoids represent the most significant achievement in the dermatological therapy since the introduction of corticosteroids. The problem of toxic effects of these compounds is the major setback for the wide application of retinoid treatments. Although endogenous retinoids are essential for a broad spectrum of normal physiological processes, the same compounds are highly toxic in excessive amounts. The toxic effect of excessive retinoids in human have been known for more than 100 years (Kamm et al., 1984). The primary targets of retinoid toxicity are the mucocutaneous, skeletal, and central nervous systems (Yob and Pochi, 1987). The effect can be acute, subchronic, and chronic, depending on the dosage and duration of the administration, as well as the pharmacokinetic property of the drug (Chytil, 1984). The major symptoms of vitamin A toxicity, termed as "Hypervitaminosis A Syndrome", are reviewed in a recent article by Lippmann and co-investigators (Lippman et al., 1987). The major symptoms manifested in humans include cheilitis, severe headaches, conjunctivitis inflammation, nausea, vomiting, dryness and scaling of skin, tenderness of bone, and bulging fontanelles in infants (Lippman et al., 1987). Clinically important synthetic

retinoids include all-*trans*-retinoic acid (Tretinoin, Retin-A), 13-*cis*-retinoic acid (Isotretinoin, Accutane), Etretinate (Tigason, Tegison) and Acritretin (Neotigeson, Soriatane). These compounds have proven highly effective in the treatment of severe forms of psoriasis, particularly generalized pustular psoriasis. These synthetic retinoids, however, have similar adverse effects. Reported side effect in the oral use of Tretinoin and Isotretinoin include headache, dizziness, cheilitis, xerosis, anoresia, abdominal pain, conjunctivitis, and excessive thirst. Topical application of Tretinoin can result in skin irritation and reversible hypopigmentation. The most serious toxic effect of retinoids is their teratogenic properties, which have been observed in laboratory animals and more recently in humans. Retinoids at high concentrations are cytotoxic because they damage lysosomal membranes via a detergent-like action (Lotan, 1980). The teratogenic effect of retinoids is often characterized by the malformation of the face, thymus, and great vessels, reflecting the failure of tissues to reach their normal size and shape (Geelen, 1979). The embryopathy of isotretinion was studied recently by Webster and co-workers in animal models (1986). Their study indicated that the drug directly interfered with the development of cranial neural crest cells. One of the metabolites, 4-oxo-isotretinion was found to be more toxic than the parent compound, 13-*cis*-retinoic acid. Retinoids developed recently, such as heteroarotinoids, possess comparable therapeutic effects but less toxicity (Dawson et al., 1984; Metha et al., 1986; Gale, 1988). Increased hydrophilicity and altered metabolic oxidative pathways due to the structural characteristics of the heteroarotinoids may partially contribute to the therapeutic improvement (Spruce et al., 1991).

Several assay systems have been developed to evaluate the biological activities of retinoids, especially of newly synthesized retinoids (Sporn, 1984). Two popular assays, the ornithine decarboxylase (ODC) assay and the hamster tracheal organ culture (TOC) assay, have been widely used to examine the activities of synthetic retinoids. In ODC, the ability of a test substance to inhibit the biosynthesis of the enzyme ornithine decarboxylase is measured. This inhibitory effect reflects the extent to which the substance inhibits skin

tumor promotion (Verma et al., 1978, 1979). The standard hamster tracheal organ culture assay permits measurement of biological activity of test retinoids to reverse keratinization of basal cells in the tracheobronchial epithelium of vitamin A-deficient hamsters. The TOC bioassay has been extremely valuable for providing information on retinoid structure-activity relationships. Heteroarotinoids have shown remarkable activities, in comparison with that of retinoic acid, in a number of these tests, including TOC, ODC, and in the induction of human promyelocytic cell (HL-60) differentiation assay (Waugh et al., 1985; Spruce et al., 1987; Spruce et al., 1991).

In conclusion, retinoids, especially synthetic retinoids, represent a group of compounds that have profound therapeutic values for a variety of diseases, most importantly for cancerous diseases. The goal of clinical studies of the relationship between retinoids and cancer prophylaxis is to search for retinoids that have minimal toxicity at effective dose levels.

Structure and Biological Activity

Numerous comparative studies on analyzing structure-function relationships of the retinoids have demonstrated that vitamin A activity is eliminated or reduced by modifications such as the saturation of a side chain double bond, removal of the methyl group at C(13), shortening at the side chain, and the oxidation of either the C(4) position or the C(5, 6)-double bond (Newton et al., 1980; Zile et al., 1980; Sietsema and DeLuca, 1982a). These metabolic pathways are generally considered to be degradation and/or inactivation processes. Presumably, arotinoids and heteroarotinoids are more active because the aromatic ring in conjunction with the cyclohexyl ring may block the oxidative degradation at C(4) position and at the C(5,6)-double bond (Loeliger et al., 1980, Spruce et al., 1987). In addition, heteroarotinoids with oxygen substituting the carbon atom at C(4) eliminates the oxidation at this position (Spruce et al., 1987). Therefore, increases in the metabolic half-lifes of arotinoids and heteroarotinoids, due to the blockage of the oxidative

degradation processes, may contribute to their higher therapeutic potencies observed in the screening tests. In addition, incorporation of an aryl ring on the side chain maintains C(13) in a fixed *cis* arrangement has been predicted to be a favorable configuration for improved biological activities (Loeliger et al., 1980; Dawson, et al., 1989).

The electron density at C(4) has been suggested as an important factor for the biological functions of retinoids because differences in electron density at C(4) may alter the cellular transport of a retinoid or may interfere with its interaction at critical sites (Miller et al., 1985). Oxidation at C(4) reduces the electron density at this position, resulting in the loss of activity. It has been predicted that compounds having comparable electronegative functional groups or atoms at this position may have similar activities (Miller, et al., 1985). The oxygen or sulfur atoms in heteroarotinoids alter the electron densities at this position , which may account for the increased activities of these compounds.

Mode of Action

The molecular basis for the broad range of biological activities of retinoids is largely unknown, except for that in the visual cycle. The concept of vitamin A activity is constantly changing. The profound functions of retinoids upon many different types of cells are widely demonstrated, but are often confusing and inconsistent (Pawson, 1982; Sporn, 1984). This phenomenon reflects the complexity of retinoid activities. There have been a number of mechanisms proposed for the action of retinoids, such as the enhancement of the immune response by retinoids, controlling membrane permeability, and the regulation of gene expression (Aszalos, 1981). To date a substantial amount of research data have firmly indicated that interaction between retinoids and genetic machinery of the cells is the most fundamental mechanism through which retinoids manifest their many cellular effects such as those on immunology and membrane changes. The major routes that retinoids modulate behavior of cells are (1) influencing growth rates, (2) altering differentiation state of cells, and (3) changing cell surface properties (Pawson et al., 1982).

Retinoids are thought to exert their effects by interacting with a complex system of cytoplasmic binding proteins and nuclear receptors, resulting in the ultimate regulation of the target gene expression (Raysdale and Brockes, 1991).

Retinoids are transported from the intestine through the blood stream to target cell nuclei. The transportation pathways are specific, yet fully understood (Blomhoff et al., 1990). Chylomicron reminants, formed in the general circulation, contain almost all the intestinally absorbed retinol and are taken up mainly by hepatocytes during the early stages of hepatic uptake of retinyl esters (Blomhoff et al., 1990). The retinyl esters are then hydrolyzed at the plasma membrane, and the retinol compounds are taken into the endosomes by a receptor-mediated endocytosis and consequently transferred to the endoplasmic reticulum (ER) (Blomhoff et al., 1985).

The involvement and importance of cellular retinoid-binding proteins for retinoid functions has been indicated in many studies (see review by Blomhoff et al., 1990 and Sani, 1991). The ER in the liver is rich in retinol-binding proteins (RBPs). Retinol binds to RBP. The retinol-RBP complex is then transported into the hepatic satellate cells, which may secrete retinol-RBP directly into blood plasma (Blomhoff et al., 1990). The plasma transport of all-*trans*-retinoic acid (RA) is believed to occur through the binding of RA to serum albumin (ALB) (Goodman and Blaner, 1984). The exact mechanisms of cellular retinoid uptake are unclear. Specific RBP receptor-mediated retinol uptake and spontaneous transfer of RA from RA-ALB to a membrane-bound cellular retinoic acid binding protein have been suggested among various possible mechanisms (Blomhoff et al., 1990; Sani, 1991). In the cell cytosol, retinol and RA bind to the cellular retinol-binding protein (cRBP) and the cellular retinoic acid-binding protein (cRABP), respectively (Sani, 1991).

The cellular retinoid-binding proteins, cRBP and cRABP, were first identified by Chytil and Ong (1978). They proposed that these binding proteins function like steroid receptors in that they transport retinoids into the nucleus, where retinoids interact with

transcriptional factors to modulate gene expression. Indeed, retinol-cRBP and retinoic acid-cRABP complexes have been found to bind specificly to cell nuclei (Takase et al., 1979; Mehta et al., 1982, McCormick et al., 1984), which was followed by specific binding of retinol or retinoic acid to chromatin (Lian et al., 1981; McCormick et al., 1984). Since then, many tissue-specific intracellular retinol- and retinoic acid-binding proteins have been identified (Chytil and Ong, 1984; Nashiwaki et al., 1990; Blomhoff et al., 1990). Recently, the expression of retinoid binding proteins were found to be tissue and/or cell-cycle-dependent (Tsutsumi et al., 1992). Blaner's group in Columbia University reported that adipose tissues are dynamically involved in retinoid storage and retinoid-binding protein synthesis (Tsutsumi et al., 1992). Furthermore, their study suggested that the expression of RBPs is cell-differentiation-dependent (Zovich et al., 1992).

Some studies seemed to indicate that the presence of cRBP or cRABP may not be sufficient and/or necessary for cells to exhibit retinoid responses. In a retinoic acid resistant S91 melanoma cell line, similar or even higher concentrations of cRABP were found when compared to the control S91 cells (Lotan, 1985). Other studies have shown that cells devoid of cRBP or cRABP still respond to retinoids (Chytil and Ong, 1984; Breitman et al., 1980). Recently, a number of nuclear retinoid receptors have been identified. Based on their primary structures, difference in affinity to retinoid ligands, and ability to regulate expression of different target genes, they are classified into two subfamilies, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), (Giguere et al., 1987; Petkovich et al., 1987, Mangelsdorf et al., 1990, 1991; Raysdale and Brockes, 1991). At least three isomers in each subfamilies have been discovered, designated as RARα, β and γ, and RXRα, β, and γ, respectively (Giguere et al., 1987; Petkovich et al., 1987; Brand et al., 1988; Zelent, et al.,1989, Krust et al., 1989). These receptors contain both DNA-binding and ligand-binding domains. The DNA binding domains of RARs display a high degree of homology with the DNA-binding domains of other members of the nuclear receptor family (Petkovich, 1992). The amino acid sequence of the DNA-binding

domain is also highly conserved among diversified species (Petkovich, 1992). The ligand-binding domains of RARs are almost completely identical, and bind to retinoic acid specificly and with high affinity. Each member of a receptor subfamily may have its own specific function as interspecies conservation of a member within the RAR subfamily was found to be much higher than the conservation of all three receptors within a given species. In addition, the expression of RARs was found in almost all cell types in the body, and was highly tissue-specific (Krust et al., 1989; Zelent et al., 1989). Furthermore, it seems that the tissue-specificity of RAR expression is further specified by the cell's positions on the same tissue. Schofield and co-workers demonstrated in their study with chick limb bud that the expression of retinoic acid receptor-β ($RAR\beta$) was position-dependent in that its expression was repressed at the distal tip of the bud, but was highly expressed in the proximal tissue of the bud (Schofield et al., 1992). In contrast to RARs, the expression of RXRa was most abundant in visceral tissues such as the liver and the kidneys (Mangelsdorf et al., 1990).

The retinoid receptors are ligand-dependent transcription factors (Petkovich, 1992). They are activated upon binding by specific retinoid ligands, resulting in the regulation of the expression of different genes. Two retinoic acid isomers, all-*trans* -retinoic acid and 9-*cis*-retinoic acid, were found to be the specific physiological ligands of RAR_α and RXR_α, respectively (Heyman et al., 1992). This finding implies that, as in the visual system, isomerization of retinoids may be the key step in controlling different signal transduction pathways.

In the nucleus, the RAR-RA complex binds to a short, specific sequence, termed as retinoic acid response element (RARE) in the enhancer of target DNA. The expression of the target gene is regulated through the interaction between the RA-RAR complex and the RARE (Evans, 1988). The nature of the interaction between the receptor and the RARE has not been thoroughly studied. Various RAREs have been identified that are responsive specificly to different RARs (Vasios et al., 1989; de The et al., 1990). Based on the

findings supportive of this model, it appears quite convincing that the diversity of the biological effects of retinoids are the result of regulation of gene expression through complex cellular signal transduction processes (Heyman et al., 1992).

It is clear from the above discussion that the broad range of biological responses triggered by retinoids are not mediated by a common process involving all-*trans*-retinoic acid. The existence of various retinoid-binding proteins, distinct RAR and RXR subfamilies, their various isomers, their specific response to different retinoids, and specific interactions with various types of RAREs, indicate clearly that cellular responses to retinoids are carried out by many very complex, but specificly organized signalling pathways. This inference is consistent with the enormous diversity of biological responses that retinoids elicit.

Retinoid-modulated Cellular Enzymes and Effectors

The number of cellular enzymes and effectors whose expression and/or activation are modulated by retinoids is numerous. Among them are ornithine decarboxylase and transglutaminase, cyclic AMP and cyclic AMP-dependent protein kinases, prostaglandins, plasminogen activator, collagenase, surface antigens, keratins, and glycoconjugates including glycoproteins, glycolipids and proteoglucans (see reviews by Sporn, 1984 and Chytil, 1984). The exact mechanisms by which retinoids exert their effects on the expression of these enzymes and effectors are largely unknown. The most well studied system thus far is the protein kinase C (PK-C) cascade, which is believed to play an important role in the carcinogenic process. The mechanism of retinoids in modulating PK-C system may explain the preventive effect of retinoids in promoter-induced carcinomas (Lippman and Meyskens Jr., 1988). Phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are the major tumor promoting agents. Thus, PK-C, the phorbol ester receptor, plays a critical role in the carcinogenic processes. Recent studies (Einat et al., 1985; Nishizuka, 1986; Jetten and Shirley, 1986) have suggested that PK-C may mediate

many phorbol ester-promoted actions such as the induction of oncogenes, c-myc, c-fos, β-actin, ornithine decarboxylase (ODC), which affects intracellular polyamine levels and epidermal growth factor (EGF) receptor down regulation. Exposure of quiescent fibroblasts to phorbol esters moves the cells out of the G_0 phase of the cell cycle, into the G_1 phase (Pledger et al., 1978). Further stimulation by EGF drives the completion of the G_1 phase, keeping fibroblasts in the division cycle rather than in a quiescent state. It has been hypothesized that retinoids may exhibit their anticancer activity by modulating PK-C activity (Jetten and Shirley, 1986, Lippman et al., 1987). Support of this hypothesis has come from a number of studies (Cope et al., 1986; Gensler et al., 1986; 1987). RA-cRABP complex has been shown to inhibit PK-C activity (Cope et al., 1986). The ability of some retinoids to prevent phorbol ester-induced ODC activity was found in correlation with their ability to inhibit tumor promotion (Verma et al., 1979). More convincingly, it was demonstrated that retinoic acid can block TPA-induced activation of PK-C (Taffet et al., 1983; Cope and Boutwell, 1985)

CHAPTER III

GROWTH-PROMOTING ACTIVITY

Introduction

Promoting normal growth in a biological system is one of the major functions of vitamin A (Coward, 1938; Wolbach, 1947). The phenomenon of growth is the result of cell growth and cell division. The importance of vitamin A in the early stage of embryonic development of birds and mammals has been known for decades (Juneja et al., 1964; Thompson et al., 1969). Depressed growth is one of the earliest and most reliable external signs of vitamin A deficiency. Vitamin A deficiency can be induced easily in early life, but is difficult to induce in mature individuals solely by dietary restriction of vitamin A (Johnson and Baumann, 1948). The use of a growth assay for the evaluation of vitamin A activity began in the early 1920s, and became a standard approach for evaluating the adequacy of vitamin A in diets (see review by Underwood, 1984). It is still popular even today (Anzano et al., 1974; Goodman et al., 1974; Stephens-Jarnagin et al., 1985; Goswami and Basumatari, 1988). Goswami and Basumatari (1988) reported that the initial vitamin A-deficiency led to the cessation of growth or weight loss in freshwater fish. Supplementation of vitamin A to the fish compensated the loss of body weight. Laboratory animals fed a vitamin A-deficient diet cease to grow when their initial liver reserves of vitamin A in the body are exhausted (Underwood, 1984; Goswami and Basumatari, 1988). After reaching a weight plateau, the animals rapidly lost weight and, if continued on vitamin A deficient diet, they eventually died. The initial decline in weight-gain may be the consequence of inefficient utilization of nutrients required for cell proliferation due to vitamin deficiency (Zile et al., 1981). Various kinds of physical, physiological and clinical

stresses further increase the body requirement of vitamin A. For full utilization of vitamin A, other dietary components, such as vitamin E, proteins, fat, and zinc, must also be adequately provided (see review by Underwood, 1984). A recent study by Mobarhan and co-investigators (1992) demonstrated that hepatic cRBP was greatly reduced in zinc deficient rats. They suggested that zinc may be an essential element for the intra-cellular transport of vitamin A, in addition to its well-established role in the intercellular transport of vitamin A (Solomons and Russell, 1980; Smith et al., 1973).

Comparative studies conducted using conventionally reared rats and germ-free rats fed with the same vitamin A-deficient diet strongly demonstrated that cessation of growth is the direct consequence of vitamin A deficiency (Beaver, 1961; Rogers et al., 1969). However, it is often difficult to separate the secondary physical signs of general health deterioration from those of vitamin A deficiency *per se* (Beaver, 1961). Commonly, animals do not die of vitamin A deficiency, but rather due to secondary infections (Rogers et al., 1971; DeLuca, 1979). This view was supported by the findings that animals suffering vitamin A deficiency were more susceptible to bacterial (Scrimshaw et al., 1968), viral (Nauss et al., 1985; Underdahl and Young, 1956), and parasitic (Sturchler et al., 1985; Stoltzfus et al., 1989) infections. Furthermore, antibiotic supplements significantly reduced the rate of mortality (Anzano et al., 1979).

In the present study, the ability of three synthetic heteroarotinoids, ethyl (*E*)-4-[2-(3,4-dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate (abbreviated as DHA that stands for diaryl heteroarotinoid), ethyl (*2E,4E,6E*)-3,7-dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoate (abbreviated as MHA-I that stands for monoaryl heteroarotinoid-I), and (*2E,4E,6E*)-3,7-dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic acid (abbreviated as MHA-II that stands for monoaryl heteroarotinoid-II), in supporting the growth of vitamin A-deficient rats was tested. Two known growth-supporting vitamin A compounds, all-*trans* retinoic acid (RA) and all-*trans* retinyl palmitate (RP) were included in the study as positive

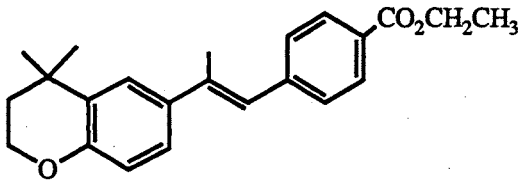
controls. The structures of these three heteroarotinoids, along with the two control retinoids RA and RP, are shown in Figure 3. Synchronous vitamin A deficiency was introduced in all rats before testing. Two approaches, namely periodic feeding and continuous feeding, were employed.

Materials and Methods

Animals and Animal Care

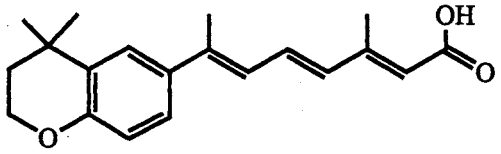
For the periodic feeding experiment, 24 male rats (Fisher-344, Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts) were obtained one week after weaning. They were then 4 weeks of age, and were at their early growth plateau (weighed about 60 g). They were housed in groups of 2 in plastic cages, and were weighed every 1-3 days. The weighing was done at approximately the same time of the day to avoid diurnal variations. The rats were reared on a vitamin A-deficient diet (Teklad Test Diets Inc., Madison, Wisconsin) available *ad libitum* . This vitamin A-deficient diet was specially formulated and the components are listed in Table I. A second growth plateau was reached when the rats were near maturity and weighed about 300 g. The rats were then separated individually into clear polycarbonate cages, and continued on the vitamin A-deficient diet for an additional 3 days.

For the continuous feeding experiment, 40 male rats (Fisher-344, Sasco Inc., Omaha, Nebraska) were obtained at the age of 17 days with their dams (10 pups per dam). The dams were fed with the same vitamin A-deficient diet immediately after arrival. The pups were weaned at the age of 19 days, and maintained on the same vitamin A-deficient diet. The weanling rats were housed in groups of ten in plastic cages for another 2 1/2 weeks before they were separated into individual cages They were weighed every 1-3 days to monitor their vitamin A-deficiency status. This early vitamin A-deprivation period continued until they reached a growth plateau. They were then 6 weeks of age, and average about 160 g.



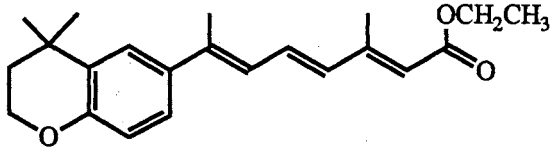
DHA

Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-
-1-benzopyran-6-yl)-1-propenyl]benzoate



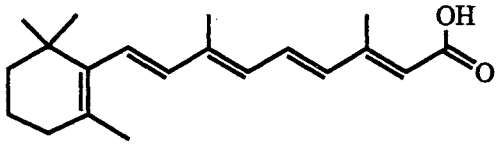
MHA-I

(2*E*,4*E*,6*E*)-3,7-Dimethyl-7-(1,2,3,4-tetrahydro-
-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid



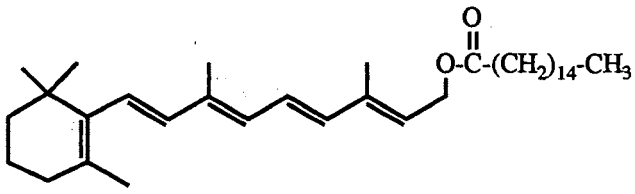
MHA-II

Ethyl (2*E*,4*E*,6*E*)-3,7-Dimethyl-7-(1,2,3,4-tetrahydro-
-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoate



RA

All-*trans*-retinoic Acid



RP

All-*trans*-retinyl Palmitate

Figure 3. The Structures of Heteroarotinoids (DHA, MHA-I and MHA-II), All-*trans*-retinoic acid (RA), and All-*trans*-retinyl Palmitate (RP).

TABLE I

THE VITAMIN A-DEFICIENT DIET
(CUSTOM FORMULA # TD 83081)

COMPONENTS	g/Kg Feed
Casein, "Vitamin-Free" Test	193.0
DL-Methionine	3.0
Corn Starch	665.1343
Cottonseed Oil	50.0
Fiber (cellulose)	50.0
Mineral Mix, AIN-76 (CAT. #170915)	35.0
Biotin	0.0004
Vitamin B_{12} (0.1% trituration in mannitol)	0.0297
Calcium Pantothenate	0.0661
Choline Dihydrogen Citrate	3.4969
Folic Acid	0.002
Menadione Sodium Bisulfite 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

All the animals were kept in a special animal room, and were cared for according to the Guide for the Care and Use of Laboratory Animals (Prepared by The Institute of Laboratory Animal Resources, NIH Publication No. 86-23, Revised 1985). The room was controlled at a constant room temperature (about 72-75^{o} F), and in a 12 hr light-12 hr dark daily cycle. Humidity was maintained between 30-70%. Air was well circulated with 10-15 complete air changes per hour. Fresh food and water were given daily and were accessible *ad libitum* . Cages were bedded with hardwood Sani-Chips (P.J. Murphy Forest Products Co., Rochelle Park, New Jersey). This type of bedding chips are free of all splinters, shavings, slivers, excessive dust and all foreign matter. No detrimental effect of this bedding material on liver functioning has been found in tests. A wire base was also placed in each cage so that feces could drop through. The cages were placed side-by-side for visual and odor socialization, and were switched frequently in a random fashion to avoid any geographic variations. The room was cleaned daily and the cages were changed twice a week.

Chemicals

The heteroarotinoids DHA, MHA-I and MHA-II were synthesized and crystallized by the research group headed by Dr. Berlin (Waugh et al., 1985; Spruce et al., 1987). They were tightly capped under a N_2 atmosphere, wrapped in aluminum foil, and kept in the dark at -20^{o} C. All-*trans*-retinoic acid (RA) was a gift from Dr. W. E. Scott, Hoffmann La Roche, Inc. (Nutley, New Jersey), and was kept in the dark in a desiccator at room temperature. Another form of RA was stabilized gelatin beadlets containing RA at a level of 10.3% (Hoffmann-La Roche Inc., Nutley, New Jersey). RP was fed in stabilized gelatin-sugar-starch beadlets that contained not less than 4.45 mg RP per kilogram (Rovimix A-325, Hoffmann-La Roche Inc., Nutley, New Jersey).

Introduction of Synchronous Vitamin A-deficiency

To induce a reliable synchronous vitamin A-deficiency, rats were fed first with diets supplemented with RA (45 μg RA/rat/day, in the form of RA beadlets) and then with diets void of RA for two consecutive weight-gain and weight-loss cycles (Lamb et al., 1974). RA beadlets were premixed with the vitamin A-deficient diet at a level of 15 mg RA beadlets/Kg feed. Before the feeding experiments, a final period of RA beadlets supplementation was implemented to negate signs of the deficiency from the preceding deprivation phase. The gross appearances and body weight of the rats were closely monitored during this final period to ensure that none of the rats were inadvertently deficient and all of them had resumed normal growth. For continuous feeding, the final RA supplementation period was 8 days. Most of the rats employed in the periodic feeding experiment were ill at the end of the long vitamin A-depletion period. Therefore, twelve to fourteen days of RA supplementation was required for the rats to recover. Rats were then grouped randomly into five groups (4 to 5 rats in each group) for the feeding assay.

Stock Solutions and Feeding Procedures

Stock solutions of the test compounds were prepared in aldehyde-free ethanol at a concentration of 500 μg/ml. A 4-5 day supply was made each time. Stock solutions were tightly capped and stored at -20^{o} C except at the times of diet preparation. To determine the average food consumption, 30 g of diet was given to each rat on the first day of the measurement, and residual food was weighed before the next feeding at approximately the same time in the next day. The average food consumption was the mean difference obtained by subtracting the weight of the residual diet from 30 g. It was estimated that each rat, on the average, consumed 25 g of diet per day in periodic feeding and 20 g per day in continuous feeding. A rat's appetite was directly related to its general health, which was directly affected in this experiment by the physiological vitamin A status (Anzano et al., 1979), and was manipulated by the feeding patterns designed in the experiments.

Therefore, adjustments in the amount of diet given to the rats were made accordingly, depending on the appetites of the animals in a certain group.

Fresh diets with various amount of drug supplementations were prepared daily by mixing measured volumes of stock solutions into the vitamin A-deficient diet while stirring. The ratio of vitamin A-deficient diet (g):volume of stock solution (μl) was determined by the level of dosing and the estimated consumption of diet per day per rat in a certain group. The diet was thoroughly mixed by stirring for about 2-3 min to ensure that all ethanol was evaporated.

The experimental procedure of periodic feeding is shown in Figure 4. The group designations are explained as follows. Group RA was designated to the animals that were fed on diets with periodic supplementation or withdrawal of retinoic acid (100 μg/day). The time length of each supplementation or withdrawal period was determined by the status of the animals' weight-change. In like manner, rats of group RP, group DHA, and group MHA-I were fed on diets with supplementation or withdrawal of retinyl palmitate (28 μg/rat/day), DHA (100 μg/rat/day), and MHA-I (100 μg/rat/day), respectively. Rats of Group CF (Cross-fed Group) were fed on diet supplemented daily with 100 μg of RA, DHA, and MHA-I alternatively after each weight-loss phase.

The experimental procedure of continuous feeding is shown in Figure 5. Compounds RA, DHA and MHA-II* were given to the animals in the amounts of 10, 100, or 200 μg/rat/day for an extended time period of 21-24 days. For example, rats of Group DHA-10 were given a diet supplemented with 10 μg of DHA per day.

The rats were weighed daily immediately prior to feeding. Occasionally in the periodic feeding experiment, weighing was done once every few days when rats were in steady weight-gaining periods. The weighing and feeding were done each time at

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* The use of MHA-II, in stead of MHA-I, in the continous feeding experiment was simply because of the shortage of the MHA-I supply. Owing to the existance of many hydrolytic enzymes in the digestive system, MHA-I and MHA-II should have the same effect since MHA-II is simply the acid form of MHA-I.

Figure 4. Diagrammatic Presentation of the Periodic Feeding Experiment.

RA: All-*trans*-retinoic Acid.

RP: All-*trans*-retinyl Palmitate.

DHA: Diaryl Heteroarotinoid Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate

MHA-I: Monoaryl Heteroarotinoid Ethyl (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoate

CF: Cross-feeding.

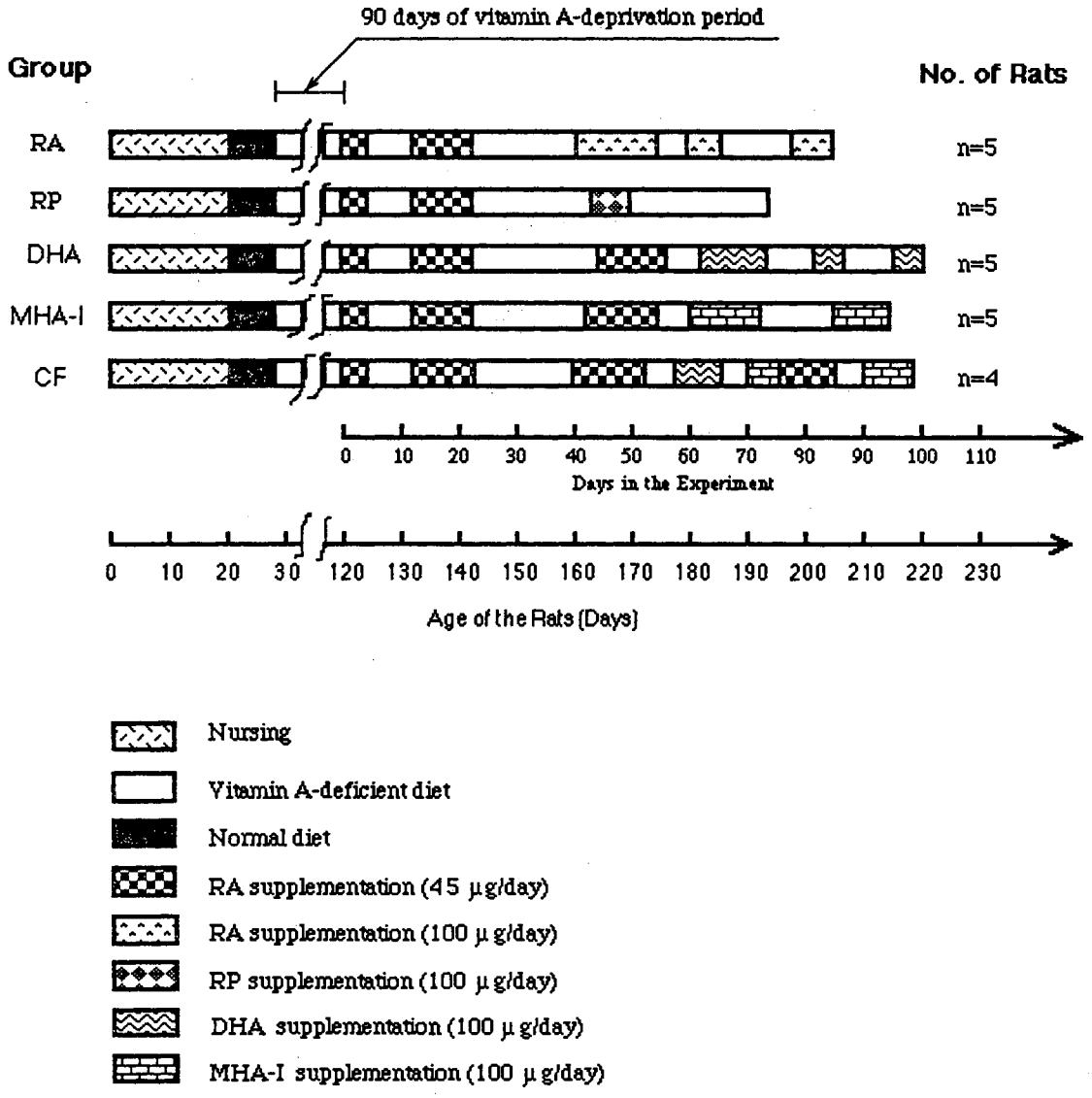
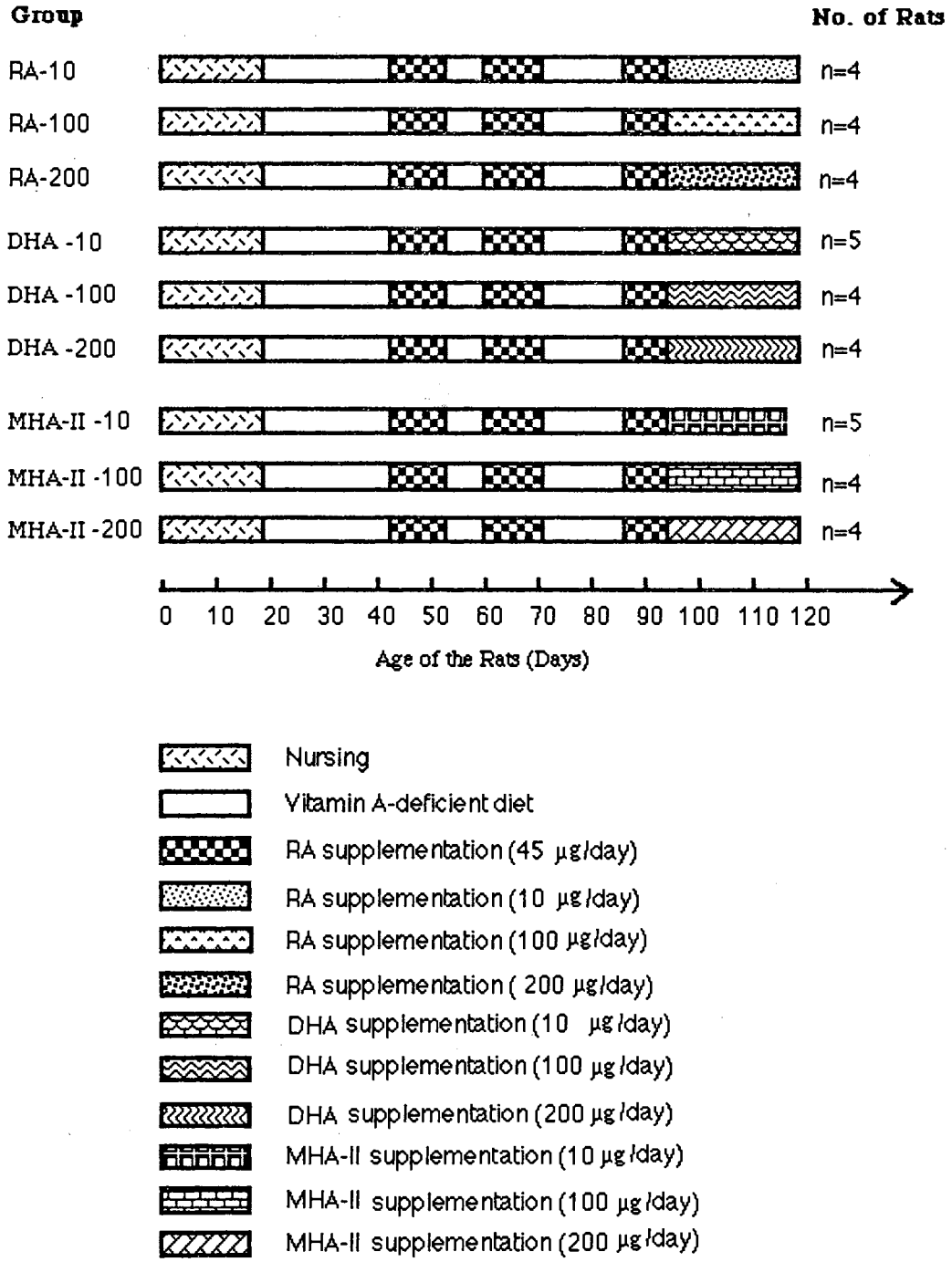

90 days of vitamin A-deprivation period
Group
No. of Rats
RA
RP
DHA
MHA-I
CF
n=5
n=5
n=5
n=5
n=4
0 10 20 30 40 50 60 70 90 90 100 110
Days in the Experiment
0 10 20 30 120 130 140 150 160 170 180 190 200 210 220 230
Age of the Rats (Days)
Nursing
Vitamin A-deficient diet
Normal diet
RA supplementation (45 μg/day)
RA supplementation (100 μg/day)
RP supplementation (100 μg/day)
DHA supplementation (100 μg/day)
MHA-I supplementation (100 μg/day)

Figure 5. Diagrammatic Presentation of the Continuous Feeding Experiment.

RA: All-*trans*-retinoic Acid

DHA: Diaryl Heteroarotinoid Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate

MHA-II: Monoaryl Heteroarotinoid *(2E,4E,6E)*-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.


Group
No. of Rats
RA-10
n=4
RA-100
n=4
RA-200
n=4
DHA -10
n=5
DHA -100
n=4
DHA -200
n=4
MHA-II -10
n=5
MHA-II -100
n=4
MHA-II -200
n=4
0 10 20 30 40 50 60 70 80 90 100 110 120
Age of the Rats (Days)
Nursing
Vitamin A-deficient diet
RA supplementation (45 µg/day)
RA supplementation (10 µg/day)
RA supplementation (100 µg/day)
RA supplementation (200 µg/day)
DHA supplementation (10 µg/day)
DHA supplementation (100 µg/day)
DHA supplementation (200 µg/day)
MHA-II supplementation (10 µg/day)
MHA-II supplementation (100 µg/day)
MHA-II supplementation (200 µg/day)

approximately the same time of the day to avoid possible diurnal variations. Any noticeable physical signs of vitamin A deficiency were recorded.

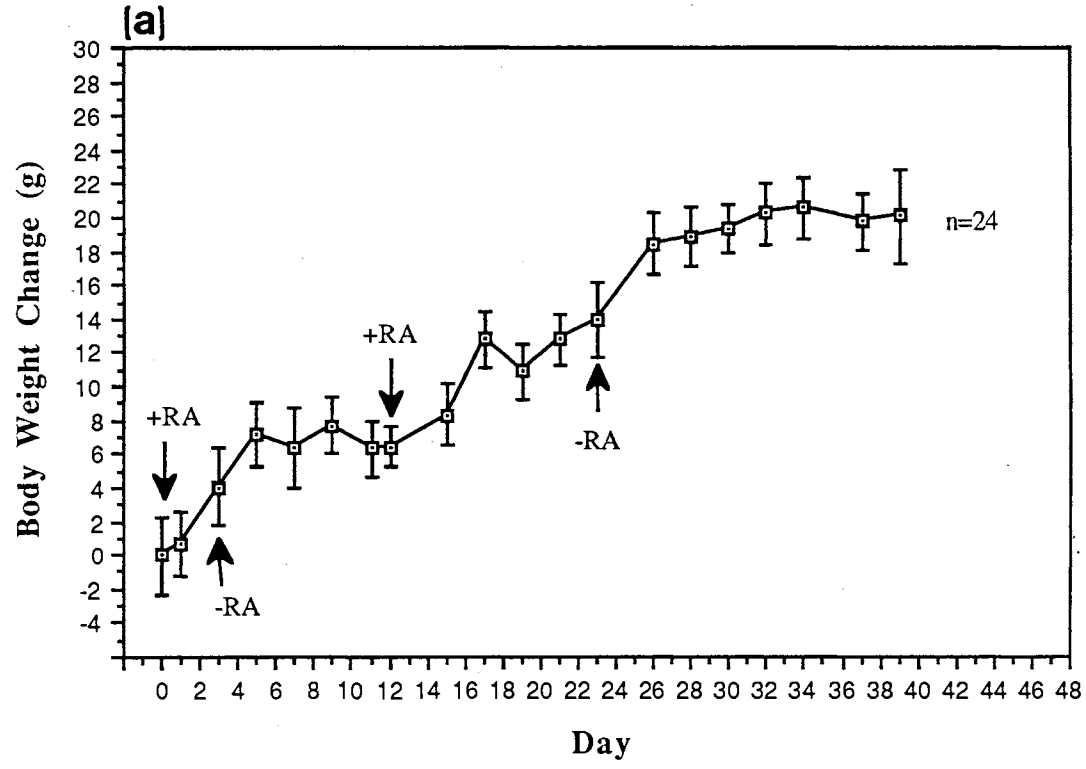
Results and Discussions

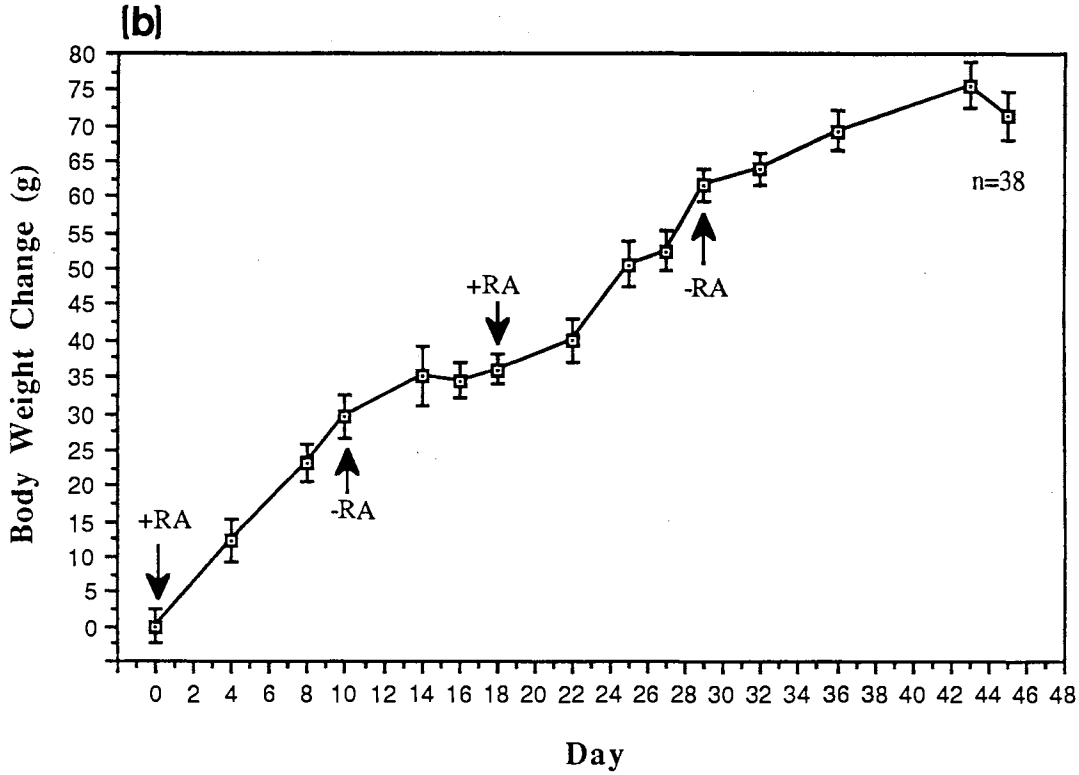
Synchronization Curves of Vitamin A Deficiency Induction

In this study, we used the synchronous vitamin A deficiency induction method described by Lamb, et al. (1974). This method provided a practical approach in terms of the speed and the synchronism for inducing the deficiency status in the entire laboratory animal population. It also offered reliable cause-effect sequences in terms of the flexibility in controlling the onset of vitamin A-deficiency and the quick responsiveness of the animals to the changes of the dietary supplements. These two aspects are the two most important elements in establishing reliable methodologies in this type of study.

Figure 6(a) and (b) show the synchronous vitamin A-deficiency curves of the rats used in the periodic feeding and continuous feeding experiment, respectively. Although the rats were in different stages of their life spans (rats in the periodic feeding experiment were twice as old compared to the rats in the continuous feeding experiment), the durations of each weight-gain and weight-loss phases and the patterns of the synchronization curves were very similar. It is clear that at a dietary RA supplementation of 45 μg/day/rat, the vitamin A-deficient rats quickly resumed normal growth. The rats in continuous feeding, for example, resumed an average growth rate of 3.1 g/day during RA supplementation, which was very close to the normal 3.8 g/day measured before the first growth plateau was observed. Withdrawal of RA resulted in the onset of the vitamin A-deficiency, which was indicated in the growth curves by a short plateau or a slightly declining phase. Normal growth resumed quickly (in about 1-2 days) after RA was added back to the diet. Such supplementation-deprivation cycles subsequent to the primary growth plateau were needed to promote a systemic, rather than a peripheral, vitamin A deficiency (Lamb et al, 1974). Residual amounts of growth-supporting retinoids in extrahepatic tissues were sufficiently

Figure 6. Synchronization Curves of Vitamin A-deficiency Introduction. (a) Periodic Feeding Experiment. (b) Continuous Feeding Experiment.
n: Number of Rats.
RA: All-*trans*-retinoic Acid.


(a)
Body Weight Change (g)
Day
+RA
-RA
+RA
-RA
n=24


(b)
Body Weight Change (g)
Day
+RA
-RA
+RA
-RA
n=38

eliminated after two cycles with 45 μg of daily retinoic acid supplementation in each cycle. At the end of the last cycle, rats were synchronously vitamin A-deficient as their average body weight-gain had dropped to 0.9 g/day, and many of them had lost weight.

Early nutritional treatment of animals is an important factor in the induction of vitamin A deficiency. Rats that were used in the continuous feeding experiment were subject to a diet deficient in vitamin A from 19 days of age. This restriction in vitamin A intake during early nourishment resulted in a much earlier onset of vitamin A deficiency when compared to the animals employed in the periodic feedings. The rats used in the periodic feeding experiment had not been on a vitamin A-deficient diet until they reached the first growth plateau. Due to the relatively high vitamin A storage in their bodies, these rats did not show a distinctive vitamin A deficiency until near maturity. This result was consistent with the earlier observations by Mason and Wolf (1935). They reported that when rats with a moderate storage of vitamin A and average weight of 40 g were put on vitamin A-deficient diet, the onset of the vitamin A-deficiency of these rats was much earlier than that of rats that had not been fed a vitamin A-deficient diet until they weighed around 50-75 g. Others also reported that induction of vitamin A deficiency by stringent dietary restriction of vitamin A was difficult in fullgrown individuals (Johnson and Baumann, 1948).

Growth Patterns of Rats with Periodic Feedings

Figure 7 to 11 show the growth curves of the vitamin A-deficient rats in response to periodic dietary supplementation and withdrawal of test compounds. Each experiment was carried out on a group of 5 animals and each data point given on the curves represents the mean of the weight-changes measured from five rats. Results in Figure 9 clearly indicated that DHA supplementation of 100 μg per rat per day was sufficient to promote a normal growth. The average growth rate of rats under DHA supplementation (3.8 g/day, extrapolated from the two DHA supplementation periods in Figure 9) was even higher that

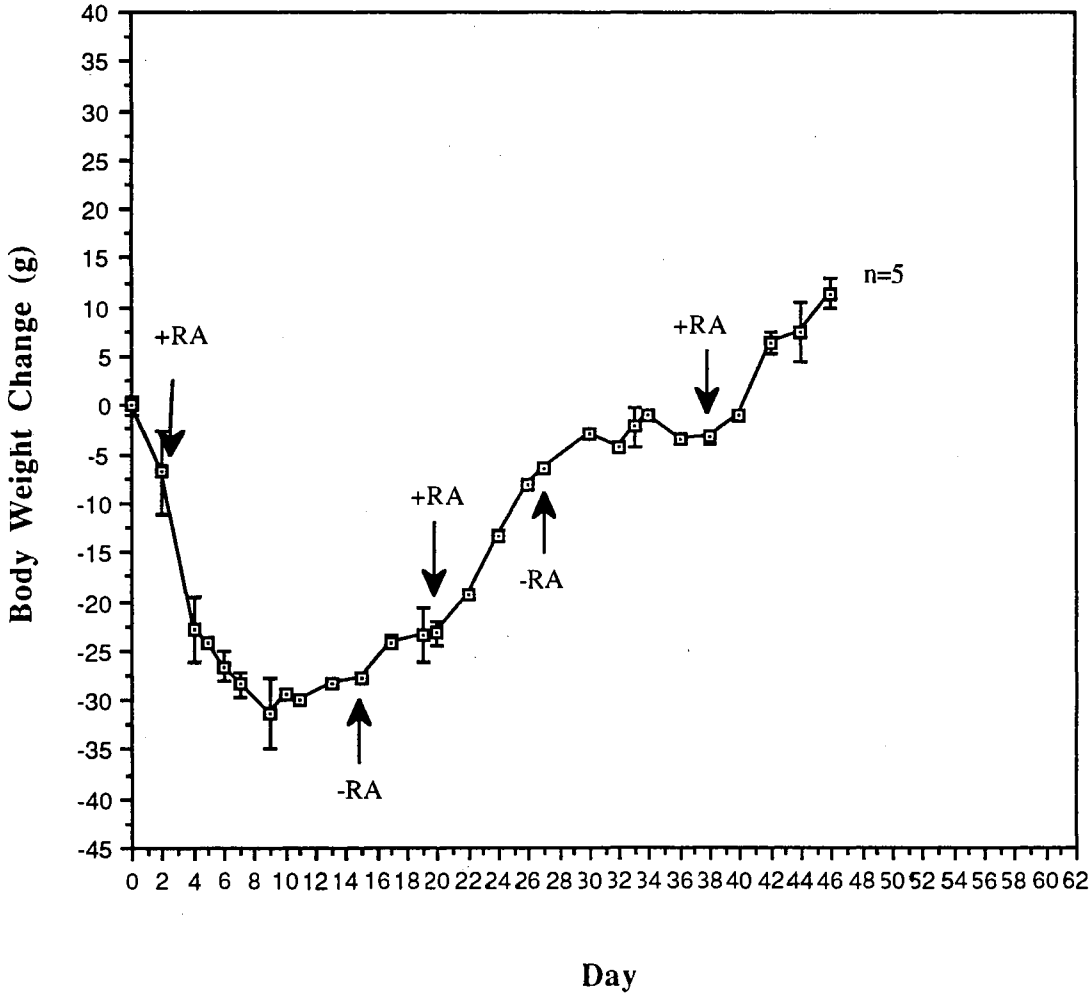


Figure 7. Growth Curve of the Vitamin A-deficient Rats Fed with Periodic Supplementation (100 µg/day/rat) and Withdrawal of All-*trans*-retinoic Acid (RA).

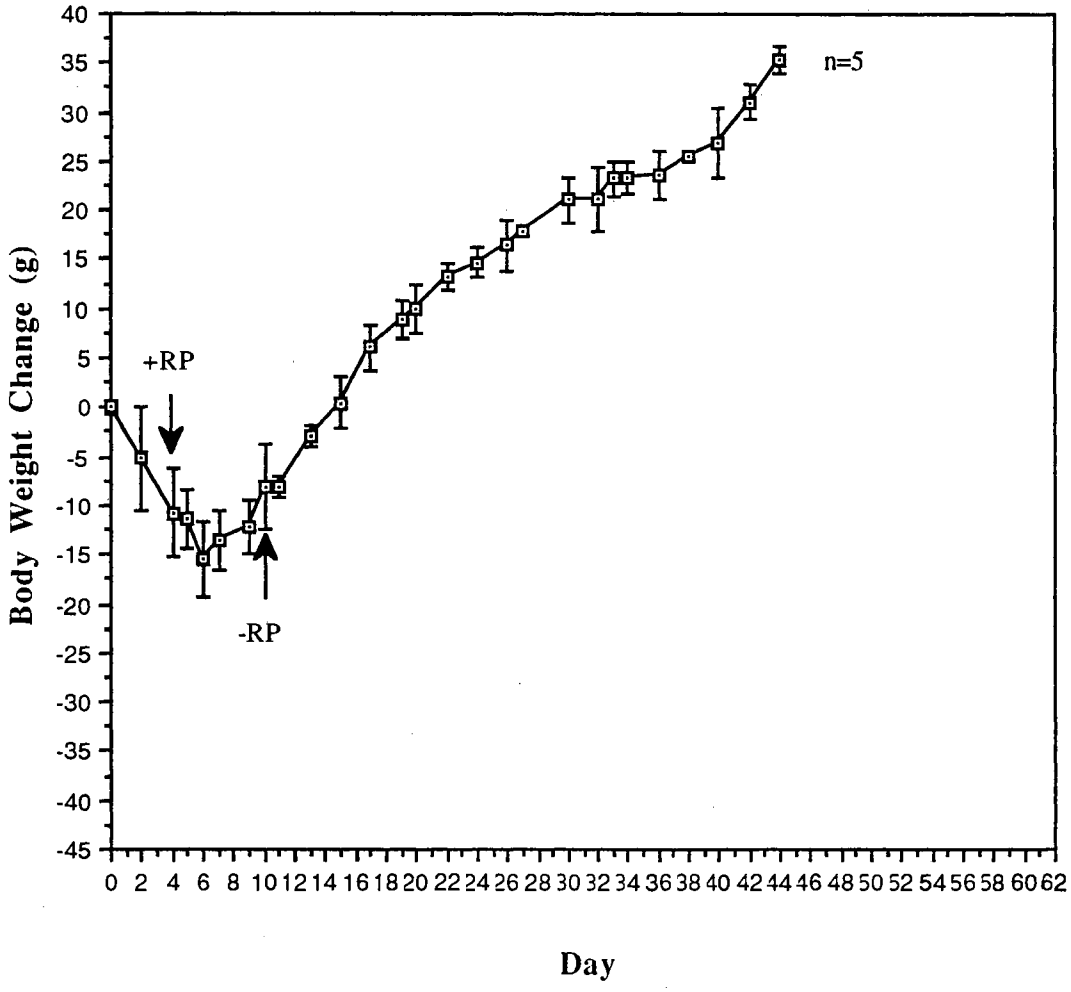


Figure 8. Growth Curve of the Vitamin A-deficient Rats Fed with Periodic Supplementation (28 μg/day/rat) and Withdrawal of All-*-trans*-retinyl Palmitate (RP).

Figure 9. Growth Curve of the Vitamin A-deficient Rats Fed with Periodic Supplementation (100 μg/day/rat) and Withdrawal of a Heteroarotinoid, Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate (DHA).

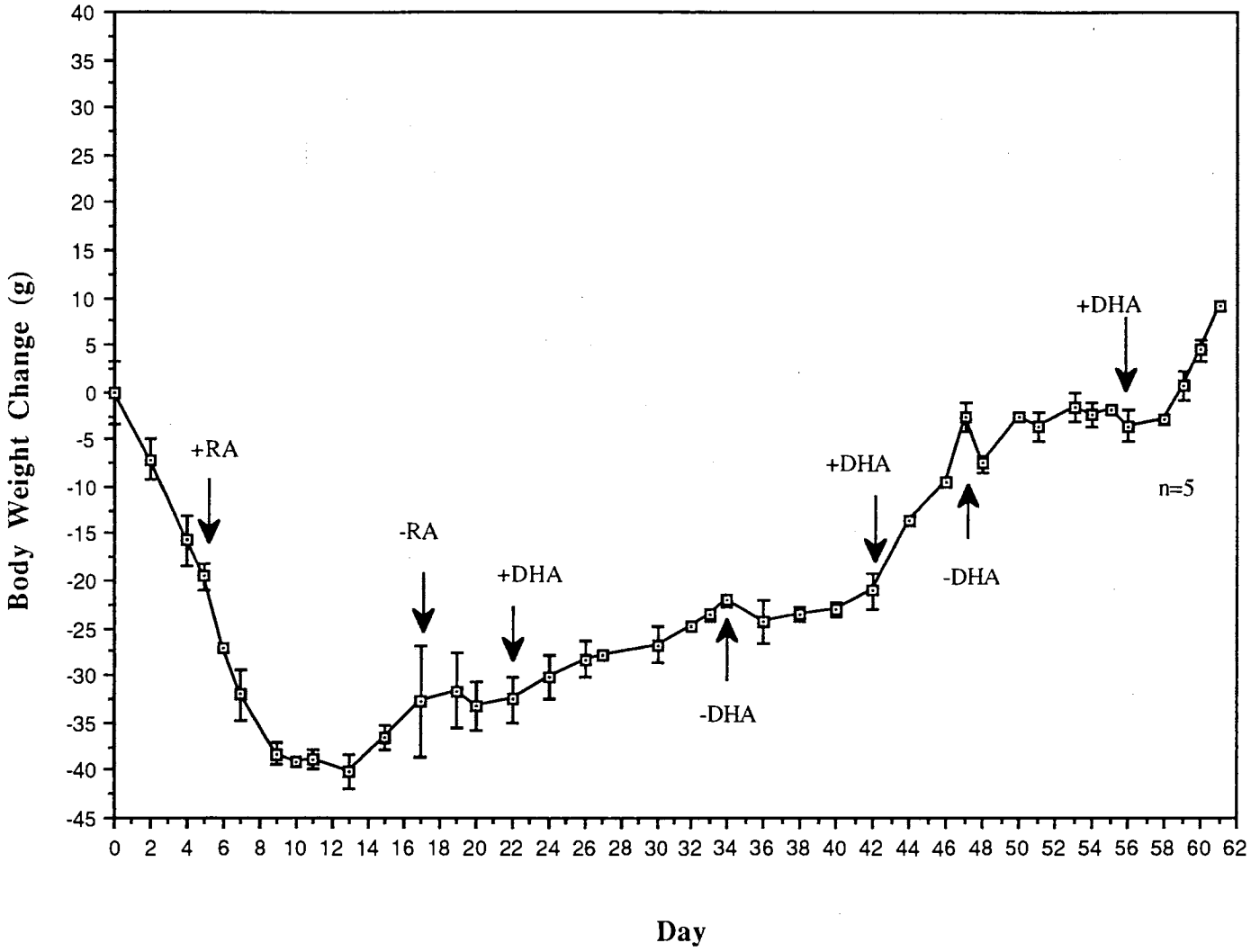

Body Weight Change (g)
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62
Day
+RA
-RA
+DHA
-DHA
+DHA
-DHA
+DHA
n=5

Figure 10. Growth Curve of the Vitamin A-deficient Rats Fed with Periodic Supplementation (100 μg/day/rat) and Withdrawal of a Heteroarotinoid, Ethyl (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoate (MHA-I).

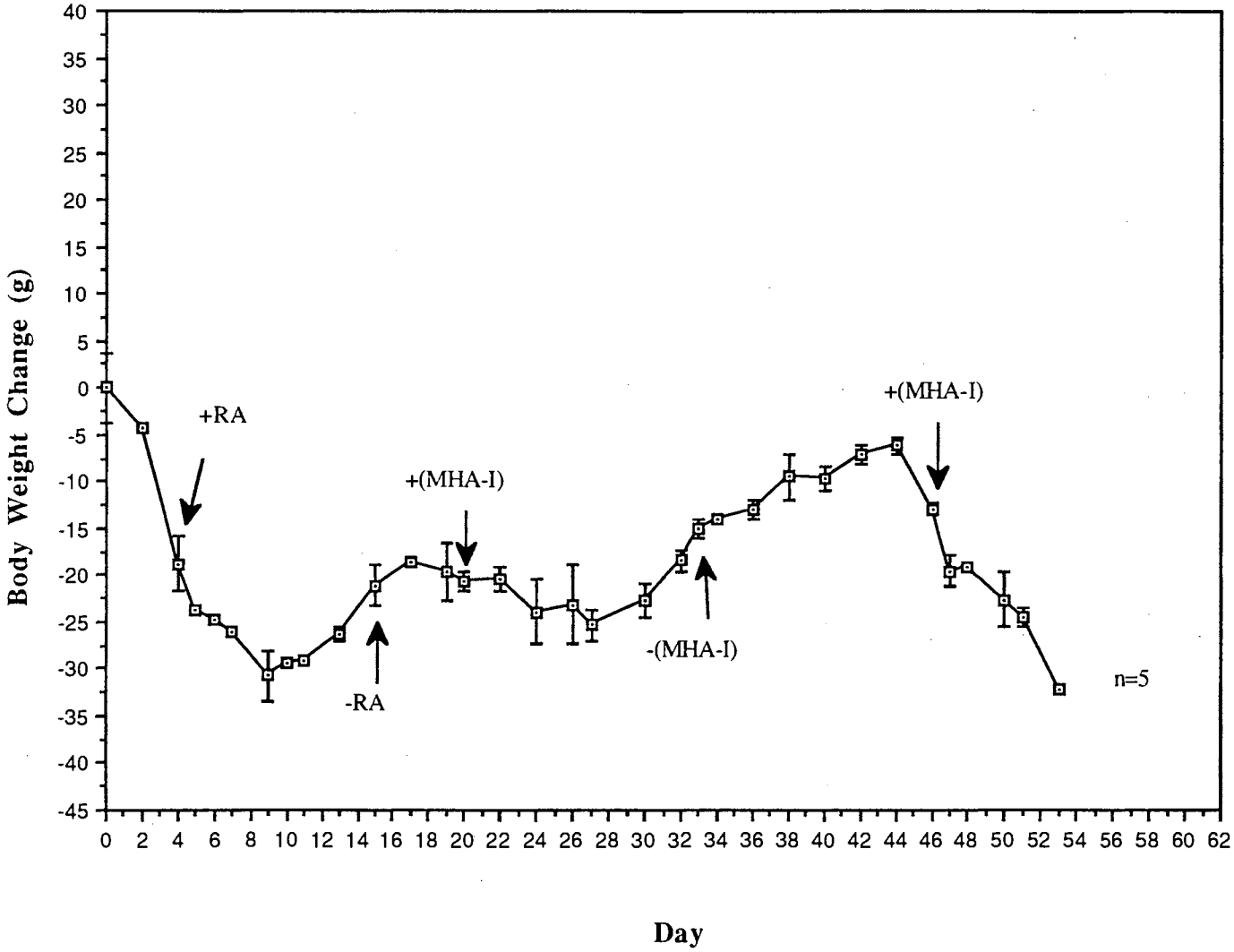
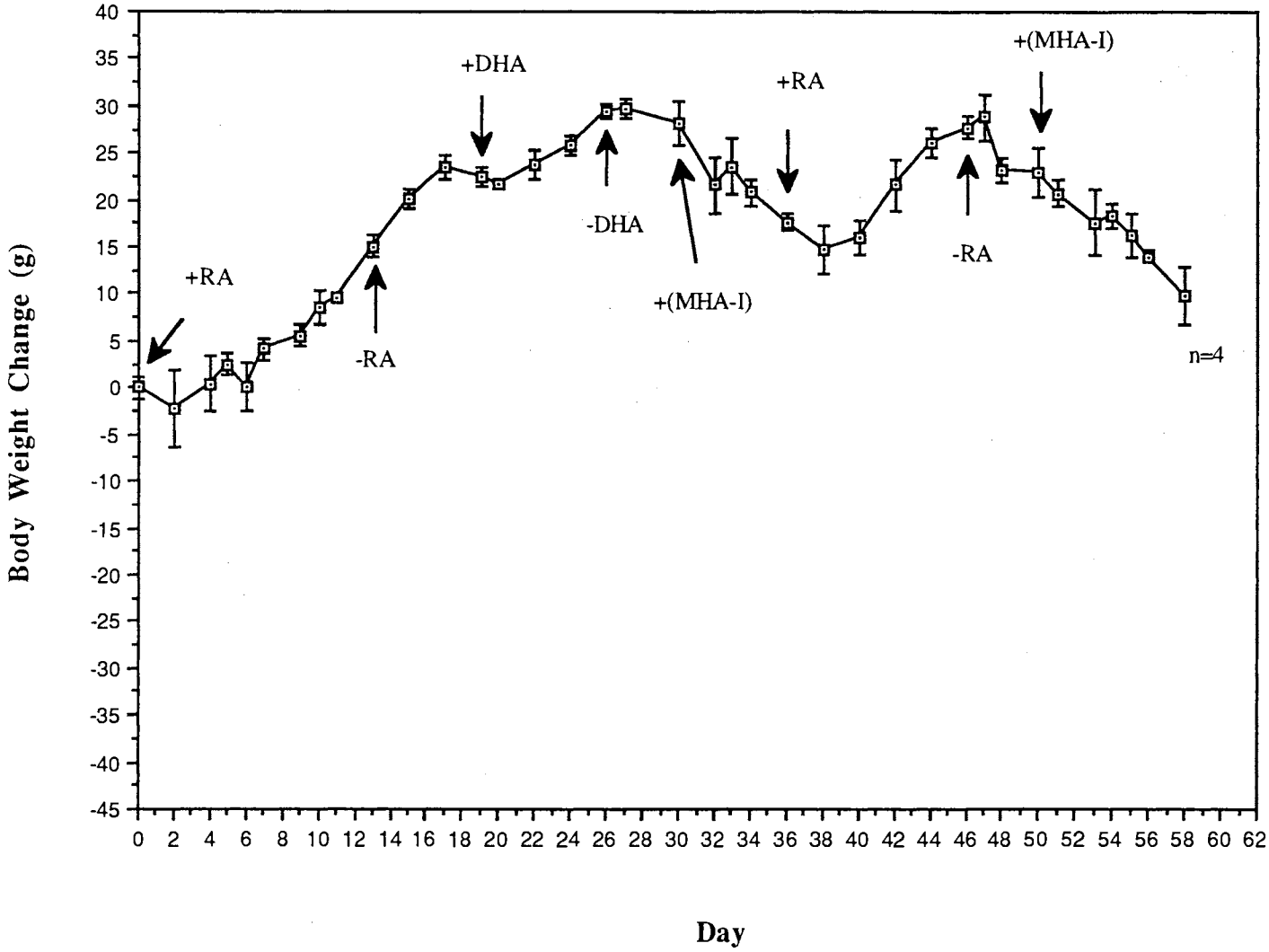

Body Weight Change (g)
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62
+RA
+(MHA-I)
-RA
-(MHA-I)
+(MHA-I)
n=5
Day

Figure 11. Growth Curve of Cross-fed (CF) Rats. The Rats Were Supplemented with 100 μg RA, DHA, and MHA-I Alternatively After Each Weight-loss Phase.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate

MHA-I: Ethyl (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoate.


Body Weight Change (g)
Day
+RA
-RA
+DHA
-DHA
+(MHA-I)
+RA
-RA
+(MHA-I)
n=4

the growth rate obtained from RA supplementation (2.4 g/day, extrapolated from the two RA supplementation periods in Figure 7 in the same manner). An irregular growth pattern, however, was observed in rats supplemented periodically with MHA-I (Figure 10). The rats either gained or lost weight irregularly or lost weight steadily in MHA-I supplementation periods. This distinctive difference between rats in response to DHA feeding and to MHA-I feeding was again observed in cross-fed rats (Figure 11). During the two MHA-I supplementation periods, the rats lost weight dramatically, while during the DHA feeding, the rats gained weight. The positive growth rate of DHA feeding in cross-fed rats was 1.7 g/day, again higher than that of RA feeding (1.3 g/day), although the difference is insignificant in this case. A different growth pattern emerged from rats supplemented with retinyl palmitate (RP). It seemed that rats could live normally and indefinitely after only a 5-day supply of RP at 28 μg per day, if other nutrients were held sufficient (Figure 8). This result demonstrated that retinyl palmitate was a very good source of vitamin A, and could be stored as a long-term vitamin A source. By the same token, however, RP was not a good control agent in this type of study. Its long-term and nearly irreversible effect makes it undesirable since flexibility in controlling growth pattern by manipulating dietary supplements is required in this type of experiments. RP, therefore, was not used in the later experiments.

Growth Patterns of Rats with Continuous Feedings

Figure 12, 13 and 14 show the growth curves of the vitamin A-deficient rats supplemented daily with either 10, 100 or 200 μg of test compounds. Over a continuous 25-day dietary supplementation period, neither DHA nor MHA-II at the level of 10 μg/day/rat was sufficient to support growth (Figure 12). While DHA apparently sustained positive growth for about 2 weeks, rats fed with 10 μg PIII per day began to lose weight 5-6 days after the feeding started. Despite the difference in timing, the rates of weight-loss between the two groups were very similar, with -2.4 g/day for DHA-fed rats and -2.0

Figure 12. Growth Curves of Vitamin A-deficient Rats Each Fed with Continuous Daily Supplementation of 10 μg of RA, DHA, or MHA-II, Respectively.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

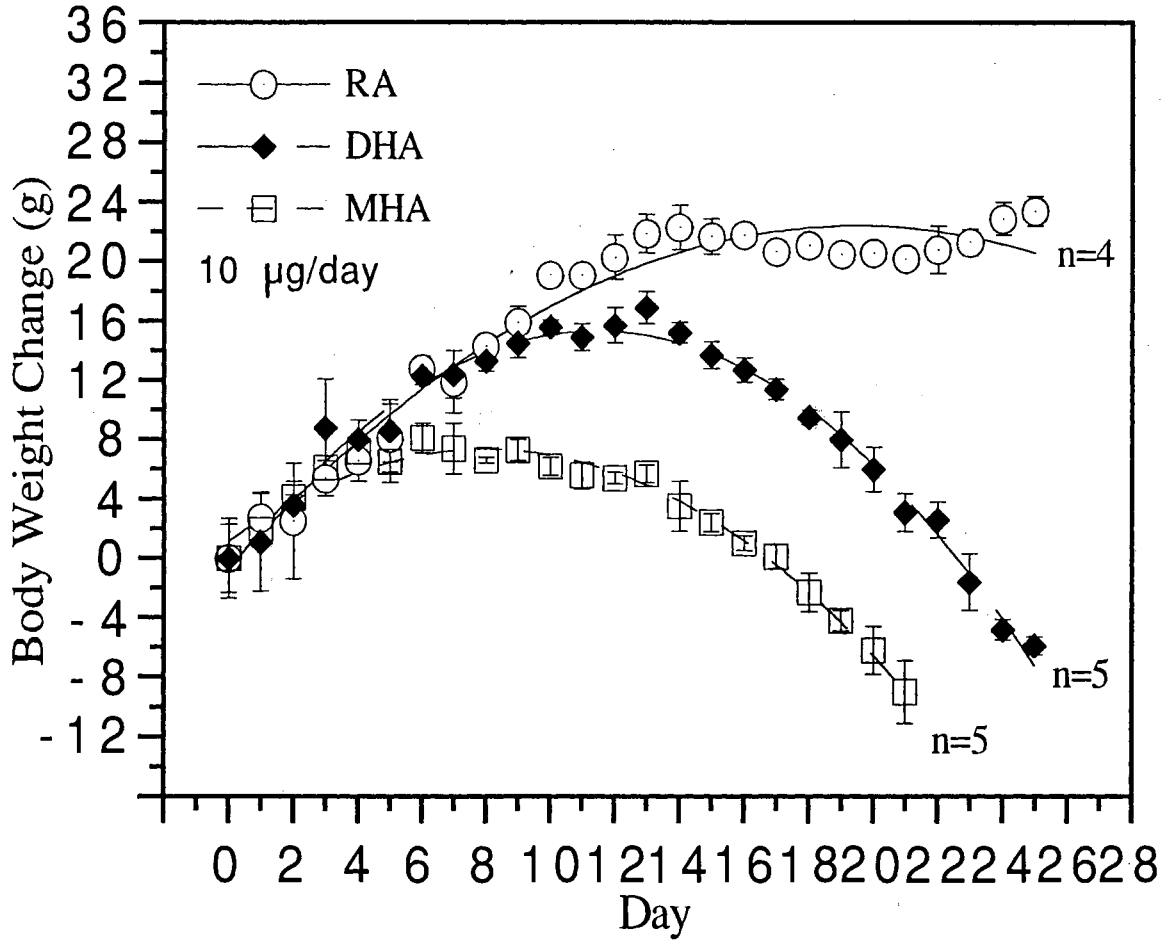

RA
DHA
MHA
10 µg/day
n=4
n=5
n=5
Body Weight Change (g)
36
32
28
24
20
16
12
8
4
0
- 4
- 8
-12
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day

Figure 13. Growth Curves of Vitamin A-deficient Rats Each Fed with Continuous Daily Supplementation of 100 μg of RA, DHA, or MHA-II, Respectively.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-Dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

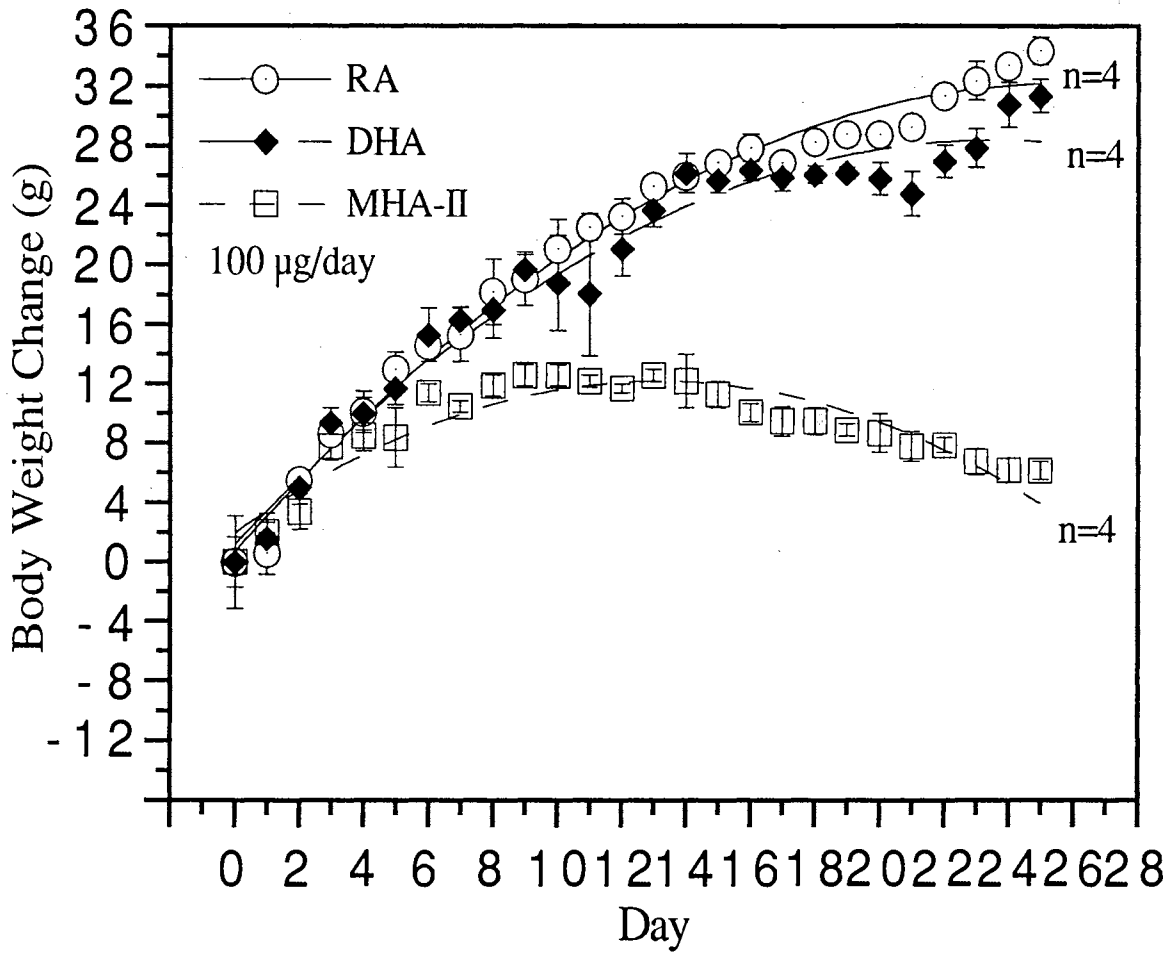
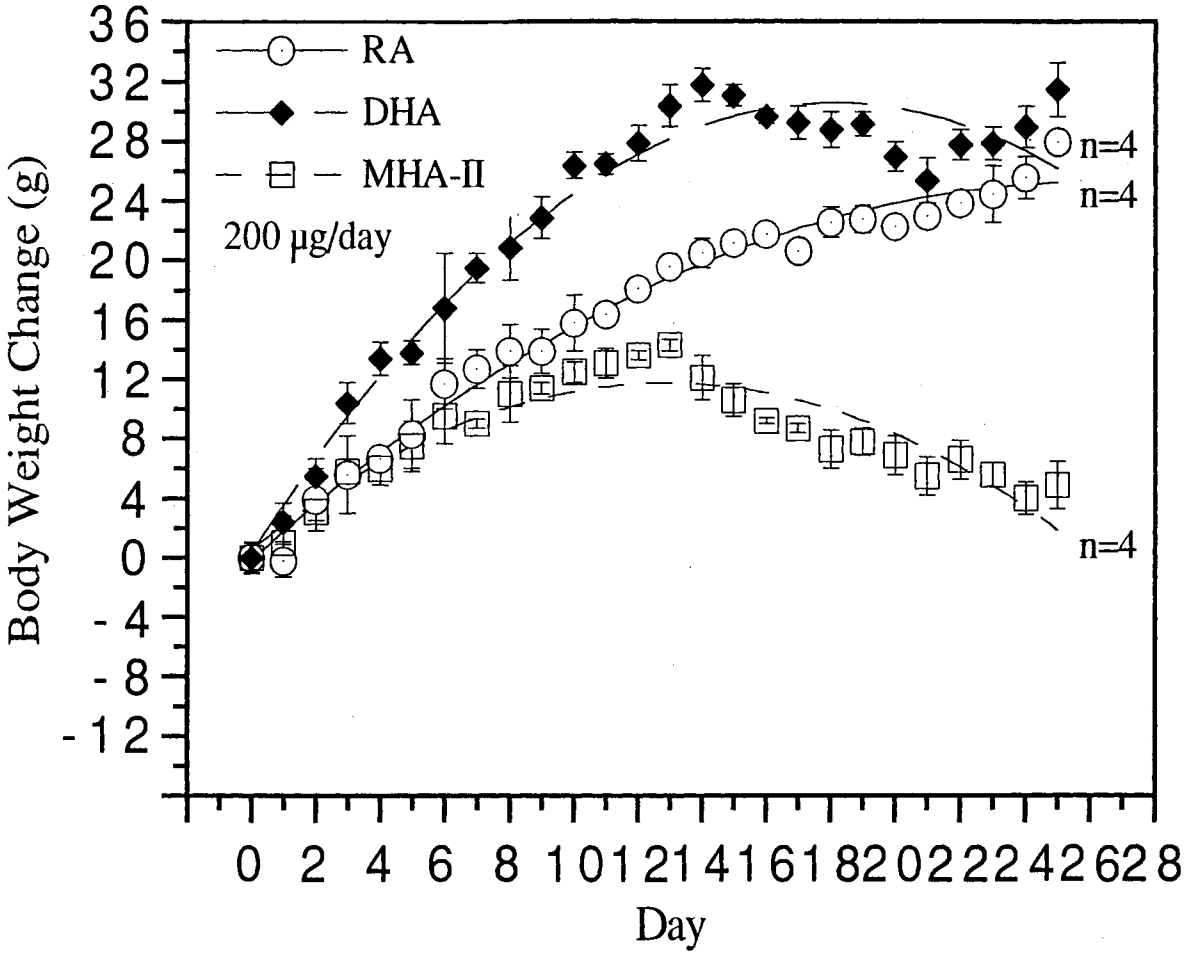

RA
DHA
MHA-II
100 µg/day
n=4
n=4
n=4
Body Weight Change (g)
36
32
28
24
20
16
12
8
4
0
- 4
- 8
- 12
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day

Figure 14. Growth Curves of Vitamin A-deficient Rats Each Fed with Continuous Daily Supplementation of 200 μg of RA, DHA, or MHA-II, Respectively.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6--chromanyl)-2,4,6-heptatrienoic Acid.


RA
DHA
MHA-II
200 µg/day
n=4
n=4
n=4
Body Weight Change (g)
36
32
28
24
20
16
12
8
4
0
- 4
- 8
-12
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Day

g/day for MHA-II-fed rats, respectively (growth rates were extrapolated from the growth curves). It is very likely that a 10 μg daily supply of MHA-II did not support growth at all since the rats were pretreated with 45 μg RA per day for 8 days before switching them on the test compounds (see previous description in Materials and Methods). The apparent growth for the 10 μg MHA-II-fed rats in the first 5-6 days may only reflect the residual effect of RA that remained in the body. The results also suggested that supplementation of RA at 10 μg/day level was not sufficient to maintain the normal growth although it was reported that the weight-maintenance dose for RA was 2 μg/rat/day by injection (Stephens-Jarnagin et al., 1985). The results obtained in this study probably did not conflict with the previous report, however, as the actual intake of RA for rats that were allowed to eat *ad libitum* was probably a great deal less than 10 μg owing to their generally poor health and accompanying poor appetite. In addition, some RA activity might be lost or reduced due to the oxidation of the compounds exposed to air and light.

In contrast, a different picture emerged when rats were given 100 μg drug/day (Figure 13). The growth-promoting activity of DHA was very similar to that of RA at this level. The initial rate of weight-gain was 2.3 g/day for RA-fed rats and 2.1 g/day for DHA-fed rats. The growth pattern of the rats given 100 μg of MHA-II daily was basically not different from that of the rats fed 10 μg MHA-II per day except for a delay in the beginning of weight-loss (about 13 days after the feeding started), and a lowered rate of weight-loss (-1.3 g/day compared to -2.0 g/day for 10 μg MHA-II-fed rats).

Figure 14 shows the growth curves of the rats with daily supplementation of 200 μg of drugs. Both RA and DHA supported growth at this level. The average growth rate of the rats fed 200 μg of DHA per day (2.3 g/day) was actually higher than that of the rats fed 200 μg of RA per day (1.8 g/day). Two hundred micrograms of daily supplement of DHA stimulated growth in rats substantially, especially in the early stage with an initial rate of 3.6 g/day. In contrast, the growth curve obtained from the feeding of MHA-II at the level of 200 μg/day was almost identical to that of MHA-II feeding at 100 μg/day (see

Figure 13). The results again demonstrated that MHA-II was not an effective growth promoter, although supplementation of MHA-II at high dosages (>100 μg/day) maintained growth in rats for a short period of 1-2 weeks (at least partially the result of RA supplementation before MHA-II feeding), no substantially long-term effect in terms of growth-supporting activity was demonstrated.

It was noted that rats fed with 200 μg RA/day did not gain more weight when compared to the rats fed with 100 μg RA/day (see Figure 13 and 14). Supplementation at the level of 200 μg RA/day resulted growth retardation when compared to the growth curve of 100 μg RA/day. This phenomenon of lower weight-gain at higher supplementation level may reflect the toxicity of RA. Likewise, fourteen days after the feeding began, there was a 7-day weight loss period in rats supplemented with 200 μg DHA/day (Figure 14). Although the rats in this group appeared to gain weight again after day 21, the overall slow-down in weight gain after two weeks of DHA supplementation may also indicate a toxic effect at the level of 200 μg/day. Should the feeding period be extended, the rats in Group RA-200 and DHA-200 might display steady weight loss if this speculation of toxicity were true. Further experiments are needed to evaluate this possibility.

Conclusions

Results from both the periodic feeding and the continuous feeding experiment clearly demonstrated the potent growth-promoting activity of compound DHA. The activity of DHA was very similar to, in some cases even higher than that of RA. Firstly, both compounds at levels of greater than 10 μg/day/rat sustained steady weight-gain in vitamin A-deficient rats when their diet was supplemented either periodically or continuously with these two compounds. Secondly, similar growth rates were obtained with 100 μg of continuous daily supplementation of either RA or DHA, while greater growth rates were demonstrated in rats given 100 μg of DHA periodically or 200 μg of DHA continuously per day when compared to RA supplementations at the same levels. Thirdly, growth in rats

was depressed within 1 to 2 days after the withdrawal of either 100 μg of RA or DHA. This temporary growth depression could be rapidly reversed after dietary RA or DHA supplementation was resumed, and the normal growth rate was restored. These comparative effects of RA and DHA held true in cross-fed rats. In contrast, two monoaryl heteroarotinoids (MHA-I and II) apparently did not support normal growth effectively, even at a supplementation level as high as 200 μg/day . Neither DHA nor RA could effectively support growth for more than two to three weeks when supplemented through diet at low dosage (10 μg/day/rat), although RA appeared to be able to sustain growth slightly longer. Results also indicated possible toxic effect when RA and DHA were supplemented at the level of 200 μg/day.

CHAPTER IV

VAGINAL-SMEAR ASSAY

Introduction

The vaginal and uterine epithelia of mammals exfoliates continuously in response to the hormonal surges of the estrous cycle. Three basic types of cells are routinely found in vaginal washes: nucleated epithelia cells, cornified epithelial cells, and neutrophils (polymorphyl leucocytes). The estrous cycle consists of four successive stages, namely proestrus, estrus, metestrus, and diestrus, and normally requires four to five days in rats for completion. Each stage of the estrous cycle is identified by the predominant representative type(s) of cells present as well as those absent from vaginal washes. During the stage of proestrus, the vaginal smear is dominated by nucleated epithelial cells. The stage of estrus is the period of heat that lasts from 9 to 15 hours. During estrus, ovulation occurs and the epithelial lining of the vaginal mucosa is hyperplastic. As new cells accumulate in the epithelium, the superficial layers become squamous and cornified. The cornified cells are eventually lost into the lumen of the vagina. The vaginal smear at this stage therefore, consists of mainly cornified epithelial cells. Shortly after ovulation, metestrus occurs, lasting from 10 to 14 hours. A large number of neutrophils along with a few cornified cells are present in the vaginal lumen during metestrus. In the following diestrus stage, the vaginal mucosa is thin, and neutrophils migrate through the cell layers easily, producing a vaginal smear consisting almost entirely of neutrophils. Diestrus lasts from 60 to 70 hours. As diestrus progresses, neutrophils become fewer in number and show degenerative changes.

The timing of the estrous cycle can be influenced by external factors such as temperature, stress, presence of males, and nutritional status. Mason et al. (1935) reported that, as the result of vitamin A deficiency, the estrous cycle in rats became irregular, lengthened, and was associated with growth retardation . It has been known since the 1920s that vaginal epithelial cornification is a very sensitive indication of early vitamin A deficiency, and that the retinoids, which have keratinization suppressing activity, reverse the cornification effect resulting from vitamin A deficiency (Evans and Bishop, 1922; Evans, 1928; Macy et al., 1927). A vaginal-smear method was developed by a number of researchers (Coward, 1929; Schmidt and Schmidt, 1930; Bauman and Steenbock, 1932; Coward et al., 1938) for the bioassay of vitamin A substances. The method, however, was considered insensitive and unreliable because of the endogenous ovarian hormone interference and oral administration of the retinoids. In the early applications of this method, the vaginal cornification could be either the result of the stage of estrus (estrogen influence) or vitamin A deficiency. Oral administration was associated with reduced bioavailability because much of the substance being tested might be poorly absorbed or destroyed in the gastrointestinal tract. In addition, direct effects of the test compounds on the vaginal epithelium could not be determined since a large percentage of the drugs absorbed would be distributed to all the tissues.

The methodology and the sensitivity of this *in vivo* assay was greatly improved by using ovariectomized vitamin A-deficient animals (Pugsley et al., 1944; Clarke and Todd, 1957), and by direct topical application of the test compounds (Kahn, 1954). In ovariectomized rats the cornification status of the vaginal epithelium is solely determined by their physiological vitamin A status. In the early practice of this method, Mason and Wolfe reported that ovariectomy did not significantly change the time of the onset of the vitamin A-deficiency in ovariectomized rats when compared to their intact litter-mates given the same treatment (Mason and Wolfe, 1935). Therefore, the continual vaginal cornification reported by those authors was not the result of the hormonal impairment, but a direct

consequence of vitamin A deficiency. Topical application of compounds exhibiting vitamin A-like activity upon the epithelium of the vagina reversed this effect, and the results correlated directly with the efficacy of the test substances (Mason and Wolfe, 1935). These findings promoted wide applications of this method in the quantitative determination of vitamin A and related compounds. With the development of more sensitive and accurate spectrophotometric methods for the quantitative determination of vitamin A compounds, the vaginal smear assay became nearly obsolete. Since the early 1980s, however, DeLuca and coworkers have used this method repeatedly in their studies (Sietsema and DeLuca, 1982; Skare et al., 1982; Miller et al., 1985). They reported success in the application of this method for testing the relative activities of small amounts of substances with vitamin A activity. Although a variety of *in vitro* assays have been developed which are 30- to 100-fold more sensitive than the vaginal smear assay (Sporn, 1984), this *in vivo* method still has great merit as a screening method in retinoid research.

The vaginal smear assay was used in the present study to test the ability of heteroarotinoids to reverse vaginal cornification and to compare the activity of heteroarotinoids with that of all-*trans*-retinoic acid (RA). The method was modified in an attempt to increase sensitivity and accuracy.

Materials and Methods

Chemicals

The heteroarotinoids DHA and MHA-II (see their structures in Figure 2) were synthesized and crystallized by the research group headed by Dr. Berlin (Waugh et al., 1985; Spruce et al., 1987). They were tightly capped under a N_2 atmosphere, wrapped in aluminum foil, and kept in the dark at -20° C. All-*trans*-retinoic acid (RA) was a gift from Dr. W. E. Scott, Hoffmann La-Roche, Inc. (Nutley, New Jersey), and was kept in the dark at room temperature.

Animals and Diets.

A diagrammatic representation of the feeding and the experimental procedure is shown in Figure 15. Forty female rat pups (Fisher-344, Sasco Inc., Omaha, Nebraska) 17 days of age, were obtained in three separate batches along with their dams. The dams were fed a vitamin A-deficient diet (TABLE I) immediately after arrival. The young rats were weaned at the age of 19 days and fed the same vitamin A-deficient diet. The animal housing facilities and care procedures were the same as previously described (See Materials and Methods in Chapter III). Their growth and vitamin A-deficiency status were monitored every 1-2 days by examining the vaginal smears (see below for detailed procedures) and by checking their weight changes. The rats were maintained on the vitamin A-deficient diet until their weight-change and their vaginal smears indicated that they had become vitamin A-deficient (about three to four weeks). The primary signs of vitamin A-deficiency included continuously cornified vaginal epithelium, xerophthalmia (tearless, swollen and bloody eyelids), and cessation of growth, etc. Ovariectomy was performed near the end of this vitamin A-deprivation period.

Ovariectomized vitamin A-deficient rats were used in the vaginal smear assays. At the completion of each assay, the rats were brought back to their normal rate of weight-gain by dietary supplementation with 45 μg RA/day/rat for 7-8 days. Withdrawal of this RA supplementation resulted in vitamin A-deficiency again in about 10-14 days, at which time the rats could be used in another round of VSA. None of the rats, however, were used more than three times.

Surgical Procedures of Ovariectomy

Estrogen-dependent vaginal opening in rats occurs at about 6 weeks of age, marking the onset of regular estrous cycling under the control of ovarian hormones (Baumann and Steenbock, 1932; Clarks and Todd, 1957). Estrous cycling was monitored by the vaginal smear assay (see below for detailed procedures). Ovariectomy was

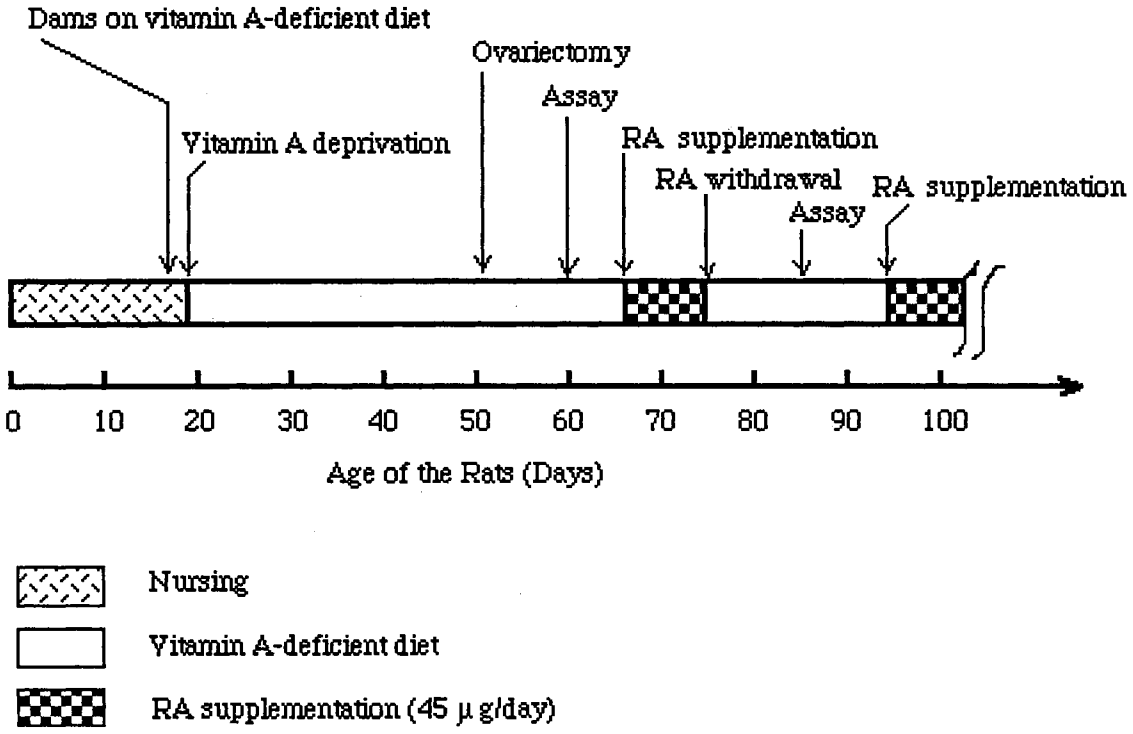


Figure 15. Diagrammatic Presentation of the Experimental Procedure of the Rat Vaginal-smear Assay (VSA).
RA: All-*trans*-retinoic Acid.

performed when rats were 7.5-8 weeks of age, by which time almost all rats had started estrous cycling. Their average body weight was 100 ± 1.2g. Ovariectomy was performed as follows. Rats were lightly anesthetized with diethyl ether (EM Sciences, Gibbstown, New Jersey) in a closed container. They were then removed from the jar and placed on their back with anesthesia continued via an ether nose cone. The abdominal area was wetted with a 1:15 dilution of chlorohexidine diacetate (Nolvasan solution, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). A 3 x 3 cm square of abdominal hair coat, beginning just posterior to the umbilicus and centered on the mid line, was shaved. All surgical instruments were cold sterilized in chlorohexidine diacetate solution. The skin and linia alba, beginning approximately 0.5 cm distal to the umbilicus and extending 2.0 cm, were separately incised. The animal was rotated to lie on its right flank, the abdominal muscle elevated and the periovarian fat pad grasped. The ovarian pedicle with ovarian artery and the distal tip of the fallopian tip were clamped as one and tired with 4-0 silk (black braided type B, Ethicon, Inc., Somerville, New Jersey). The ovarian tissue and the adherent fat were severed distal to the ligature. The procedure was repeated on the right site by turning the animal on its left flank. Excised tissue was evaluated for completeness of removal of ovarian tissues.

The abdominal muscles were closed with continuous interlocking sutures of 4-0 nylon (1.5 Metric Black Monofilament Nylon, Cyanamid Canada Inc., Montreal, QUE., Canada). The skin was closed with simple interrupted 4-0 nylon sutures spaced at 4 mm intervals. The incision area was gently cleaned with diluted chlorohexidine diacetate, patted dry and sprayed with furazolidone (Topazone Aerosal Powder, Norden Laboratories, Inc., Lincoln, Nebraska). Skin sutures were removed in 7 days.

Vaginal-smear Assay

Smear Preparation. The procedure used in this study was a modification of the method described by Sietsema et al. (1982). Rats were slightly anesthetized with ether.

Vaginal smears were taken using a glass disposable pipet (Pasteur Pipet, 5 3/4 inches, VWR Scientific, Toronto, Ontario, Canada). The pipets were briefly fired at the tip prior to use to narrow the openings and smooth the edges. The vagina was gently flushed 2 to 3 times with approximately 0.5 ml of distilled water. A small amount of the resulting cell suspension was placed on a microscope slide . Isotonic saline was not used in sampling because salt crystals remaining on the slides interfered with the examination of the cells. Smears were allowed to air dry undisturbed for about 3-4 hours, stained with 0.9% (mg/ml) methanol solution of Wright Stain (Sigma Chemical Co., St. Louis, Missouri) for 2 min, and neutralized in pH 6.4, 0.1M phosphate buffer for 1 min. Slides were then rinsed gently with a slow steady flow of tap water, air dried completely, and covered with cover slips. Xylene (Fisher Scientific, Grade R, Fair Lawn, New Jersey) and Cytoseal 280 (Staphens Scientific, Denville, New Jersey) were used as mounting solution and mounting medium, respectively. A phase contract microscope (Nikon 72739, Tokyo, Japan) was used for low (10x) and medium (43x) power examination. Another phase contract microscope system (Nikon Biological Microscope Optiphot with Photomicrographic Attachment Microflex Model UFX-II, Tokyo, Japan) was used for further examination and photomicroscopy.

Scoring Procedure. A numerical scoring system (Table II) was developed in an attempt to quantitatively measure the cellular changes in the rat epithelium. Scores (0-6) represent certain typical combinations of cells in the vaginal smears. This scoring system was a modification from the scoring systems used by Robson (Robson, 1938) and Sietsema et al. (1982). The modification consisted in the addition of scores given to situations where the content of nucleated epithelial cells was more than 25%. No scores were given to such situations in the study reported by Sietsema and co-workers (1982). From the author's experience, any given vaginal smear could be scored by one of the 13 typical cell combinations shown in Table II. The greater the concentration of the cornified epithelial cells in a vaginal smear, the higher the score that was given to the smear sample.

TABLE II

VAGINAL SMEAR SCORING SYSTEM

Epithelial Cells			
Neutrophils (%)	Nucleated (%)	Cornified (%)	Score
$75 < x < 100$	$0 < x < 25$	$x < 1$	0
$25 < x < 75$	$0 < x < 25$	$0 < x < 25$	1
$x \cong 100$	$x < 1$	$x < 1$	1
$x < 1$	$20 < x < 80$	$0 < x < 20$	1
$75 < x < 100$	$x < 1$	$0 < x < 25$	2
$50 < x < 75$	$0 < x < 25$	$0 < x < 50$	2
$50 < x < 75$	$x < 1$	$25 < x < 50$	3
$0 < x < 25$	$0 < x < 25$	$25 < x < 75$	3
$x < 1$	$50 < x < 80$	$20 < x < 50$	3
$x < 1$	$25 < x < 50$	$50 < x < 75$	4
$25 < x < 50$	$x < 1$	$50 < x < 75$	4
$x < 1$	$5 < x < 25$	$75 < x < 95$	5
$10 < x < 25$	$x < 1$	$75 < x < 90$	5
$x < 1$	$0 < x < 5$	$95 < x < 100$	6
$0 < x < 10$	$x < 1$	$90 < x < 100$	6

Highly cornified vaginal epithelium in ovariectomized rats indicated a severe vitamin A deficiency status. Vitamin A-like compounds that achieved physiological concentrations in the vaginal epithelium would reverse the cornification in a dose and potency dependent manner. In this situation, increased numbers of nucleated epithelial cells and neutrophils would be observed in the vaginal lumen. The relative activity of such a compound, at certain times after a specific dose was applied, was quantitated by a score that reflects the relative percentages of these cells in the entire vaginal lumen content. Therefore, the numerical scores represent progressive cellular changes in relation of one vaginal smear to another.

A group of 4 to 5 rats was used for each test compound at each dosing level. As no significant correlation was found between the initial body weight of the animals and their response to a test substance in a preliminary study (Clarke and Todd, 1957), the animals were allocated to different treatment groups randomly regardless of their individual weight. The numbers of neutrophils, nucleated epithelial cells, and cornified epithelial cells were carefully counted in three different viewing fields for each slide. The fields were chosen by initially determining the first viewing field at low power setting (100x), which was usually at the center of the smear with cells most evenly and densely distributed, and then moving horizontally and vertically the same distance from the first viewing point to locate the second and the third field. Cells were counted at medium power (430x) in all three fields. Thus, each slide had three scores based on the cell counts in three viewing fields. The three scores were then averaged to give the mean. All slides were counted in the same manner unless small alterations were made for counting poorly distributed and/or stained smears. For smears with low cell count and/or faintly stained, fields with the best visibility of the cells were chosen while conforming to the established method as much as possible for consistency. The final score for a compound tested at a specific dosing level was calculated by averaging the mean scores obtained from all the rats in the group dosed with

the compound at that level. Standard deviations and standard errors of the means were determined. Statistical comparisons were made according to Student's *t* -test.

Drug Application Procedures

Stock Solution Preparation. For compounds RA and MHA-II (MW = 300), 150 μg (500 nmol) of each was weighed out using an automatic electrobalance (Cahn 29 Automatic Electrobalance, Cahn Instrument Company, Paramount, California) and dissolved in 100 μl of aldehyde-free ethanol to make an initial stock solution (10^{-8} mol/2μl). For compound DHA (MW = 350), 175 μg (500 nmol) was weighed out to prepare the initial stock solution. Serial dilutions of the compounds were made from these initial stock solutions (10^{-8} mol/2μl) by diluting 10-fold at each time with aldehyde-free ethanol. All dilutions, with concentrations ranging from 10^{-9} to 10^{-14} moles per 2 μl, were prepared immediately before dosing and were kept at -20° C before the next dilutions were made.

Dosing Solution Preparation and Administration. The dosing solutions were prepared by mixing 16 μl stock solution with 200 μl isotonic saline solution immediately before dosing. A total volume of 27 μl of this dosing solution (containing the test compound in the amount of 10^{-9} moles, 10^{-10} moles, and so on, at each dosing level) was administered to each test rat with the exception of the 10^{-8} mol application. Owing to the limited solubilities of the compounds in saline, an additional 24 μl of aldehyde-free ethanol was added when preparing the dosing solution for the 10^{-8} mol application, and a total volume of 30 μl of dosing solution (containing 10^{-8} moles of test compound) was applied to the vaginal epithelium of each rat .

The drugs were applied intravaginally using an automatic pipet immediately after the first vaginal smear was taken (at time 0). Rats were under light ether anesthesia during dosing and in all the subsequent smear-taking procedures. The second vaginal smears were taken from the rats 18 hours after the drug application, and then every 24 hours

thereafter. A stretch of 6-7 days of continuous sampling was usually needed for each round of assay.

Controls. Assay controls were run concurrently with the drug-testing assays. A blank dosing solution, a mixture of 25 µl saline and 2 µl aldehyde-free ethanol, was applied to each of the control rats with all the experimental procedures and treatments done in exactly the same manner. This negative control assay was done twice, with the first one at the beginning and the second one at the end of the entire VSA study (2.5 months apart). Results from the assays with RA, tested at the same and each dosing level as to the heteroarotinoids, served as reference points for comparisons between the three test compounds. Furthermore, each rat was its own control with respect to the cornification status since they had exhibit vaginal smear scores of 5 or 6 (indicating complete or near complete cornification status) for at least two consecutive days before they were employed in the assays.

Results and Discussions

Growth Rates

The average weight-gain of the young rats during the last two weeks of the vitamin A deprivation period (before ovariectomy was performed) is plotted in Figure 16. The average rate of growth (2.1 ± 0.2 g/day) reflected a retarded growth since normal growth rate for young rats of 5-6 weeks of age is usually around 5 g/day. However, the growth rate indicated that the rats were still gaining weight quite steadily during this period. Random sampling of the vaginal smears indicated continuous estrous cycling. About a week later, the growth had almost ceased. The vaginal smears taken at this time showed continual cornification and the rats were ready for the assay. The average weight-gain was 0.2 ± 0.3 g/day at the beginning of an assay.

The length of time that each round of vaginal-smear assay required was usually between 6 to 7 days, during which time the vaginal smears were taken daily and the rats

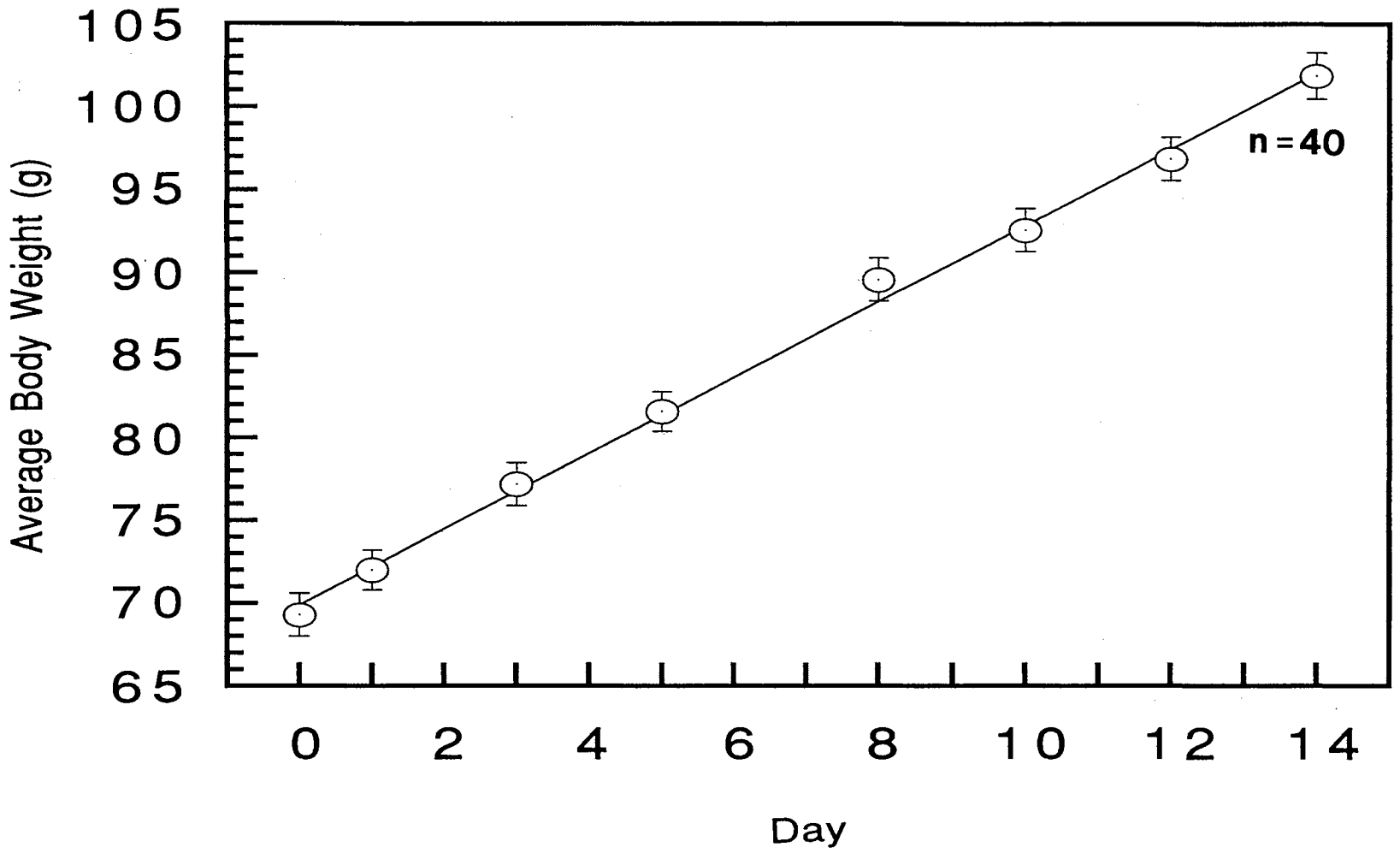


Figure 16. The Average Weight-gain of the Rats Before Ovariectomy.
n: Number of Rats.

were kept in a vitamin A-deficient status Towards the end of this time, the severity of the deficiency had worsened so that many rats appeared very sick. Most of them lost weight and showed swollen, bloody eyelids (the symptoms of xeropthalmia). Some rats developed respiratory difficulties, and diarrhea. Retinoic acid, equivalent to 45 μg per day in stabilized gelatin beadleds, was given to each rat immediately at the completion of an assay. On the average, about one week of retinoic acid supplementation reversed the vitamin A-deficient conditions and restored growth rate to normal. Two rats died of vitamin A deficiency-initiated complications. The average growth rate was 1.3 ± 0.1 g/day two days after the completion of an experiment. Though lower than the normal growth, this rate indicated the beginning of recovery from vitamin A deficiency. The rate of growth increased to 3.0 ± 0.1 g/day by one week after the rats were on the RA supplemented diet. The signs of xeropthalmia disappeared for most rats at this time. The above observations were consistent with the early observations reported by others (Baumann and Steenbock, 1932; Aberle, 1933).

Repetitive Use of Rats

After the rats recovered from vitamin A-deficiency, RA was removed from the diet to prepare the rats for another assay. On the average, the rats became vitamin A-deficient again in 10-14 days. In earlier studies of the vaginal smear assay, rats were used repeatedly for up to eight times (Pugsley et al., 1944). Although it was reported that the differences in the previous treatments, as well as the number of the previous treatments, had no significant effect on the successive assays, the time required to re-deplete the rats following the removal of a "maintenance diet that devoid of vitamin A" was decreased with successive assays (Clarke and Todd, 1957). The reason may simply be the fact that body retinol storage was further decreased, or even exhausted, in the successive assays. This phenomenon was not obvious in this study, perhaps due to the fact that no rats were used more than three times.

Vaginal Smears

As described earlier, the vaginal smear score is a relative measurement of the vaginal epithelial cornification status (see the section of Scoring Procedure for detailed description). Figure 17 through Figure 20 present the photomicrographs of four representative rat vaginal smears obtained from this study. The identities of these smears along with their scores are given in the legends.

At the beginning of this study, the scoring system presented by Siesma and co-workers (1982) was used. It was found later that their scoring system was insufficient in the present study because it does not include situations in which the percentage of nucleated epithelial cells exceeds 25% of the entire cellular content in the lumen of rat vagina. In a more recent work done by the same research group (Miller, 1985), a modified scoring system was used, in which the number of nucleated epithelial cells were not quantitatively differentiated from that of the neutrophils. The total content of these two types of cells in a vaginal smear was measured as non-cornified cells. This newest scoring system, although it is much simpler and less time consuming, was not adapted for the present study. A new scoring system was developed in order to measure those situations in which nucleated epithelial cells exceeded 25% of total cells present in the smears. In fact, these situations were often encountered (see Figure 18) and might be of additional physiological significance.

It was reported that the absolute number of nucleated epithelial cells was reduced in ovariectomized rats as compared to that of intact rats (Mason and Wolfe, 1935). Nucleated epithelial cells were found predominant in the vaginal smears after the administration of vitamin A-active substances (De Venarizi and Montenegro, 1945). There are four possibilities that may result in large numbers of nucleated epithelial cells. a) Vitamin A-like activity of test compounds stimulated both the epithelial differentiation and proliferation in such a way that large numbers of nucleated epithelial cells appeared in the epithelial tissues as the ultimate result of the activity. Recent studies have demonstrated that cell

Figure 17. A Representative Vaginal Smear That Contains Entirely the Cornified Epithelial Cells.
Magnification: 400x
Identity: Control-II at Hour 42.
Score: 6.0

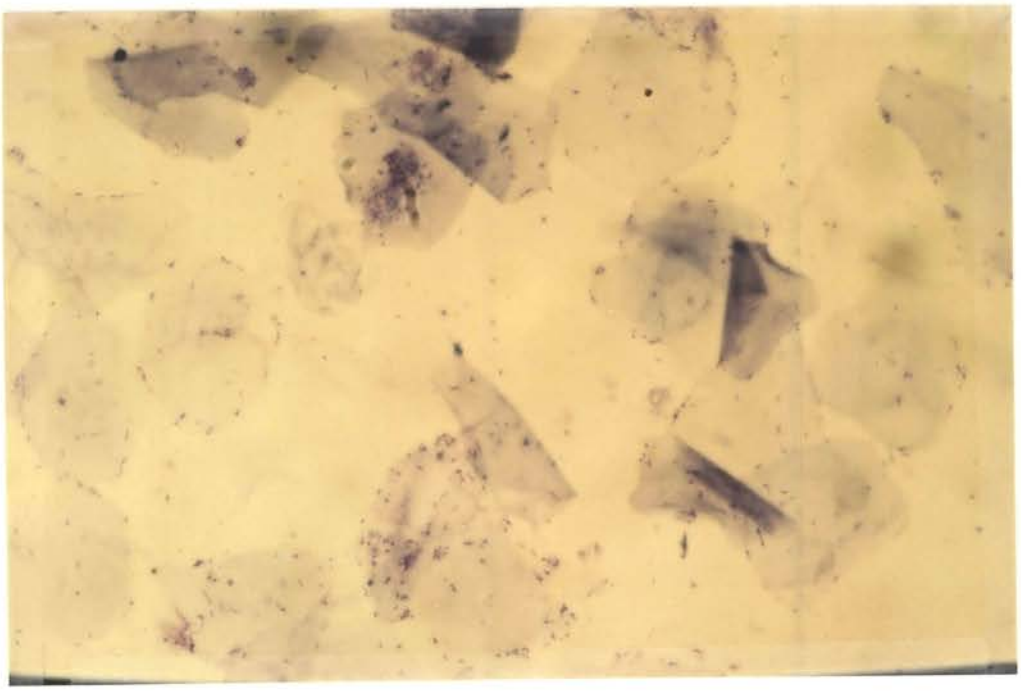


Figure 18. A Representative Vaginal Smear That Contains a Large Number of the Nucleated Epithelial Cells.
Magnification: 400x
Identity: RA^{-13} mol/rat at Hour 42.
(RA: All-*trans*-retinoic Acid)
Score: 4.3

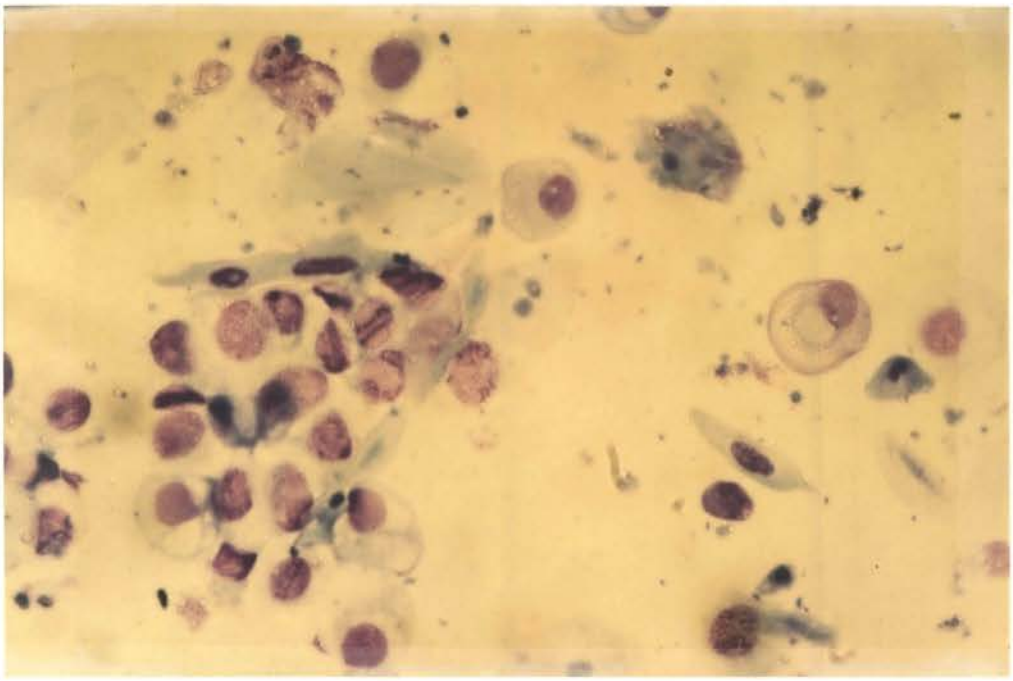


Figure 19. A Representative Vaginal Smear That Contains a Majority of Neutrophils.

Magnification: 400x

Identity: DHA^{-8} mol/rat at Hour 66.

(DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate)

Score: 1.0

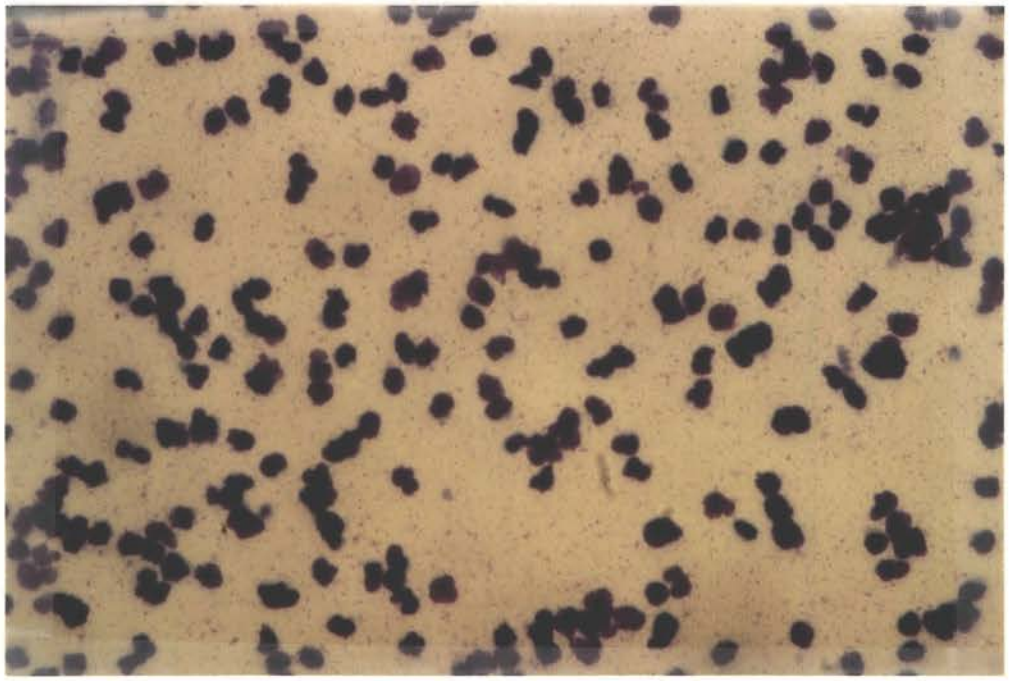
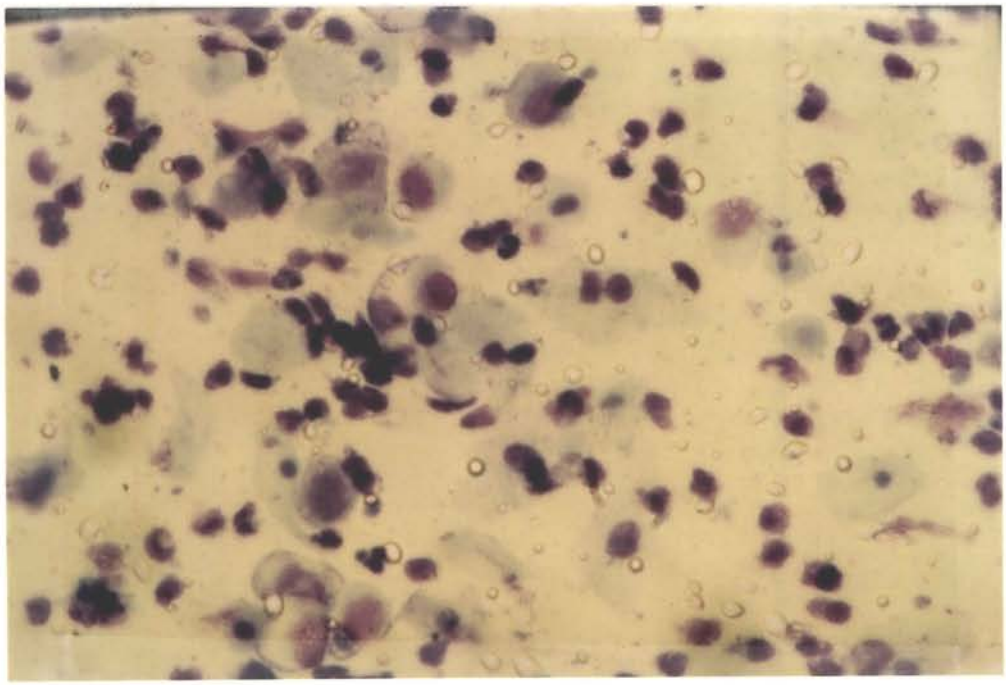


Figure 20. A Representative Vaginal Smear with the Co-existence of Three Cell Types: Neutrophils, the Nucleated Epithelial Cells and the Cornified Epithelial Cells.
Magnification: 400x
Identity: DHA^{-14} mol/rat at Hour 138.
(DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate)
Score: 2.7.



proliferation and differentiation can be mediated concordantly by retinoids, rather than only reciprocally as commonly believed (Marcelo and Madison, 1984). b) The test compounds might exhibit estrogen-like or progesterone-like hormonal effect. A situation similar to metestrus might be observed if the test compounds could partially mimic the action of estrogen on the estrus cycle. Robson (1937) reported that the vaginal cornifying action produced by estrogens could be inhibited by the simultaneous administration of progesterone. Since the ligand-binding domain of recently discovered retinoic acid receptors (RARs) is similar to that of the steroid hormone receptors, speculations that the actions of retinoids may share some characteristics of steroid hormones may have some validity. DHA and MHA-II might bind to steroid receptors to mimic some of the actions of steroid hormones, resulting in large number of nucleated epithelial cells. c) Estrous cycling might continue to some extent due to estrogen (or other luteal hormones, such as progesterone) released from extra-ovarian sites, such as adrenal glands. Normally, small amounts of estrogen and progesterone are released from the adrenal (Turner, 1963). The removal of ovaries posts a negative feedback that stimulates the luteal hormone production in adrenal glands. d) Estrous cycling might continue due to incomplete removal of ovarian tissue. However, the possibility of a residual hormone-controlled estrous cycling is not likely in the present case as the typically persistent, rather than periodic, cornification of vaginal epithelial tissue was observed for all the animals used in the assays. In addition, the time periods required for de-cornification by dietary RA supplementation after the completion of an assay and for re-cornification following the removal of RA supplement were almost the same for all treated rats. Such synchronous responses would not be possible if residual ovarian and/or adrenal tissue of each individual rat was supplying any estrogenic stimulus.

Time-course Curves of Vaginal Epithelial Response

In order to directly compare the relative activities of RA, DHA, and MHA-II, the time-course curves of vaginal response to the test compounds at each dose level are plotted in Figure 21 through Figure 27. The control curves are shown in Figure 28. The statistical analysis of the vaginal smear scores was summarized in Table III. Statistical significances in relative activities were compared with respect to that of time 0 for each compound at each dose level ($p<0.1$, 0.05, and 0.005).

No response was observed in the control rats (Figure 28), which indicated that dosing solution had no effect on cornified vaginal epithelial. All three compounds, RA, DHA and MHA-II, were active, and their activities were comparable (Figure 21 to Figure 27). The comparable activities of these three compounds were obvious, especially at high dosages. Overall, MHA-II was slightly less active than RA and DHA. At dose ranging from 10^{-8} mol/rat to 10^{-10} mol/rat, the peak activity of MHA-II was consistently lower than that of RA and DHA. Furthermore, the duration of the response did not always correlate with the dosages as other investigators have reported for some other retinoids (Sietsema, et al., 1982).

Late Reappearance of Neutrophils

The activity curves obtained at lower doses ($< 10^{-11}$ mol/rat) revealed two phenomena that were not observed at high dosing levels (Figure 25 to Figure 27). First, at low dosing levels, the peaks of the activity shifted towards later time points, especially for the two heteroarotinoids. Secondly, there were repeatedly no clearly defined peaks of activities in the curves of low doses as compared to those obtained at the higher doses. The activity levels did not drop noticeably after the highest activity points were reached. The activity levels remained at about the same level, or in some cases increased, with the time basically because of the reappearance of large numbers of neutrophils in the smears. In some cases, excessive neutrophils appeared in only one smear. In others, the reappearance

Figure 21. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-8} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

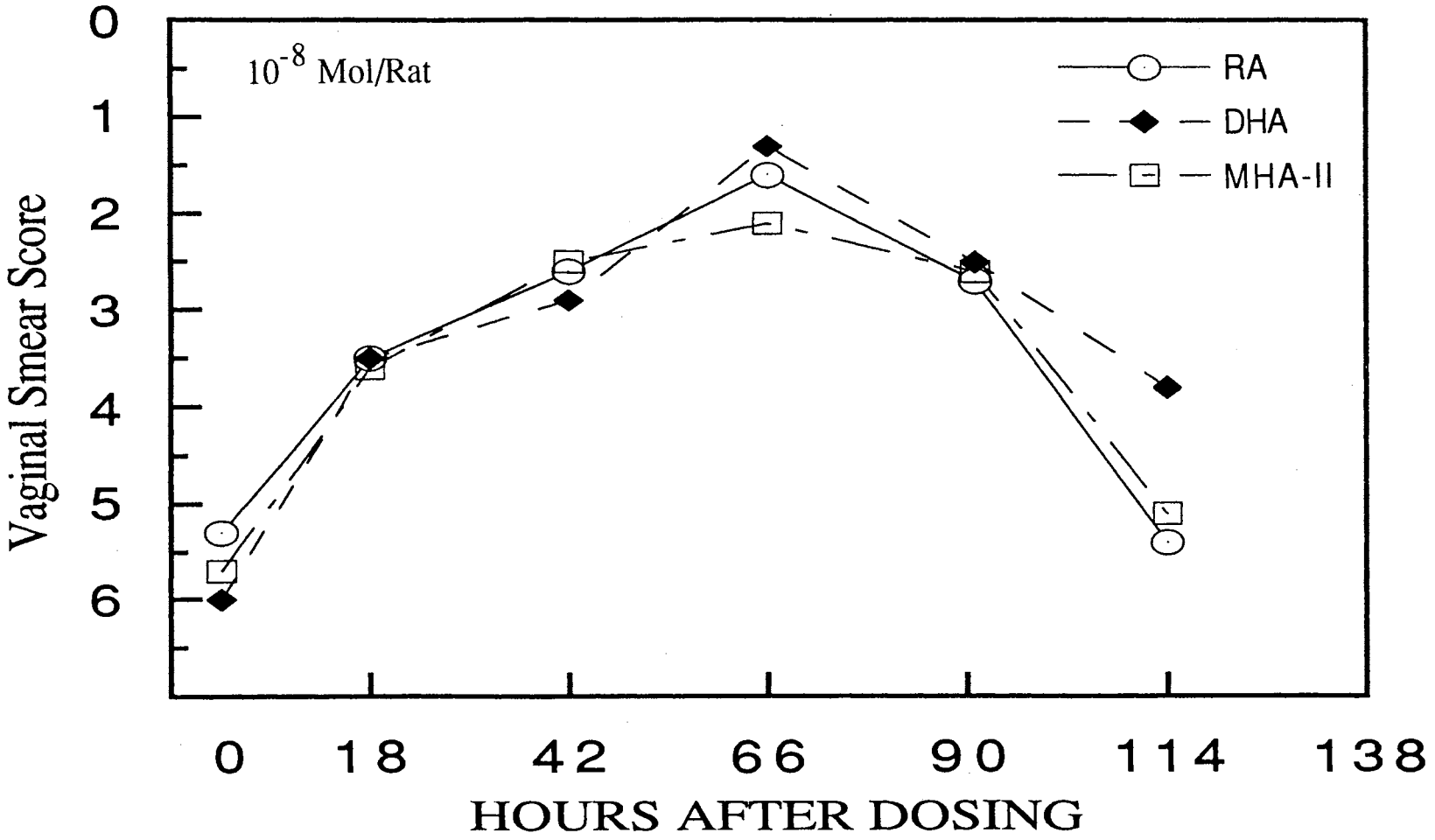

10^{-8} Mol/Rat
RA
DHA
MHA-II
Vaginal Smear Score
0
1
2
3
4
5
6
0
18
42
66
90
114
138
HOURS AFTER DOSING

Figure 22. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-9} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

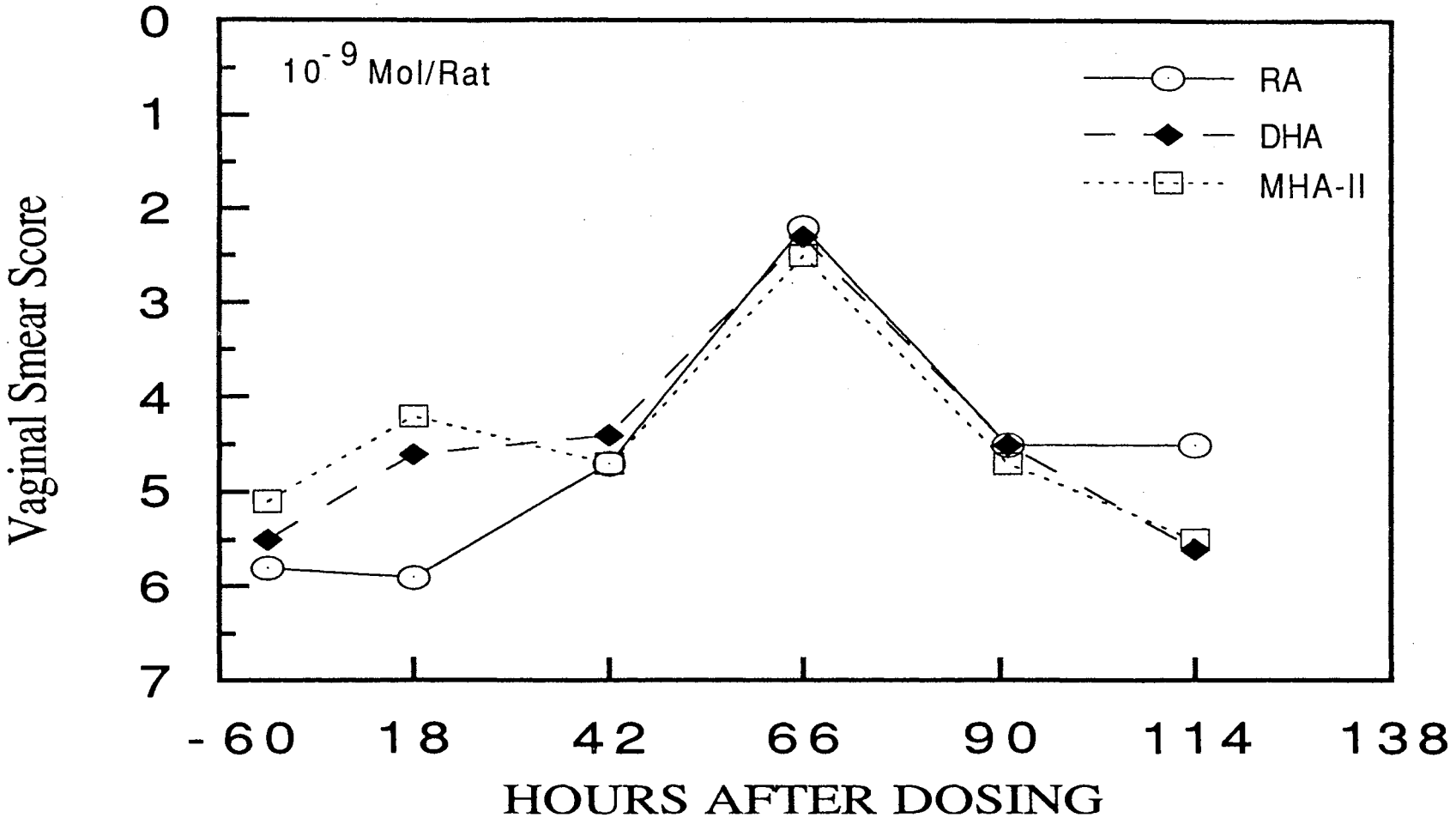

0
1
2
3
4
5
6
7
Vaginal Smear Score
10- 9 Mol/Rat
RA
DHA
MHA-II
-60
18
42
66
90
114
138
HOURS AFTER DOSING

Figure 23. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-10} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

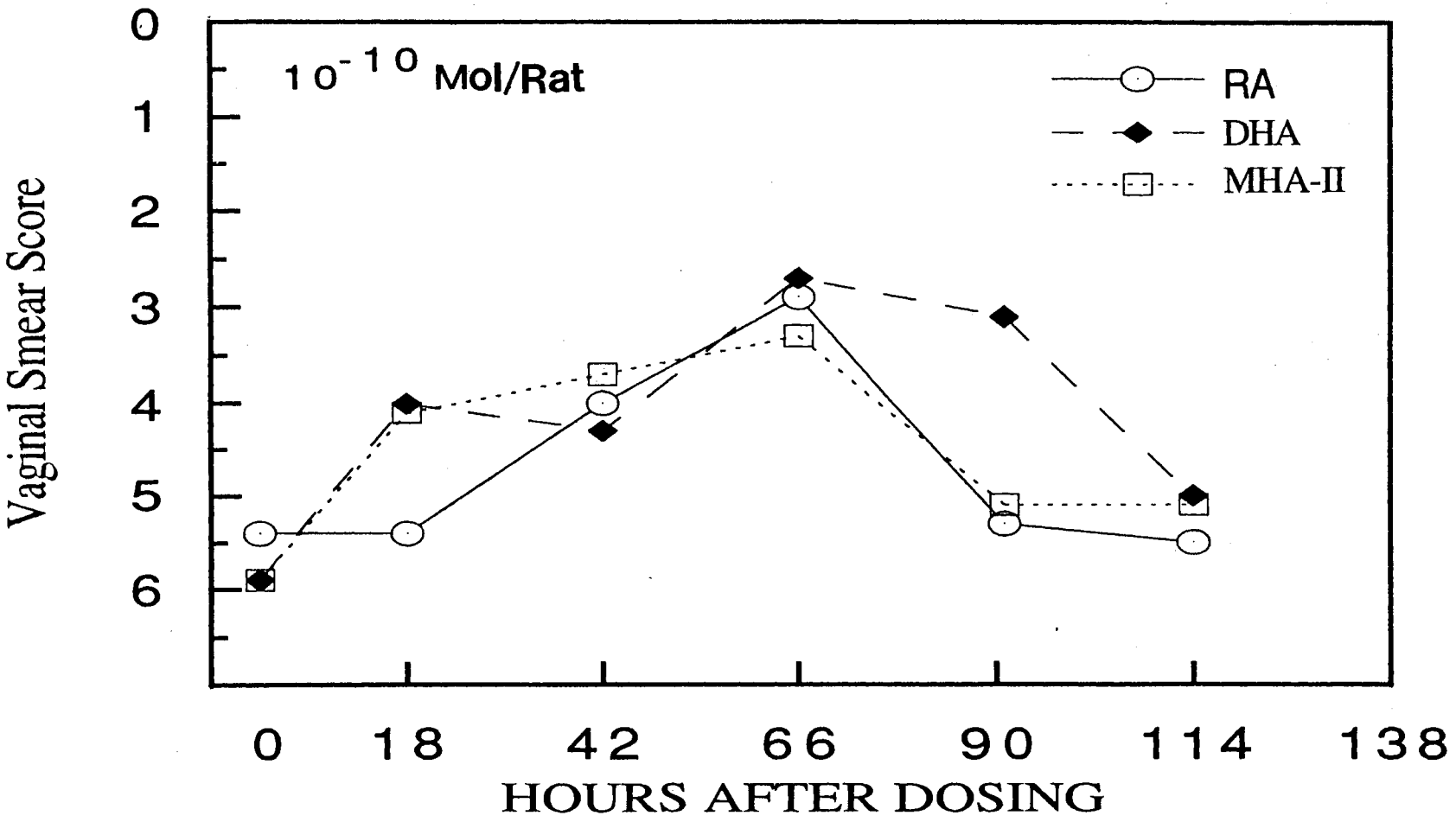

Vaginal Smear Score
0
1
2
3
4
5
6
10^{-10} Mol/Rat
RA
DHA
MHA-II
0
18
42
66
90
114
138
HOURS AFTER DOSING

Figure 24. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-11} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

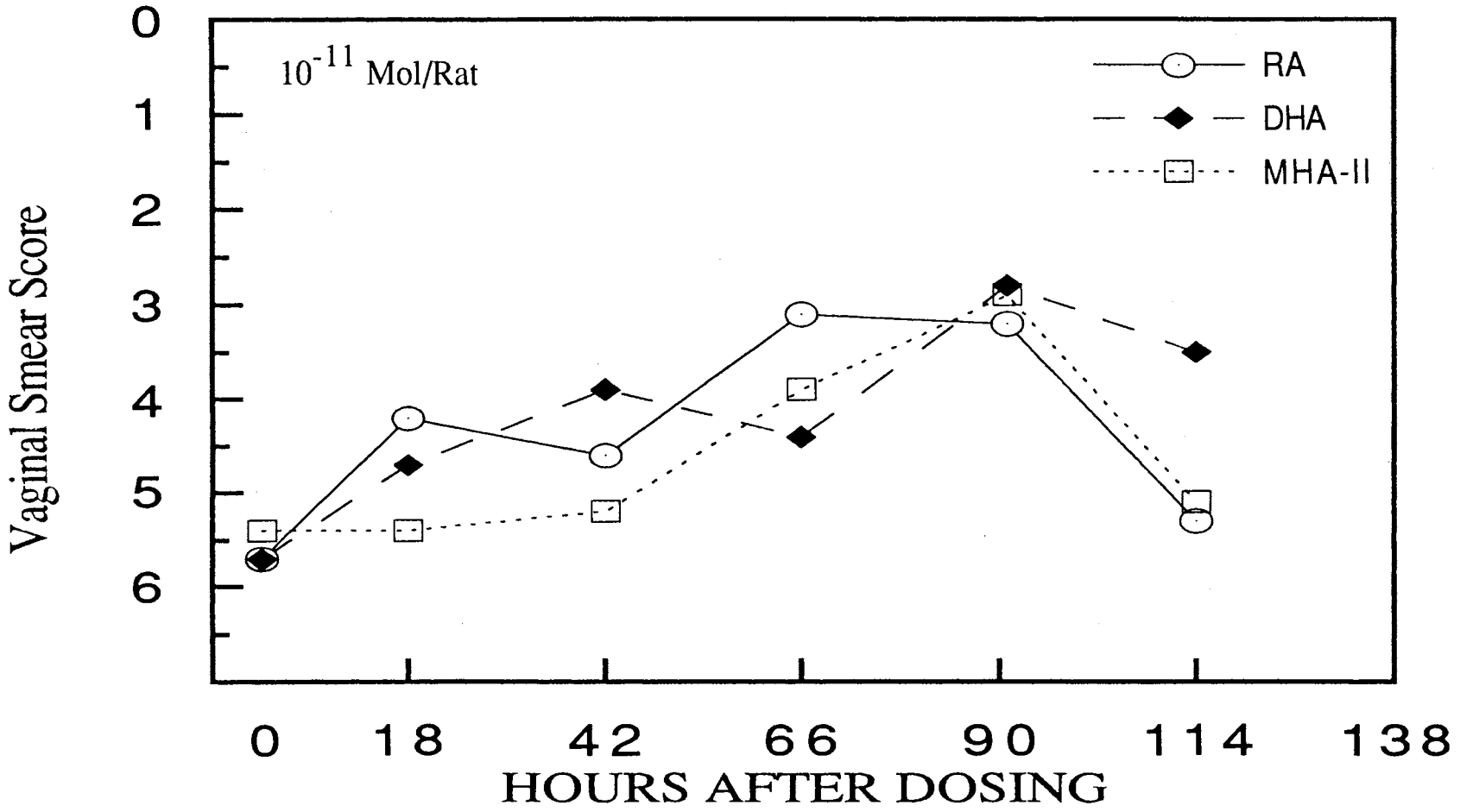

10^{-11} Mol/Rat
RA
DHA
MHA-II
Vaginal Smear Score
0
1
2
3
4
5
6
0
18
42
66
90
114
138
HOURS AFTER DOSING

Figure 25. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-12} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

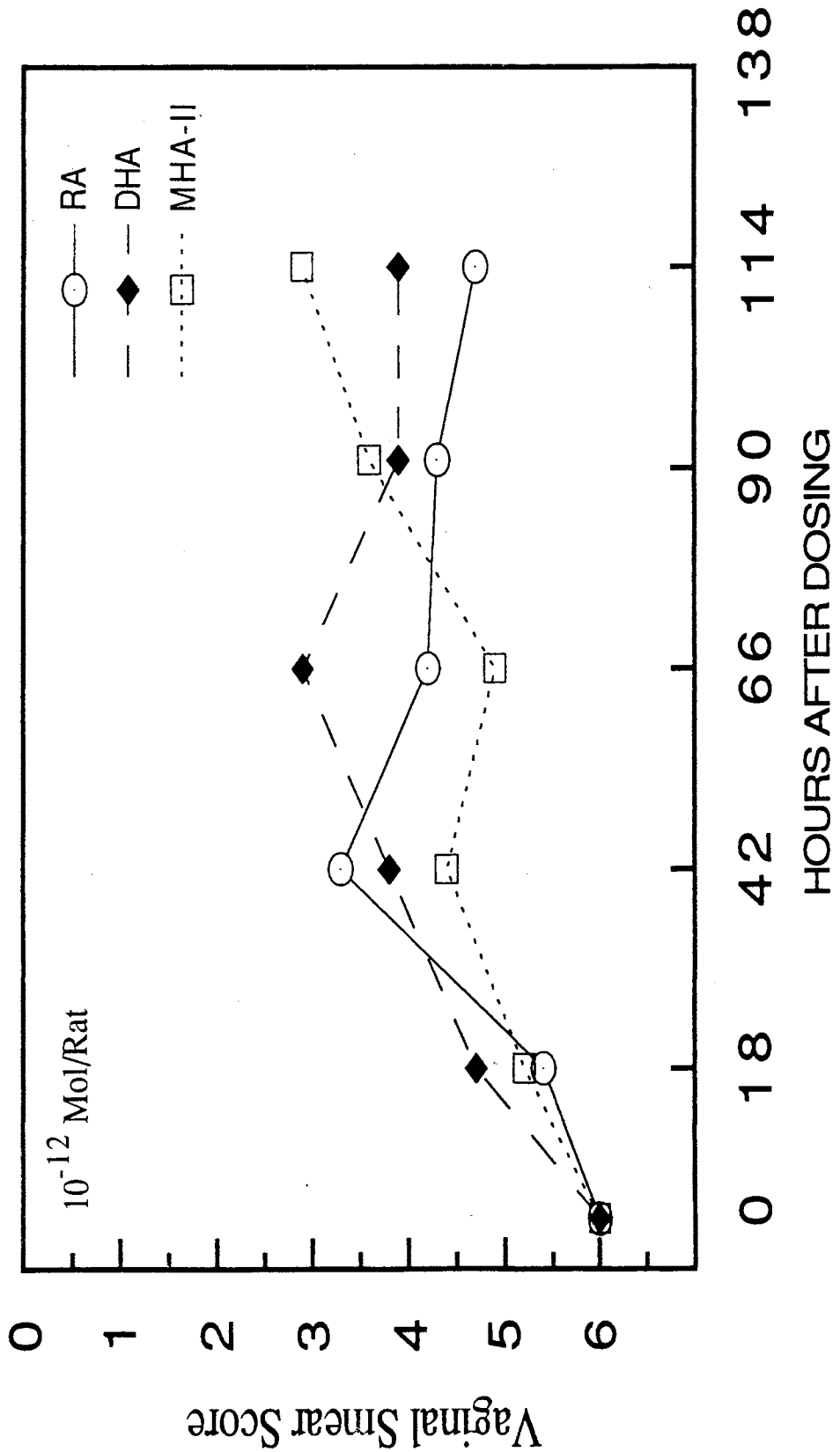

Vaginal Smear Score
0
1
2
3
4
5
6
10^{-12} Mol/Rat
RA
DHA
MHA-II
0
18
42
66
90
114
138
HOURS AFTER DOSING

Figure 26. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-13} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

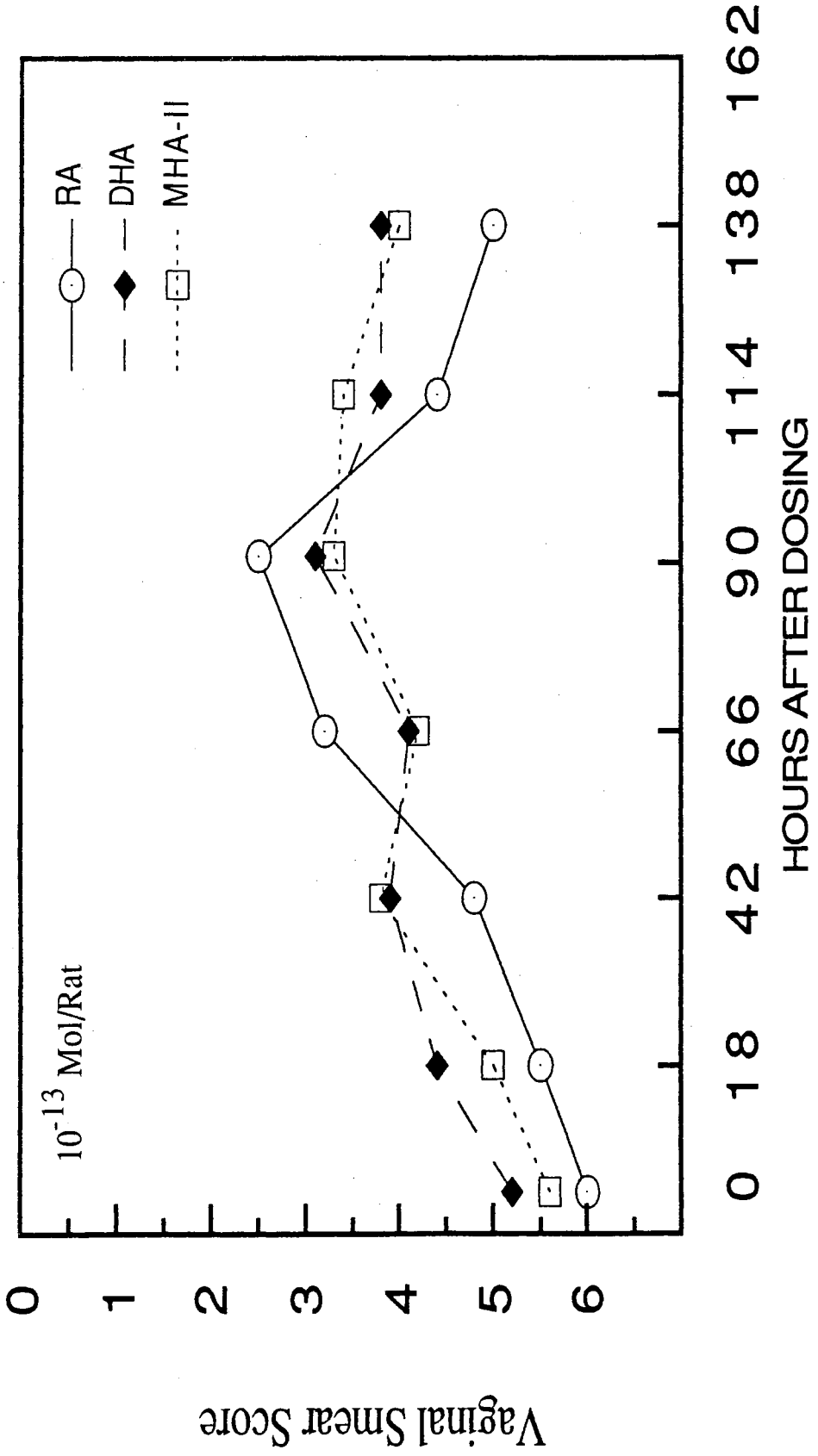

RA
DHA
MHA-II
10^{-13} Mol/Rat
0
1
2
3
4
5
6
0
18
42
66
90
114
138
162
HOURS AFTER DOSING
Vaginal Smear Score

Figure 27. Time-course Curves of the Vaginal-smear Assay at the Dose of 10^{-14} mol/rat.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

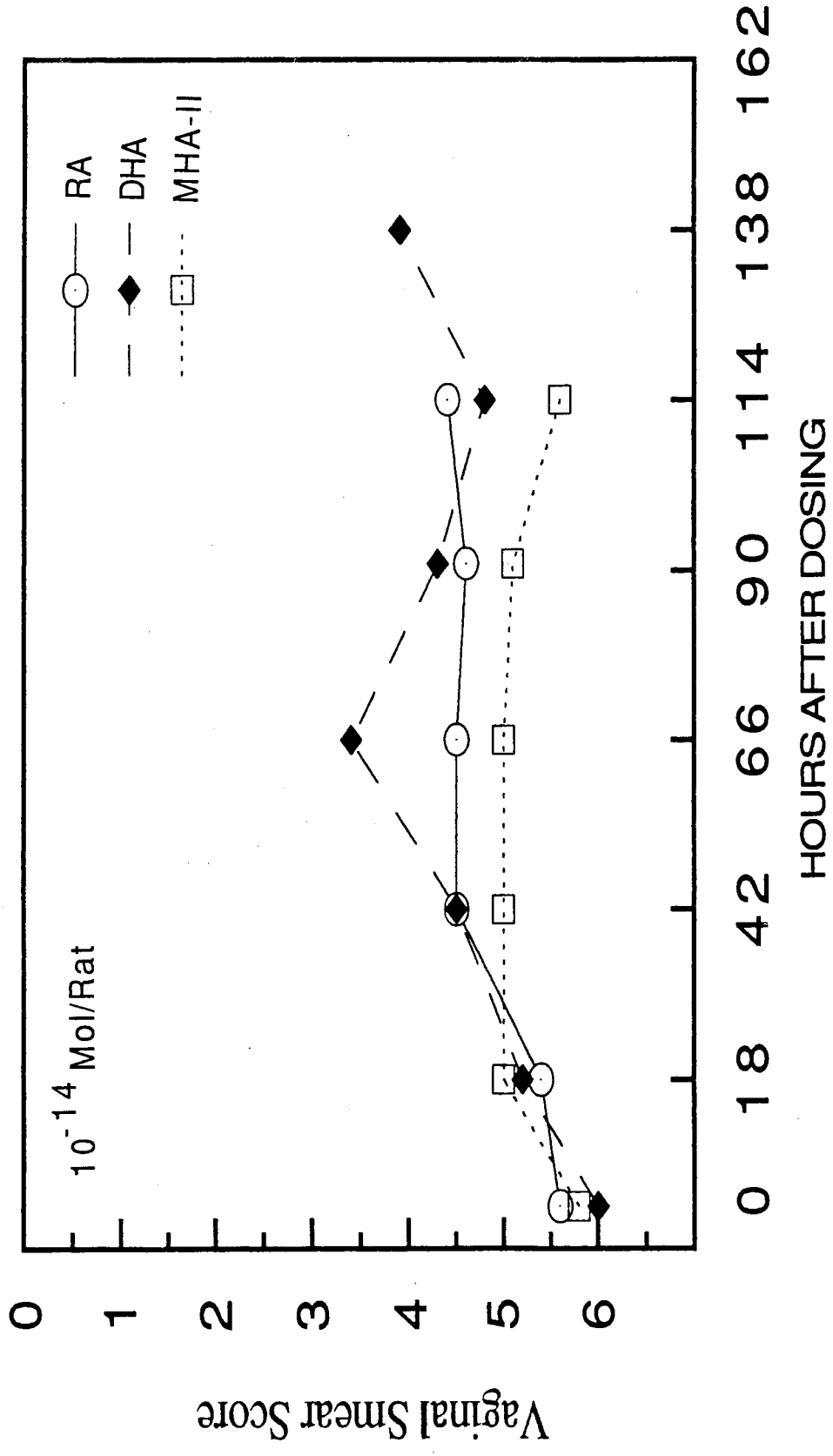

Vaginal Smear Score
0
1
2
3
4
5
6
10^{-14} Mol/Rat
RA
DHA
MHA-II
0
18
42
66
90
114
138
162
HOURS AFTER DOSING

Figure 28. Time-course Curves of the Vaginal-smear Assay Controls.
The Controls Were Done Concurrently with the Drug-testing Assays with All the Experimental Procedures and Treatments Done in Exactly the Same Manner. Vaginal Smears Were Taken After the Administration of a Blank Dosing-solution: a Mixture of 25μl of Normal Saline and 2μl of Aldehyde-free Ethanol.

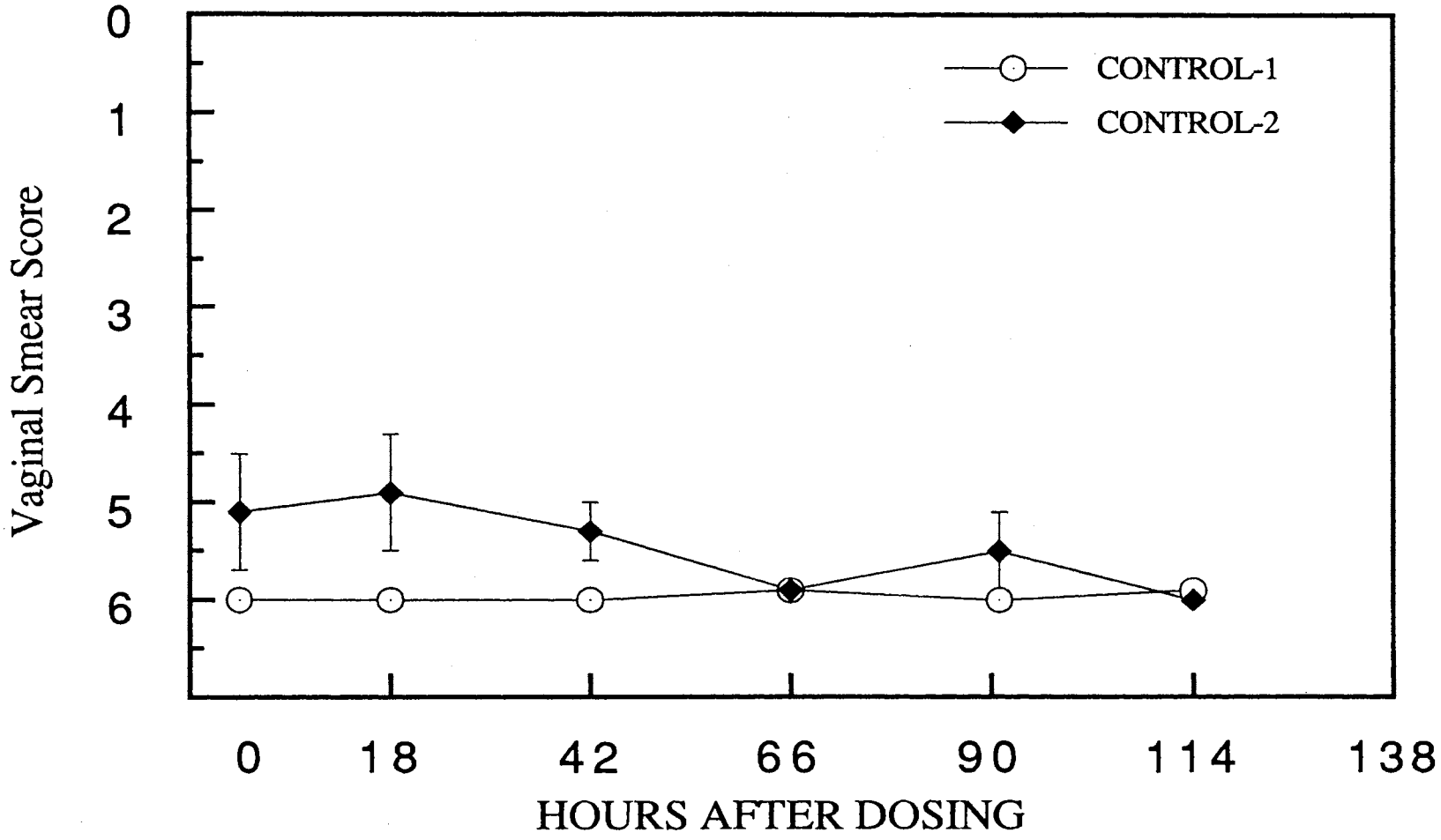

Vaginal Smear Score
0
1
2
3
4
5
6
CONTROL-1
CONTROL-2
0
18
42
66
90
114
138
HOURS AFTER DOSING

TABLE III

SUMMARY OF VAGINAL-SMEAR ASSAY

Test Compound	Dose (Mol/Rat)	No. of Rats	Mean VSA Scores at Time (hour) 0	18	42	66	90	114	138
Control-1	N/A	5	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	5.9 ± 0.1	6.0 ± 0.0	5.9 ± 0.1	
Control-2	N/A	5	5.1 ± 0.6	4.9 ± 0.6	5.3 ± 0.3	5.9 ± 0.1	5.5 ± 0.4	6.0 ± 0.0	
RA	10^{-8}	5	5.3 ± 0.4	3.5 ± 0.5[b]	2.6 ± 0.5[a]	1.6 ± 0.5[a]	2.7 ± 0.9[a]	5.4 ± 0.4	
	10^{-9}	4	5.8 ± 0.2	5.9 ± 0.1	4.7 ± 0.7[c]	2.2 ± 0.7[a]	4.5 ± 0.4[b]	4.5 ± 0.2[b]	
	10^{-10}	4	5.4 ± 0.3	5.4 ± 0.5	4.0 ± 0.9[a]	2.9 ± 0.8[a]	5.3 ± 0.5	5.5 ± 0.2	
	10^{-11}	4	5.7 ± 0.2	4.2 ± 0.7[c]	4.6 ± 0.5	3.1 ± 0.8[a]	3.2 ± 0.7[a]	5.3 ± 0.4	
	10^{-12}	4	6.0 ± 0.0	5.4 ± 0.3	3.3 ± 0.2[a]	4.2 ± 0.6[a]	4.3 ± 0.4[a]	4.7 ± 0.6[b]	
	10^{-13}	4	6.0 ± 0.0	5.5 ± 0.3	4.8 ± 0.4[b]	3.2 ± 0.5[a]	2.5 ± 0.4[a]	4.4 ± 0.4[a]	5.0 ± 0.6[c]
	10^{-14}	4	5.6 ± 0.2	5.4 ± 0.4	4.5 ± 0.6	4.5 ± 0.8	4.6 ± 0.9	4.4 ± 0.5	
DHA	10^{-8}	5	6.0 ± 0.0	3.5 ± 1.0[a]	2.9 ± 1.0[a]	1.3 ± 0.2[a]	2.5 ± 1.1[a]	3.8 ± 1.1[c]	
	10^{-9}	4	5.5 ± 0.5	4.6 ± 0.9	4.4 ± 0.3	2.3 ± 0.9[a]	4.5 ± 1.0	5.6 ± 0.3	
	10^{-10}	5	5.9 ± 0.1	4.0 ± 0.9[b]	4.3 ± 0.3[b]	2.7 ± 0.8[a]	3.1 ± 0.5[a]	5.0 ± 0.2	
	10^{-11}	5	5.7 ± 0.1	4.7 ± 0.4	3.9 ± 0.7[b]	4.4 ± 0.7[c]	2.8 ± 0.5[a]	3.5 ± 0.4[a]	
	10^{-12}	4	6.0 ± 0.0	4.7 ± 0.8	3.8 ± 0.2[b]	2.9 ± 0.9[a]	3.9 ± 1.0[b]	3.9 ± 0.5[b]	
	10^{-13}	4	5.2 ± 0.4	4.4 ± 0.7	3.9 ± 1.0	4.1 ± 0.7	3.1 ± 0.5[b]	3.8 ± 0.5	3.8 ± 0.6
	10^{-14}	4	6.0 ± 0.0	5.2 ± 0.5	4.5 ± 0.3[b]	3.4 ± 0.3[a]	4.3 ± 0.5[b]	4.8 ± 0.7[c]	3.9 ± 0.6[a]

TABLE III (Continued)

Test Compound	Dose (Mol/Rat)	No. of Rats	Mean VSA Scores at Time (hour) 0	18	42	66	90	114	138
MHA-II	10^{-8}	5	5.7 ± 0.2	3.6 ± 0.8[b]	2.5 ± 0.5[a]	2.1 ± 0.1[a]	2.6 ± 1.2[a]	5.1 ± 0.6	
	10^{-9}	5	5.1 ± 0.4	4.2 ± 0.2[c]	4.7 ± 0.4	2.5 ± 0.9[a]	4.7 ± 0.6	5.5 ± 0.3	
	10^{-10}	5	5.9 ± 0.1	4.1 ± 0.5[b]	3.7 ± 0.6[a]	3.3 ± 0.1[a]	5.1 ± 0.1	5.1 ± 0.2	
	10^{-11}	4	5.4 ± 0.3	5.4 ± 0.5	5.2 ± 0.6	3.9 ± 0.7[c]	2.9 ± 0.9[a]	5.1 ± 0.1	
	10^{-12}	4	6.0 ± 0.0	5.2 ± 0.8	4.4 ± 0.6[c]	4.9 ± 0.4	3.6 ± 0.7[a]	2.9 ± 0.5[a]	
	10^{-13}	4	5.6 ± 0.2	5.0 ± 0.4	3.8 ± 0.4[b]	4.2 ± 0.5[c]	3.3 ± 0.6[a]	3.4 ± 0.5[a]	4.0 ± 0.8[b]
	10^{-14}	5	5.8 ± 0.1	5.0 ± 0.3	5.0 ± 0.8	5.0 ± 0.7	5.1 ± 1.0	5.6 ± 0.2	

VSA - Vaginal-Smear Assay

RA: All-*trans*-retinoic Acid

DHA: Diaryl Heteroarotinoid Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate

MHA-II: Monoaryl Heteroarotinoid *(2E,4E,6E)*-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6--heptatrienoic Acid

N/A - Not Applicable

[a] Significantly Different Compared with the Score at Time 0 at 99% Confidence Level

[b] Significantly Different Compared with the Score at Time 0 at 95% Confidence Level

[c] Significantly Different Compared with the Score at tTme 0 at 90% Confidence Level

of neutrophils was persistent for a few days. A typical smear of such a case is shown in Figure 29. This phenomenon was apparently more obvious for the two heteroarotinoids, DHA and MHA-II.

A review of the available literature involving the vaginal smear assay made it clear that the phenomenon regarding the reappearance of neutrophils was more common than expected. Similar observations were reported by a number of investigators since the early 1930s (Baumann and Steenbock, 1932; Aberal, 1933; Pugsley et al., 1944; DeLuca, 1985). Speculative rationale, however, was lacking in those reports. The neutrophils observed at this time were distinguishable from those in the high neutrophils-containing smears that represented high decornification activities (compare the neutrophils shown in Figure 19 and Figure 29). The reappearing neutrophils were morphologically more degenerative, and were often accompanied by large amounts of debris and colloidal materials. In another words, they may very likely reflect some pathologic changes as the result of vitamin A-deficiency.

Aberle (1933) reported that when vaginas of vitamin A-deficient rats were sectioned, 15% of the rats had developed necrosis in the mucosa. Leukocytes could be seen migrating between the cornified cells in 60% specimens of the intact mucosae. It is possible that a new and different mechanism might have been involved. Another possibility is that an excessive number of neutrophils might be the result of vaginal infection (Pugsley et al., 1944). When the epithelium becomes cornified, the vagina becomes very dry. Although every precaution was exercised when taking the lavage in order to prevent trauma to the mucosa of the vagina, the tissue might still become irritated and infected. Regional infection could be the cause of the presence of a large number of neutrophils migrating into the lumen of the vagina. Whatever the cause might be, since the scoring system does not distinguish neutrophils at different stages of development, this phenomenon was sometimes quite misleading in the measurement of vitamin A-like activities by the scoring system used in this study.

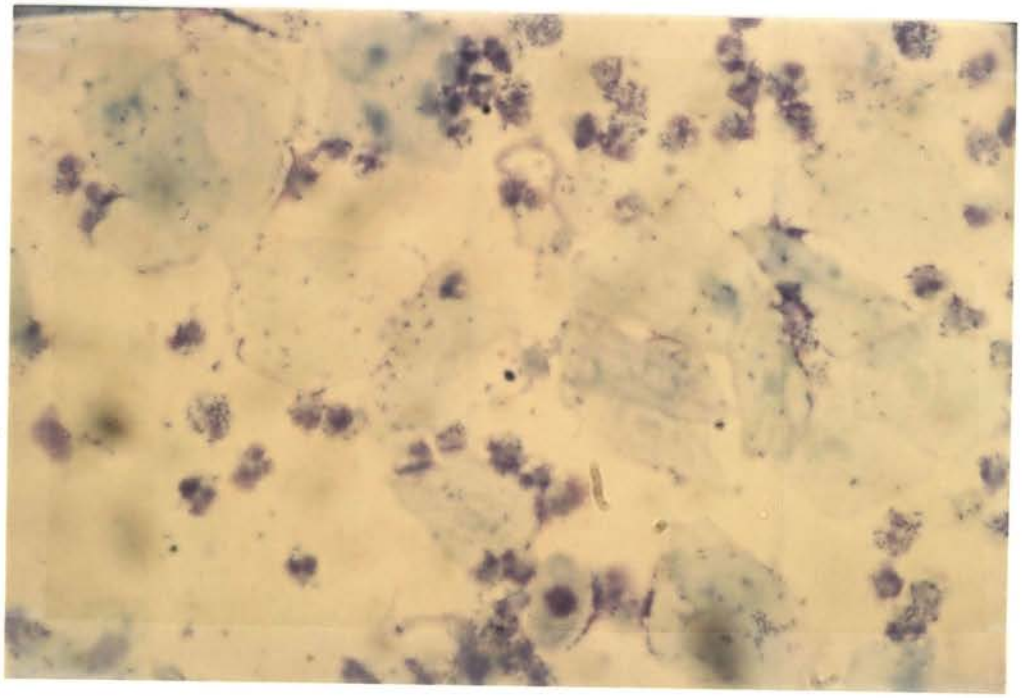
Figure 29. A Representative Vaginal Smear with Reappearance of Neutrophils.

Magnification: 400x

Identity: DHA^{-14} mol/rat at Hour 138.

(DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate)

Score: 4.0



Dose-response Curves

The responses of the rat vaginal epithelia to the compounds at 18 hours after the drug administration were generally not significant statistically except for those at the high dosing level (see Table III). Tissue irritations, resulting from drug applications, might contribute to the apparent responses at this early stage. In most cases, the responses first became significant at 42 hours, and peaked at about 66 hours (see Table III). The dose-response curves of the test compounds at 66 hours after dosing are plotted in Figure 30. It is demonstrated from these computer-generated best-fitted curves that, overall, compound DHA was more active than RA, while compound MHA-II was less active than RA. The difference in activity was more significant at lower dosing levels. The relative activities of the test compounds were compared by means of their ED_{50} values, the effective dose at 50% maximum activity level. ED_{50} is often used in drug evaluations. An ED_{50} value in this study is the molarity of a retinoid required to reverse the vaginal epithelial cornification by 50% from its maximum cornification status. The ED_{50} values were determined on the dose-response curves from the point at which the curve crosses the vaginal smear score of 3, and were listed in Table IV. The relative potencies determined from the ED_{50} indicated that the biological activity of compound DHA, in terms of stimulating epithelial differentiation in rat vaginal tissue, was comparable to that of RA, while that of MHA-II was about 10-fold less active when compared to RA.

Mechanism of Action

That vitamin A plays an important role in protein synthesis and protein metabolism was suggested nearly 57 years ago to explain the action of vitamin A-active compounds observed in the vaginal smear assay (Mason and Ellison, 1935). However, to date no systematic research data addressing this problem are available. Extensive investigations, however, have revealed substantial evidence regarding the regulatory effect of retinoids on epithelial differentiation of other tissues, most representitive of which is trachea (Shapiro,

Figure 30. Dose-response Curves of the Vaginal-smear Assay at 66 Hours After the Drug Administration.

RA: All-*trans*-retinoic Acid.

DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

MHA-II: (*2E,4E,6E*)-3,7-Dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic Acid.

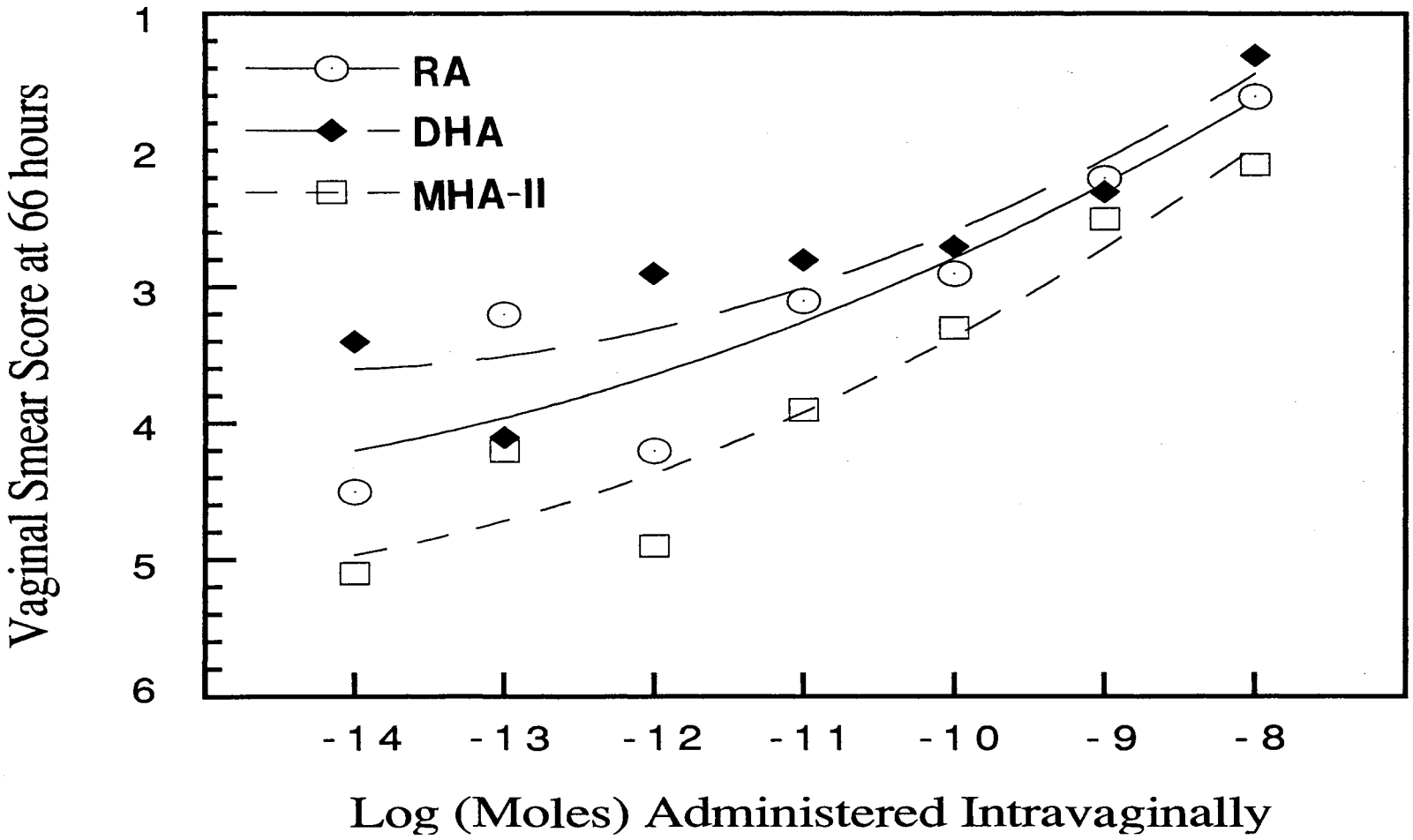

Vaginal Smear Score at 66 hours
1
2
3
4
5
6
RA
DHA
MHA-II
-14
-13
-12
-11
-10
-9
-8
Log (Moles) Administered Intravaginally

TABLE IV

RELATIVE POTENCIES OF THE TEST COMPOUNDS OBTAINED FROM THE VAGINAL-SMEAR ASSAY

Test Compound	ED_{50} (mol/rat)	Relative Potency
RA	4.0×10^{-11}	1
DHA	1.0×10^{-11}	4
MHA-II	4.0×10^{-10}	0.1

RA: all-*trans*-retinoic acid

DHA: ethyl (*E*)-4-[2-(3,4-dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1--propenyl]benzoate

MHA-II: (*2E,4E,6E*)-3,7-dimethyl-7-(1,2,3,4,-tetrahydro-4,4-dimethyl-6--chromanyl)-2,4,6-heptatrienoic acid

1986; Jetten, 1987). The process of cornification and the process of keratinization in the terminal differentiation of epithelia are complex series of events. The role of retinoids in these processes is not fully resolved. Nevertheless, the production of glycoproteins, mucin in particular, and keratin species appears to be directly affected by vitamin A status (Shapiro, 1986).

Keratins are proteins that form the 8-10 nm intermediate intracellular filaments of epithelial cells. There are approximately 20 keratins, a subset of which is expressed in any given epithelial cell type depending on its state of growth and differentiation (Edmondson, et al., 1990). Therefore, the expression of certain keratins serves as a biological marker reflecting the type and the differentiation state of epithelial cells. The normal tracheobronchial epithelium consists of three major cell types: ciliated cells, basal cells and a variety of secretory cells. During Vitamin A deficiency, the epithelium undergoes hyperplasia followed by squamous metaplasia at the levels of the basal cells. The outmost layer of these squamous cells constitutes a fully cornified surface with numerous keratin filaments (Wong and Buck, 1971; Evans et al., 1986). A recent *in vitro* study with hamster tracheal epithelial (HTE) cells indicated that the complex keratin synthesis pattern with the expression of specific keratins characteristic of lesions of squamous metaplasia was observed under vitamin A-deficient conditions. In contrast, fewer and simpler species of keratins were formed in HTE cells cultured in vitamin A-supplemented medium (Edmondson et al., 1990). Synthetic retinoids have also been shown to affect epithelial keratinization processes. Madison et al.(1981) reported that the aromatic retinoid Ro 10-9395 inhibit both epidermal keratinocyte proliferation and specific differentiation *in vitro*.. Arotinoid Ro-1698 was shown to significantly reduce the synthesis of non-covalently and disulfide cross-linked keratins, while enhancing the synthesis of keratohyalin granule-associated proteins that were thought to be relevant in the effectiveness of retinoid treatment of psoriasis (Stadler et al., 1984).

An antagonist effect of retinoids on estrogen-induced keratinization is also possible. Kahn pointed out that the inhibition of vaginal response to estrogen by vitamin A may suggest certain intimate relationship between the hormone and the vitamin (Kahn, 1954). The recent discovery of the structural similarities between nuclear retinoic acid receptors (RARs) and steroid hormone receptors may suggest that retinoids and steroids might share certain receptors, although there are no apparent chemical similarities between retinoids and steroid hormones. This possible antagonism between retinoids and estrogen was also suggested in a very recent study. Fontana et al (1992) demonstrated that retinoic acid inhibited estradiol-induced expression of the transforming growth factor α (TGF-α) and pS2 gene in a breast carcinoma cell line. On the other hand, the expression of retinoic acid receptor transcripts was reduced by estradiol. The presence of retinoic acid, however, prevented this reduction.

Retinoids modulate the synthesis and secretion of many cell surface glycoconjugates (Shapiro, 1986). The effect of vitamin A on cell surface mucous glycoproteins (mucins) was studied by Clark and Marchok with rat tracheal epithelial tissues (1979a and 1979b). They found that, overall, vitamin A stimulated mucin production, but the quantity and identity of mucin species were dependent upon the vitamin A concentrations in the media. In the absence of vitamin A, the epithelium became keratinized and squamous with little mucus secretion. Similar effects were also observed by Jetten and co-workers (Jetten et al., 1986). A culture system using rabbit tracheal epithelial cells were developed to investigate the stimulatory effect of secretory phenotypes by vitamin A and its synthetic analogs. Release of mucous glycoproteins was demonstrated upon retinoid stimulation and was concentration dependent. Most strikingly, the arotinoid Ro 13-7410 was shown to be effective at concentrations as low as 10^{-12} M.

From the above discussion, it appears quite clear that epithelial tissues, primarily tracheal epithelium, produce secretory cells in the presence of vitamin A-active retinoids, and become squamous and cornified when depleted of vitamin A-active retinoids. The

effects of retinoids on the synthesis of keratins and mucins are important aspects responsible for this phenomenon.

Only MHA-II was tested in this assay because of the shortage in MHA-I supply. It is believed, however, that the effect of MHA-I and MHA-II should be very similar in this assay because MHA-II is the acid form of MHA-I. Miller and co-workers (1985) reported that methyl retinoate was equipotent compared with all-*trans*-retinoic acid in vaginal smear assay. They suggested that either the methyl ester is as active as retinoic acid without change or sufficient esterases are present in the vaginal lumen to convert methyl retinoate into the free acid.

Conclusions

The vaginal smear assay measures the changes occurring in the sensitive vaginal epithelial tissues in response to the regional supply of vitamin A-like compounds. The vaginal epithelium of a vitamin A-deficient ovariectomized rat is marked by persistently cornified epithelial cells that can be clearly observed in the vaginal smears. The increased thickness of the vaginal epithelium as the result of vitamin A deficiency is in accord with the increase in mitotic activity in the vaginal epithelium (Aberle, 1933), and with metaplastic alterations observed in other epithelial tissues (Wolbach and Howe, 1925).

The anti-keratinization activity of heteroarotinoids was clearly demonstrated in this study. The reversal of keratinization, which led to the thinning of the vaginal epithelium and the occurrence of diapedesis, was measured by the disappearance of cornified epithelial cells and the appearance of large numbers of neutrophils and nucleated epithelial cells after a single application of a heteroarotinoid. Both monoaryl and diaryl heteroarotinoids are active. Cornified vaginal epithelium as the result of vitamin A deficiency was reversed by dose levels as low as 10^{-14} mol/vagina. The relative activity of DHA is comparable or even slightly higher than all-*trans*-RA, while MHA is 10-fold less active that all-*trans*-RA.

Neutrophils were occasionally observed in the lumen of rat vagina 6-7 days after the drugs were applied. This phenomenon was more profound when animals were treated with the heteroarotinoids and at low dosing levels. The cause of this effect is unclear.

The major desirable features of the vaginal smear assay can be summarized as follows:

(1) Vaginal epithelial cornification of ovariectomized rats occurs before the appearance of other symptoms of vitamin A-deficiency, such as weight loss and xeropthalmia. Therefore, severe vitamin A-deficiency can be avoided.

(2) The onset of vitamin A deficiency judged by the continual vaginal cornification is more uniformly manifested among all test animals, i. e., less variation in the timing of the symptom appearance when compared to that of other signs of vitamin A deficiency, for instance, the eye changes.

(3) The use of ovariectomized vitamin A-deficient rats and direct topical administration of the test compounds makes the method more sensitive, more specific and reliable in terms of cause-effect relations involved in this assay. Vaginal smear cornification is not significantly influenced by other factors such as inherent growth capacities of the animals, general debility, and extraneous causes of irritations and infections.

(4) The response is rapid and easy to recognize.

(5) The method is economical as rats are reusable, and the relatively simple procedure requires very little access to complicated and expensive equipment.

(6) The method is simple to perform.

The major setbacks of the method are:

(1) Time consuming. The entire assay requites a considerable amount of tedious work.

(2) Relative inaccuracy indicated by the relatively large standard deviations when compared with other *in vivo* bioassays such as growth assay. This problem was observed

in this study as well as in some early reports (Coward et al., 1935). A few differences may be accountable for this relative inaccuracy. First of all is the difference among rats. Because the rats were not all at exactly the same stages of vitamin A deficiency, they might have responded to the same treatment differently. The response was also affected by the general health of the rats. Secondly, variations occurred in the counting process. The actual counting of the cells is more error-bound than theoretically described in the method. The cellular content in the lumen of the rat vagina is complicated. There are more than three types of cells in the lumen of the vagina. Often it is quite difficult to distinguish one cell type from another, and the identities are not always easy to ascertain. The cells are also not evenly distributed. Therefore, the proportions of the cells can be quite different from one field to another. Furthermore, the cells vary in their degenerative stages, which is not distinguished in the scoring system. The inclusion of internal standards, such as retinoic acid used in this study, can alleviate the variations to a certain extend. More importantly, reducing the number of individuals who are involved in the actual "bench-work" of the experiment would be more effective in eliminating possible human bias. To be consistent, only one person should be assigned to the counting. In conclusion, the vaginal smear assay should not be used as a primary method in quantification. It is more desirable to be used in the measurement of relative activities.

CHAPTER V

HISTOPATHOLOGICAL EXAMINATION OF THE EFFECTS OF RETINOIC ACID AND TWO HETEROAROTINOIDS ON VARIOUS EPITHELIAL TISSUES

Introduction

Pathologic alterations of various epithelial tissues as the result of vitamin A deficiency has been extensively investigated in the classic study of Wolbach and Howe (1925). They concluded that the effect of vitamin A deprivation is "the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraocular glands and the genitourinary tracts". In general the sequence in which different organs exhibit tissue follows the order: the respiratory tract, the salivary glands, eye, the genitourinary tract, the paraocular glands and the pancreas. No changes were observed in the liver, parenchyma of the kidney, stomach, and intestine (Wolbach and Howe, 1925). According to Wolbach and Howe (1925) and Anzano et al.(1980), as the vitamin A deficiency progresses, widespread keratinization with or without squamous metaplasia of the ducts and antrophy of gland tissues proceed, followed by edema, degeneration of secretory acinar cells of the submaxillary gland, and reduction of mucin formation in acinar cells of the sublingual gland. The epithelial cells become separated from their acini, the nuclei of the cells degenerate, the cells become rounded, vacuolated, and eventually cornified. Manifestations of atrophy include an increase in interlobular spaces, fibrosis, dilatation of ducts, and eventually cyst formation and

suppuration. Infiltration of lymphocytes, plasma cells and polymorphnuclear leucocytes usually indicates a late stage of vitamin A deficiency, when infections often occur.

Wolbach and Howe also concluded that the stratified keratinizing epithelia (epidermoid metaplasia) primarily resulting from vitamin A deficiency are identical in appearance in all locations, and the keratinized cells arise from focal proliferation of basal cells. In addition, increases of chromatin and in the size of nuclei are among the early morphological changes (1925, 1928). This premise was challenged later when marked decrease in the replication of columnar secretory (mucous) cells of the tracheal epithelium were observed, while the replication of the basal cells continued (McDowell et al., 1984a). Therefore, the "hyperplastic" appearance of basal cells was simply the result of a disproportional growth between the columnar secretory cells and basal cells. The same research group also reported that the flattening (from columnar to cuboidal cell type) and reduced DNA synthesis of the secretory cells, accompanied by multinucleation and dispersion of nuclear heterochromatin, are all primary effects of vitamin A deficiency (McDowell et al., 1987). They demonstrated that the basal cells clustered in groups, but did not differentiate into any cell types. It was the altered secretory cells that expressed the epidermoid (squamous) phenotype. They argued that epidermoid metaplasia is a secondary event that occurs late during vitamin A deficiency as an attempt for epithelial regeneration because morphologically it shares many characteristics of rapidly induced metaplastic lesions following injuries. In prolonged vitamin A deficiency, however, the restoration of a mucociliary state from metaplastic state is blocked, and the metaplastic phenotype persists (McDowell et al., 1987).

Studies have shown that retinoids are important in maintaining normal growth, shape and differentiation of epithelial tissues, as well as in the recovery of epithlial tissues from pathologic injuries following vitamin A deprivation (Wolbach and Howe, 1932, Anzano et al, 1980; McDowell et al., 1984b). The present study reports the effect of two heteroarotinoids in comparison with that of all-*trans*-retinoic acid in this regard. Vitamin A-

deficient rats were fed through dietary supplementation with three compounds at various dosing levels for an extended period of time. Animals were then sacrificed. Tissues containing representative epithelial linings were stained sectioned. Morphological and/or pathological effects on various epithelial tissues resulting from the primary vitamin A deficiency, the ability or the inability of the test compounds to replace the complete physiological effects of vitamin A, and the direct toxicity of the compounds were evaluated. The effects of the different test compounds (inter-compound comparisons), and of the same compound but with different dose levels (intra-compound comparisons) were compared both quantitatively and qualitatively.

Materials and Methods

Animal Preparation

The rats that were used in the continuous feeding experiment (Chapter III) were employed in this study. Detailed description of the rats, animal care measures, and feeding procedures of the vitamin A-deficient rats are referred to Materials and Methods in Chapter III. Briefly, weanling rats (Fisher-344, Sasco Inc., Omaha, Nebraska) were weaned 19 days post-natal and were maintained on a vitamin A-deficient diet (TABLE I) until they were 6 weeks of age. Rats ceased growth at the end of this vitamin A-deprivation period. They were then maintained on the same diet but first supplemented with and then lacking retinoic acid (RA 45 μg/rat/day) for two consecutive weight-gain and weight-loss cycles. After a final 8-days of RA supplementation, the animals were randomly grouped into nine groups with 4-5 rats in each group. Retinoic acid and two heteroarotinoids, DHA and MHA-II (see Figure 3 for the structures), were given to the rats daily as dietary supplementations at dose levels of 10, 100 or 200 μg per rat per day. Each group of rats received only one type of supplement, for instance 10 μg DHA/day/rat (Group DHA-10), for a period of 25-40 days. At the end of this period, the animals, at 4 to 4 1/2 months of age, were killed by diethyl ether overdose (rats in Group MHA-II-10 were terminated on

Day 21 because they were moribund). Control animals were age-paired rats of the same strain (Fisher-344, Retired breeders, Sasco Inc., Omaha, Nebraska). They had been fed with normal laboratory rodent chow (Ralston Purina, St. Louis, Missouri).

Necropsy Procedures

An animal was washed on the external surface to thoroughly wet the haircoat. A midline incision through the skin and subcutaneous tissue was made from the apex of the mandible to the pubis. The skin was separated from the underlying facia of the external muscles and bluntly dissected laterally. The muscle layers were then incised from the apex of the mandible to the pubis, exposing the abdominal viscera and the thoracic cage. Heavy scissors were used to puncture the diaphragm and cut the rib cage, exposing the thoracic viscera. Throughout this chapter, fixative will always refer to phosphate buffered (pH 7.4) 10% formalin.

The trachea was grasped just anterior to the larynx, and the trachea and esophagus were freed from adherent tissue with dissection posteriorly into the thoracic cavity. Here mediastinal attachment was dissected free, and the pluck was freed with the severance of the posterior vena cava and distal esophagus. The pluck was placed in a cold saline wash and then placed *in toto* into fixative. Hemostatic forceps were placed across the esophagus just distal to the diaphragm and across the distal-most portion of the colon to contain intestinal content. Tissue was severed on the distal side of each clamp. With elevation of the anterior forceps, the intestinal tract was freed from the omental attachments and dissected free from the abdominal cavity, and then placed in a cold saline wash and then placed *in toto* in fixative. Submandibular salivary glands were excised from both the right and left sides of jaw, hemisected in several places and placed *in toto* into fixative. The left and right adrenal glands were dissected free of mesenteric attachments and each gland was hemisected for penetration of fixative and placed in formalin. The left and right kidneys were freed of mesenteric tissue, the capsule was elevated off the surface of both organs.

The kidneys were halved from pole to pole and then three horizontal incisions were made in each to provide better penetration of fixative. The ventral prostatic tissue was dissected from its attachment to the urinary tract, hemisected and then placed *in toto* into the fixative. The urinary bladder was excised at the neck, incised for 3/4 of its length and placed *in toto* into the fixative. Each of the testis was removed from the scotral sac with a portion of the vas deferens. One half to 1 ml of formalin was injected into each of the testis before being placed *in toto* into the fixative. The left caudal lobe of the liver was excised and several generous longitudinal incision were made deep into the parenchyma to allow for optimal fixative penetration. After two weeks in fixative, the organs of interest were removed, washed with tap water and small sections of tissue were taken for paraffin impregnation. When possible, 3 to 5 mm thick sections were made, with the knife-facing surface, the surface that was exposed to fixative originally. Sectioning was done to obtain 6 micron thick specimens and routinely stained with hematoxylin and eosin.

While many organs were fixed, including the brain, spleen, pancreas and salivary glands, only those tissues with significant epithlial components and/or known to be affected by vitamin A deficiency were evaluated in this study. The tissues and the sectioning of the tissues are listed in Table V.

Results and Discussions

Gross Pathology

The external appearances of the rats varied in response to the different treatments. Rough, dull haircoat was generally observed in heteroarotinoids supplemented rats, but not in the RA supplemented rats. Crusted eyes with no secretions, pale lips, pinnea, and feet were mainly observed in Group DHA-10, occasionally in other groups. Elevated intra orbital area was repeatedly observed in Groups DHA-10 and MHA-II-10. Thickened soft tissues in these ares were not obvious in post mortem examination. Although skeletal changes in the scull were not measured, this phenomenon was believed to be the result of

TABLE V

TISSUES AND THE SECTIONING OF THE TISSUES
IN THE HISTOPATHOLOGICAL STUDY

TISSUE	SECTIONING
Kidney (rt)	Mid-portion, through pelvis, perpendicular to the long axis
Testis	Five mm from caudal pole, perpendicular to long axis
Cuput Epididymus	Three mm from cranial pole, perpendicular to long axis
Prostate (ventral)	Five mm from cranial margin, transversely
Urinary Bladder	Two mm distal to the rounded apex, transversely
Trachea	Five mm distal to the larynx, transversely
Lung (rt middle lobe)	Five mm caudal to branch of right middle lobar bronchus

TABLE V (Continued)

TISSUE	SECTIONING
Liver	Five mm distal to attachment of right madial lobe to right lateral and right caudate lobes
Gastrointestinal Tract	
a. Stomach	Across junction of glandular and non-glandular portion
b. Duodenum	One cm distal to pyloric sphincter
c. Jejunum	Midway between pylorus and ileocecal valve
d. Ileum	One cm proximal to the ileoceral valve
e. Cecum	An 1 cm x 1 cm section on antimesenteric border
f. Colon	One cm distal to the cecocolic valve
g. Esophagus	Included in the cross section of trachea

an increased cerebrospinal fluid (CSF) pressure due to vitamin A deficiency (Nelson et al., 1964). In studying young vitamin A-deficient rabbits that suffered hydrocephalus, Millen and Wollam (1956) reported that distorted bone growth in the skull resulted in the abnormal expansion of the animal's frontal area due to increased CSF pressure. The gaits were generally quite normal. Humped posture was seen in a number of rats. Teeth appeared normal in all the rats, but gums of three DHA-10 rats were gray in color. Post mortom examinations revealed atrophic adrenal glands in almost all heteroarotinoid treated rats, and sometimes in RA treated rats as well, regardless of dosages. The adrenal glands were mushy, much smaller than normal, and bead-shaped instead of normal triangle-shaped. The situation was worse in rats treated with MHA-II. Very little or none abdominal fat was observed in DHA treated rats.

Histopathology

The specific tissue pathology is described as follows.

Respiratory Tract

Trachea. The effects of the drug treatment viewed in the tracheal sections were unremarkable. In one DHA-treated animal, some degenerate neutrophils were seen in the lumen but did not appear to have come from the local area. The lung section of this animal did not provide an etiology. In all groups there were representative animals which displayed some mild lymphocytic infiltration in the tracheal mucosa and occasionally the submucosa. There appeared to be good integrity of all cellular elements, with no evidence of squamous metaplasia.

Lungs. In the lung sections viewed, no evidence of squamous metaplasia was found. Most treated and control rats displayed some degree of peribronchiolar lymphoid accumulation. In most cases, these were lymphoid in character but occasionally neutrophils and/or eosinophils were present in increased numbers. In none of these

sections could an etiology be located in the lumen of the bronchiole. A lack of capsule suggested that these were not the recognized peribronchiolar associated lymphnodes because the latter are extra-parenchymous and few in number.

Male Genital Tract

Prostate. In the cross section of the prostate gland (ventral prostate and occasionally including some of the lateral prostate), cells of the epithelial lining were mostly squamous to cuboidal. They were evaluated for lesions of the epithelial linings as well as the presence of secretory material in the cell lumen. While not enumerated, the number of mitotic figures were subjectively assessed for the different groups.

All sections of the tissues from RA-treated rats had a homogeneous, acellular, eosinophilic material in the duct lumen (Figure 31). It was probably the normal fluid product of the epithelium. There was no inflammatory response in the RA rats and the low dosage (10 μg/day/rat) DHA rats. However, 50% of the DHA-100 and 100% of the DHA-200 rats exhibited necrotic granulomatous lesions (small areas of granulated tissues associated with infections) within the tubular lumen. Inflammatory cells were essentially neutrophils or neutrophils and plasmacytes (Figure 32). In 50% of the DHA-200 rats, the tubular lumen was obliterated by hyperplastic growth of epithelial cells of a metaplastic nature. One out of four rats of the MHA-II-100 showed a granulocytic response mixed with metaplasia, and 75% of the MHA-II-200 rats showed a mixed granulocyte/lymphocyte inflammatory response with one of them (1/4) exhibiting metaplasia (Figure 33).

In all drug-treated rats, the number of mitotic figures in the secretory epithelium of the prostate was greatly reduced or essentially absent, the number of cells with secretory substance was reduced to zero in most cases, and the epithelium tended to be much more squamous to cuboidal in nature rather than cuboidal to columnar when compared to the control group. In addition, the cell linings along the duct were thickened in heteroarotinoid-treated rats.

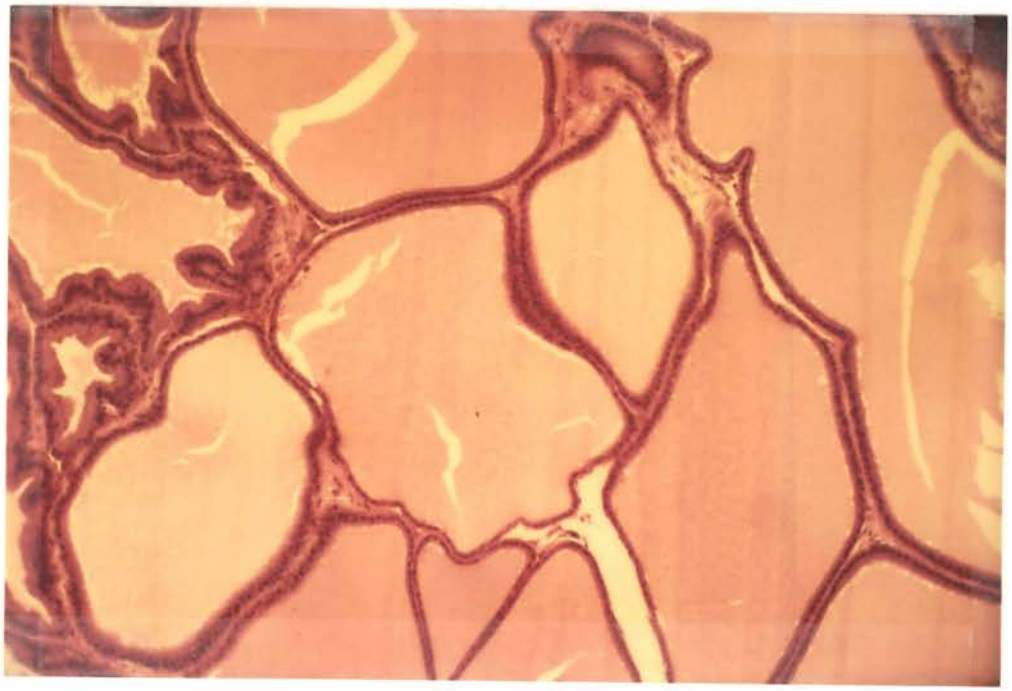


Figure 31. The Epithelial Tissue of the Rat Prostate Gland.
Identity: RA-200
Magnification: 100x
RA: All-*trans*-retinoic Acid

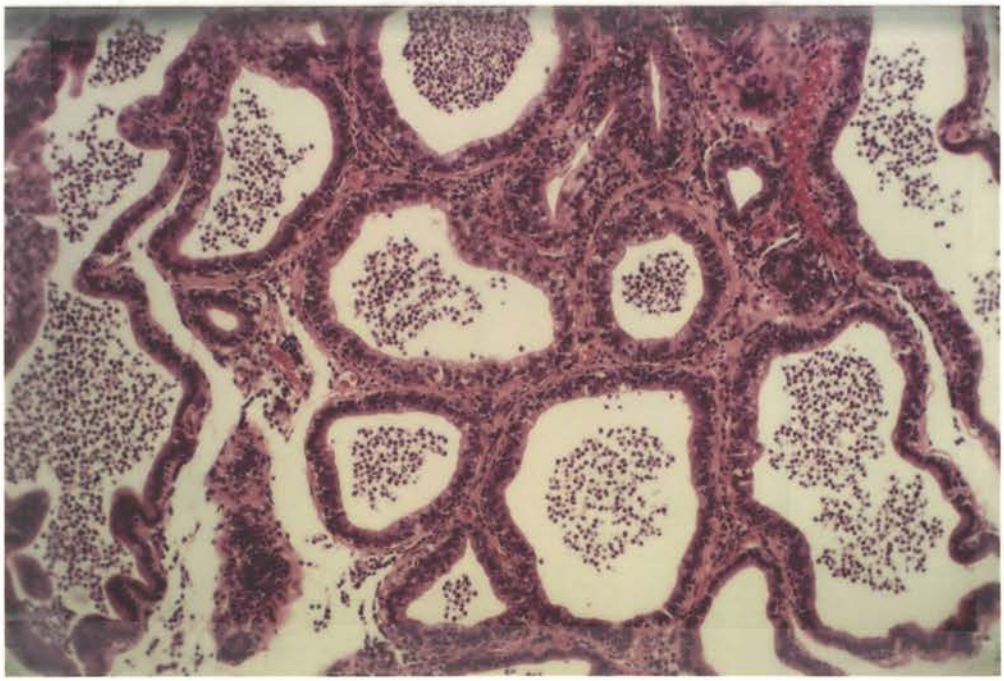


Figure 32. The Epithelial Tissue of the Rat Prostate Gland.
Identity: DHA-100
Magnification: 100x
DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-
-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate

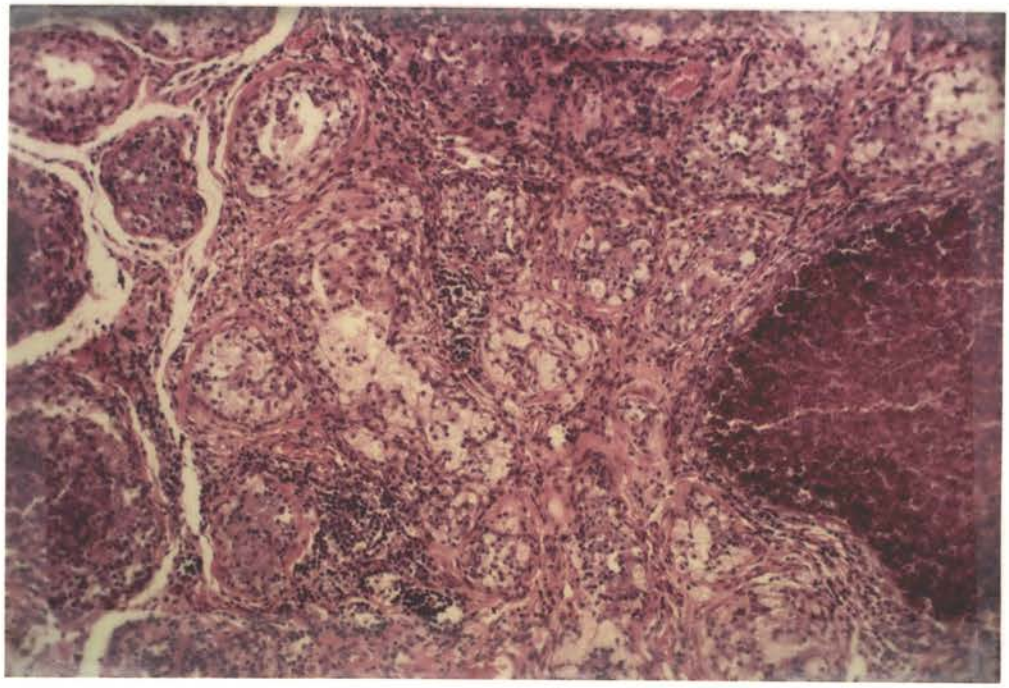


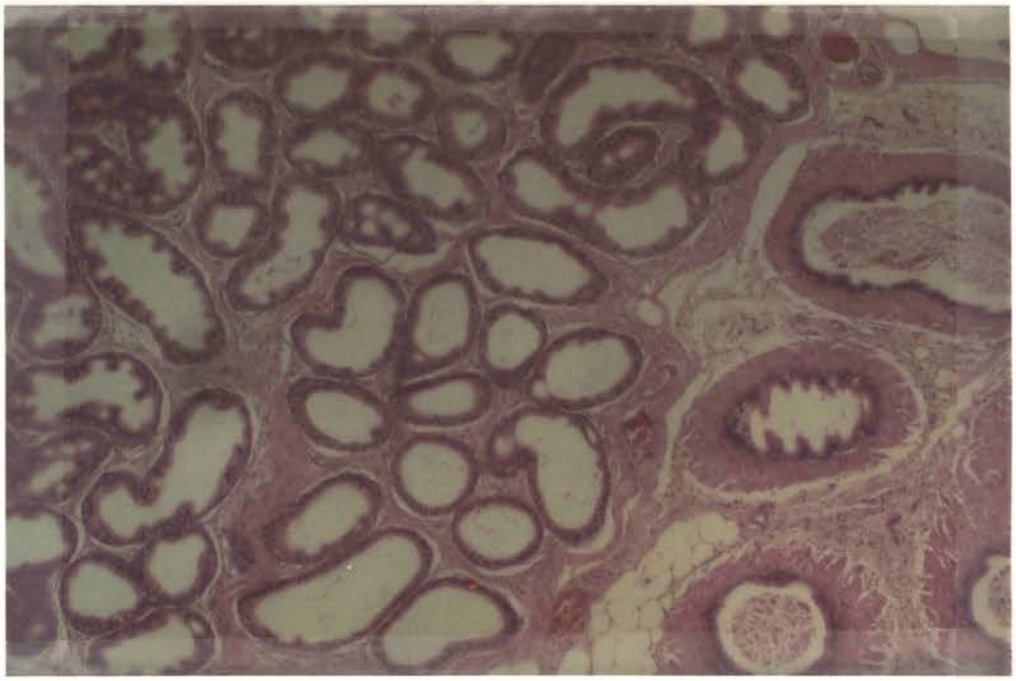
Figure 33. The Epithelial Tissue of the Rat Prostate Gland.
Identity: MHA-II-200
Magnification: 100x

In evaluating these findings, one must consider that lesions may be due to: (i) a lack of vitamin A activity of a particular drug; (ii) the absence of adequate testosterone (based on paucity of Leydig cells in the testes); (iii) direct toxic effects of the agents. Obviously, combinations of these factors could exist with variations dependent upon dose levels. Chopra and Wilkoff reported that carcinogen-induced hyperplasia in the mouse prostate organ cultures was inhibited and/or reversed by a variety of retinoids (1977a and b). A more recent study showed that excessive vitamin A increased the risk of prostate cancer in elderly men (Kolonel et al., 1987). These varied accounts suggested that the effects of retinoids at different dose levels might be dramatically different.

Epididymus. In this tissue, the epithelial lining was the focus of attention with secondary interest in the vascular endothelium. Inflammatory reactions of any nature were uniformly absent from all of the drug groups. Degenerate epithelial lining cells from the seminiferous tubules along with degenerate spermatazoa were uniformly present in either the epididymal tubular lumen and/or the vas tubular lumen. A peculiar interlumenal cystic structure (Figure 34) was found in representative rats of all the drug-treated groups at all dose levels. Epithelial pillars growing into the lumen closed upon themselves to form multiple cystic and ciliated-cell lined structures within the original lumen. All RA-treated animals, regardless of dose levels, showed a 100% incidence. A 75% incidence was seen in DHA-10, DHA-200 and MHA-II-100 rats. A 50% incidence was seen in DHA-100 and MHA-II-200 rats.

The interlumenal cystic structure seen in this study resembled the morphological changes in the epididymis that Wolbach and Howe observed in their 1925 study, although they did not show the picture. The epithelial cells of the epididymis in vitamin A deficient rats increase in number, they described, "...inasmuch as they form a deep epithelium layer, often with large vacuoles between cells and occasionally with secondary gland-like formation of cells with cuticular borders bounding a lumen." If this is indeed the case, this

(a)



(b)

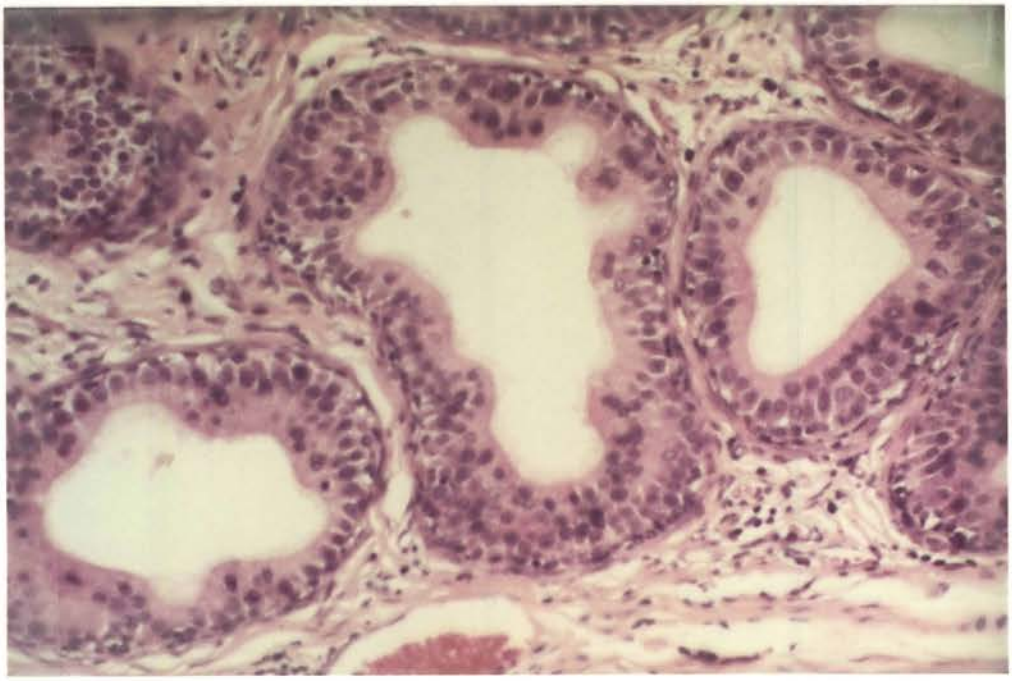


Figure 34. The Epithelial Tissue of the Rat Epididymus.
Identity: RA-200
Magnification: (a) 40x
(b) 200x
RA: All-*trans*-retinoic Acid

observation seems to indicate that all three compounds at the doses tested are unable to function as vitamin A for maintaining a healthy epididymal epithelium in the rats.

Testis. Overall, the size and volume of the testes of all groups appeared to be quite comparable. The drug treated groups, however, exhibited testes that were tan-brown when viewed from the external surface while those of the control group were white. In all drug treated groups, seminiferous tubules were essentially void of viable cellular elements and the basement membranes were collapsed into the center of the lumen. If cells were present, they were usually primary spermatogonia rather than Sertoli cells, based on morphology. These desquamated, degenerate cells were found in the lumen of the epididymis along with degenerated sperm. The intersitital spaces were acellular to a great extent though the capillary density appeared to be the same as in controls. Leydig cells were difficult to identify in many preparations and appeared to be devitalized based on staining properties.

To summarize, the changes seen in the testes indicated atrophic alterations in the tissue. The phenomenon described above seems similar to the lesions resulting from vitamin A deficiency, which was described as such: when edema and atrophy of the testes reached extremes, the seminiferous tubules "seemed floating in a liquid medium" (Wolbach and Howe, 1925).

Gastrointestinal Tract

Esophagus. Measuring keratin layers of the esophagus epithelium did not demonstrate any significant difference in any treatment group. There was no evidence of squamous metaplasia.

Stomach. The level (average thickness) of keratinization of the non-glandular portion of the stomach (extension from the esophagus) was evaluated at its junction with the glandular portion. One test animal, which was clinically ill and moribund at the time of necropsy, had severe hemorrhagic gastritis, though this lesion was not considered an adverse effect of the drug as it appeared in a low dose animal but not at higher doses.

Duodenum. Evaluation of the villi length, mitotic numbers/villus and ratio of mucosa to submucosa in thickness, were unaffected by treatment when compared to controls.

Jejunum. Evaluation of villus length, ratio of mucosa to submucosa and the occurrence of mitotic figures/villus were unaffected by treatment when compared to control animals.

Ileum. Evaluation of length of villi, the number of mitotic figures per villus and the ratio of the depth of mucosa to the depth of submucosa were unchanged parameters when compared to the control animals. Peyer's patches did appear to be greatly enlarge in all treatment groups compared to the control animals. The numbers of mucoid secreting cells in the drug-treated animals did not appear different from control animals.

Cecum and Colon. There were no differences in the histology of these structures in the treated animals when compared to the control animals.

It was noted that, for the majority of the animals in the treatment groups, there were no bacteria adherent to the mucosa or present in the lumen of the intestine, irrespective of the identity of the drug. In contrast, large colonies of various bacteria were found present in the lumen of the control animals (given regular rat chow as opposed to the synthetic diet), and were adherent to the mucosal surface.

Others

Liver. Cross sections of the liver were evaluated with respect to the epithelial cells of the bile ducts, the endothelial cells of the vessels (arterioles and veins), epithelial cells of the liver capsule and cell vitality in the periportal area (those most sensitive to xenobiotic and oxygen deprivation toxicity).

There were no identifiable lesions with respect to the structures of interest in any of the three drug groups, irrespective of the levels of the agent in the feed. One animal in the

DHA-100 group had some focal fatty changes but this is consistent with animals of this age. Some were also seen in the control animals.

Kidney. In all heteroarotinoid-treated rats, renal tissues with tan-brown, amorphous concretions that were intracellular in the proximal tubular cells as well as in the lumen of this segment of the tubule and collecting ducts were seen at all dose levels. While there was occasional eosinophilic, amorphous, non-cellular casts in a few of the control animals, the presence of these casts was greatly intensified in DHA, MHA-II and, to a lesser extent, RA treated rats. In all dose levels of the heteroarotinoids, there were scattered, focal areas of cellular basophilia and pyknosis indicative of dying or aged cells. These foci were not associated with inflammatory reactions and primarily were located in the cortical regions of the proximal tubular networks. In rats of DHA-treated groups, there was ample evidence of interstitial edema accumulation identified as clear, eosinophilic staining.

Urinary Bladder. In this tissue, the transitional epithelium of the bladder was the primary focus. All RA animals showed focal, mild to moderate desquamation (inappropriate shedding or loss) of epithelial cells (Figure 35). In 75% RA-100 and 25% RA-200 rats, there was microscopic hemorrhage into the lumen of the bladder. All DHA-10 rats had normal epithelial cells while there was mild focal desquamation in 50% DHA-100 and 50% DHA-200 rats. Of the MHA-II-100 rats, 75% showed normal epithelia, while 25% had focal, very mild desquamation and clusters of cells with very pyknotic nuclei. Seventy five percent of the MHA-II-200 rats also appeared normal, while 25% showed metaplastic activity and some amorphous, granular eosinophilic material in the lumen.

Rapid growth suggestive of neoplastic potentiality and desquamation was observed by Wolbach and Howe (1925) in vitamin A-deficient rats. However, extensive keratinization and dermoid cyst-like formation identified by these authors as pathological changes of vitamin A-deficiency were not displayed in this study.

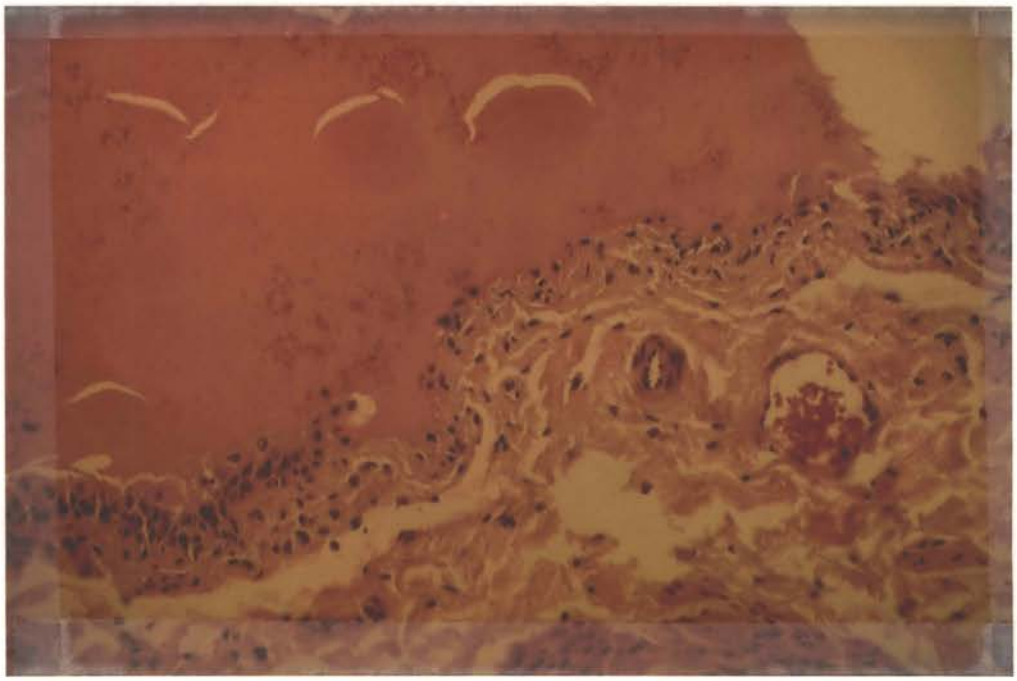


Figure 35. The Epithelial Tissue of the Rat Urinary Bladder.
Identity: RA-200
Magnification: 200x
RA: All-*trans*-retinoic Acid

Conclusions

It was the purpose of this study to examine the effect of dietary supplementation of three drugs, all-*trans*- retinoic acid (RA) and two heteroarotinoids (DHA and MHA-II), on the epithlial tissues that are most susceptible to vitamin A deficiency. Since all rats used in this study were vitamin A deficient prior to the drug treatment, normal epithelia observed in this study in the organ tissues that were reportedly injured by vitamin A deficiency would indicate recovery from the lesions. The findings are summarized as follows:

1) Epithelia of all organs in gastrointestinal tract, respiratory tract and liver appeared normal.

2) Slight atrophy in the kidney manifested as edema, amorphous concretions and cellular pyknosis in the proximal tubular networks was seen primarily in heteroarotinoid-treated rats, and only occasionally in RA-treated rats. Edema was only seen in DHA-treated rats.

3) Moderate desquamation of epithelial cells was the main pathology observed in the urinary bladder. The situation was most severe in RA-treated animals, and less severe in DHA-treated animals than MHA-treated rats. In addition, the effect was more profound in rats treated at high doses. Twenty five percent of the rats dosed with MHA-II manifested slight atrophy.

4) Severe pathologies of metaplastic, atrophic and inflammatory reaction were overwhelming in all three male genital organs examined. While no inter-compound differences could be demonstrated in the testes, the damage in the prostate gland was more severe in heteroarotinoid-treated rats than RA-treated animals, and the damage in the epididymis was greater in RA-treated rats than heteroarotinoid-treated rats.

5) Intra-compound differences were generally not obvious.

The characteristics of the changes observed in this study, such as edema, inflammation, atrophy and metaplastic growth, were all indications of vitamin A-deficiency; however, the location and the severity of the lesions are different from those simply

resulting from vitamin A deficiency. The changes seen in this study may reflect the effects of the drugs, or may be due to the lesions remaining from the previous vitamin A-deficiency with the drugs causing no effects on them. It appears quite likely that heteroarotinoids have activities similar to that of RA in terms of their functions on epithelial tissues. The main organs where neither RA nor the heteroarotinoids tested herein could maintain the integrity of their epithelial tissues were the eye and those located in the genitourinary tract.

CHAPTER VI

MASS SPECTROMETRY ANALYSIS OF THE SYNTHETIC STANDARDS

Introduction

The metabolism of xenobiotic substances is essential to pharmacological and toxicological research. The metabolic pathways are usually complex, and the quantity of the metabolites is very small, often in a trace amount (Pudewicz and Straub, 1986). The identification of the metabolites is further complicated by the existence of complex biological matrixes (Pudewicz and Straub, 1986). A number of analytical methodologies have been developed to study drug metabolism (Frigerio and Ghisalberti, 1977; Coutts and Jones, 1980). Conventional methods usually involve many steps of metabolite separation, isolation and purification. HPLC is a good technique in purification,and separation, but it requires detectors of sufficient specificity to provide structural information (Hines, 1984). Application of any single type of spectroscopic analysis, such as NMR, usually requires substantial quantity of purified metabolites. The size, solubility restrictions, nonvolatile and unstable nature of most drugs and their metabolites, limit the utilization of the powerful technique of capillary gas chromatography/mass spectrometry (CGC/MS) in structure determination (Newton, 1990; Bellar and Budde, 1988).

The complementary combination of HPLC separation and MS detection (LC/MS) with ultimate structural identification provides the method of choice for the analysis of complex drug metabolites (see review by Tomer and Parker, 1989). Various types of interfaces coupling HPLC and MS, such as thermospray and direct liquid introduction, have been designed and applied in the metabolism study of many types of drugs (Vestal, 1984; Tomer and Parker, 1989). The rapidity and power of the LC/MS technique in

providing detailed structural information of drug metabolites was clearly demonstrated in the structure elucidation of various types of drug metabolites including N-oxides, sulfates, glucuronides, sulfoxides, decarboxylated products, taurine conjugates and dealkylation products (see review by Tomer and Parker, 1989). One of the greatest advantages of LC/MS is the direct analysis of the metabolites within a complex biological matrix without excessive clean-up and compound isolation (Blake and Beattie, 1989).

The first application of mass spectrometry in the study of the metabolism of vitamin A (all-*trans*-retinol) and its related compounds was reported by Vecchi and co-workers (1967). The mass spectra of vitamin A and a number of its analogs were also obtained using the direct inlet technique (Lin et al., 1970; Reid et al., 1973). Although mass spectrometry served as a major technique in the identification of many metabolites of vitamin A and its analogs, isolation and derivatization of the unknown compounds were usually required prior to the application of mass spectral analysis (Skare et al., 1982a).

An on-line HPLC-plasmaspray liquid chromatography/mass spectrometry system (LC/MS) was developed for its application in the analysis of synthetic heteroarotinoid standards, individually and in a mixture. A liquid secondary ion mass spectrometry technique (LSIMS) was used in the analysis of individual standards.

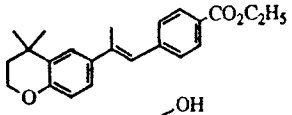
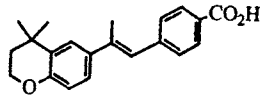
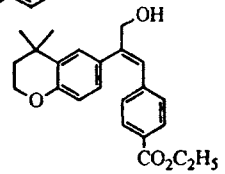
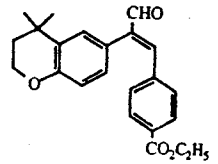
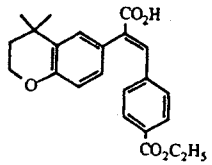
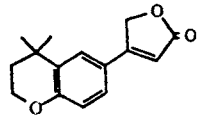
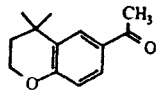
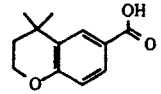
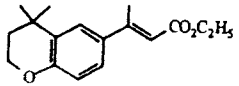
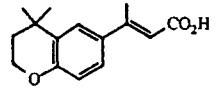
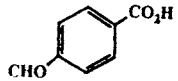
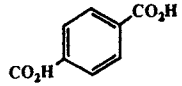
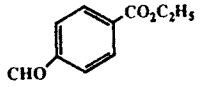
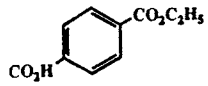
Materials and Methods

Chemicals

Based on the general pathways of drug metabolism, and the pathways of retinoid metabolism in particular, more than a dozen potential metabolites of DHA (see Figure 3 for the structure) have been designed and synthesized by Dr. Berlin's group in the Department of Chemistry, Oklahoma State University (Waugh et al., 1985; Sunthankar et al., 1991). Some of those synthetic standards (Table VI) were used in this study. The designations (S1, S2, and so on with S stands for "standard") used in this writing are for the purpose of simplicity and clarity only. They are not the formal designations used in any publications,

TABLE VI

STRUCTURES OF THE SYNTHETIC HETEROAROTINOIDS

Designations	Structures	MW	Designations	Structures	MW
DHA		350.4	S-1		322.4
S-2		366.4	S-3		364.4
S-4		380.4	S-5		244.3
S-6		204.3	S-7		206.3
S-8		274.4	S-9		246.3
S-10		150.1	S-11		166.1
S-12		178.1	S-13		194.2

MW: Molecular Weight.

nor do they indicate any significance of any kind. These compounds, with a wide range of polarities, served as standards in establishing proper separation and detection protocols that were used in the analysis of real metabolites.

The other reagents used in sample preparation and analysis were methanol (Optima grade, Fisher Scientific, Fair Lawn, New Jersey), water of either Optima grade (Fisher Scientific, Fair Lawn, New Jersey) or laboratory doubly distilled, and acetic acid (Tracemetal grade, Fisher Scientific, Fair Lawn, New Jersey). Compressed N_2 gas (HPLC grade, Sooner Supplies, Inc., Shawnee, Oklahoma) was used for sample condensing, and He gas (HPLC grade, Sooner Supplies, Inc., Shawnee, Oklahoma) was used for solvent degasing.

Principle and Apparatus.

Plasmaspray LC/MS An on-line HPLC-Plasmaspray Liquid Chromatography / Mass Spectrometry technique (LC/MS) was developed and employed in the study. The basic principle of plasmaspray ionization can be briefly described as such: the eluant from the HPLC system enters the plasmaspray interface through a 1/16 union mounted on the base of the HPLC and passes through a length of fused silica capillary which has the ground voltage. The eluant then passes through a further union into a stainless steel capillary termed "the vaporizer". The vaporizer is electronically heated (3210 v) and the eluant enters the ion source block (2700 v) as a fine spray of droplets. A different voltage is applied between the vaporizer and the ion source block to create a DC plasma of sample and solvent ions. In this study, a 510 v plasma voltage was applied on the cathode (the steel capillary) with respect to the anode (the ion source), causing a low discharge ionization (Figure 36).

The analysis of a mixture of twelve standards was performed on a LC/MS system, which included a VG Tritech TS-250 mass spectrometer equipped with a VG Tritech Plasmaspray interface (VG Tritech, Manchester, United Kingdom), a Waters-600MS

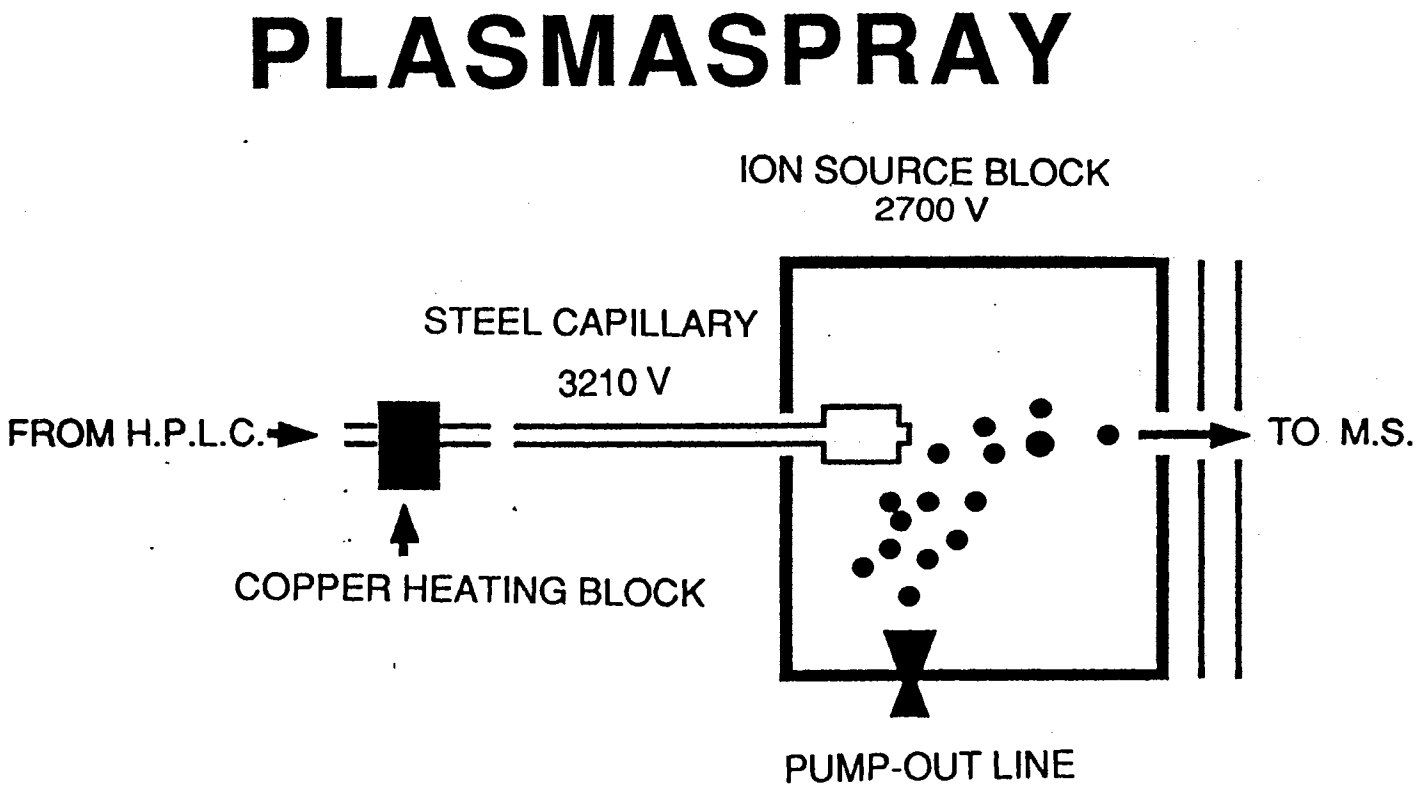


Figure 36. Diagrammatic Illustration of the Plasmaspray Interface in the On-line HPLC-Plasmaspray Liquid Chromatography/Mass Spectrometry System.

multisolvent delivery system, a Waters 6000-MS system controller,and a Waters U6K injector (Waters Associates, Milford, Massachusetts). A C_{18} reversed phase column (P/10 ODS 3, 0.47 x 23.5 cm, Particle size 5 μm, Whatman Inc., Clifton, New Jersey) and a six-port sampling value (N60, Valco Instruments Co., Inc., Houston, Texas) were used in this system. Each standard was also analyzed individually through by-passing the HPLC portion of the system, and was directly introduced into the plasmaspray interface.

Liquid Secondary Ion Mass Spectrometry (LSIMS) LSIMS was performed on a VG-ZAB-2SE mass spectrometer (VG Tritech, Manchester, United Kingdom) using cesium ions at 35 Kv for the ionization. LSIMS mass spectra of the standards were obtained on $LSIMS^+$ or $LSIMS^-$ (both modes were used for some standards) depending on the relative positive ionization and negative ionization potentials of the standards. Standards were first dissolved in methanol (5-10 μg standard / μl CH_3OH), and then loaded on the LSIMS probe by dripping 3 μl solution on the tip of the probe using thioglycerol as the matrix. Linked scan mass spectral analysis (MS/MS) in a magnetic section/electric section geometry (B/E) was also performed by selecting the known molecular ion of the standards. Fragment ions (daughter ions) specific to each selected molecular ion were detected through collisionally activated dissociation (CAD) using He as the activating gas.

Results and Discussions

Analysis of the Standards by LC/MS

A reconstructed total ion chromatogram (TIC) of a mixture of twelve standards is shown in Figure 37 using an 120 min linear solvent delivery program (detailed analytical information including the gradient program is shown in the legend of Figure 37). The peaks representing the corresponding standards are indicated on the TIC. The mass spectra of the standards obtained from this LC/MS system are presented in Figures 38 through Figure 49. Interpretation of the spectra is limited, and is shown on each spectrum.

Figure 37. Reconstructed Total Ion Chromatogram (TIC) of a Mixture of Twelve Synthetic Standards.

Instrument: Waters-6000 HPLC system
Plasmaspray LC/MS, VG-TS 250

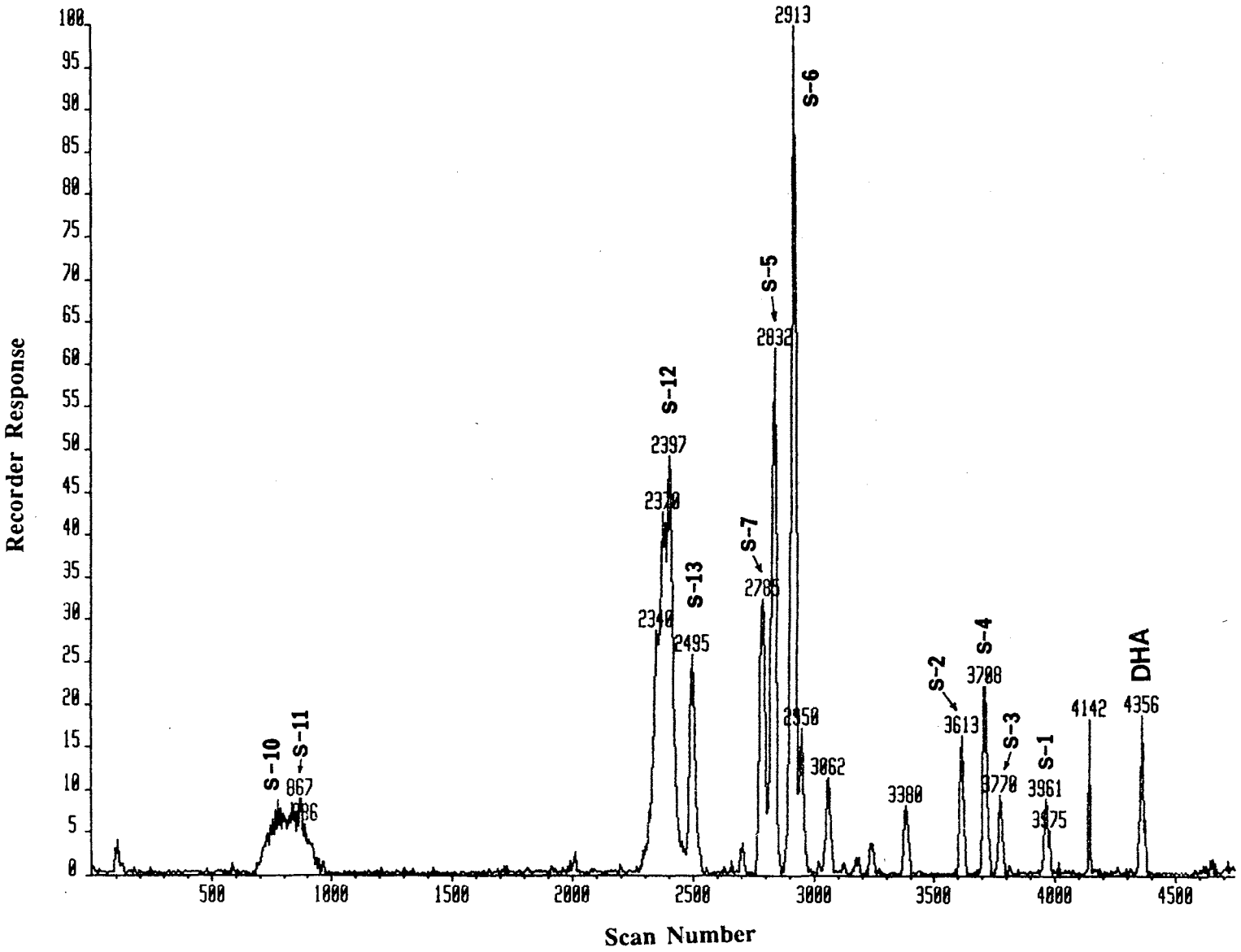
Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 80% HAC (0.01M)
20% CH_3OH Hold 10 min

Linear, 120 min

100% CH_3OH Hold 20 min


Recorder Response
Scan Number
S-10
S-11
S-12
S-13
S-7
S-5
S-6
S-2
S-4
S-3
S-1
DHA
2913
2832
2397
2495
3613
3788
4142
4356
3062
3380
3961

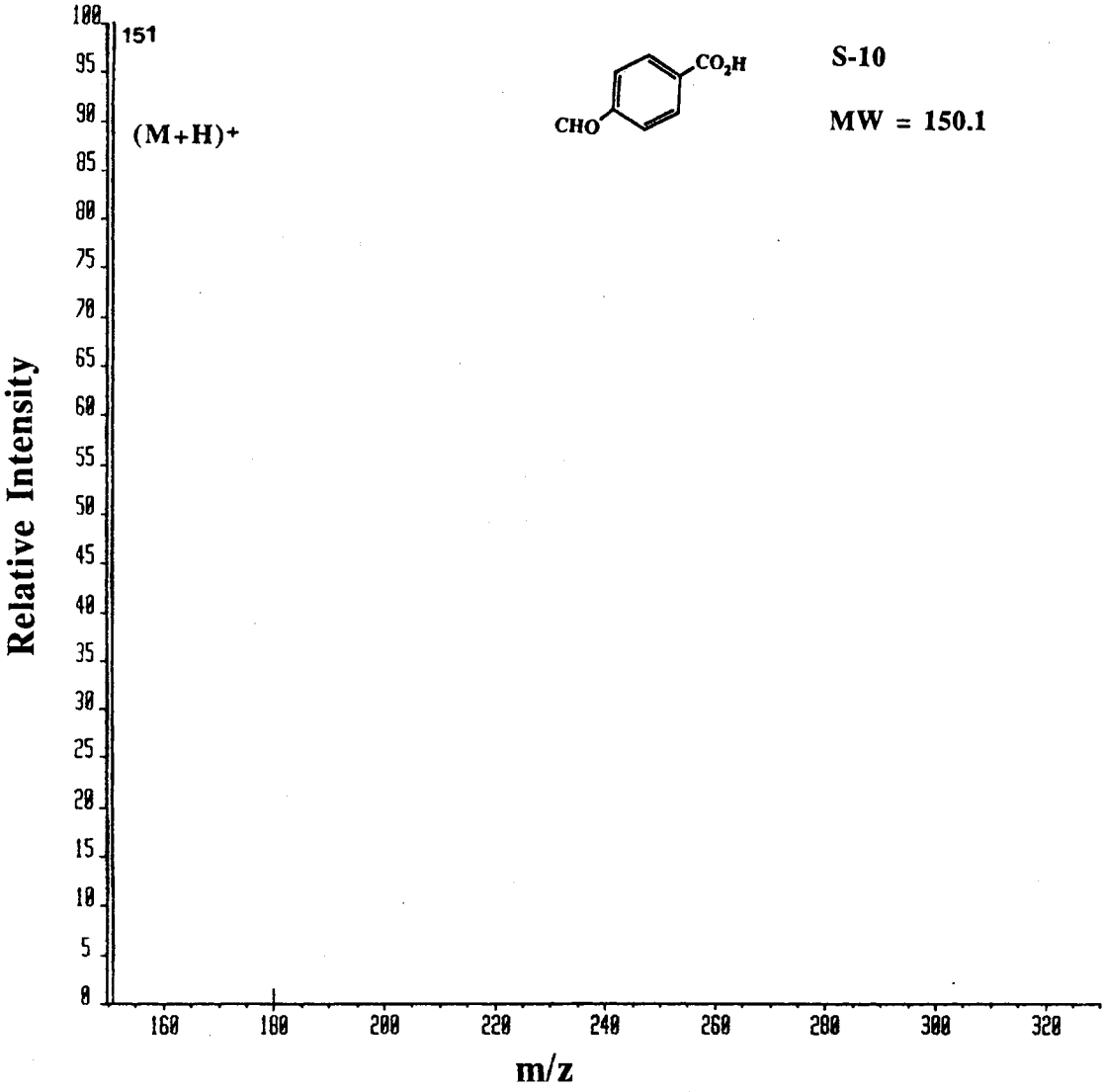


Figure 38. The Plasmaspray LC/MS Spectrum of the Standard S-10.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

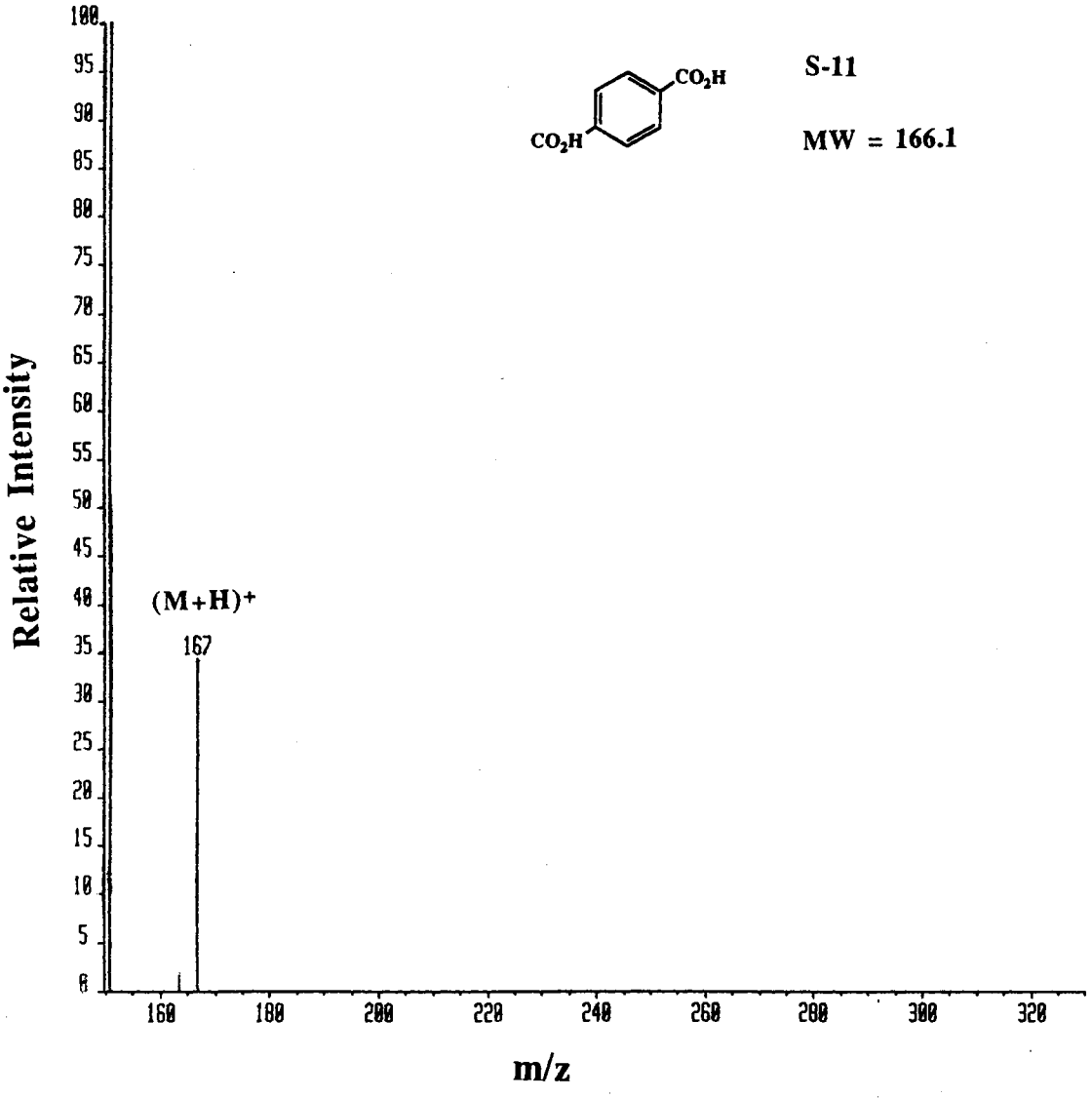


Figure 39. The Plasmaspray LC/MS Spectrum of the Standard S-11.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

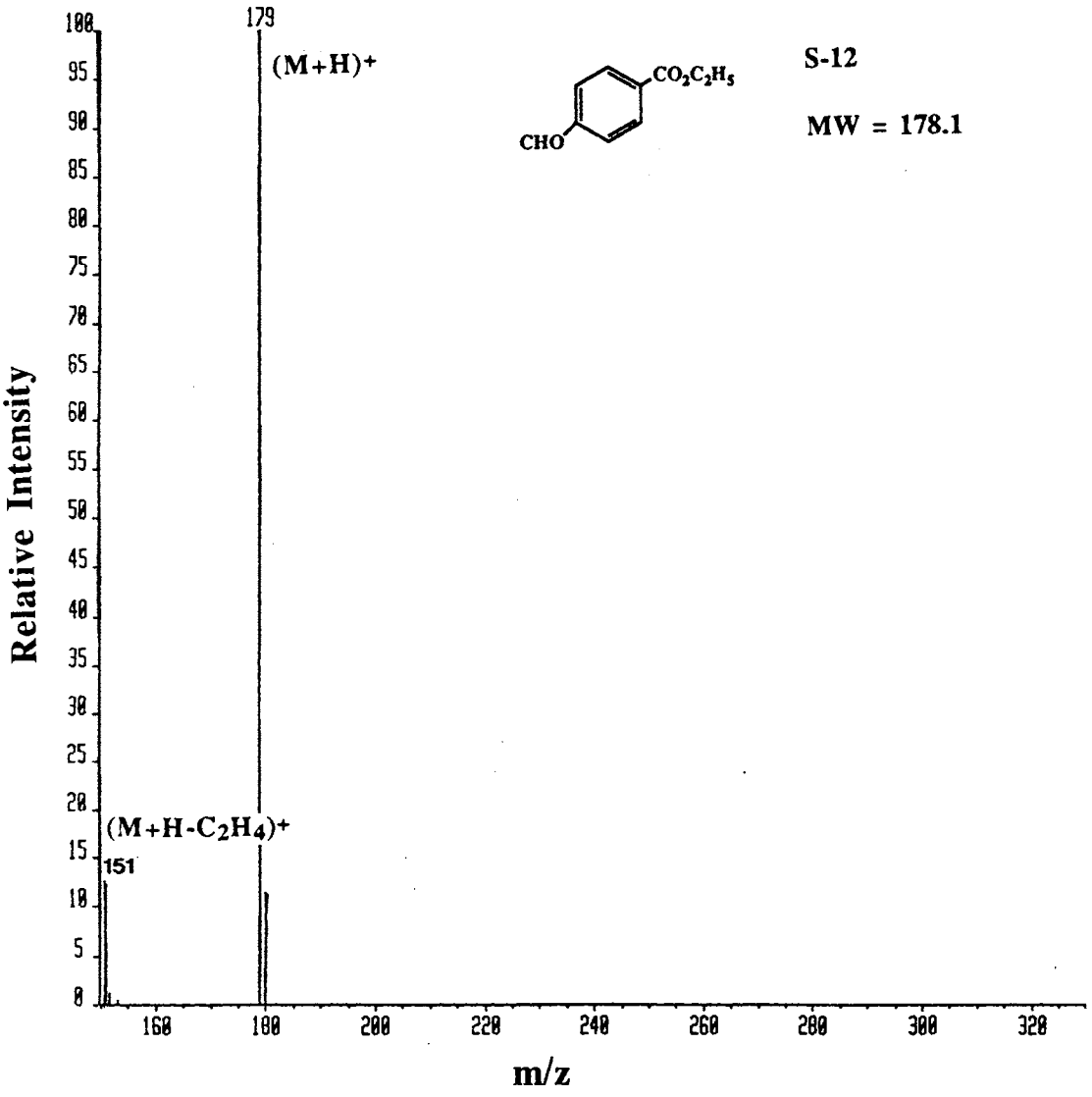


Figure 40. The Plasmaspray LC/MS Spectrum of the Standard S-12.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

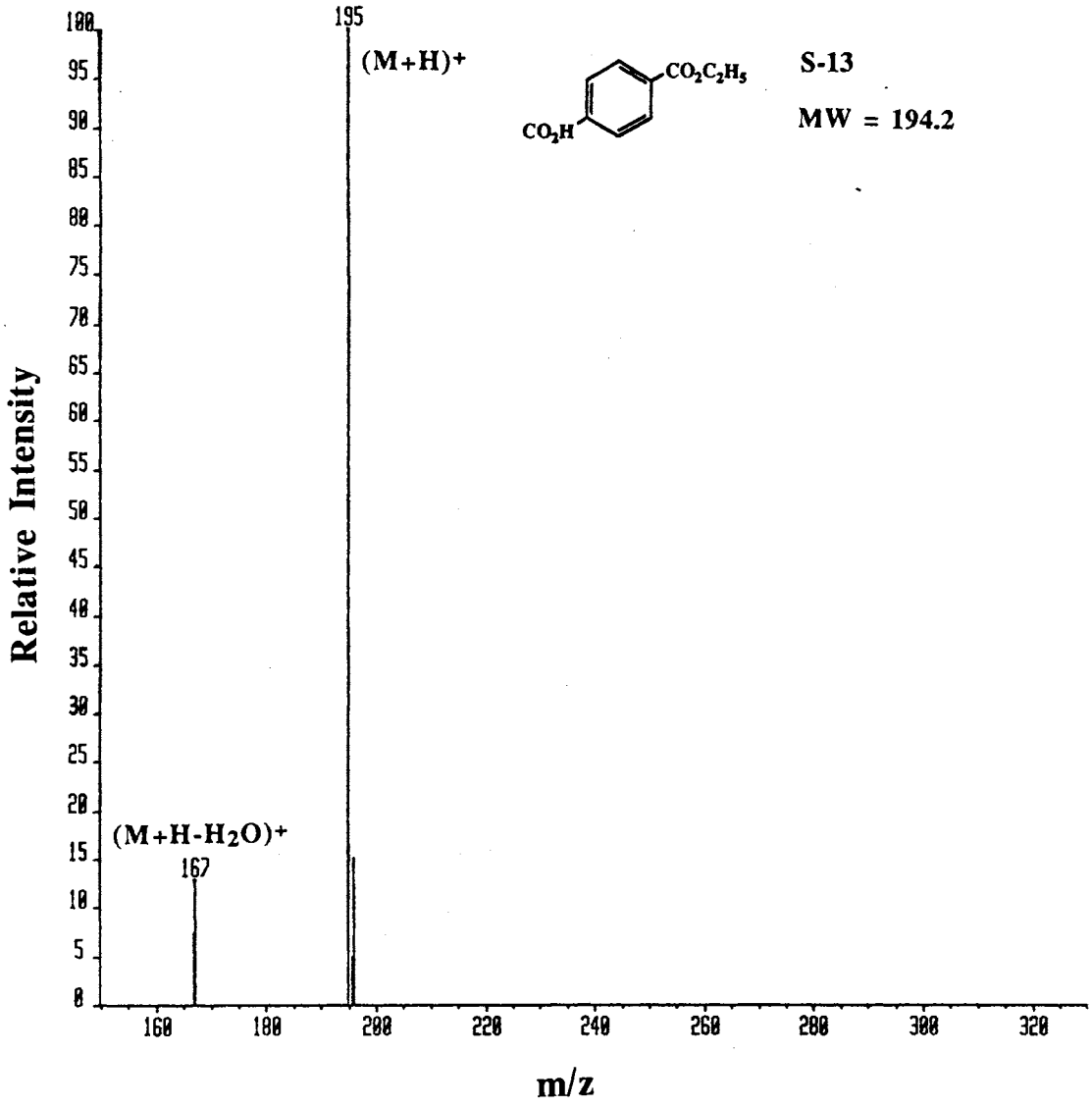


Figure 41. The Plasmaspray LC/MS Spectrum of the Standard S-13.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

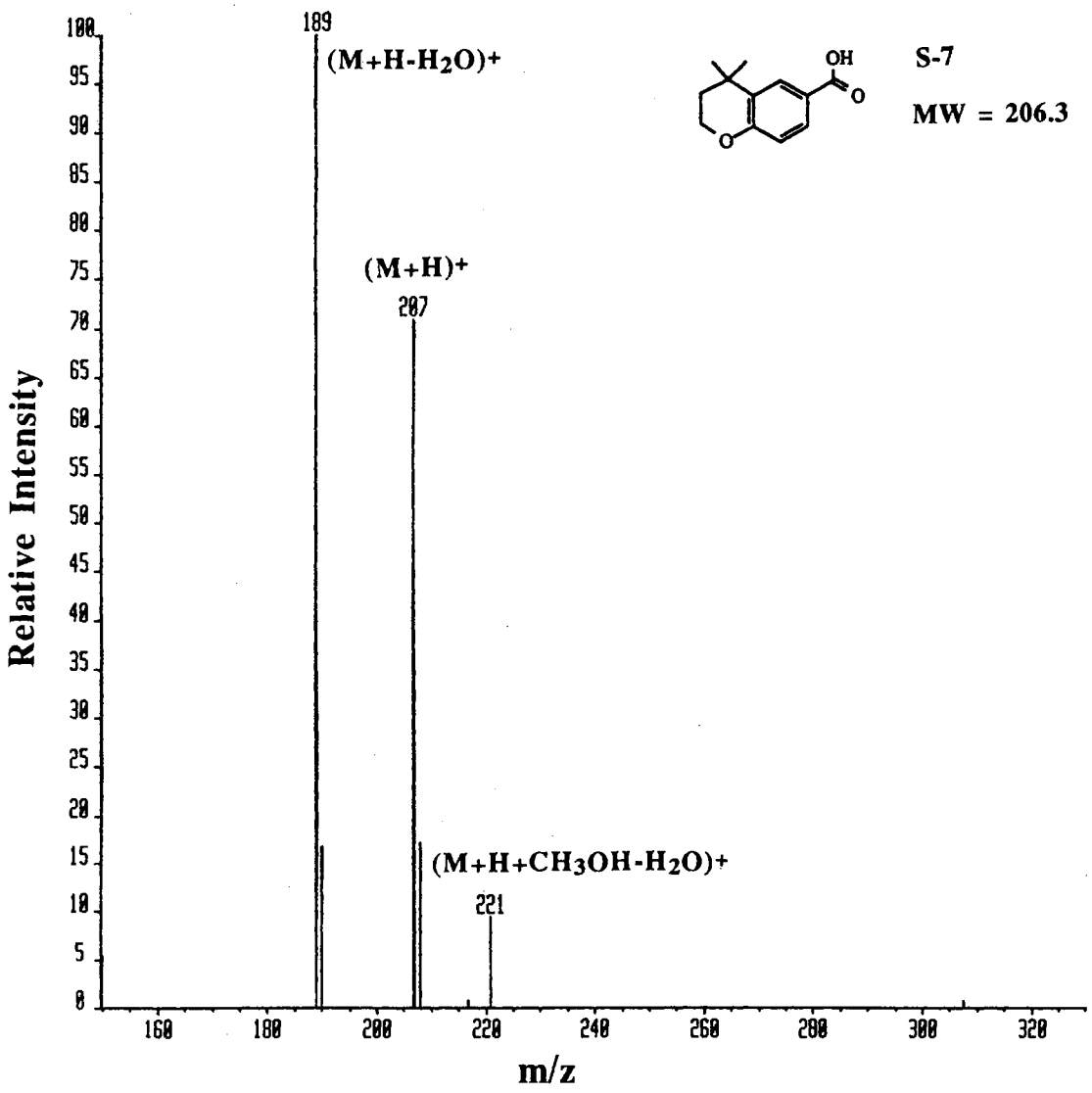


Figure 42. The Plasmaspray LC/MS Spectrum of the Standard S-7.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

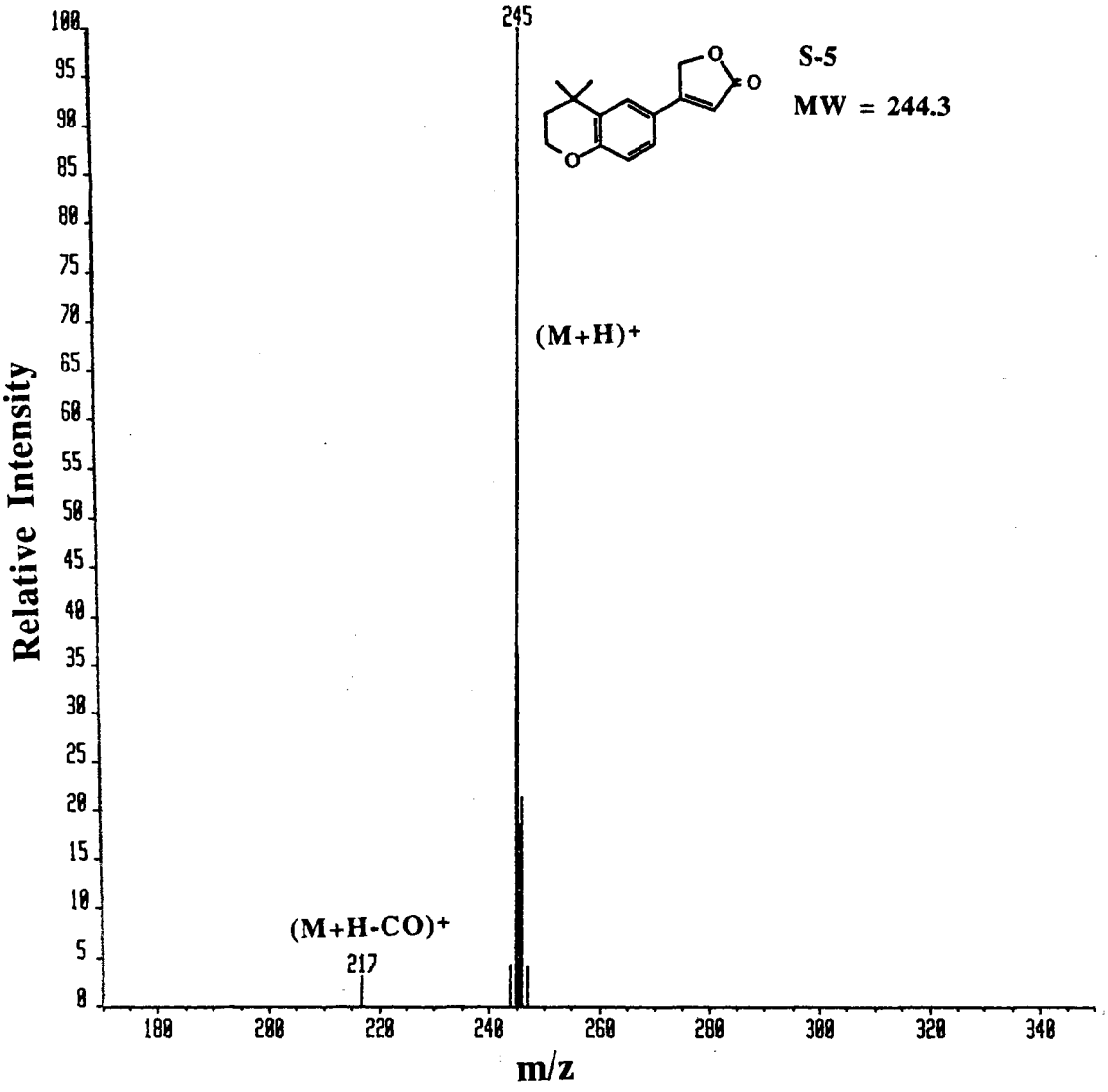


Figure 43 The Plasmaspray LC/MS Spectrum of the Standard S-5.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

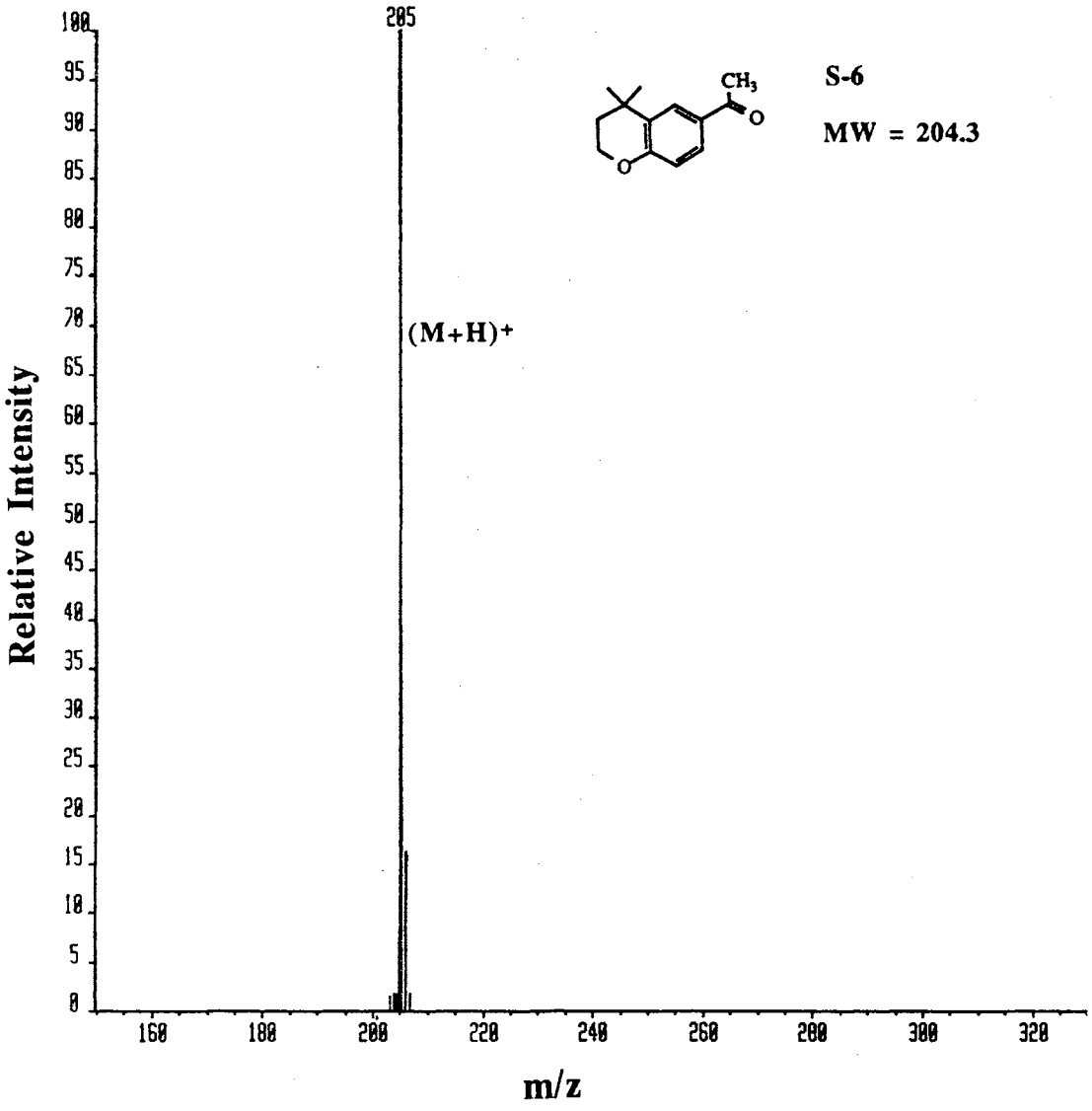


Figure 44. The Plasmaspray LC/MS Spectrum of the Standard S-6.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

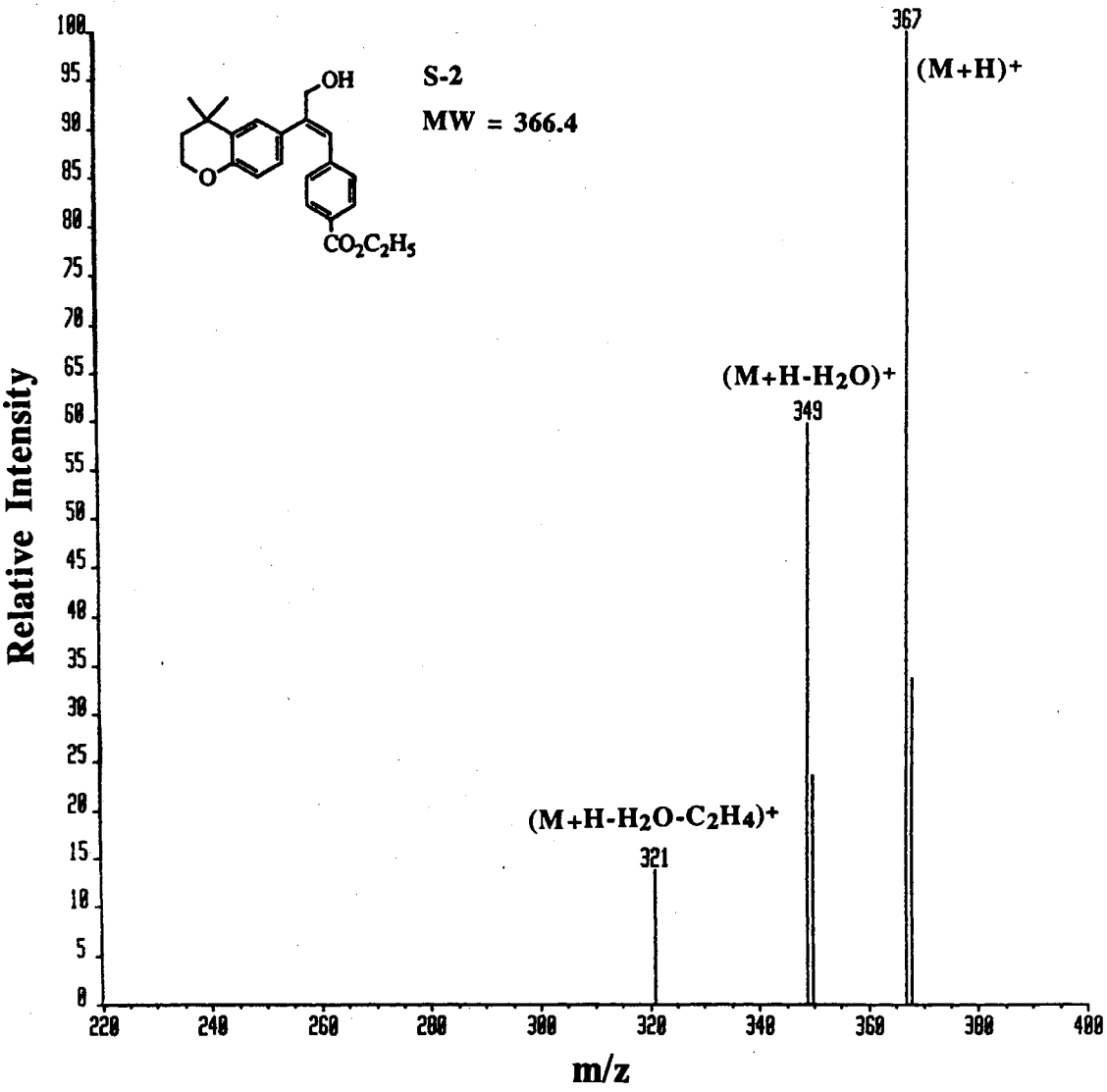


Figure 45. The Plasmaspray LC/MS Spectrum of the Standard S-2.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

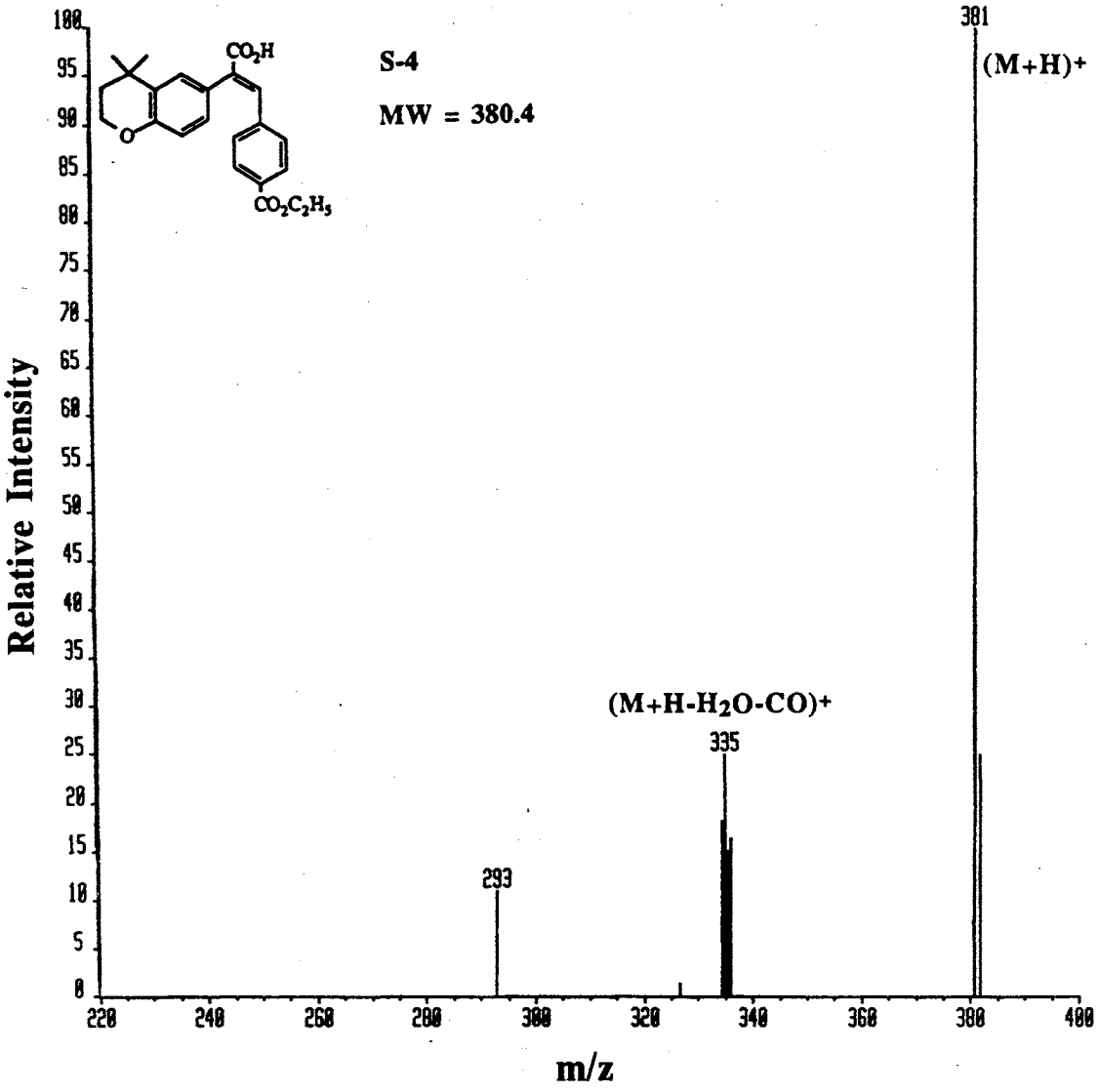


Figure 46. The Plasmaspray LC/MS Spectrum of the Standard S-4.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

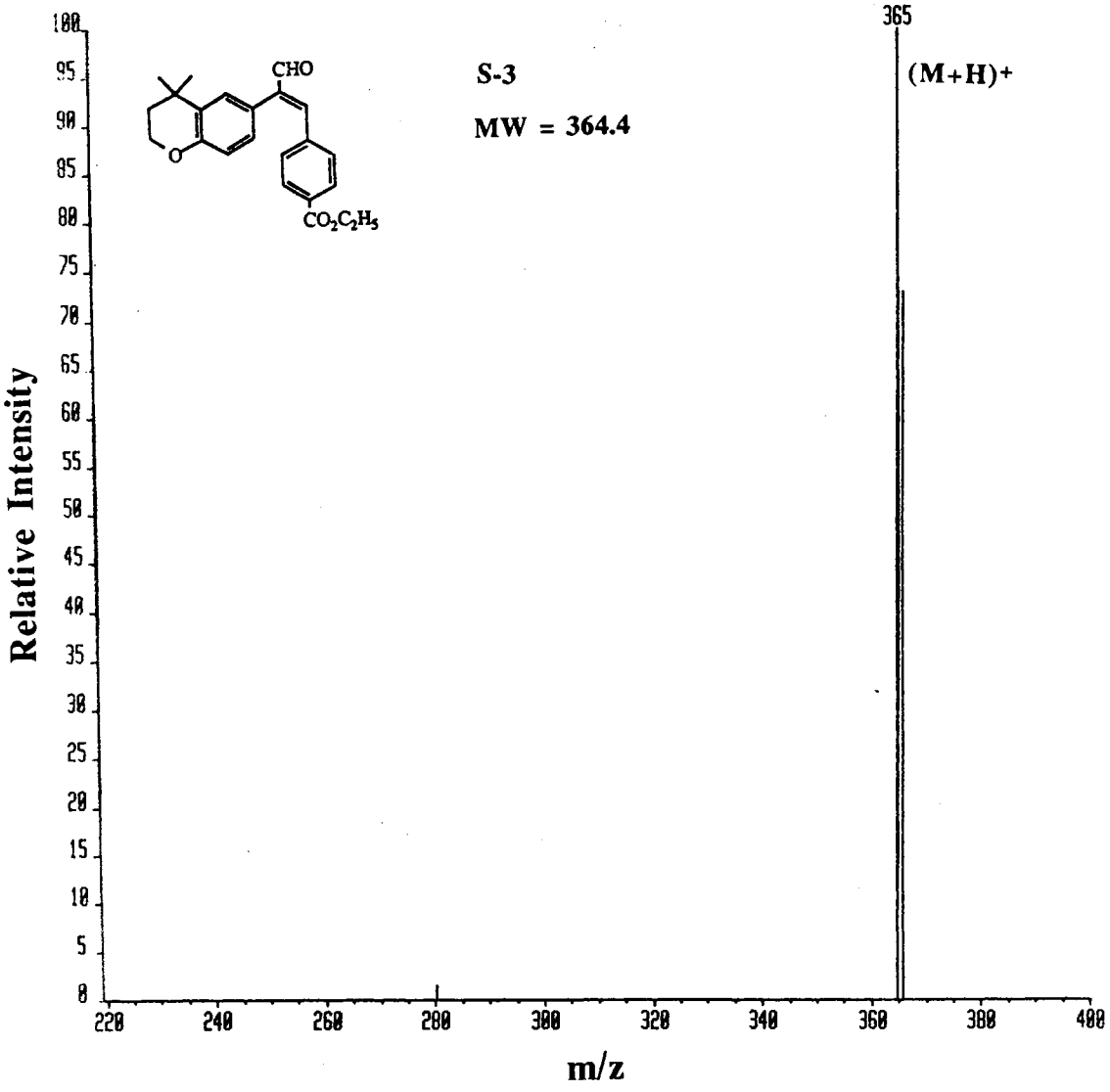


Figure 47. The Plasmaspray LC/MS Spectrum of the Standard S-3.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

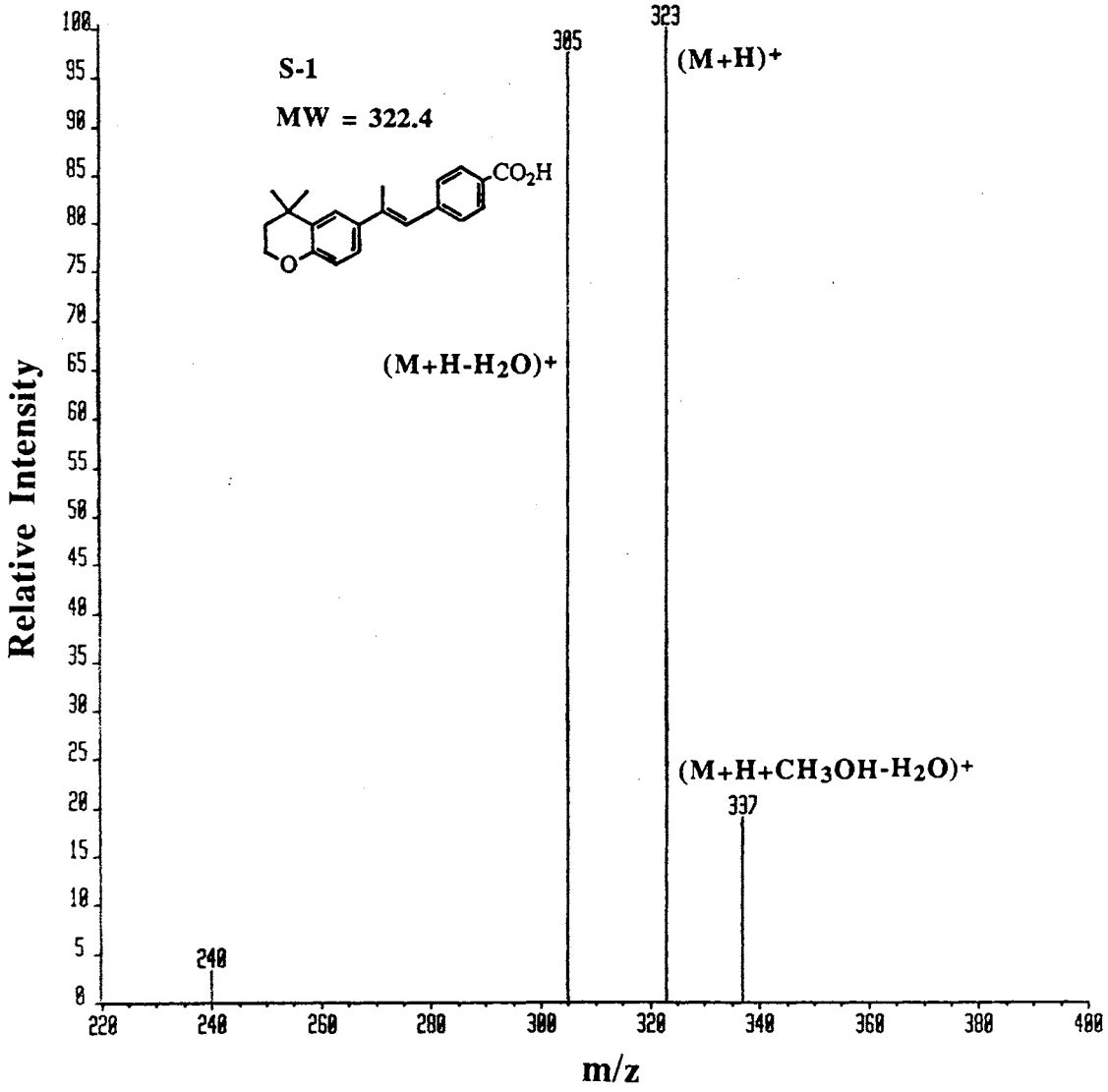


Figure 48. The Plasmaspray LC/MS Spectrum of the Standard S-1.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

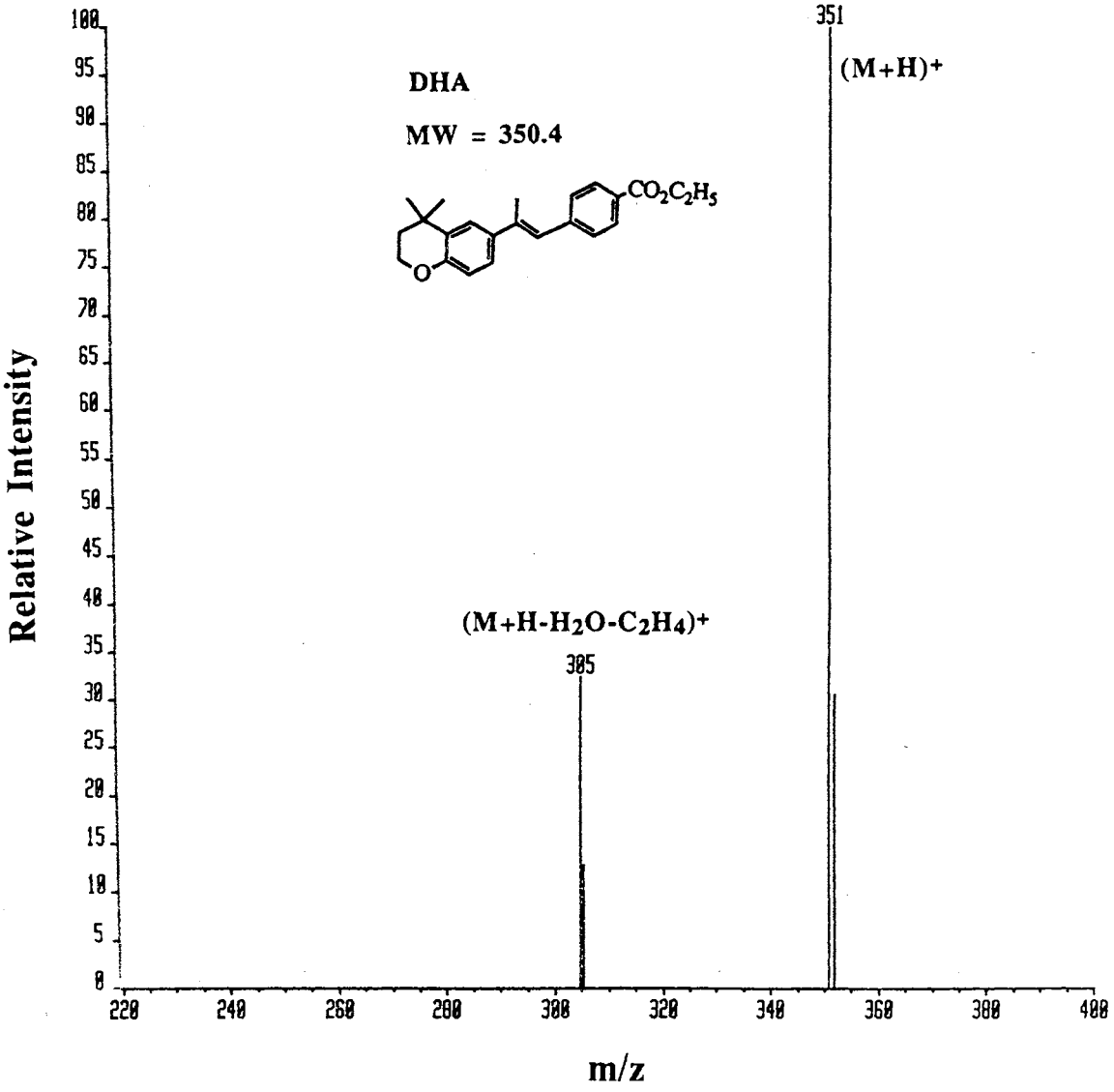


Figure 49. The Plasmaspray LC/MS Spectrum of the Standard DHA.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

Each standard was also analyzed individually with this system through HPLC by-passing. The mass spectra of the standards obtained in this manner are almost identical to those presented above, except that, in some cases, the relative intensities of the mass peaks are not the same (spectra not shown).

Analysis of the Standards by LSIMS Technique

Figure 50 through Figure 64 show the ten positive ion LSIMS spectra and five negative ion LSIMS spectra of the standards, as well as their corresponding daughter ion CAD mass spectra. As in the data presentation of LC/MS analysis, interpretation of the spectra is limited, and is shown on each spectrum.

Conclusions

The techniques of the on-line HPLC plasmaspray LC/MS, the LSIMS and the B/E linked scan mass spectrometry were successfully applied for the analysis of the synthetic heteroarotinoid standards. In most cases, LC/MS spectra provided molecular weight information, and a few major fragment ions resulting from loss of water and/or other small gaseous molecules. Methylated molecular ions were generally obtained when a free carboxylic group was present in the structure. Therefore, when methanol was used as a component of an acidic solvent mixture, methylation on free carboxylic group occurred readily in this LC/MS system.

Information on the molecular weight was well provided by the LSIMS spectra. Though LC/MS and LSIMS were both soft ionization techniques, LSIMS provided more fragment peaks when compared to the LC/MS spectra of the same compounds. The results indicated that LC/MS and LSIMS can serve as complementary methods in the structural determination of unknown metabolites. The MS/MS spectra showed fragment ions apparently resulted from some rearranged ions.

Figure 50. LSIMS Spectra of the Standard DHA.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

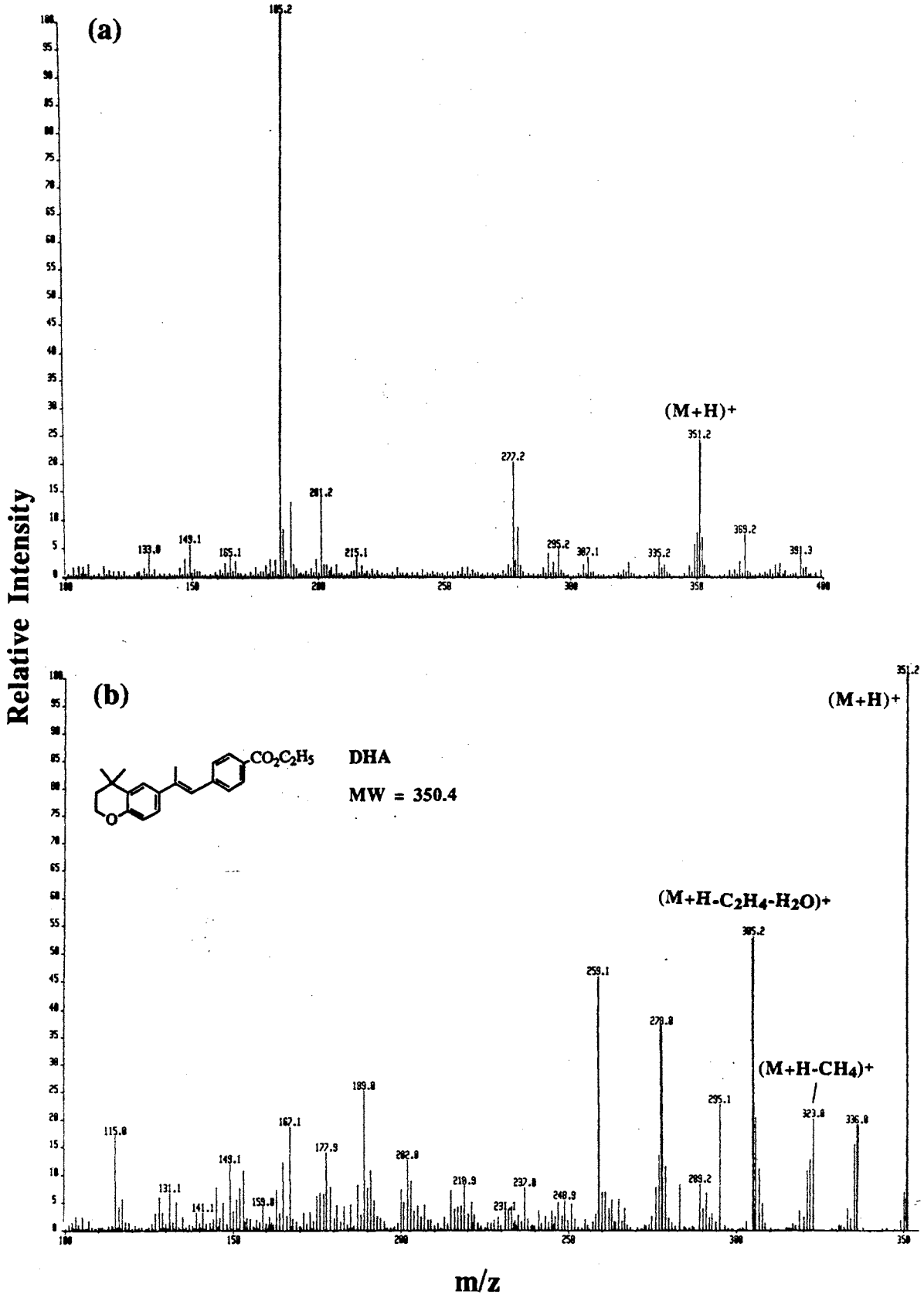

(a)
(M+H)+
351.2
277.2
201.2
185.2
149.1
133.0
165.1
215.1
295.2
307.1
335.2
369.2
391.3
(b)
CO2C2H5
DHA
MW = 350.4
(M+H)+
351.2
(M+H-C2H4-H2O)+
305.2
259.1
279.0
(M+H-CH4)+
323.0
336.0
295.1
189.0
167.1
115.0
177.9
282.0
149.1
131.1
219.9
237.0
289.2
248.9
159.0
141.1
231.1
Relative Intensity
m/z

Figure 51. LSIMS Spectra of the Standard S-1.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

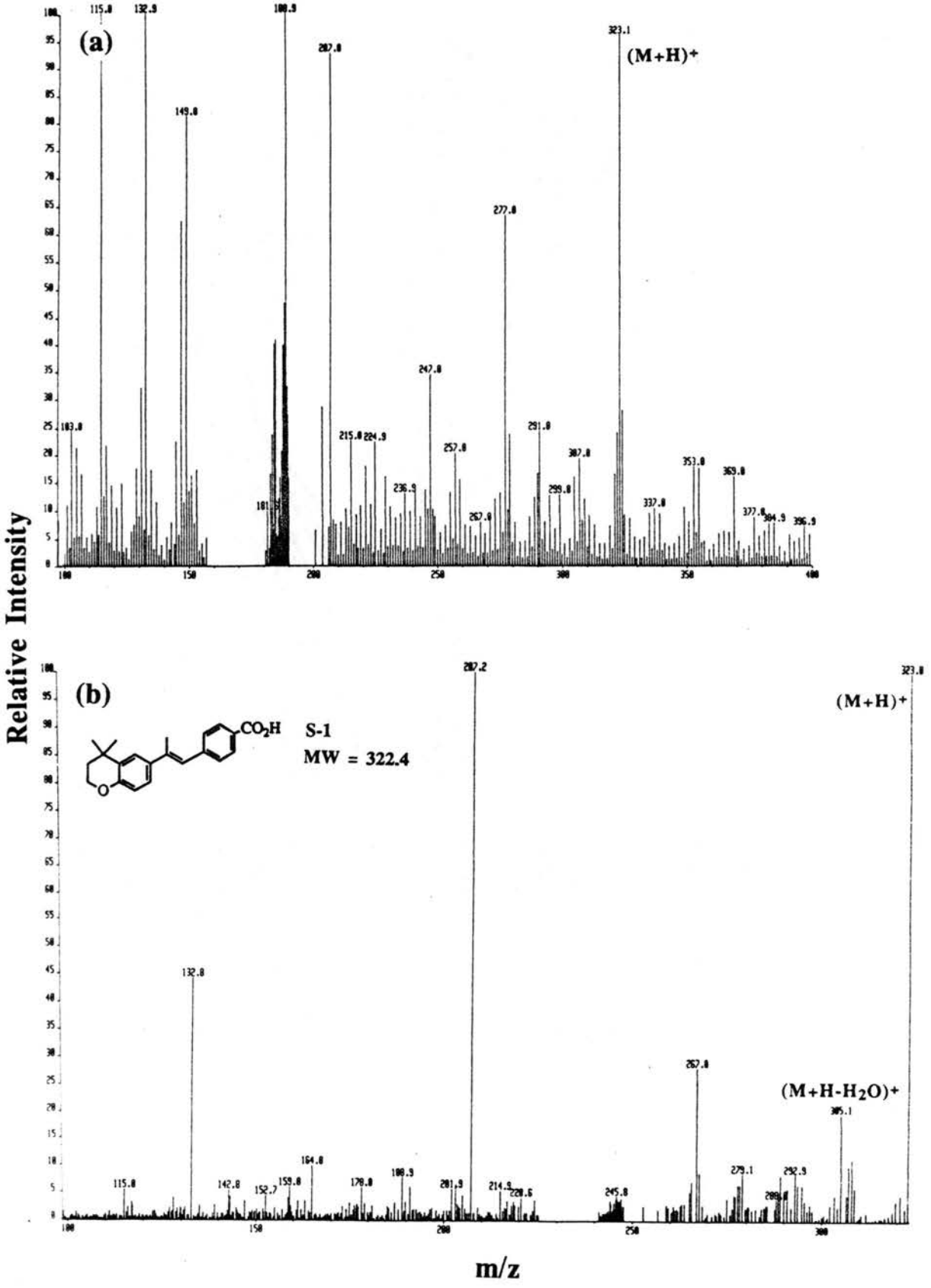

(a)
(M+H)+
323.1
(b)
S-1
MW = 322.4
CO2H
(M+H)+
323.0
(M+H-H2O)+
305.1
Relative Intensity
m/z

Figure 52. LSIMS Spectra of the Standard S-2.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

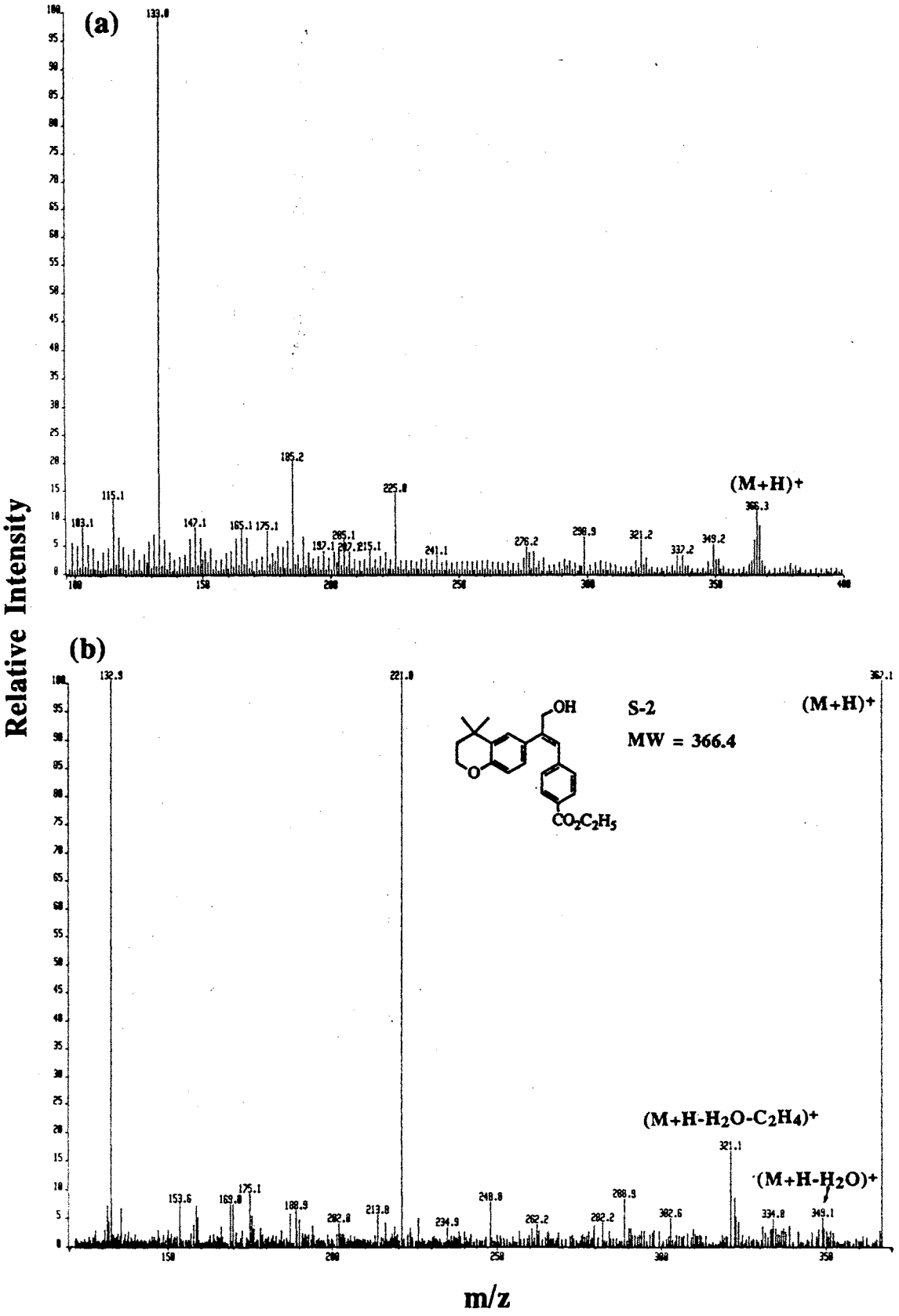

(a)
(M+H)+
(b)
S-2
MW = 366.4
(M+H)+
(M+H-H2O-C2H4)+
(M+H-H2O)+
Relative Intensity
m/z

Figure 53. LSIMS Spectra of the Standard S-3.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

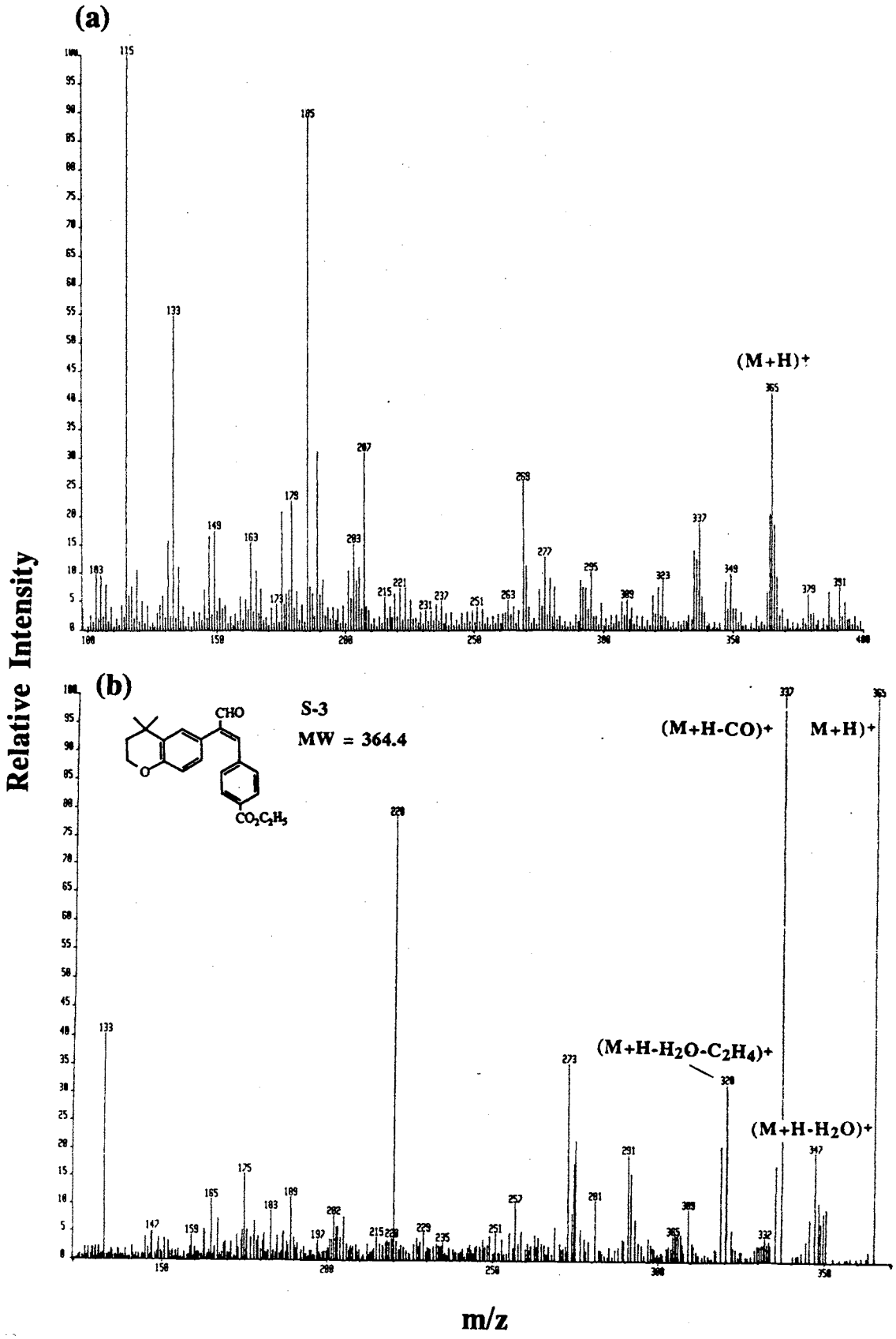

(a)
(M+H)+
(b)
S-3
MW = 364.4
CHO
CO2C2H5
(M+H-CO)+
M+H)+
(M+H-H2O-C2H4)+
(M+H-H2O)+
Relative Intensity
m/z

Figure 54. LSIMS Spectra of the Standard S-4.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

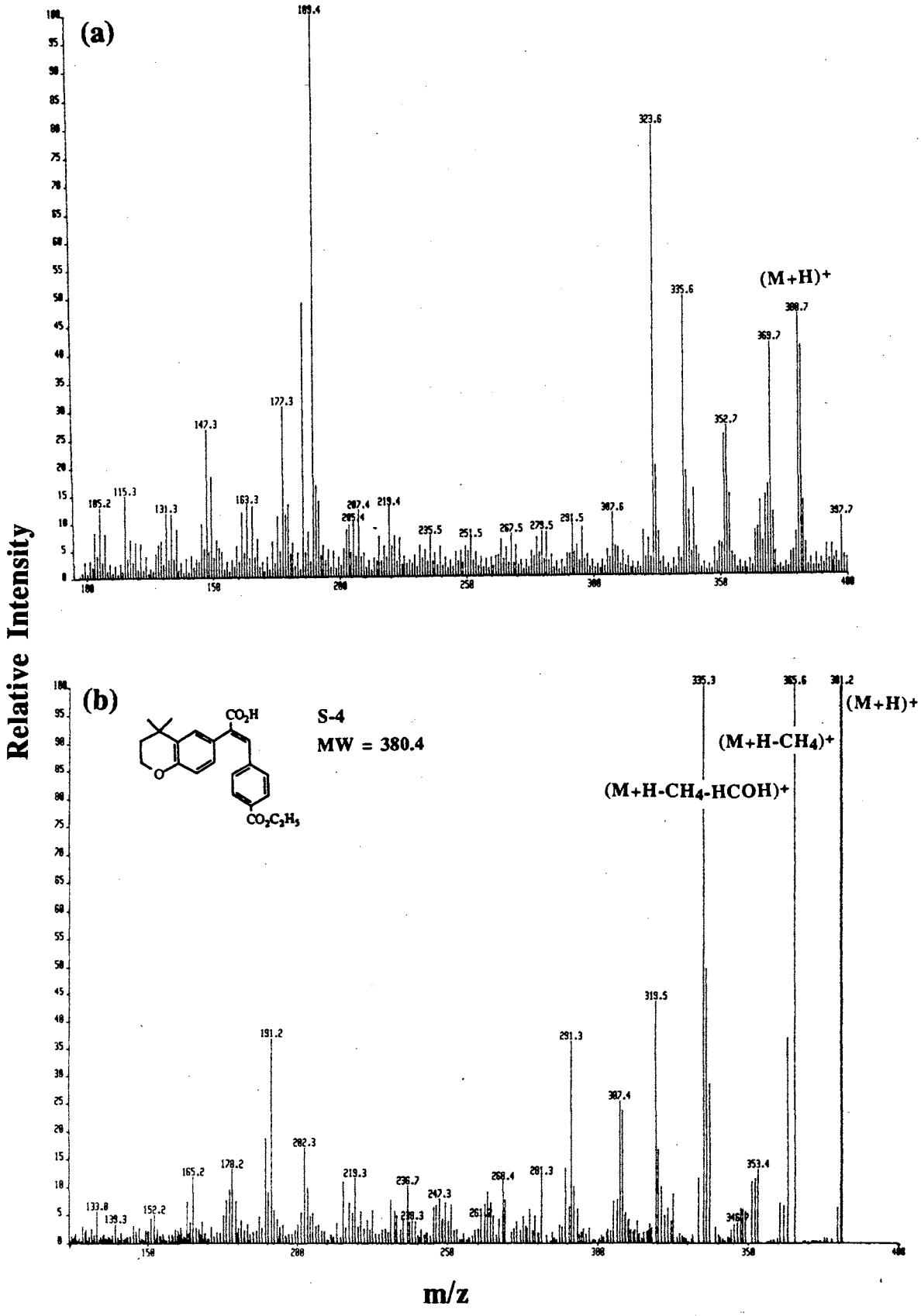

(a)
(M+H)+
(b)
S-4
MW = 380.4
(M+H)+
(M+H-CH4)+
(M+H-CH4-HCOH)+
Relative Intensity
m/z

Figure 55. LSIMS Spectra of the Standard S-5.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

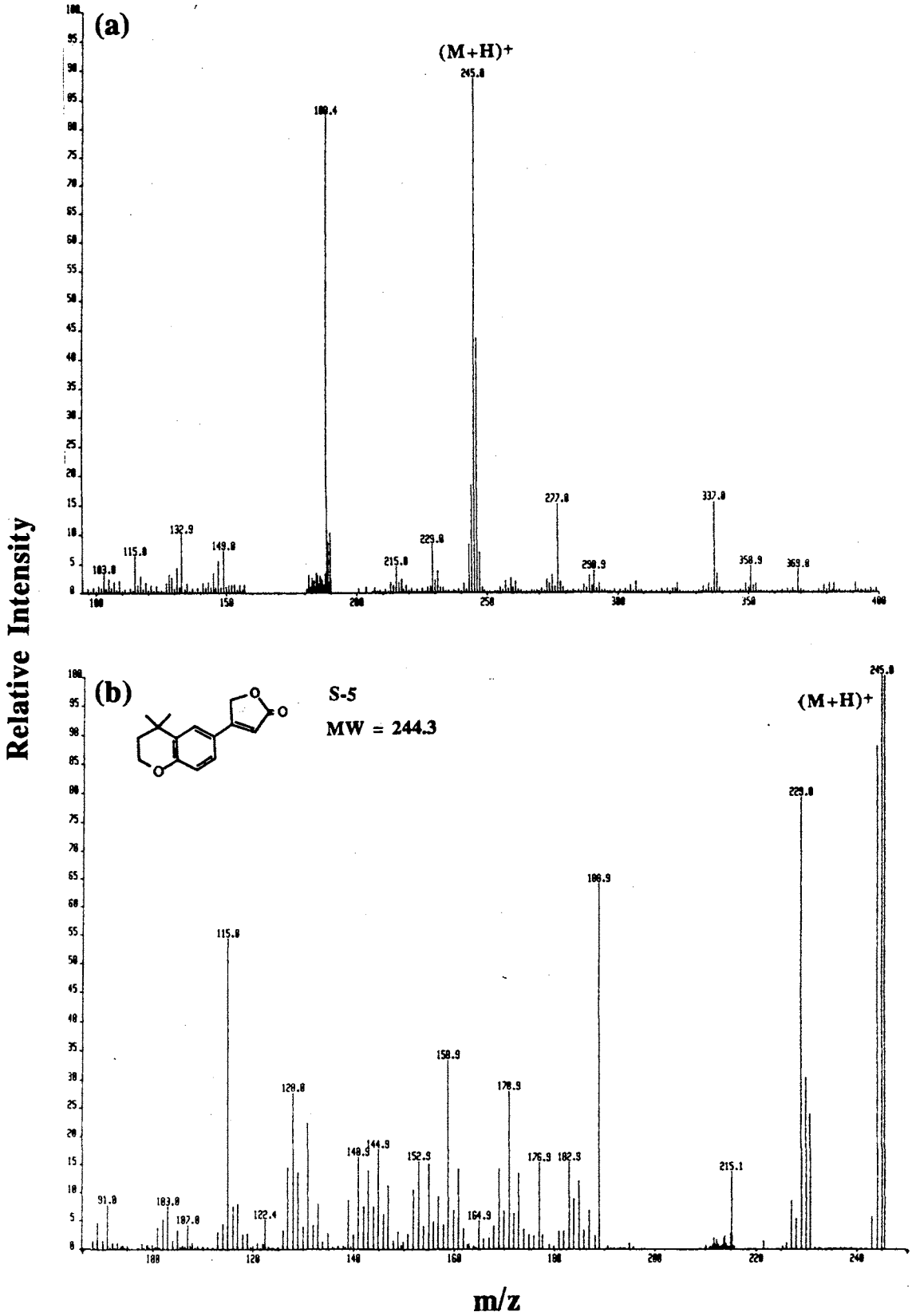

(a)
(M+H)+
245.0
189.4
277.0
337.0
132.9
149.0
115.0
229.0
215.0
298.9
358.9
369.0
183.0
(b)
S-5
MW = 244.3
(M+H)+
245.0
229.0
189.9
115.0
158.9
178.9
129.0
144.9
148.9
152.9
176.9
182.9
215.1
91.0
183.0
187.0
122.4
164.9
Relative Intensity
m/z

Figure 56. LSIMS Spectra of the Standard S-7.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

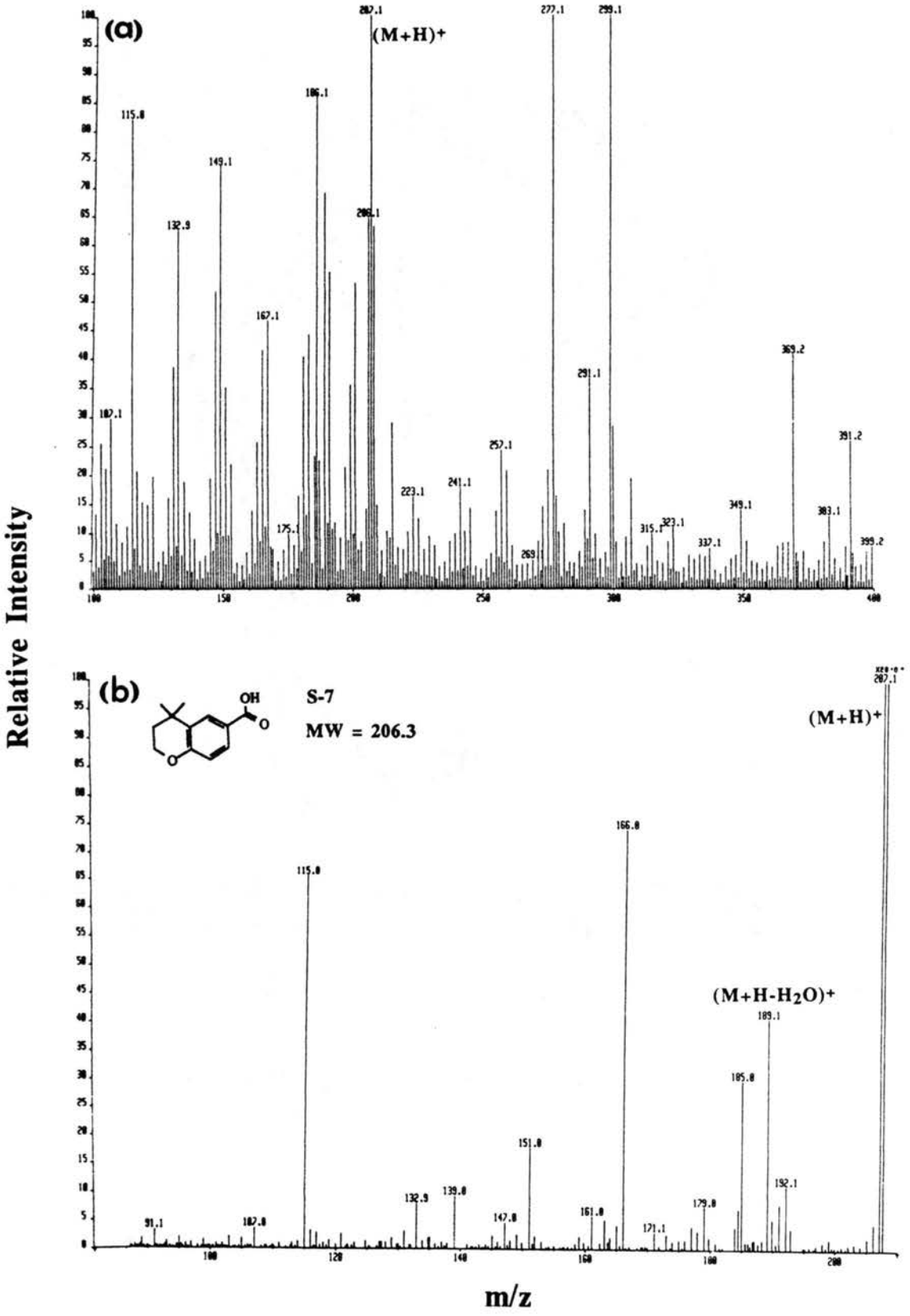

(a)
(M+H)+
(b)
S-7
MW = 206.3
(M+H)+
(M+H-H2O)+
Relative Intensity
m/z

Figure 57. LSIMS Spectra of the Standard S-9.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

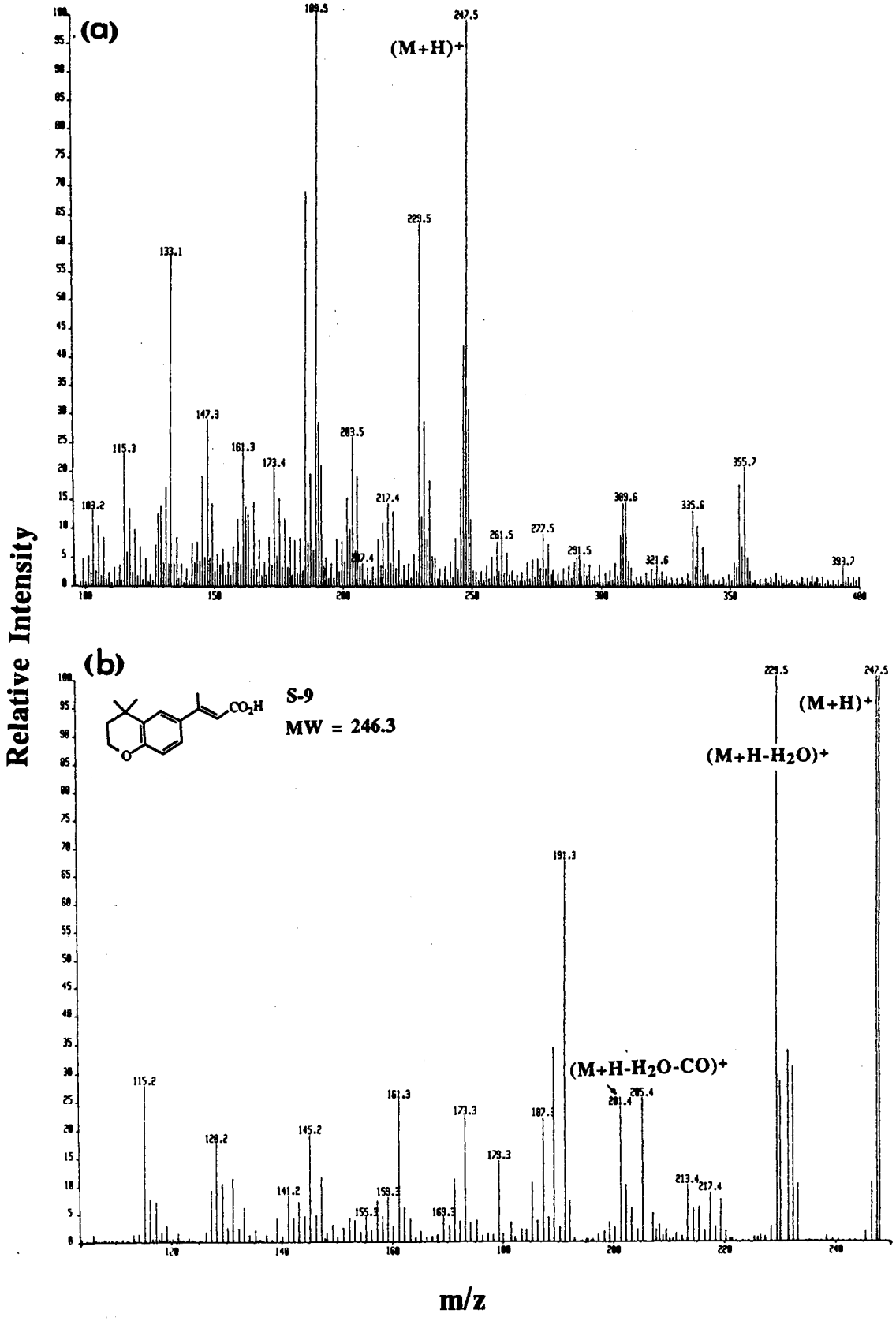

(a)
(M+H)+
247.5
229.5
133.1
147.3
115.3
161.3
173.4
203.5
217.4
183.2
261.5
277.5
291.5
309.6
321.6
335.6
355.7
393.7
(b)
S-9
MW = 246.3
CO2H
O
(M+H)+
(M+H-H2O)+
(M+H-H2O-CO)+
191.3
115.2
128.2
145.2
141.2
155.3
159.3
161.3
169.3
173.3
179.3
187.3
201.4
205.4
213.4
217.4
Relative Intensity
m/z

Figure 58. LSIMS Spectra of the Standard S-10.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

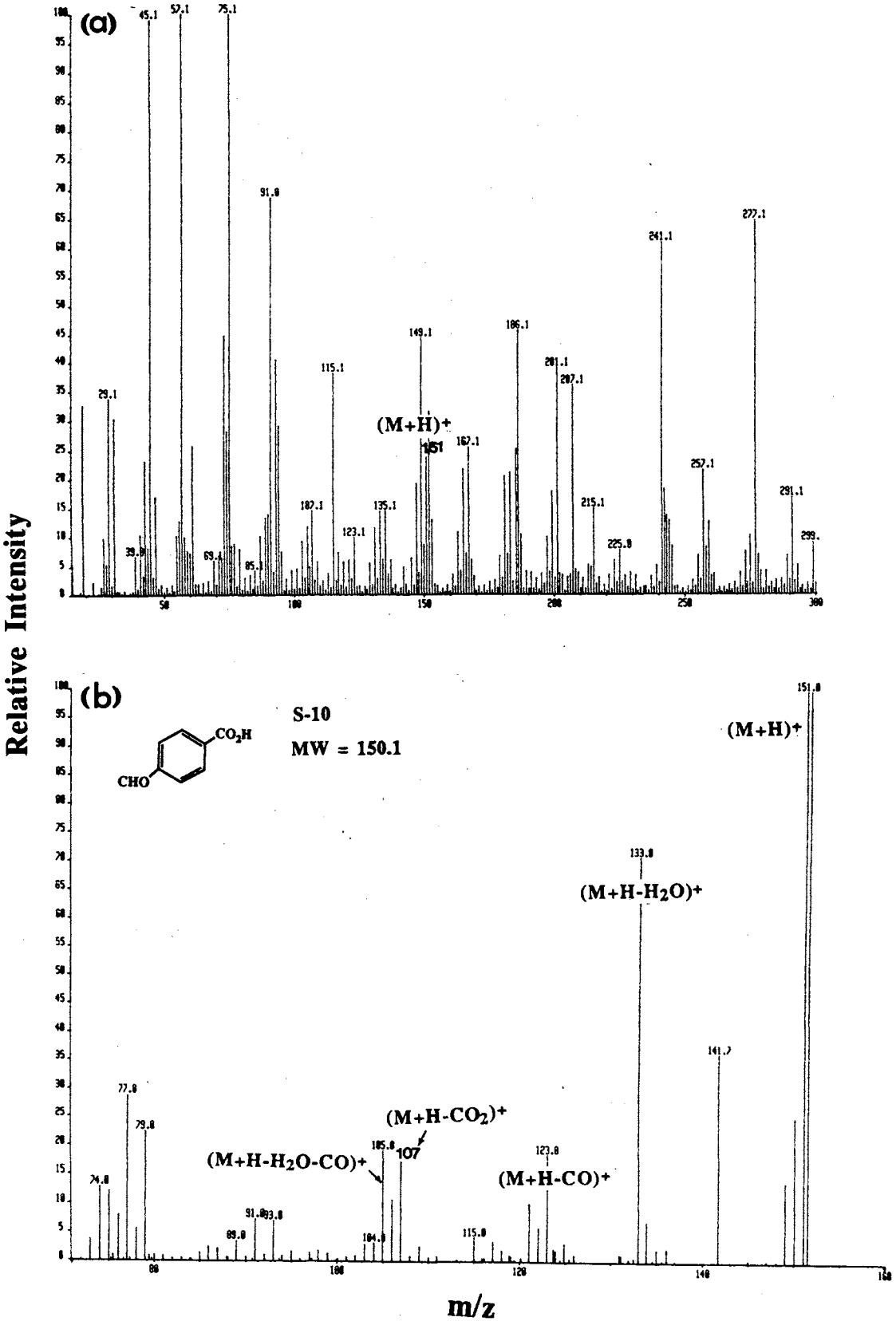

(a)
(M+H)+
151
(b)
S-10
MW = 150.1
CO2H
CHO
(M+H)+
(M+H-H2O)+
(M+H-CO2)+
107
(M+H-H2O-CO)+
(M+H-CO)+
Relative Intensity
m/z

Figure 59. LSIMS Spectra of the Standard S-13.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Positive Ionization

(b) MS/MS, B/E CAD, Positive Ionization

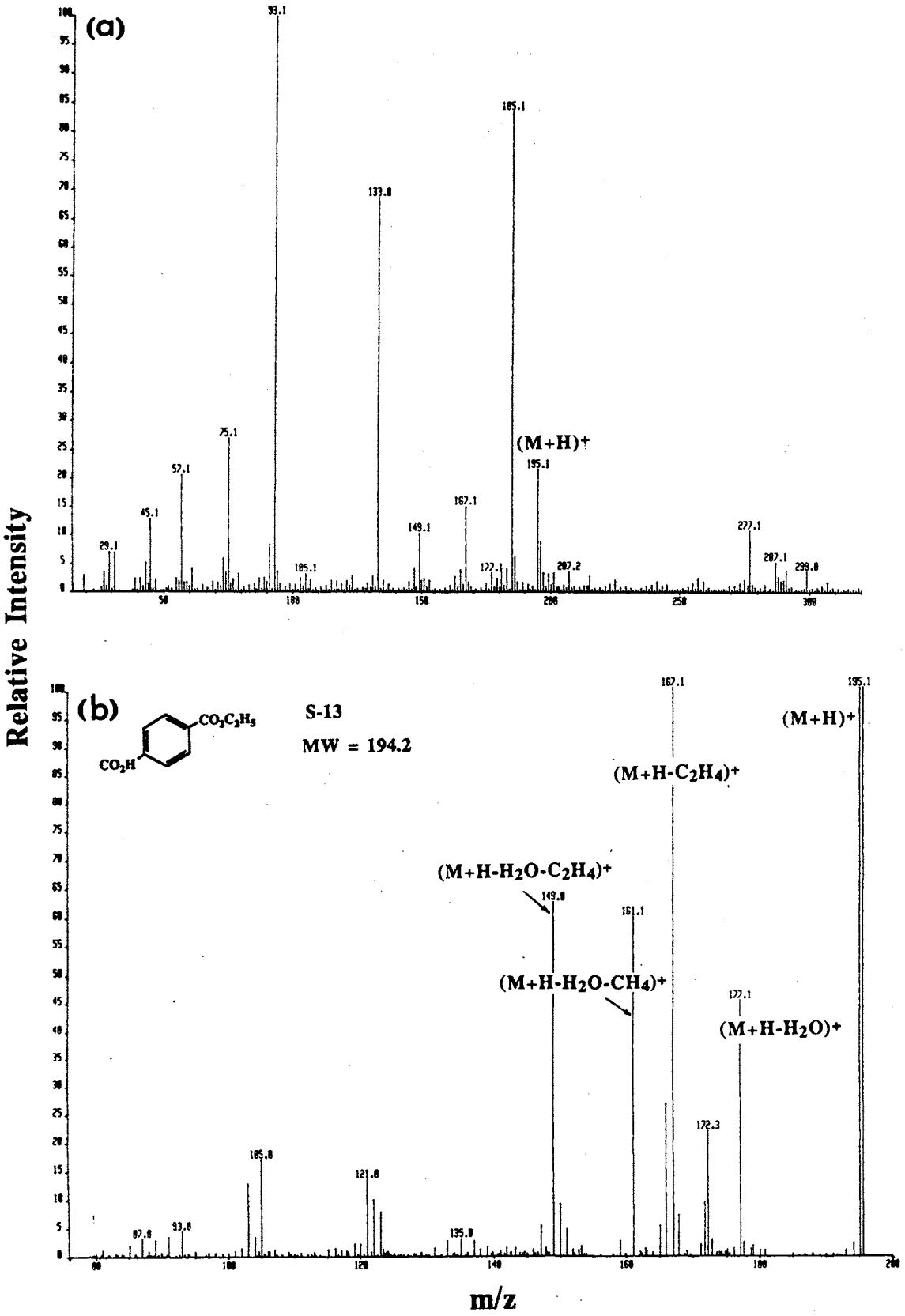

(a)
93.1
185.1
133.0
(M+H)+
195.1
75.1
57.1
45.1
167.1
149.1
277.1
29.1
185.1
172.1
287.2
287.1
299.0
(b)
CO2C2H5
CO2H
S-13
MW = 194.2
167.1
195.1
(M+H)+
(M+H-C2H4)+
(M+H-H2O-C2H4)+
149.0
161.1
(M+H-H2O-CH4)+
177.1
(M+H-H2O)+
172.3
185.0
121.0
87.0
93.0
135.0
Relative Intensity
m/z

Figure 60. LSIMS Spectra of the Standard S-2.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Negative Ionization

(b) MS/MS, B/E CAD, Negative Ionization

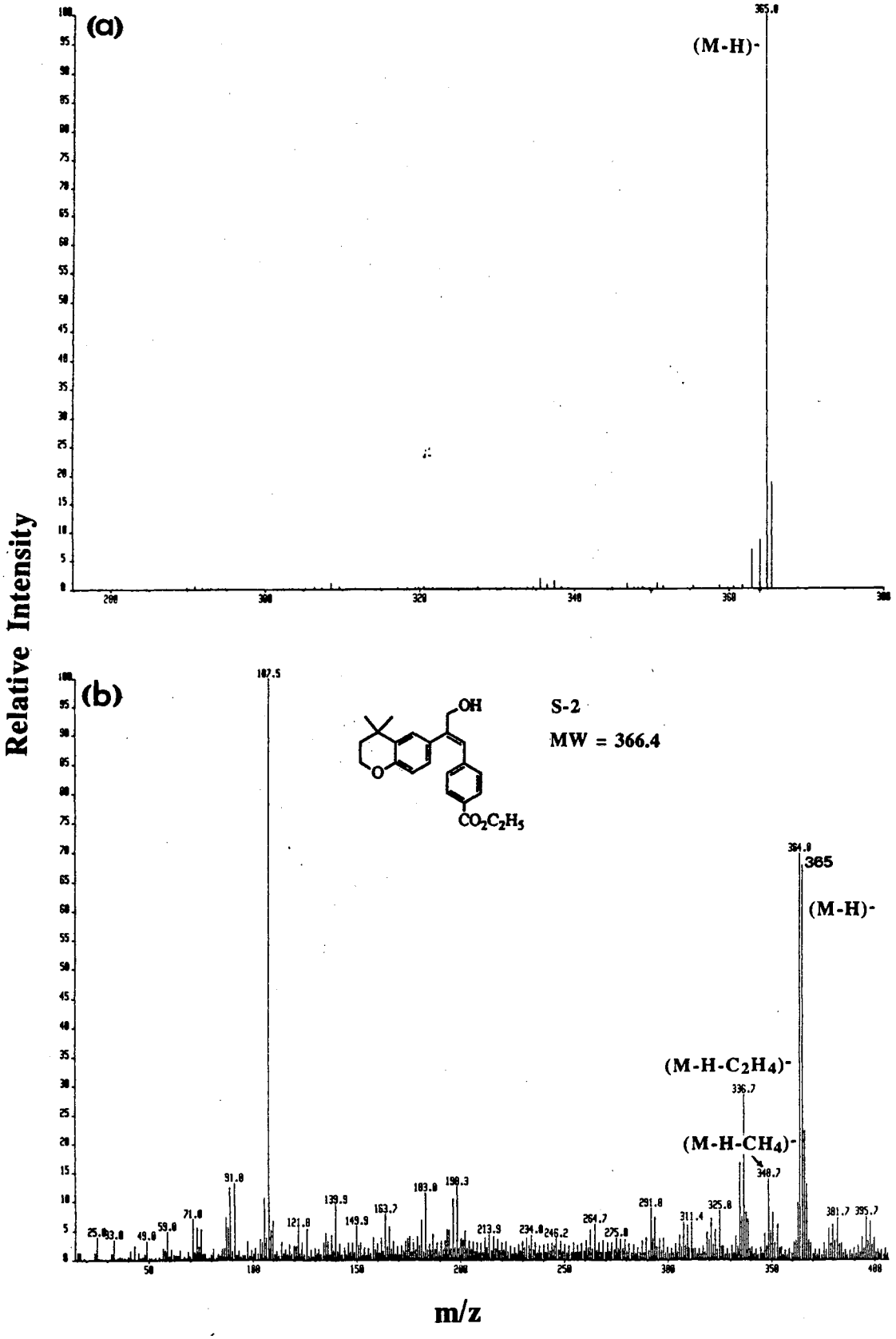

(a)
365.0
(M-H)-
(b)
182.5
OH
S-2
MW = 366.4
CO2C2H5
364.0
365
(M-H)-
(M-H-C2H4)-
336.7
(M-H-CH4)-
348.7
91.0
71.0
59.0
139.9
149.9
163.7
183.0
190.3
121.8
213.9
234.0
246.2
264.7
275.0
291.0
311.4
325.8
381.7
395.7
Relative Intensity
m/z

Figure 61. LSIMS Spectra of the Standard S-3.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Negative Ionization

(b) MS/MS, B/E CAD, Negative Ionization

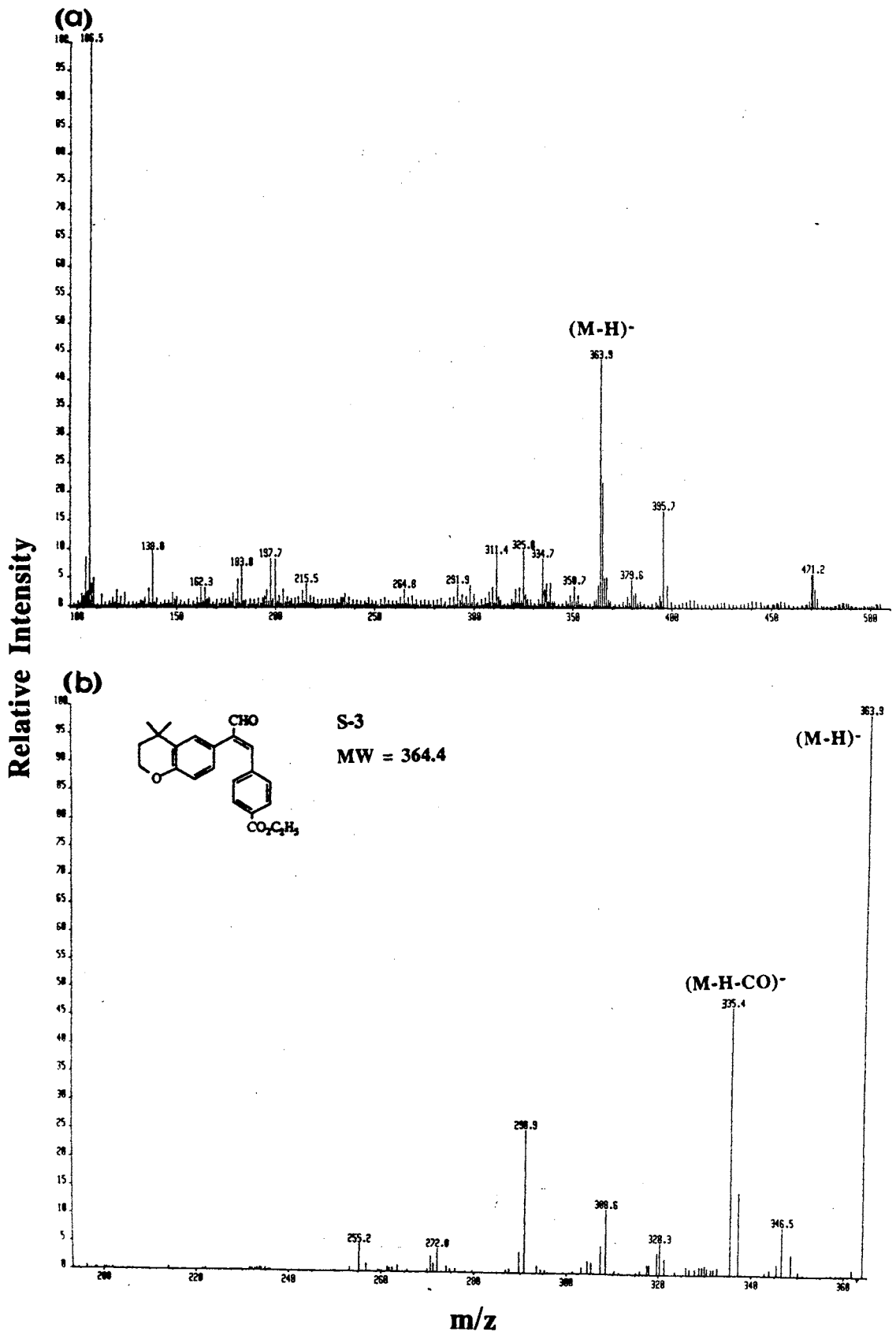

(a)
(M-H)-
363.9
395.7
471.2
(b)
S-3
MW = 364.4
CHO
$CO_2C_2H_5$
(M-H)-
363.9
(M-H-CO)-
335.4
298.9
308.6
346.5
255.2
272.0
328.3
Relative Intensity
m/z

Figure 62. LSIMS Spectra of the Standard S-4.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Negative Ionization

(b) MS/MS, B/E CAD, Negative Ionization

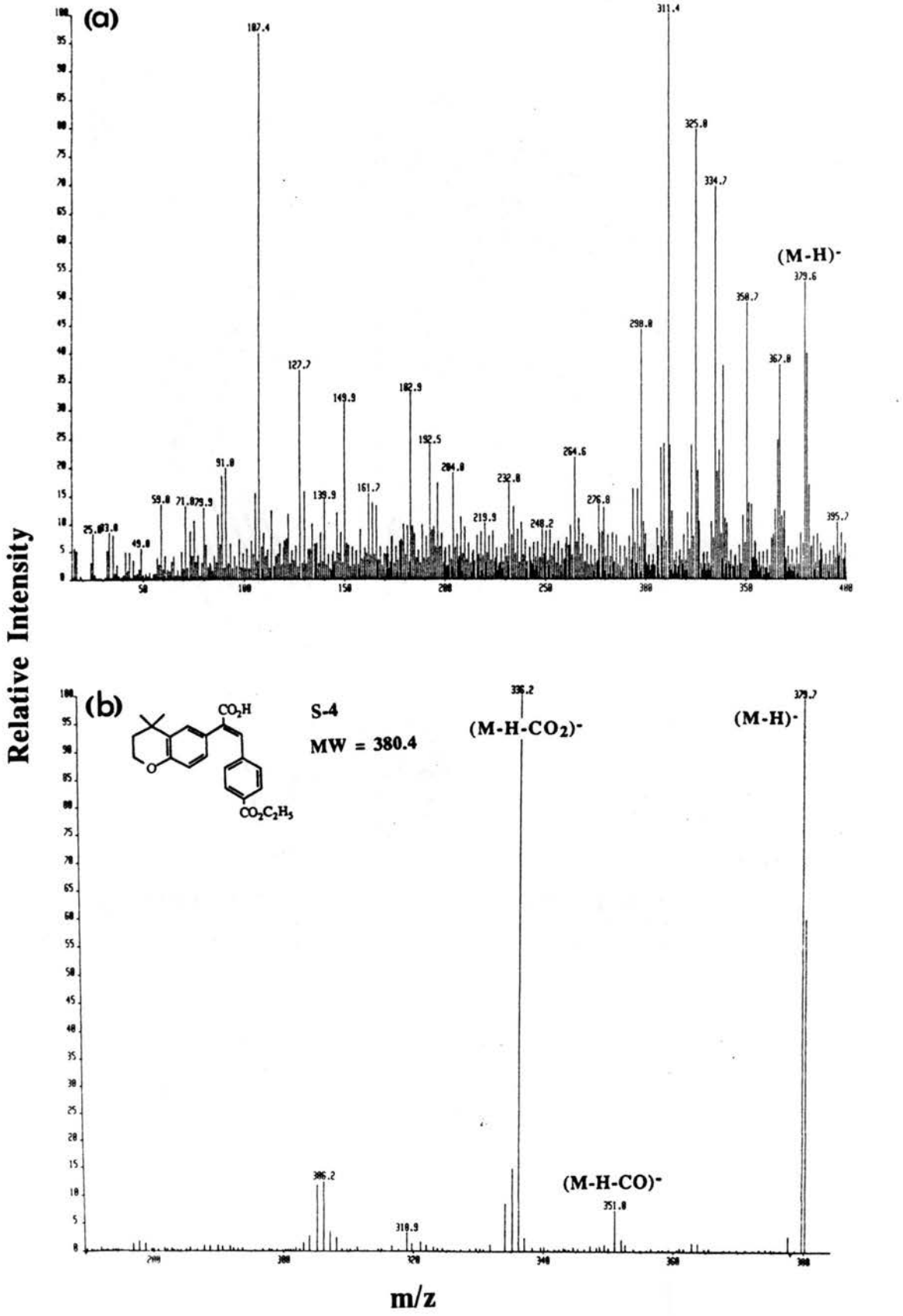

(a)
(M-H)⁻
(b)
S-4
MW = 380.4
(M-H-CO₂)⁻
(M-H)⁻
(M-H-CO)⁻
Relative Intensity
m/z

Figure 63. LSIMS Spectra of the Standard S-9.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Negative Ionization

(b) MS/MS, B/E CAD, Negative Ionization

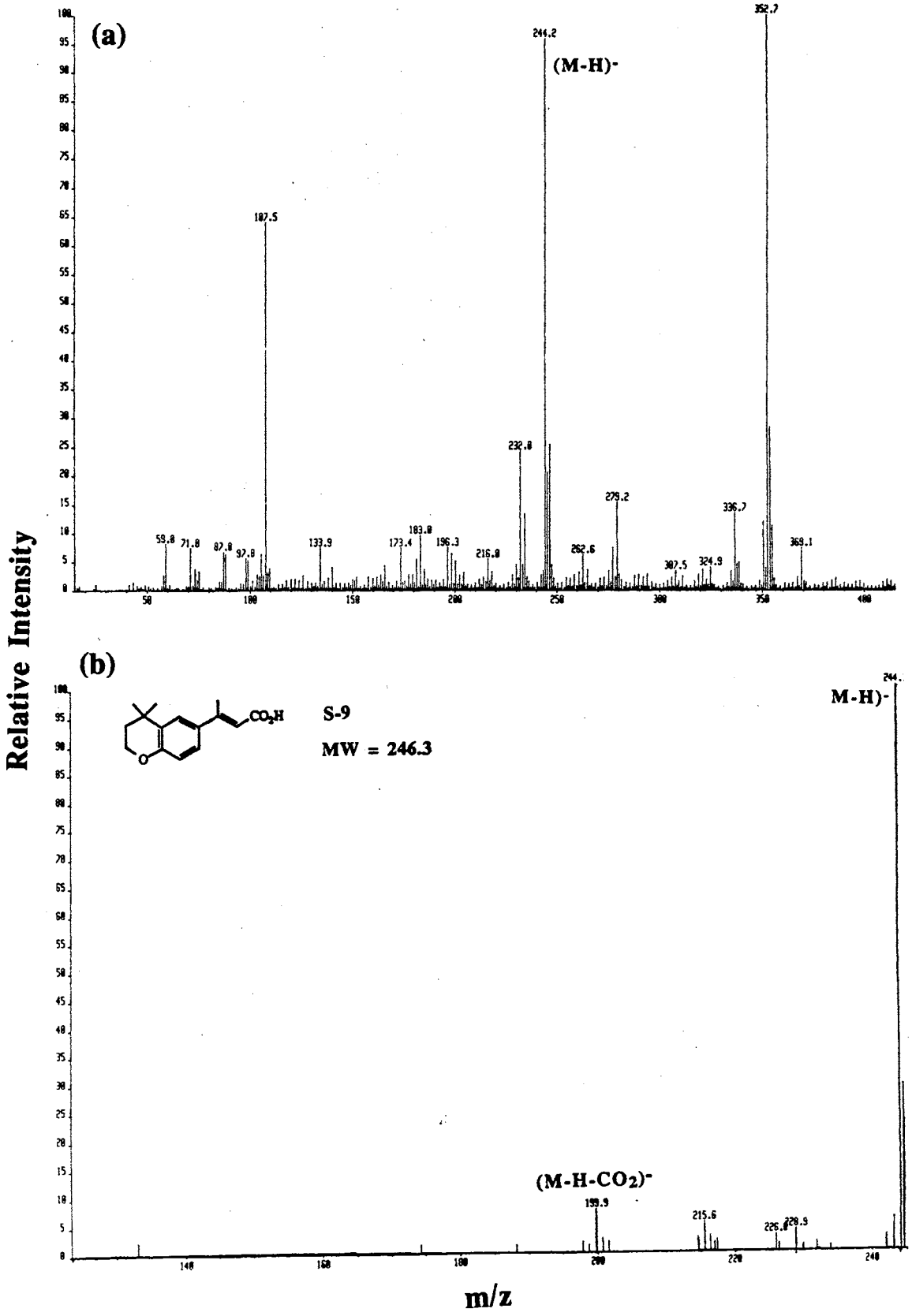
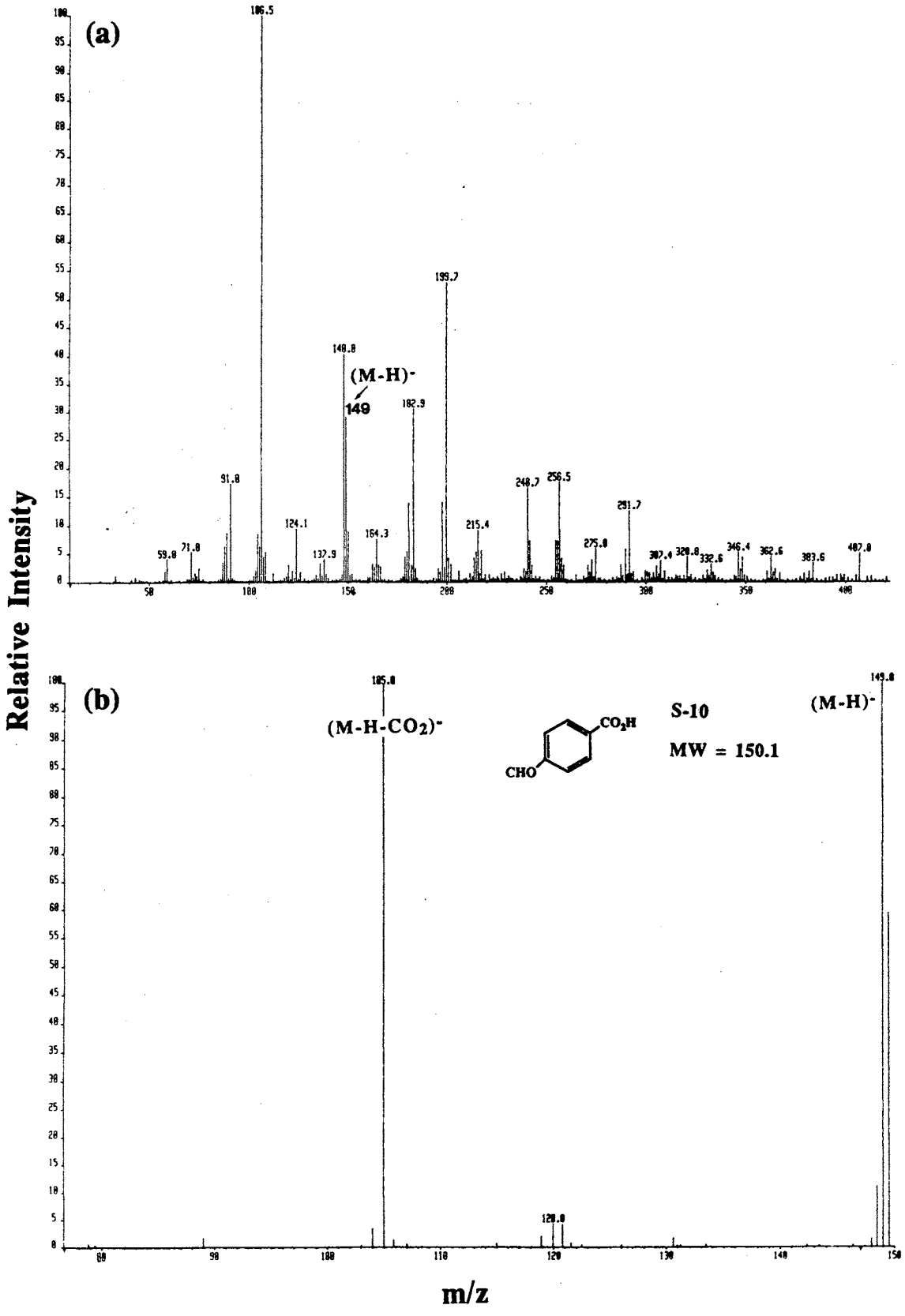

(a)
(M-H)-
244.2
352.7
187.5
232.8
279.2
336.7
(b)
S-9
MW = 246.3
CO2H
O
M-H)-
(M-H-CO2)-
Relative Intensity
m/z

Figure 64. LSIMS Spectra of the Standard S-10.

Instrument: VG-ZAB-2SE

Mode: (a) Straight, Negative Ionization

(b) MS/MS, B/E CAD, Negative Ionization


(a)
(M-H)⁻
149
(b)
(M-H-CO2)⁻
S-10
MW = 150.1
(M-H)⁻
Relative Intensity
m/z

CHAPTER VII

METABOLISM STUDY OF ETHYL (*E*)-4-[2-(3,4-DIHYDRO-4,4-DIMETHYL--2*H*-1-BENZOPYRAN-6-YL)-1-PROPENYL]BENZOATE

Introduction

Since the 1960s, major pathways of vitamin A metabolism have been discovered and recently reviewed by Frolik (1984). Although studies in this area continue, metabolites identified from many *in vitro* and *in vivo* investigations have demonstrated that esterification, conjugation, isomerization, and oxidation are the major pathways that occur during various stages of vitamin A metabolism and functioning processes (see Metabolism of Retinoids in Chapter II). As studies of synthetic retinoids have rapidly developed with the exciting prospect that they may be of clinical use in the treatment of many diseases including cancer, knowledge concerning their pharmacokinetic properties and metabolism becomes especially important. In a number of screening tests, the biological activities of the newest synthetic retinoids, the heteroarotinoids, have been shown to be comparable to or even higher than some of the retinoids that are currently being used in clinical studies (see Toxicity and Pharmacology in Chapter II). One of the most promising aspects of the heteroarotinoids is their much lowered toxicities. In the present study, methodologies in an *in vivo* study of a selected heteroarotinoid, ethyl (*E*)-4-[2-(3,4-dihydro-4,4,dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate (abbreviated as DHA in this writing, see Figure 3 for the structure), are described including the tentative identification of three major biliary metabolites.

Materials and Methods

Animals

Male rats of the Sprague-Dawley Stock (CD rats, Charles River Laboratories, Wilmington, Massachusetts) with body weights in the range of 301-325 g were purchased at least one week prior to the biliary cannulation. They were caged individually. Laboratory rodent chow (Ralston Purina, St. Louis, Missouri) and water were available *ad libitum*. The other animal housing facilities and care procedures were the same as previously described (see Materials and Methods in Chapter III).

Chemicals

The labelled and unlabeled parent heteroarotinoid, ethyl (*E*)-4-[2-(3,4-Dihydro-4,4--dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate, and its potential metabolites (standards) were synthesized by Dr. Berlin's group in the Department of Chemistry, Oklahoma State University (Waugh et al., 1985; Sunthankar et al., 1990; Sunthankar et al., 1991). The other reagents used in sample preparation and analysis were methanol (Optima grade, Fisher Scientific, Fair Lawn, New Jersey), water of either Optima grade (Fisher Scientific, Fair Lawn, New Jersey) or laboratory double distilled, and acetic acid (Tracemetal grade, Fisher Scientific, Fair Lawn, New Jersey). Compressed N_2 gas (HPLC grade, Sooner Supplies, Inc., Shawnee, Oklahoma) was used for sample condensing, and He gas (HPLC grade, Sooner Supplies, Inc., Shawnee, Oklahoma) was used for solvent degasing.

Biliary Cannulation Procedures

Detailed description of the bile duct cannulation procedures done in a standard veterinary hospital is referred to in the recent publication by Rolf et al. (1991). The same operation as conducted in a regular laboratory setting will be briefly described here. The entire procedure was done under anesthesia. Initially, the animal was lightly anesthetized

with diethyl ether in a closed container. It was then removed and placed on an operating table installed on the edge of a fume hood. The anesthesia was continued throughout the surgical procedures through a nose cone with halothane (Fluothane, Fort Dodge laboratories, Inc., Fort Dodge, Iowa).

A 4x6 cm area, centered on the midline from the xiphoid cartilage caudally, and a 1x1 cm area, centered on the points of the scapulae, were shaved with an electric clipper and scrubbed with a solution of 1:5 dilution of chlorohexidine diacetate (Nolvason, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). A 3-cm midline incision through the linea alba was made extending caudally from the xiphoid cartilage. The bile duct was located, and elevated with a mosquito hemostat. Pancreatic tissue and mesenteric fat was dissected gently with a moistened cotton-tipped applicator. A 4-0 silk (Ethicon Inc., Somerville, New Jersey) ligature was placed loosely around the distal duct to block the bile flow and cause distension of the proximal duct. An incision was made with a pair of ophthalmic scissors on the ventral surface of the bile duct. Catheters (Polyethylene, 0.58 mm I.D. x 0.96 mm O.D., Clay Adams, Parsippany, New Jersey) that were specially shaped at the tip (Rolf et al., 1991) were inserted , with one end towards the proximal aspect and the other towards the distal aspect of the bile duct. They were secured to the duct with the silk ligatures. The splinted catheters were then sutured to the body wall.

With the rat positioned laterally, a 0.5 cm stab incision was made between the scapulae. A pair of straight Crile hemostatic forceps was inserted into the incision, tunnelled down through the subcutaneous tissue, penetrated the abdominal wall, grasped the loop of the catheter, and retracted through the tunnel to the neck incision. Catheter position in the abdominal cavity was adjusted to eliminate any kinks to ensure free flow of bile.

The two incisions, one on the abdomen and one on the back of the neck, were closed with 4-0 monofilament polypropylene (Ethicon Inc., Somerville, New Jersey). The catheter segments were sutured to the skin around the neck incision with 3-0 monofilament

nylon (Ethicon Inc., Somerville, New Jersey) or silk. The catheters were trimmed to about one and a half inches in length from the neck, and the ends joined with a 22 gauge stainless steel cannula. Wounds were sprayed with Furazolidone (Topazone Aerosol Powder, Norden Laboratories, Inc., Lincoln, Nebraska).

Post-operative Animal Care

After a successful operation, the animal usually awakened within 0.5 hour, and began eating and drinking within 2 hours. The animal was given saline solution (0.45% NaCl) as its drinking water during the first 24 hours following the operation. A heating pad at medium setting was also provided for the first 1-2 nights. The recovery period took about 48 hours, during which time about 50-70 g of the body weight was lost. If no infections developed, the animals began gaining weight rapidly after the first 2 days. Skin sutures were removed in 7 days. Normal eating, drinking and steady weight-gain were the general indications of stabilized post-surgery conditions (about one week), at which time the first bile collection was performed.

The animals were under close observation daily. Any obvious problems, such as loosened or clogged tubings and minor infections, were taken care of immediately upon notification. As long as the catheter loop was positioned properly so that it did not contact the pinnae, the rats were rarely bothered by it. Close attention was paid to keep the catheter loop closed. Any spillage of bile on the skin was cleaned promptly to prevent skin irritations. Animals under good care could survive for as long as 1.5 months while bile was collected at various time intervals.

Bile Sampling Procedure

The time intervals between two bile collections were at least 3-4 days. Bile was collected for 6 hours continuously each time. Bile that was collected with no prior injection served as a blank. Exactly 500 μg of the parent compound (DHA) dissolved in 1 ml of vehicle (50% normal saline:50% aldehyde free ethanol, v:v) was injected intraperitoneally

into the rat under light sedation with diethyl ether. The metal connector of the bile-passing loop on the upper back of the rat was opened, and bile flow was immediately led into a cylinder placed in ice. The animal was allowed to move freely to retain the normal physiological status throughout the experiment. Control bile was collected in exactly the same manner after an injection of 1 ml of the vehicle.

Bile Sample Processing

The procedure for purification and isolation of metabolites from a bile sample is shown in Figure 65. A bile sample, usually about 7 ml in volume, was diluted immediately with an equal amount of methanol. The sample was either dried immediately under N_2 in a 32°C heating block or kept under a N_2 atmosphere at -20°C when storage was required. The dry bile extract was suspended in about 2 ml of methanol, and the solution was passed through a Sep-Pak Plus C_{18} Environment cartridge (Waters Chromatography Division, Millipore Corporation, Milford, Massachusetts) to remove cell wall debris and protein precipitates. The filtrate was then chilled with dry ice and filtered through a pre-chilled millipore filter (FH 0.5 μm, Millipore Corporation, Milford, Massachusetts). The filtrate was again dried under N_2 at 32°C. The 0.5 ml suspension of the sample (5% CH_3OH in 0.01 M HAC) was loaded on a pre-conditioned Sep-Pak Plus C_{18} cartridge, and eluted first with 3.0 ml of double-distilled H_2O, and then with three 1.5 ml-2.0 ml portions of solvents sequentially as indicated in Figure. 65. The rate of elution was controlled at around 0.3 ml/min. Four fractions ranging from most non-polar (Fraction-IV) to most polar (Fraction-I) were obtained. The fractions were dried under N_2 and kept either dry or in methanol at -20°C. Metabolites in each fraction were analyzed either by the on-lined HPLC Plasmaspray Liquid Chromatography/Mass Spectrometry (LC/MS) or further separated by HPLC. Individually separated metabolites were pooled and analyzed by means of UV and MS spectroscopies.

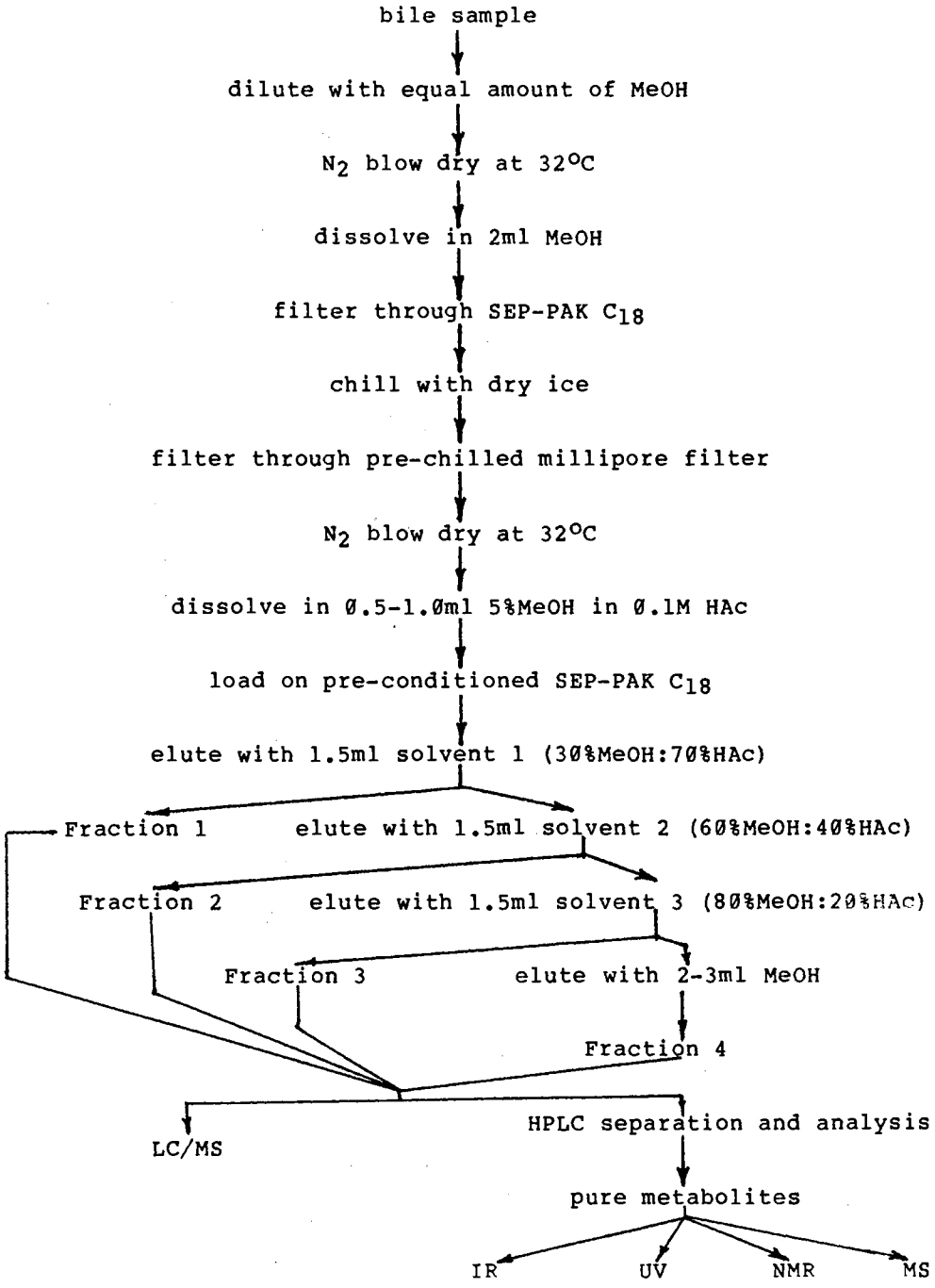


Figure 65. Procedure for Bile Sample Purification, Metabolite Isolation and Identification.

HPLC Analysis of the Synthetic Standards

Purification, separation and detection of the heteroarotinoid standards could be achieved with a Waters Associates HPLC System (Waters Associates, Milford, Massachusetts), consisting of a Model 680 automated gradient controller, 2 Model 6000A solvent delivery systems, a Model 440 dual wavelength absorbance detector (254 nm and 350 nm), and a Hewlett-Packard Model 1040A HPLC Detection System (Hewlett-Packard Co., Waldbronn, Federal Republic of Germany), consisting of an HP-1040A diode-array UV/Visible spectroscopic detector mainframe, an HP-85 Personal Computer, an HP-9021D Flexible Disc Drive and an HP-7470A Graphics Plotter. Also used in this HPLC system were a C_{18} reversed phase column (P/10 ODS 3, 0.47 x 23.5 cm, Particle size 5 μm, Whatman Inc., Clifton, New Jersey), a six-port sampling value (N60, Valco Instruments Co., Inc., Houston, Texas), and an Omni Scribe dual wavelength recorder (Model B5217-1. Houston Instrument, Austin, Texas).

Control Studies

Feasibility of the Purification Procedures. In order to test the effectiveness and the efficiency of the bile sample processing procedure presented in Figure 65, a mixture of standards was processed according to the procedure. HPLC profiles were obtained before and after the processing. The number of the peaks, the retention times and the relative intensities of the standards were compared between the two chromatograms.

Solvents. All the solvents used as the HPLC mobile phase were checked for any contamination and/or interfering UV absorption by running the solvents either individually or in gradient programs similar to those used in the real sample analyses through the HPLC analysis system.

Dosing Vehicle. The HPLC profiles of a blank bile (bile sample obtained without prior injections) and a control bile (bile sample obtained after an injection of the dosing vehicle only) from the same animal were compared to check whether or not the introduction

of the dosing vehicle (1 ml 50% normal saline:50% alcohol) interferes with the animal's normal metabolism. A principle desire of this metabolism study was to investigate the metabolism of the selected heteroarotinoid DHA under normal physiological conditions. Whenever possible, a test animal's normal physiological processes during an experiment were not to be disturbed. If the dosing vehicle interfered with the animal's normal metabolism, an altered pattern of the endogenous metabolites would be observed in comparing the HPLC profiles.

Artifacts Derived from the Parent Compound. "Artificial metabolites" might be produced simply during a bile sample processing or through interactions between the parent compound and a bile matrix at any stages of the bile sample processing procedure. Therefore, small amount of parent compound DHA was added to a control bile. The bile sample was then processed and analyzed according to the procedure. The HPLC profile was compared to that of the control bile.

Artifacts Derived from Metabolites. "Artificial metabolites" might be derived through interactions between the real metabolites and a bile matrix during sample processing. These possible interactions would result in the missing of real physiological metabolites and the detection of false metabolites. The existence of such possible artifacts was examined by adding a mixture of standards into a control bile, the mixed bile sample was then processed according to the procedure, and analyzed along with the control bile. The HPLC profiles of the mixed bile sample and the control bile were compared. Although this test could not provide an absolute answer to the possible problem since the real metabolites might react with the bile matrix differently from that of the synthetic standards. Nevertheless, before any metabolites were known, results from such a test would give a good indication of the possible existence of the problem as the standards were logically designed based on the known knowledge of the retinoid metabolism.

Variations Among Animals. Metabolism varies even among animals of the same species, resulting in different metabolites appeared in the bile samples taken from different

rats. Therefore, sample bile was always analyzed along with, and compared with the blank and control bile from the same rat.

Deterioration of Bile Samples. Alteration in the original components of a biological fluid such as bile may be due to a changing environment and/or the presence of enzymes. To prevent any possible deterioration, bile samples were always processed immediately or diluted with methanol and kept at -20°C, if storage was required. Frequent freeze-and-thaw processes were avoided whenever possible. Processed bile samples were stored dry or in methanol and purged with N_2 before storage. Samples were never left at room temperature for longer than a few minutes during analyses. Even though all these measures were taken to reduce deterioration, standards and bile samples were checked by HPLC analyses to determine the degree of deterioration.

Isomerization. One of the most distinctive nature of retinoids is that they are very sensitive to light, which induces isomerization. To prevent light-induced isomerization, all experimental procedures were done under special yellow light (Gold F40/GO, 40W, Sylvania, GTE, USA). Any samples that were brought under natural or fluorescent lighting were protected with aluminum foil.

Identification of the Biliary Metabolites by Plasmaspray LC/MS Technique

HPLC Analysis. After processing, bile samples were analyzed directly on the HPLC system described previously. A mixture of standards and the corresponding control bile samples were always included with the analyses of the biles containing metabolites. The procedure for the metabolite identification was established as follows: 1) to work out a HPLC gradient program using synthetic standards that has a good separation and is applicable to the Plasmaspray LC/MS system; 2) to distinguish metabolites of interest from the endogenous metabolites by comparing the HPLC profile of a sample bile with that of the corresponding control bile; 3) to identify the metabolites of interest by comparing their

retention times and UV spectra with that of the possible corresponding standards; and 4) to apply the same solvent gradient program in the on-line HPLC-Plasmaspray LC/MS system described earlier for mass spectrometic analysis.

Plasmaspray LC/MS Analysis. The Plasmaspray LC/MS analysis of the individual standards and a mixture of standards (see Chapter VI) established a suitable and effective program for the analysis of the biliary metabolites. Each sample bile along with its corresponding control bile and a mixture of the standards were analyzed by the same on-line HPLC-Plasmaspray LC/MS system (see Methods and Materials in Chapter VI) using the solvent gradient program developed with the standards.

Identification of the Individually Isolated Metabolites
Using LSIMS Technique

Fractionation. Owing to the malfunctioning of the on-line HPLC Plasmaspray LC/MS system after some initial identification studies described above, direct identification of metabolites in the biliary mixture became impossible. Focus was, therefore, shifted to the fractionation and isolation of individual metabolites according to the procedures shown in Figure 65. All four fractions obtained were analyzed initially by HPLC. Numerous solvent gradients were tried to establish a good program for the separation and isolation of the metabolites of interest in each fraction. For a chosen fraction, repeated separation and isolation procedures were done in a semi-preparative fashion using the HPLC system and a fraction collector (Isco Instrumentation Specialties Co., Model 328, Lincoln, Nebraska). Alternatively, fractionation was also achieved using a FOXY Fraction Collector in conjunction with a Model 2150 Peak Separator (Isco Instrumentation Specialties Co., Lincoln, Nebraska), and a Cole-Palmer Model 8373-20 Recorder.(Cole-Palmer Instrument Company, Chicago, Illinois). Fractions were immediately placed on ice and kept in the dark during a collection. The fractions containing metabolites of interest were then pooled,

and their UV spectra were obtained. Pooled samples were dried under a gentle N_2 stream in a 32° C heating block, and stored dry under N_2 at -20° C until further analysis.

Standards were grouped according to their polarities. Metabolites in each bile fraction were compared with a mixture of corresponding standards that were in the same polarity region. Both the standards and the corresponding control bile were fractionated, fractions were pooled, dried, and stored in exactly the same manner as was done to the sample bile.

Metabolites isolated from the HPLC fractionation procedures were further separated and purified by HPLC using isocratic solvent delivery programs. Two to three components were usually found in each initial peak, and were separated and collected in this step . Their UV spectra were obtained and compared with that of the standards.

LSIMS Analysis. The LISIMS technique described previously (see Methods and Materials in Chapter VI) was employed to analyze the individually isolated biliary metabolites. All isolated components in the previous HPLC separation step were subjected to the LSIMS analysis.

Desalting. In the trial LSIMS analysis of the metabolites isolated from the HPLC procedures, high content of Na^+ ions was found in the samples. As the result of this problem, no metabolite ions could be detected as the matrix-conjugated Na^+ ions overwhelmed the entire spectra (data not shown). The LSIMS analysis was, therefore, preceded by a desalting step . Considering the generally labile nature of the metabolites, a gentle rinsing instead of an ion-exchange procedure was designed to prevent any structural alterations, such as isomerization, from taking place. The desalting procedure was illustrated in Figure 66. Additionally, doubly distilled H_2O, instead of normal saline, was used for rinsing and flushing the tubings during bile sample collection to eliminate external sources of salt.

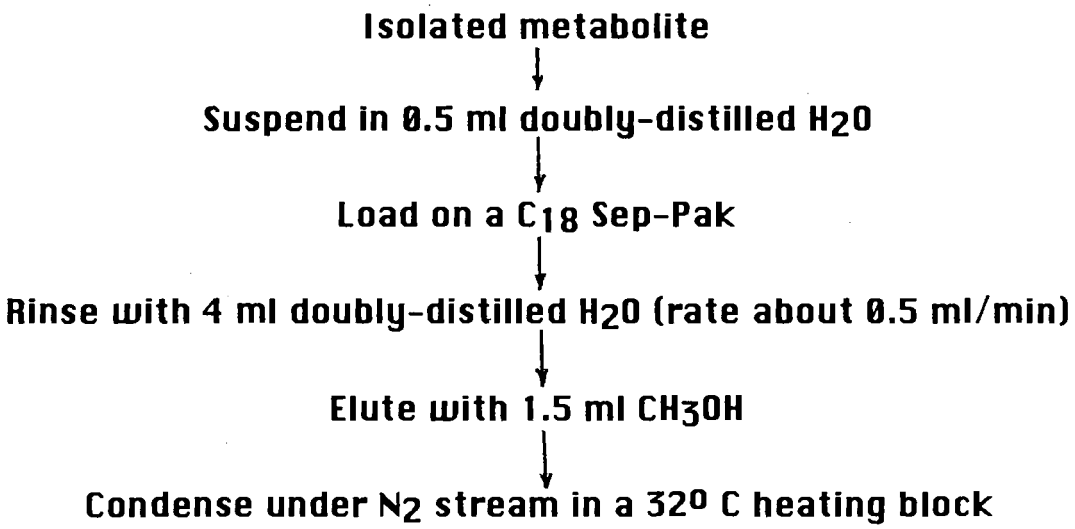


Figure 66. The Bile Sample Desalting Procedure.

Confirmatory Study Using Isotope-labelled Parent Compound

A triple-^{14}C labeled parent compound DHA* was synthesized (Sunthankar et al., 1990) and used in this preliminary study. The structure of DHA* is shown in Figure 67. The purpose of this study was to develop methodologies in metabolism studies using isotope-labelled tracers. Bile was obtained from cull chickens weighing about 2 lbs (Department of Animal Science, Oklahoma State University). No attempt was made to identify the metabolites detected in this trial because rats were to be used, according to the plan, in the main course of the metabolism study

Purification of the Labelled Parent Compound

22 mg of crude DHA* with a total radioactivity of 16.20 μci was purified by a Waters HPLC system described previously using a C_8 reversed phase column (Whatman, Spherical 5 μm, I.D. 0.46 x 12.5 cm), and an isocratic program.(70% CH_3OH:30% 0.01 M HAC). A Fisher Recordall UV recorder (Series 5000, Model D5117-1AQ, Bausch & Lomb, Houston Instrument Division, Austin, Texas) was used to record the HPLC profile. DHA* was collected at a retention time of 24 min. The DHA*-containing fractions were pooled and evaporated to dryness with a rotary evaporator (Rotavapor-R, BUCHI, Glasappatefabrik Flawil, Switzerland) in a 38° C water bath controlled by an automatic temperature controller (Buchler Instruments, Fort Lee, New Jersey). This purification process was repeated twice. Total radioactivity recovered from each purification was counted using a Packard-1900CA Liquid Scintillation Analyzer (Packard Instrument Company, Laguna Hills, California).

Administration of the Labelled Parent Compound

Purified DHA* (total radioactivity of 4.12 μci) was dissolved in 2 ml of dosing vehicle (50% normal saline:50% aldehyde free ethyl alcohol, v:v) and injected into the test chicken intraperitoneally. The control chicken was injected with 2 ml of dosing vehicle

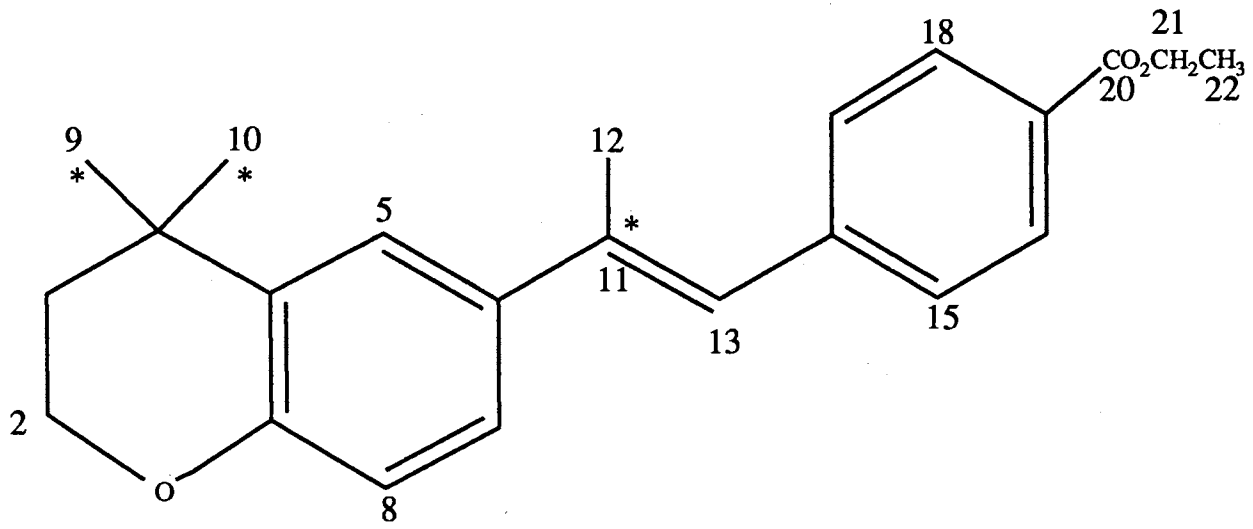


Figure 67. The Structure of a ^{14}C-labelled Diaryl Heteroarotinoid (DHA*), 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

only. The animals were given 5 ml of water through the mouth in 3 hours, and were sacrificed 6 hours after the drug administration. A total of about 1.5 ml bile and 2 pieces of liver tissue (about 20% of total liver) were collected from both chickens. A total radioactivity of 0.19 μci was recovered from the bile of the test chicken. The liver tissues were rinsed with cold distilled H_20, frozen immediately with dry ice, and stored at -20° C. All experimental procedures, including the analysis procedures described previously, were done parallell with samples collected from both the test and the control chicken.

Bile Sample Processing and Analysis

Bile samples were processed according to the procedure in Figure 65. No fractionation was done and the total bile preparation was counted as 0.095 μci. Half of the total bile preparation (containing 0.047 μci radioactive ^{14}C material) was analyzed by the same HPLC system described previously using the same C_{18} reversed phase column. About two hundred 1.0 ml fractions were collected using the automatic fraction collector , and the radioactivity of each fraction was counted by the liquid scintillation analyzer.

Liver Sample Processing and Analysis

The liver sample processing procedure is presented in Figure 68. Ten grams of frozen liver tissue from both the test chicken and the control chicken were cut into small pieces with a No.10 surgical blade and homogenized in a sequential of 15, 30, and 20 ml of mixed solvent ($CHCl_3$:CH_3OH = 2:1, v:v) for 30 seconds each time using an electric blender (Omni-Mixer, Ivan Sorvall, Inc., Norwalk, Connecticut). The homogenate was filtered through a Whatman #1 filter, and pooled. The pooled filtrate was passed through a Whatman #1 filter one more time, and a total volume of 50 ml filtrate was obtained. The liver extract was then mixed with 11 ml of normal saline in an 150ml separation funnel, shaken gently, and set for about 20 min to allow partitioning. This process was repeated three times, and the lower organic phase was separated from the upper aqueous phase.

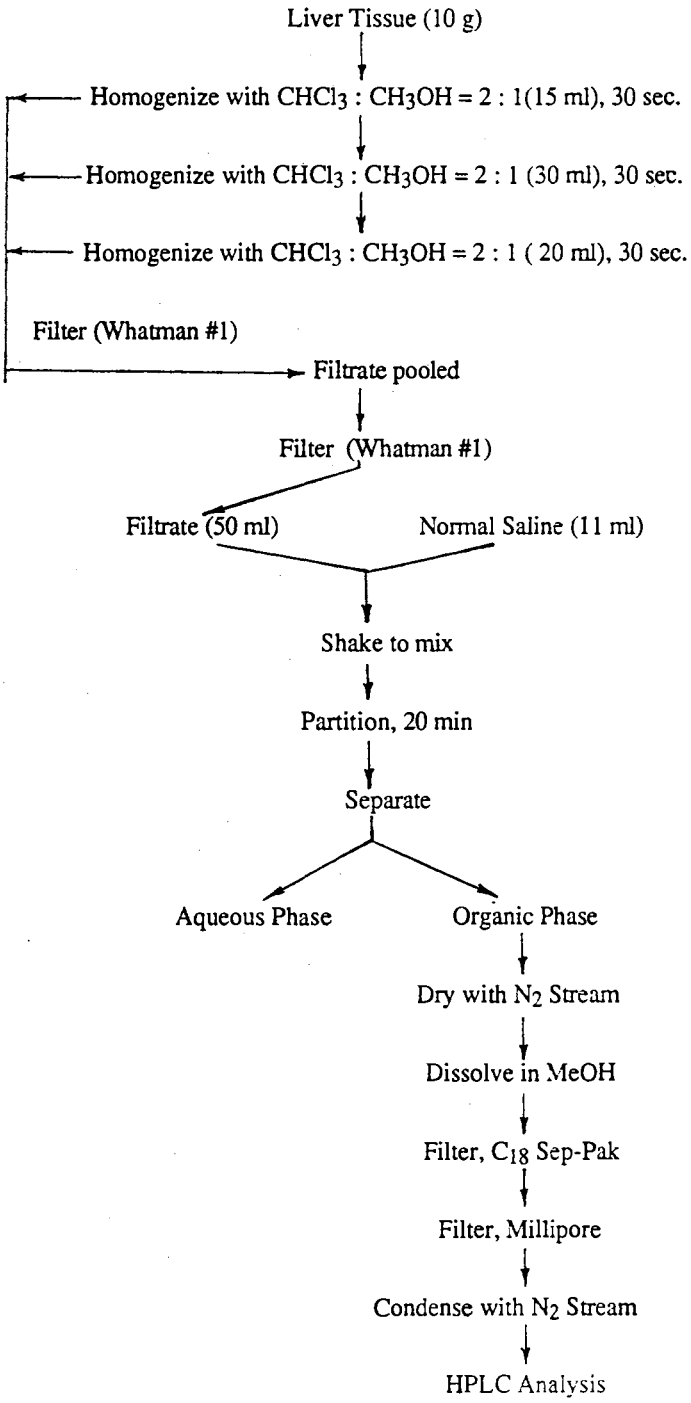


Figure 68. Procedure for the Hepatic Metabolite Extraction, Purification and Analysis.

Both phases were collected. The total volume of the organic phase was about 30 ml, and that of the aqueous phase was about 25 ml.

One milliliter out of each phase was taken to dryness under N_2 stream in a 32° C heating block. Their radioactivity was counted, and the total radioactive material in either phase was estimated. The total organic phase was taken to dryness under N_2, redissolved in CH_3OH, chilled with dry ice, and filtered through a C_{18} Sep-Pak and a millipore filter, sequentially. Sample was then condensed under N_2 at 32° C, and analyzed on HPLC using a 70% CH_3OH:30% 0.01M HAC isocratic program. Fifty seven fractions of 1ml each were collected using the automatic fraction collector, and counted in the liquid scintillation counter.

Results and Discussions

HPLC Analysis of the Synthetic Standards

All the standards listed in Table VI absorb light in the UV or near the UV region (230-320 nm). Their UV spectra were obtained using the HPLC system described previously with 254 nm and 340 nm wavelength settings on the Model 440 Dual Wavelength Absorbance Detector (spectra not shown). *Cis-* and *trans-* isomers could usually be separated and distinguished by their different UV absorbancies. Although the standards were both chemically and conformationally pure from purification procedures following their synthesis (Sunthankar et al., 1991), further purifications could be readily achieved by HPLC.

By using proper solvents and solvent gradients, mixtures of the standards were well separated and detected by the HPLC system. An example of a HPLC separation profile of a mixture of twelve standards is shown in Figure 69.

Figure 69. HPLC Separation of a Mixture of Twelve Standards.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible
Spectroscopic Detector

Detector Wavelength Setting: 254 nm

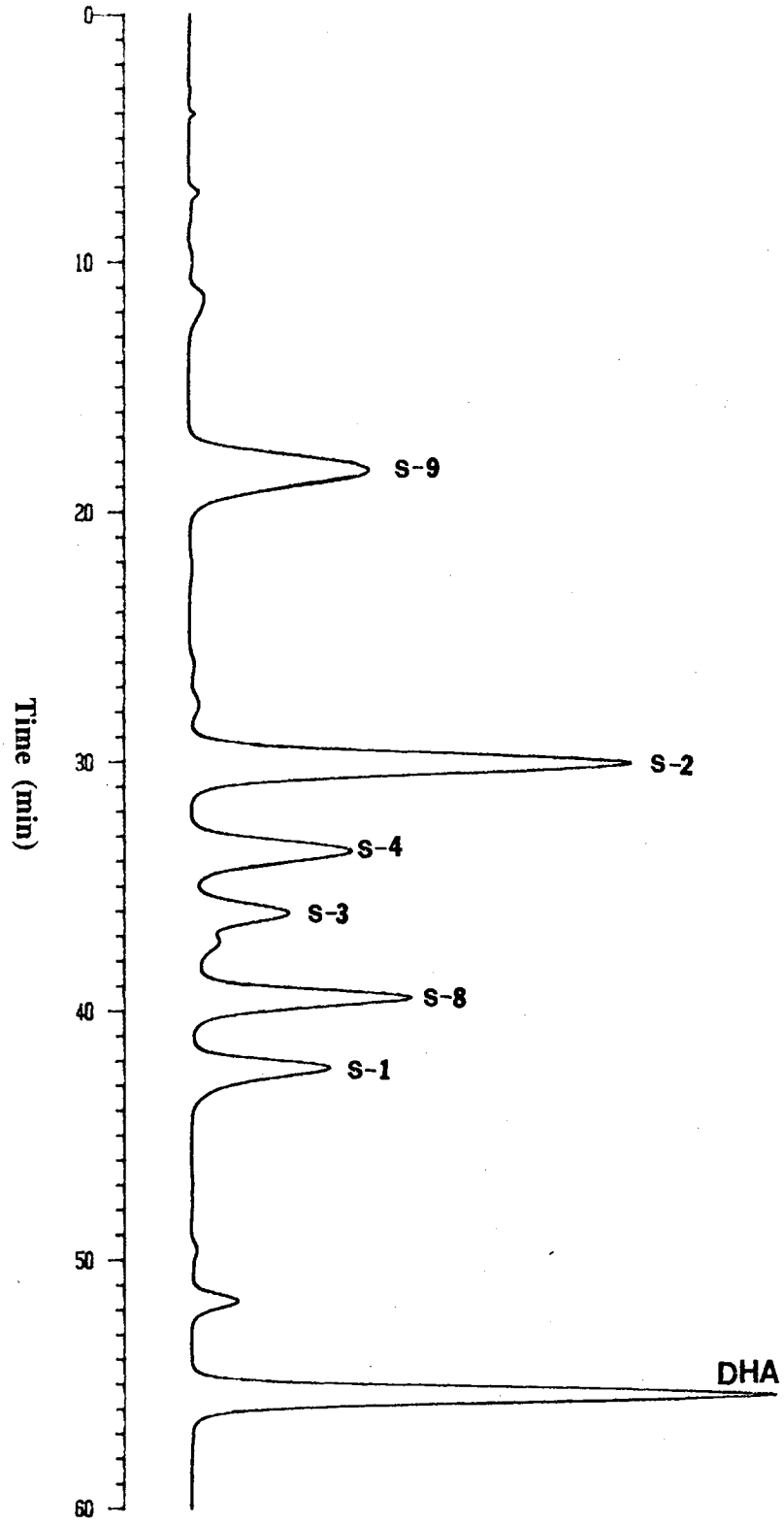
Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 72% HAC (0.01M)
28% CH_3OH Hold 15 min

Linear, 90 min

100% CH_3OH Hold 5 min


Relative Absorbance
Time (min)
0
10
20
30
40
50
60
S-9
S-2
S-4
S-3
S-8
S-1
DHA

Control Studies

The following conclusions were made from the control studies (no spectra from the control studies are shown here):

(1) The bile processing procedure shown in Figure 65 was successful when tested with standards. No contamination was introduced during the processing, and no isomerization was caused by repeating the filtration and condensation steps.

(2) Normal metabolism was not significantly disturbed by the injection of the dosing vehicle as the pattern of the major endogenous metabolites, including their retention times, and relative intensities, was not affected.

(3) No artificial "metabolites" were produced due to any interactions which might have occurred between the parent compound and the bile matrix during bile sample processing.

(4) No significant interactions occurred between the standards and the bile constituents during bile sample processing. Therefore, endogenous metabolites would not be expected to interact with the metabolites of DHA. The existence of the endogenous metabolites, however, might cause problems in the detection and isolation of the metabolites of interest since considerable overlapping was expected to occur, and most identification work was done with non-radioactive compounds.

(5) As expected, no two rats had exactly the same metabolism. It could be concluded, however, from numerous comparisons that the major endogenous components of bile samples obtained from different rats varied only slightly. More importantly, the peaks representing some major metabolites of DHA in bile from different rats remained fairly consistent in terms of the number of the metabolites, their retention times and their relative intensities. Based on these results, samples from different rats were combined after they had been individually analyzed in order to obtain large preparations of the metabolites.

(6) Deterioration of the bile samples appeared to be negligible if the measures discussed in Materials and Methods of this chapter were strictly followed. Isomerization

did not seem to be a serious problem as standards appeared to be conformationally stable if they were not exposed to light and were stored dry at -20° C.

Identification of Biliary Metabolites Using the on-line HPLC-Plasmaspray LC/MS System

Two biliary metabolites were tentatively identified, initially by HPLC analysis (Figure 70) and later confirmed by the on-line HPLC plasmaspray LC/MS technique. They are, designated as M-1 and M-2 [Figure 70(b)], identical to the standard S-1 and S-2, respectively, based on their chromatographic and spectroscopic identities. The corresponding HPLC profile of the control bile is shown in Figure 70(a). The corresponding peaks of the two standards, S-1 and S-2, can be referred to in Figure 71. The parent compound, DHA, was also recovered in the bile [peak M-P in Figure 70(b)]. The UV spectra of peak M-1, M-2, and M-P along with those of standards S-1, S-2 and DHA are presented in Figure 72 through Figure 74, respectively. The UV spectrum of the metabolite M-2 did not match the spectrum of S-2 (see Figure 73). This may be due to the fact that M-2 was not pure since the spectrum of M-2 was obtained from the analysis of the bile mixture, although it could not be excluded that M-2 and S-2 do not have the same structure. Nevertheless, the UV spectra of M-1 and M-P appeared similar to that of S-1 and DHA (Figure 72 and 74), respectively.

As a confirmatory measure, the same bile sample was spiked with a small amount of standard S-1 and S-2, respectively, and analyzed again on HPLC under the same conditions. The results supported the initial identification as the height of peak M-1 and peak M-2 increased distinctively in response to the spike with standard S-1 and S-2, respectively, [Figure 75(a) and (b)].

The bile sample was then subjected to LC/MS analysis using the same HPLC program and analytical conditions as those used in the analysis of the standards (see the previous description in Materials and Methods, Chapter VI). The reconstructed TIC of the

Figure 70. (a) Regional HPLC Profile of the Control Bile. (b) Regional HPLC Profile of the Biliary Metabolites.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible Spectroscopic Detector

Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 72% HAC (0.01M)
28% CH_3OH Hold 10 min
↓ Linear, 90 min
100% CH_3OH Hold 15 min

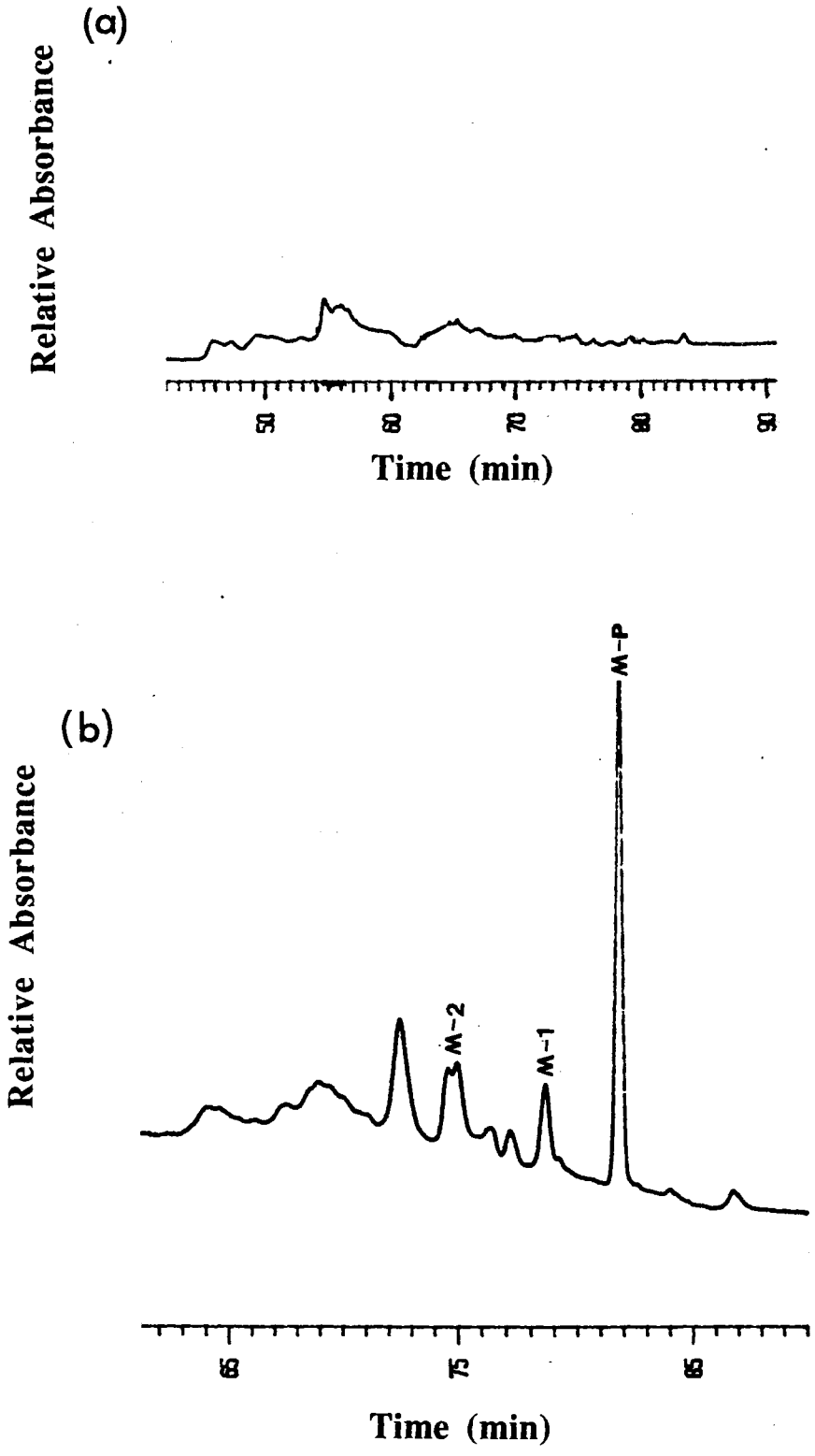

(a)
Relative Absorbance
50
60
70
80
90
Time (min)
(b)
Relative Absorbance
M-2
M-1
M-P
65
75
85
Time (min)

Figure 71. Regional HPLC Profile of the Synthetic Standards.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible
Spectroscopic Detector

Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Solvent Program: 72% HAC (0.01M)
28% CH_3OH Hold 10 min

Linear, 90 min

100% CH_3OH Hold 15 min

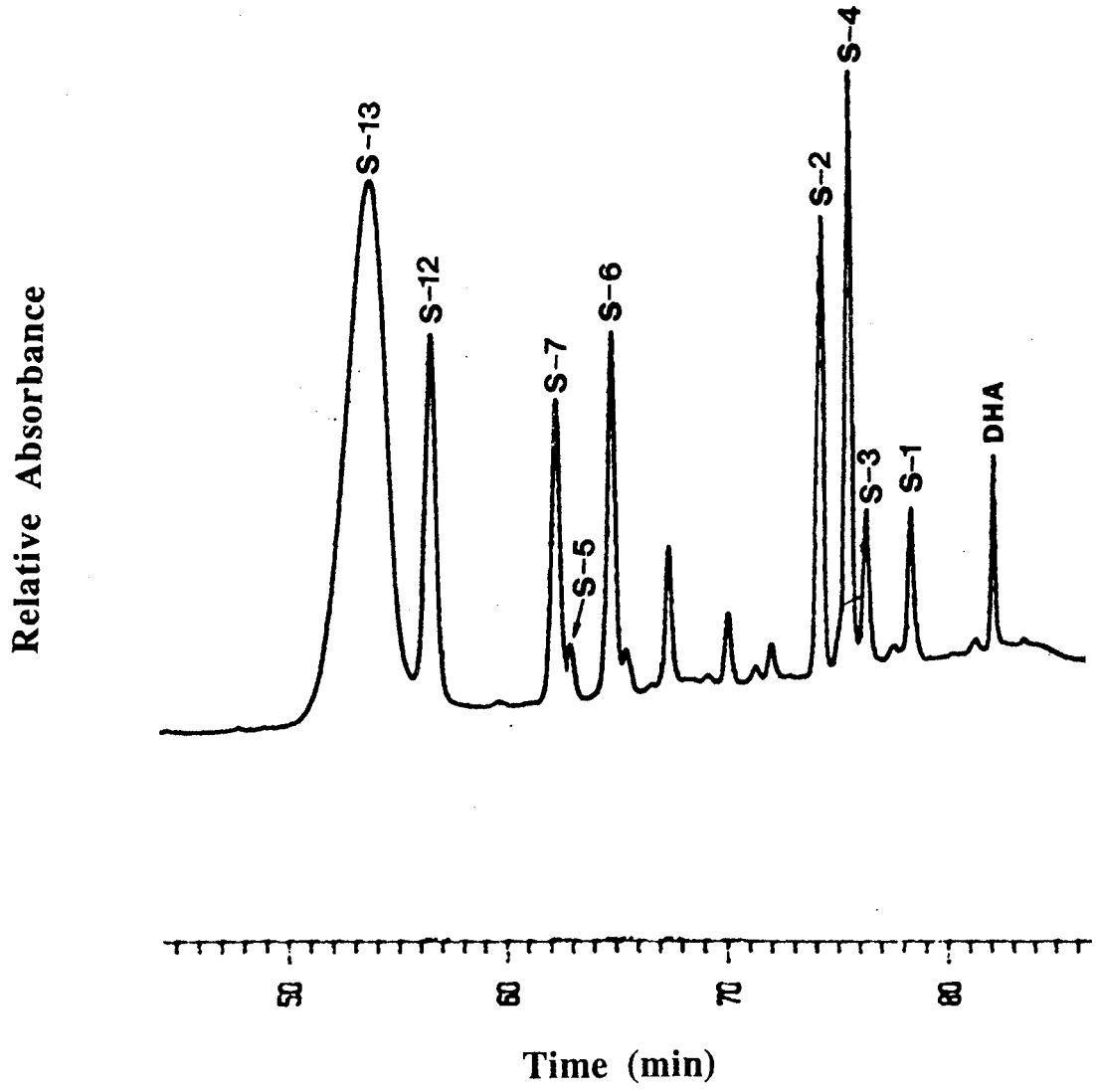

Relative Absorbance
S-13
S-12
S-7
S-5
S-6
S-2
S-4
S-3
S-1
DHA
50
60
70
80
Time (min)

Figure 72. UV Spectra of the Metabolites M-1(a), and the Standard S-1 (b).

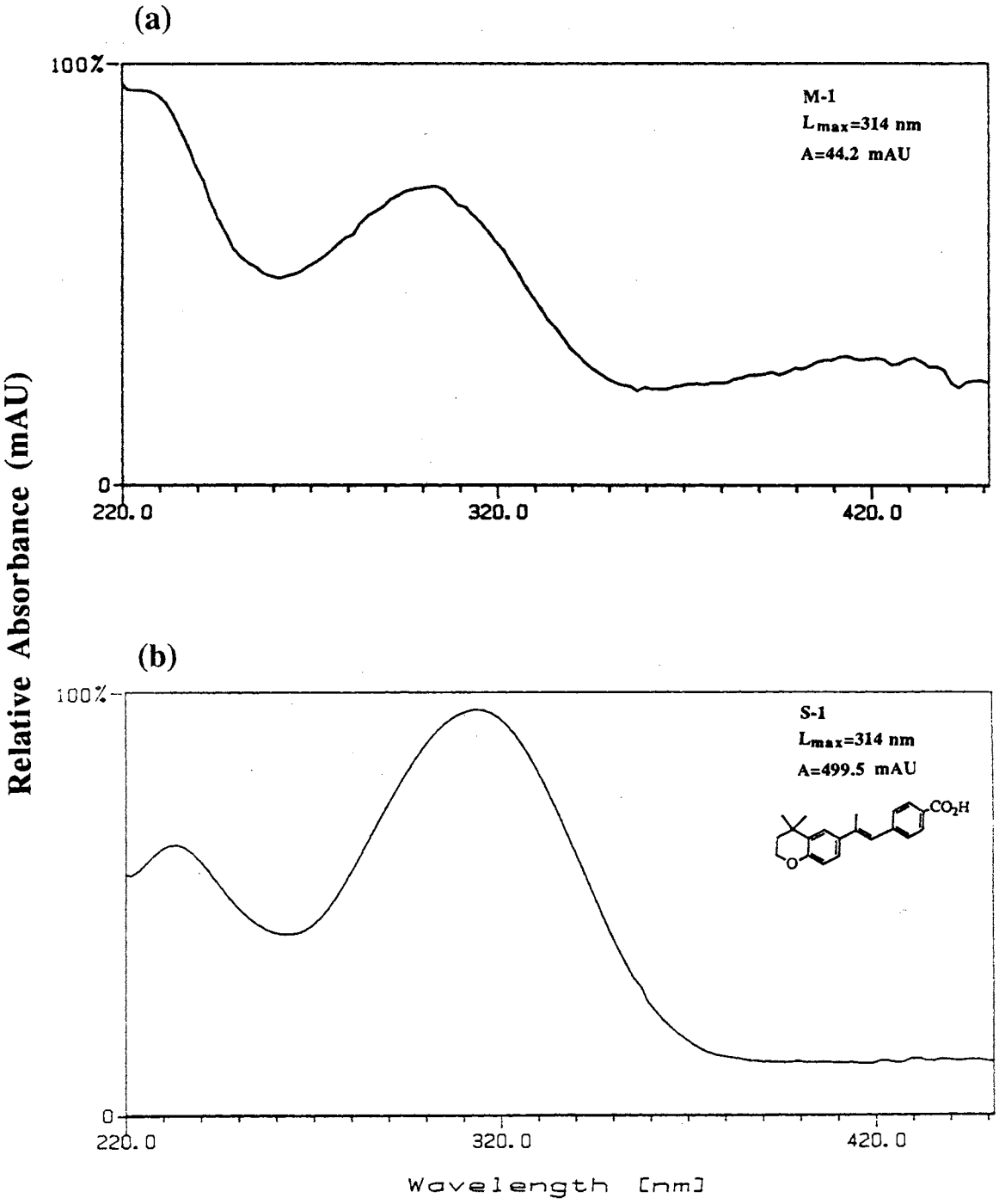

(a)
100%
0
220.0
320.0
420.0
M-1
L_{max}=314 nm
A=44.2 mAU
(b)
100%
0
220.0
320.0
420.0
S-1
L_{max}=314 nm
A=499.5 mAU
CO_2H
O
Relative Absorbance (mAU)
Wavelength [nm]

Figure 73. UV Spectra of the Metabolites M-2(a), and the Standard S-2 (b).

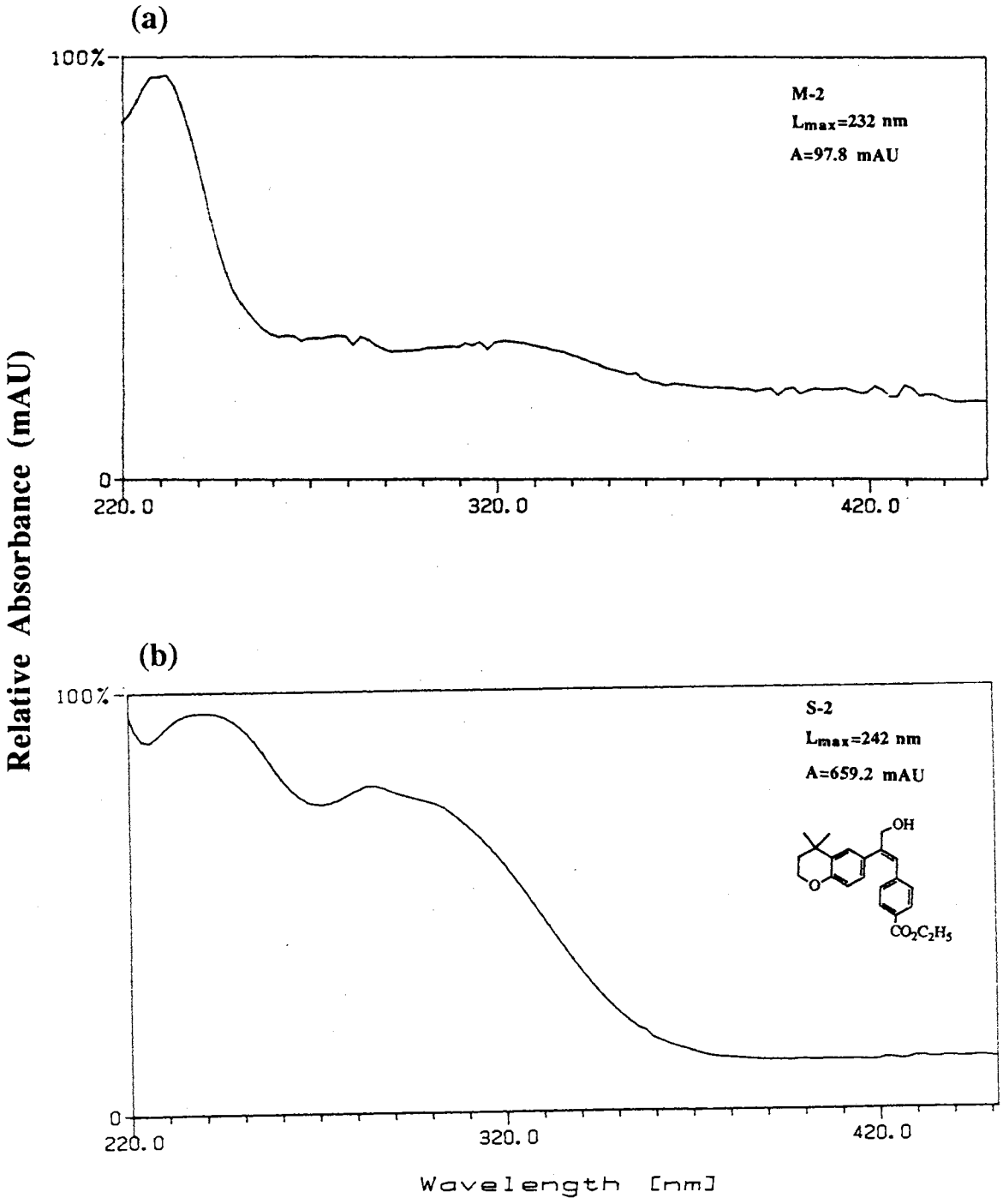

(a)
100%
0
220.0
320.0
420.0
M-2
Lmax=232 nm
A=97.8 mAU
(b)
100%
0
220.0
320.0
420.0
S-2
Lmax=242 nm
A=659.2 mAU
OH
O
CO2C2H5
Relative Absorbance (mAU)
Wavelength [nm]

Figure 74. UV Spectra of the Metabolites M-P(a), and the Standard DHA (b).

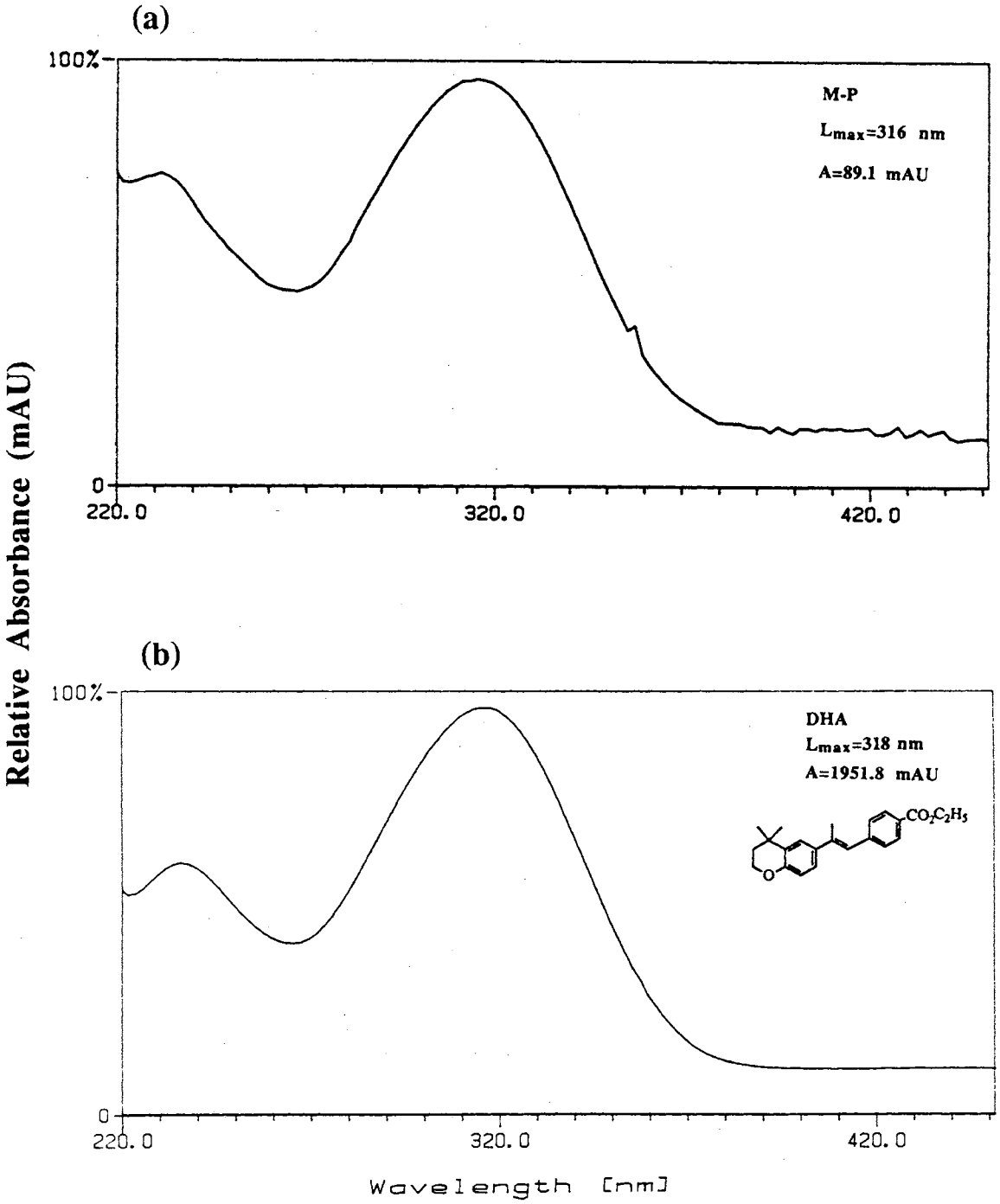

(a)
100%
0
220.0
320.0
420.0
M-P
L_{max}=316 nm
A=89.1 mAU
(b)
100%
0
220.0
320.0
420.0
DHA
L_{max}=318 nm
A=1951.8 mAU
$CO_2C_2H_5$
O
Relative Absorbance (mAU)
Wavelength [nm]

Figure 75. Regional HPLC Profiles of Biliary Metabolites Spiked with the Standard S-1 (a), and with the Standard S-2 (b).

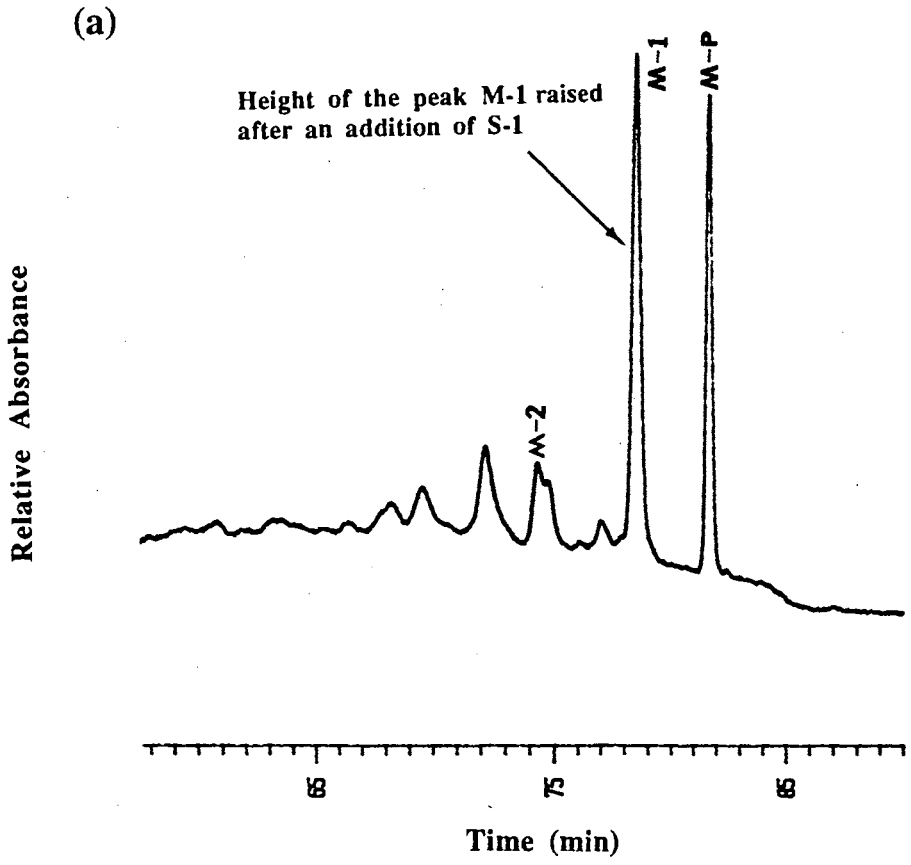
Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible Spectroscopic Detector

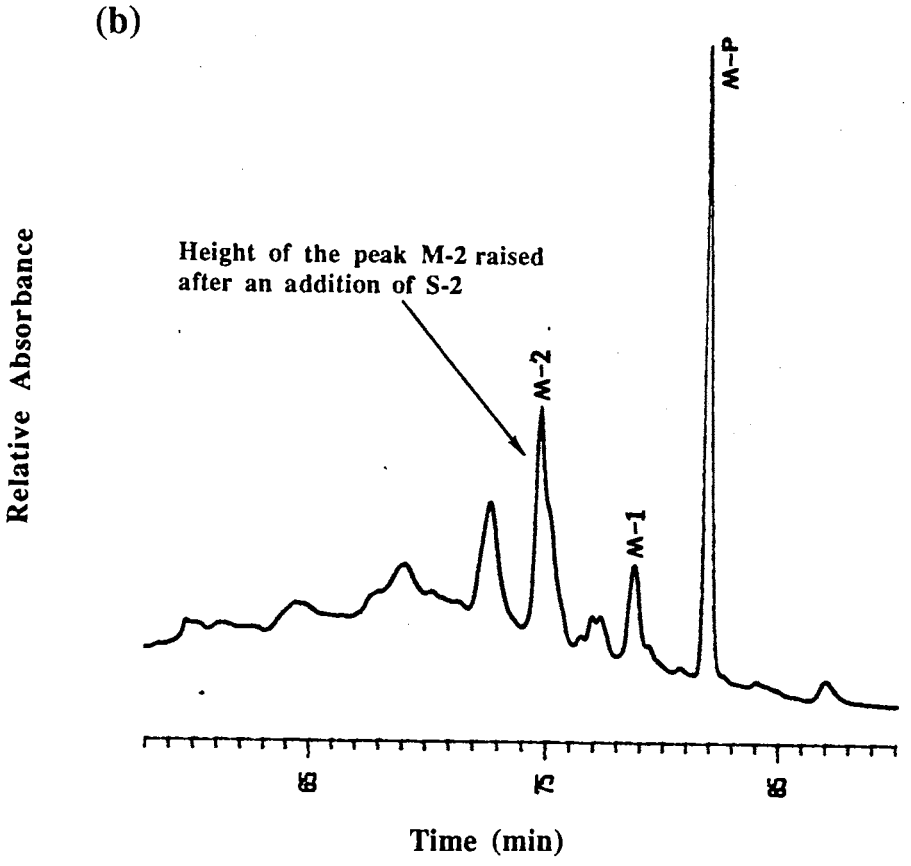
Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 72% HAC (0.01M)
28% CH_3OH Hold 10 min
↓ linear, 90 min
100% CH_3OH Hold 15 min


(a)
Height of the peak M-1 raised after an addition of S-1
M-1
M-P
M-2
Relative Absorbance
65
75
85
Time (min)


(b)
Height of the peak M-2 raised after an addition of S-2
M-P
M-2
M-1
Relative Absorbance
65
75
85
Time (min)

bile sample is shown in Figure 76. The metabolites M-1 and M-2 were detected. Metabolite M-P (recovered DHA), however, was not detected. Although Peak M-2 was relatively small and almost undetectable in the entire TIC, it could be seen in the enlarged portion of the chromatogram (see Figure 76 insert). The mass spectra of M-1 and M-2 along with the mass spectra of their corresponding standards, S-1 and S-2, are presented in Figure 77 and Figure 78, respectively. It can be seen that the mass spectrum of M-1 [Figure 77(a)], including the molecular ion m/z = 337 $[M + H + CH_3OH - H_2O]^+$, and the two major fragment ions, m/z = 323 $[M + H]^+$ (the existence of the peak at m/z = 323 is clearly seen, although the peak at m/z = 322 is more intense) and m/z =305 $[M + H - H_2O]^+$, matches well with that of the standard S-1 [Figure 77(b)]. It was noticed that the retention time of M-1 was a few minutes later (see Figure 76) than that of S-1 (see Figure 37). The delay was probably due to electronic drift , which is not uncommon, from one run to another. The retention time of M-2 is very close to that of S-2 (see Figure 76 and Figure 37). Fewer peaks are shown in the mass spectrum of M-2 when compared to that of S-2 [Figure 78(a) and (b)]. Nevertheless, the molecular ion, m/z = 367 $[M + H]^+$, and one of the major fragment peaks, m/z = 303 $[M + H - H_2O - C_2H_5OH]^+$ [Figure 78(a)], match well with those in the spectrum of S-2 [Figure 78(b)]. It was not too surprising that the spectrum of M-2 was poor, however, since the signal of M-2 was weak and might not be yielding a satisfactory spectrum.

Two other major metabolites of DHA were also detected. They were labelled as UM-1 at scan 2951 and UM-2 at scan 2250, respectively, in Figure 76 (UM stands for unknown metabolite). The mass spectra of these two unknown metabolites are shown in Figure 79 and Figure 80, respectively. Neither the retention times nor the mass spectra of these two compounds match those of the synthetic standards.

Similar results were obtained in a second analysis (data not shown), but additional trials failed owing to the malfunctioning of the Plasmaspray LC/MS system. Unfortunately, the on-line HPLC-Plasmaspray LC/MS system was never restored to

Figure 76. Reconstructed Total Ion Chromatogram (TIC) of the Biliary Metabolites.

Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm
Flow Rate: 1.0 ml/min
Solvent Program: 80% HAC (0.01M)
20% CH_3OH Hold 10 min
↓ Linear, 120 min
100% CH_3OH Hold 20 min

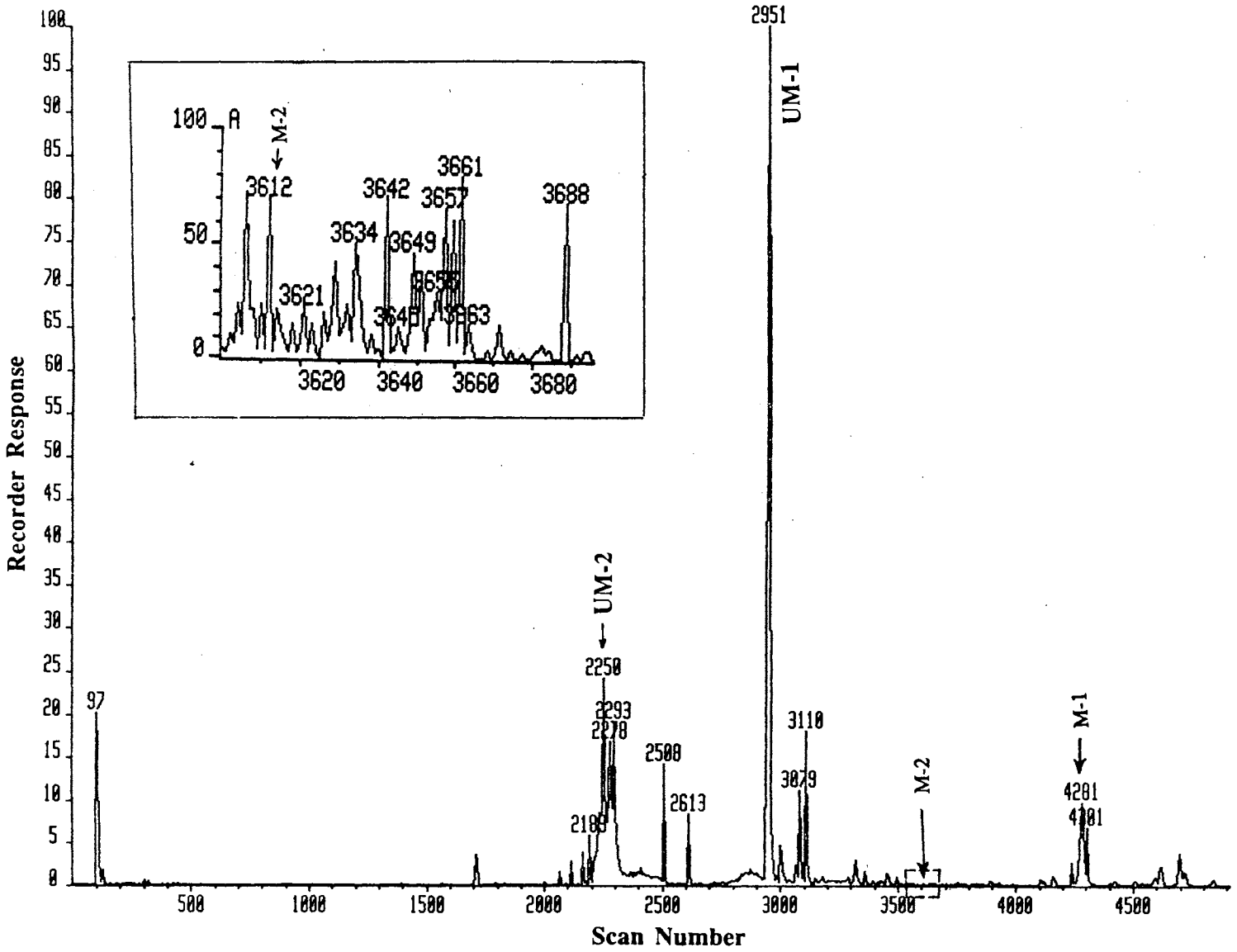

Recorder Response
Scan Number
2951
UM-1
← UM-2
2250
2293
2278
2500
2613
2188
97
3110
3079
M-2
M-1
4281
4381
A
← M-2
3612
3642
3661
3657
3688
3634
3649
3621
3645
3663
3620
3640
3660
3680

Figure 77. The Plasmaspray LC/MS Spectra of the Metabolite M-1 (a), and the Standard S-1(b).

Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250

Mode: Positive Ionization

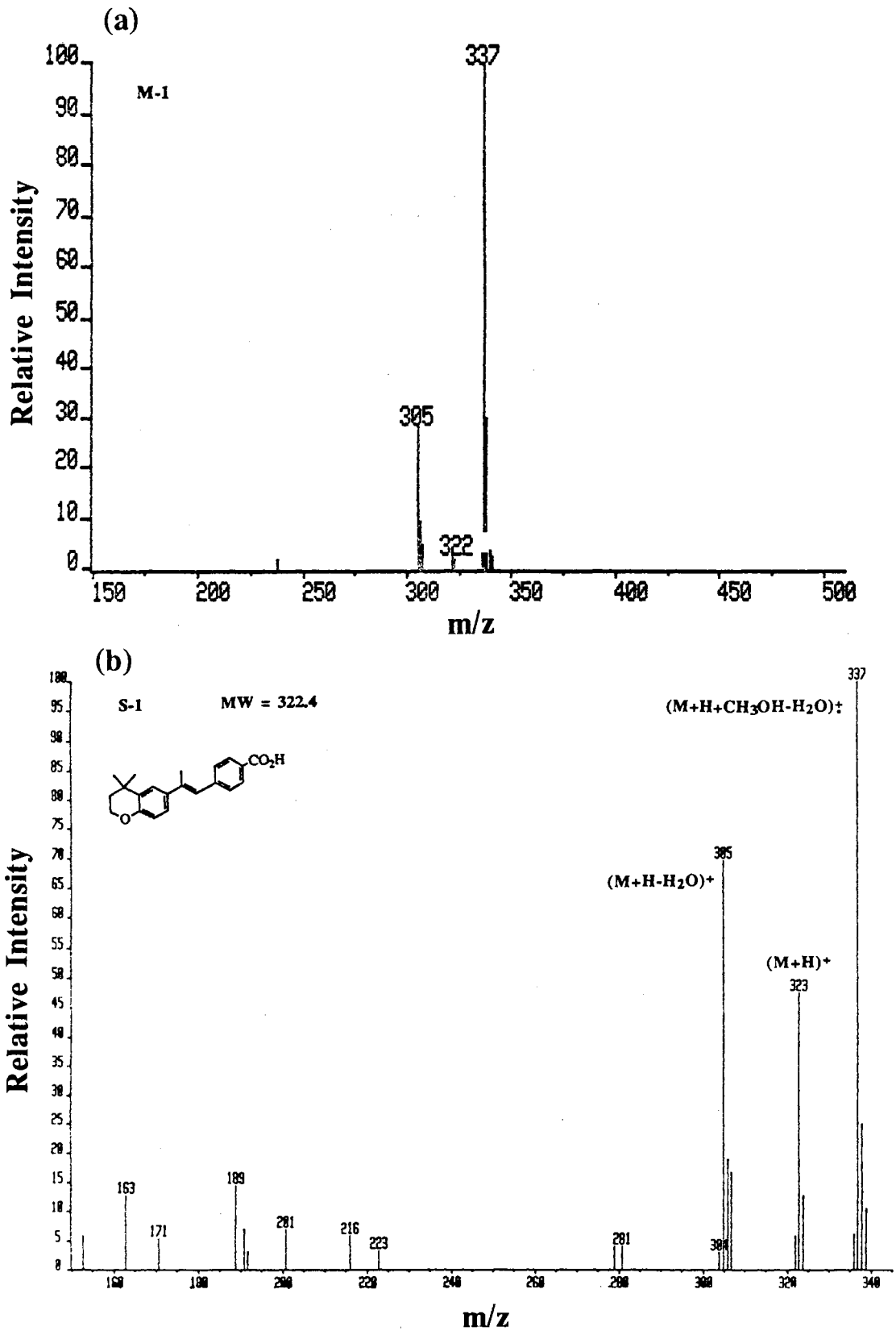
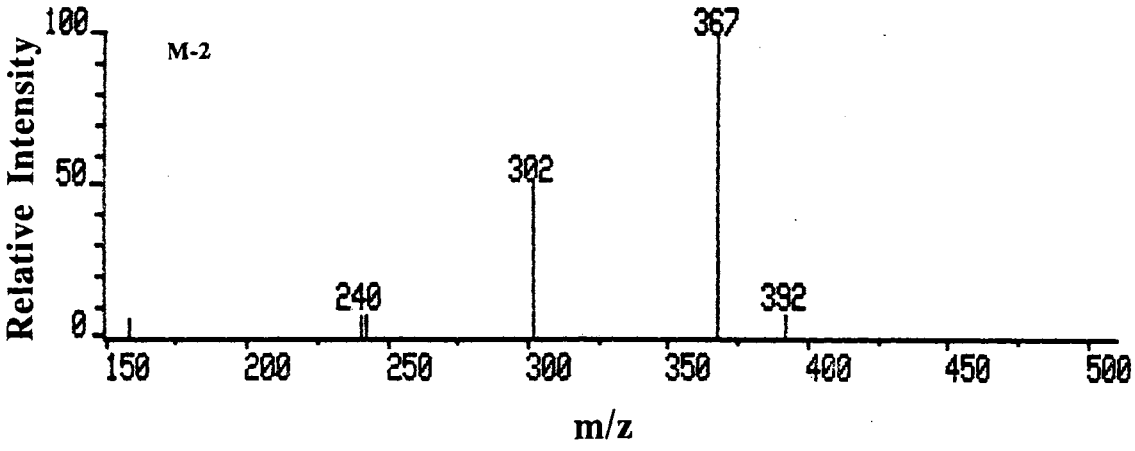

(a)
M-1
337
305
322
Relative Intensity
m/z
(b)
S-1
MW = 322.4
CO2H
(M+H+CH3OH-H2O)+
(M+H-H2O)+
(M+H)+
337
305
323
163
171
189
281
216
223
281
Relative Intensity
m/z

Figure 78. The Plasmaspray LC/MS Spectra of the Metabolite M-2(a), and the Standard S-2(b).

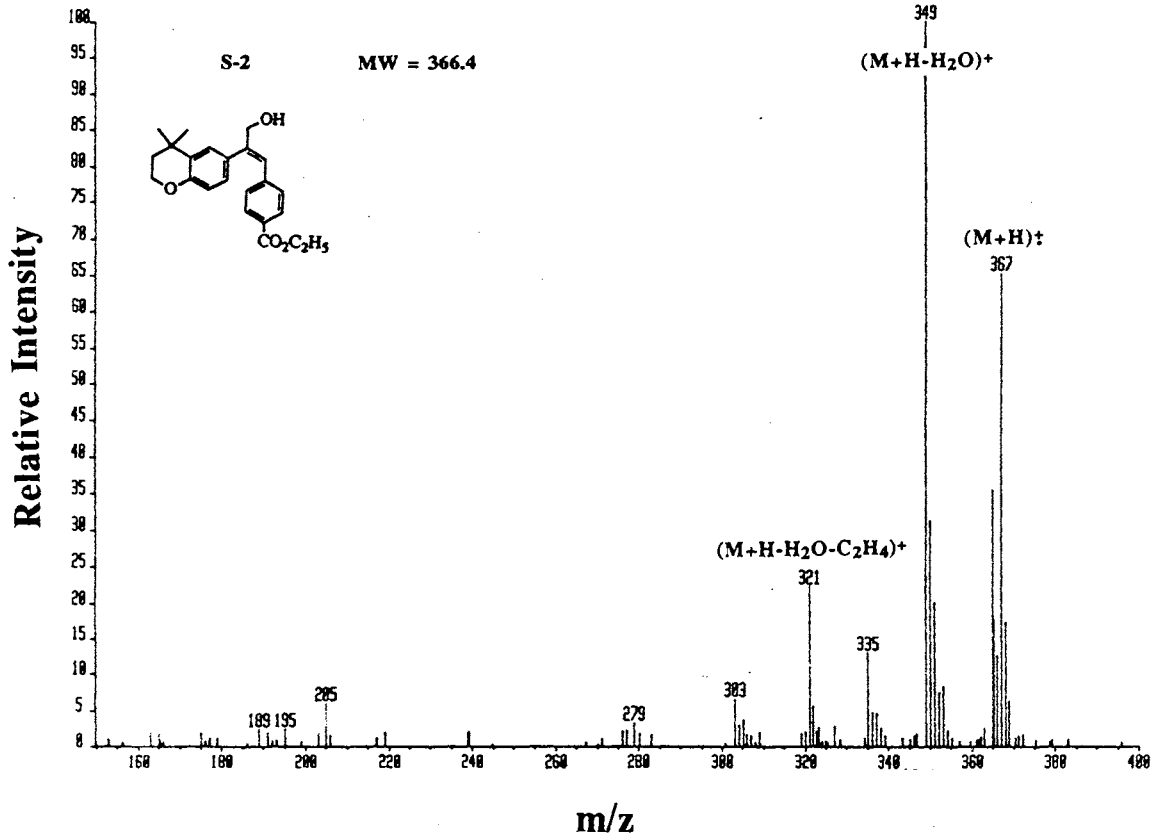
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250

Mode: Positive Ionization

(a)


Relative Intensity
100
50
0
M-2
367
302
240
392
150
200
250
300
350
400
450
500
m/z

(b)


Relative Intensity
S-2
MW = 366.4
OH
O
CO2C2H5
349
(M+H-H2O)+
(M+H)+
367
(M+H-H2O-C2H4)+
321
335
303
285
279
189 195
m/z

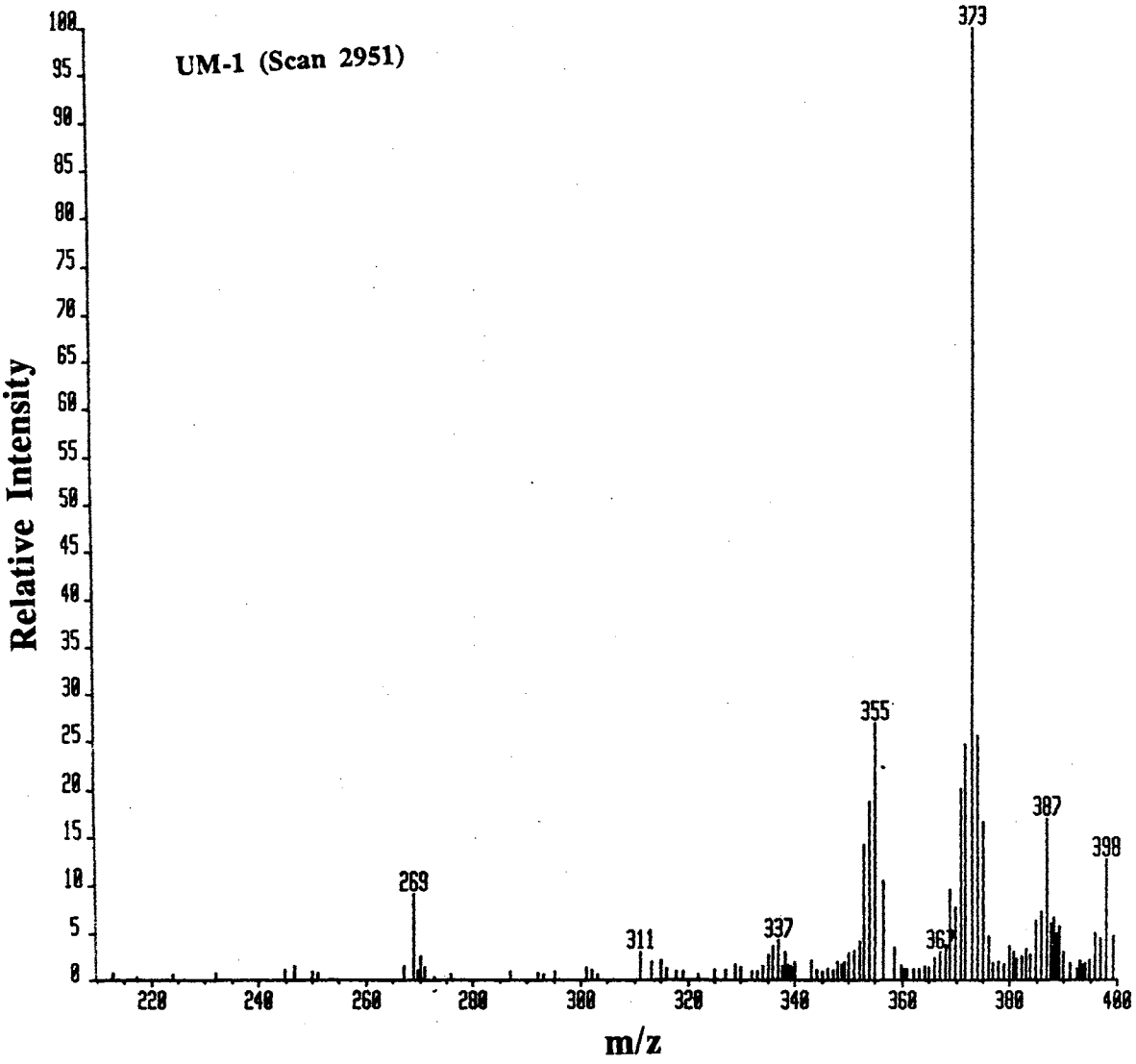


Figure 79. The Plasmaspray LC/MS Spectrum of the Metabolite UM-1.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

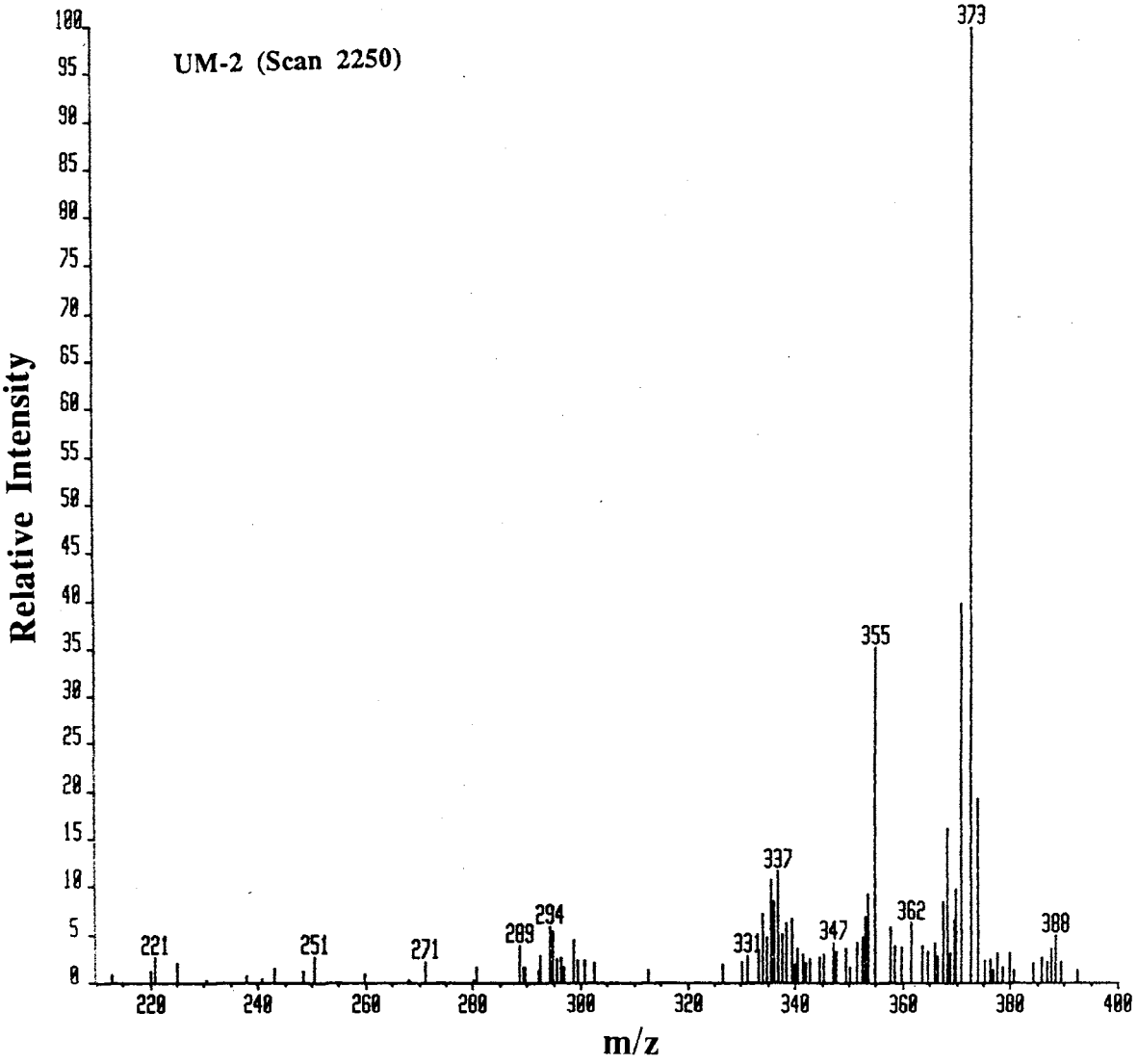


Figure 80. The Plasmaspray LC/MS Spectrum of the Metabolite UM-2.
Instrument: Waters-6000 HPLC System
Plasmaspray LC/MS, VG-TS 250
Mode: Positive Ionization

normal. Experience from these efforts, however, gave us other useful information. It was clear that direct analysis of a complicated biological mixture, such as a bile sample, by LC/MS was technically difficult although it is ideal and possible. In addition, a considerable amount of sample was needed (over a hundred times more than the amount applied on HPLC) in order for some metabolites to provide strong enough signals for MS analysis. This was a high demand since most of the metabolites existed only in trace amounts in the bile matrix.

Identification of the Individually Isolated Metabolites by LSIMS

Fractionation. According to the bile processing procedure (Figure 65), the metabolites were fractionated into four fractions based on their polarities. From the non-polar fraction (Fraction IV), only the parent compound (DHA) was detected (data not shown). The existence of drug metabolites in Fraction I and II was obvious. Identification of these metabolites without the use of radioactive tracers, however, was extremely difficult due to extensive overlapping from endogenous metabolites (data not shown).

The HPLC profile of Fraction III is presented in Figure 81. The corresponding fraction of the control bile and the standards in this region are shown in Figure 82 and 83,, respectively. A few metabolites or clusters of metabolites, labeled as BM-1 to BM-6, were apparently from the parent compound in this fraction, and they were well separated and detected (Figure 81). Direct comparison of retention times between these metabolites, BM-1 through BM-6 (Figure 81), with the corresponding seven standards (Figure 83) revealed that if the entire chromatogram of Fraction III was shifted 1-2 min towards the later time, the retention time of BM-2 was very close to that of S-2. Likewise, the retention times of BM-3, BM-4, BM-5, and BM-6 were very close to that of S-4, S-8, S-1, and DHA, respectively (see Figure 81 and 83). Repeated separation and isolations were done using

Figure 81. HPLC Profile of the Control Bile Fraction III.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible
Spectroscopic Detector

Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 35% HAC (0.01M)

65% CH_3OH Hold 10 min

↓ Linear, 60 min

100% CH_3OH Hold 5 min

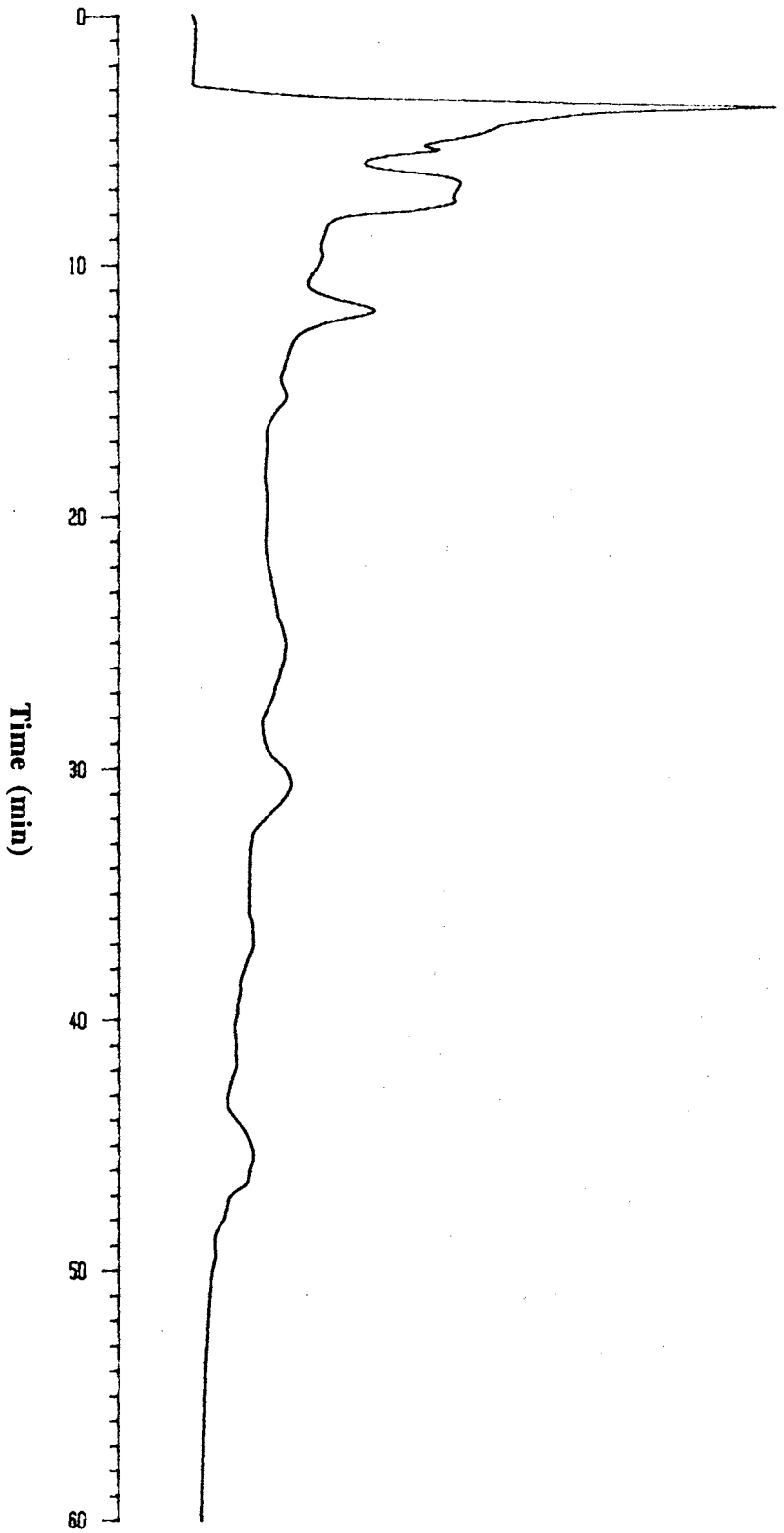

Relative Absorbance
Time (min)
0
10
20
30
40
50
60

Figure 82. HPLC Profile of the Biliary Metabolite Fraction III.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible
Spectroscopic Detector

Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

Flow Rate: 1.0 ml/min

Solvent Program: 35% HAC (0.01M)
65% CH_3OH Hold 10 min
↓ Linear, 60 min
100% CH_3OH Hold 5 min

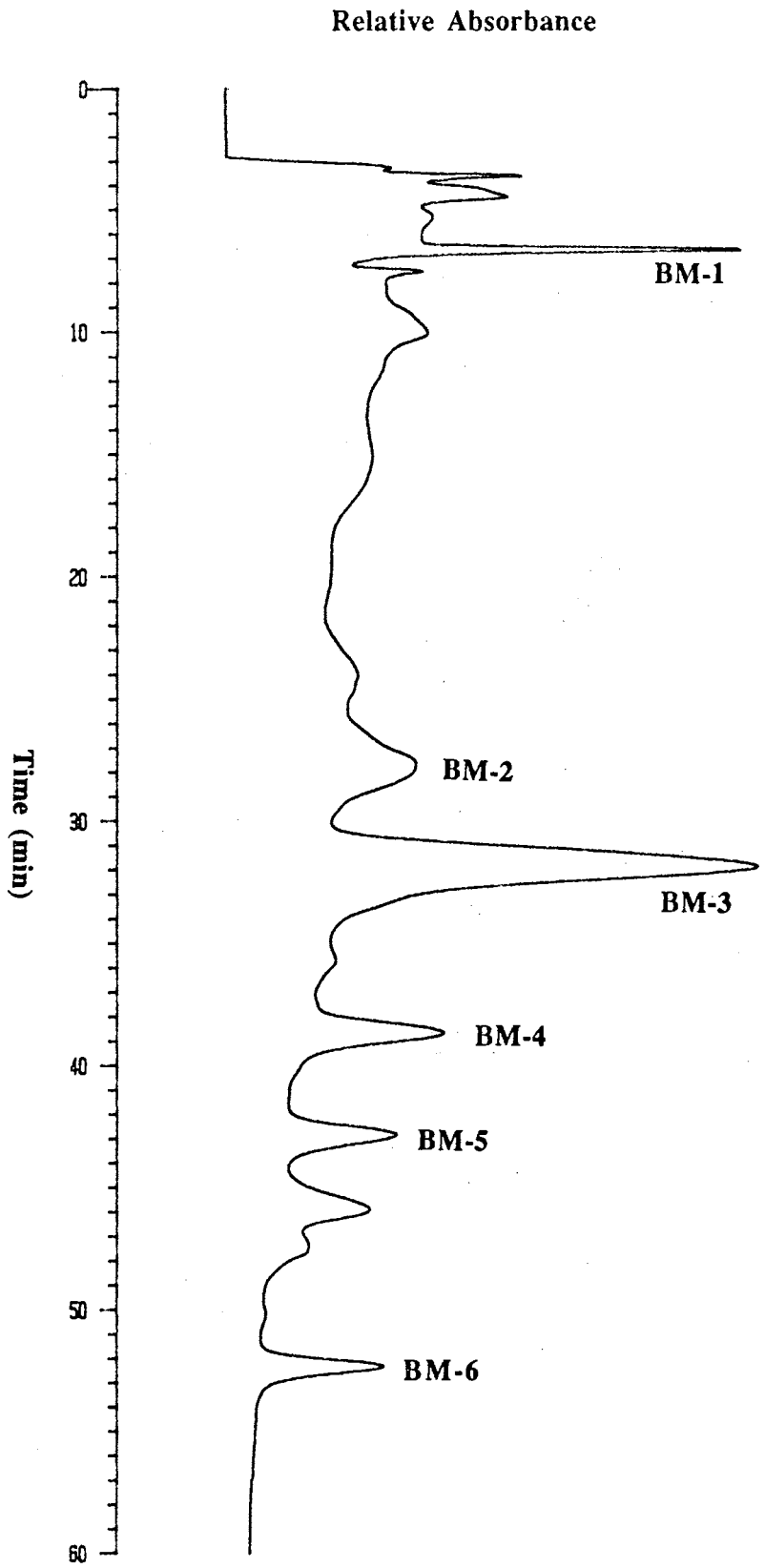

Relative Absorbance
Time (min)
0
10
20
30
40
50
60
BM-1
BM-2
BM-3
BM-4
BM-5
BM-6

Figure 83. HPLC Profile of a Mixture of Seven Synthetic Standards in the Same Polarity Region Corresponding to the Biliary Metabolite Fraction III.

Instrument: Waters-6000 HPLC System
Hewlett-Packard-1040A Diode-array UV/Visible Spectroscopic Detector

Detector Wavelength Setting: 254 nm

Column: Whatman C_{18} ODS-3, 5 μm Particle, 0.47 x 23.5 cm

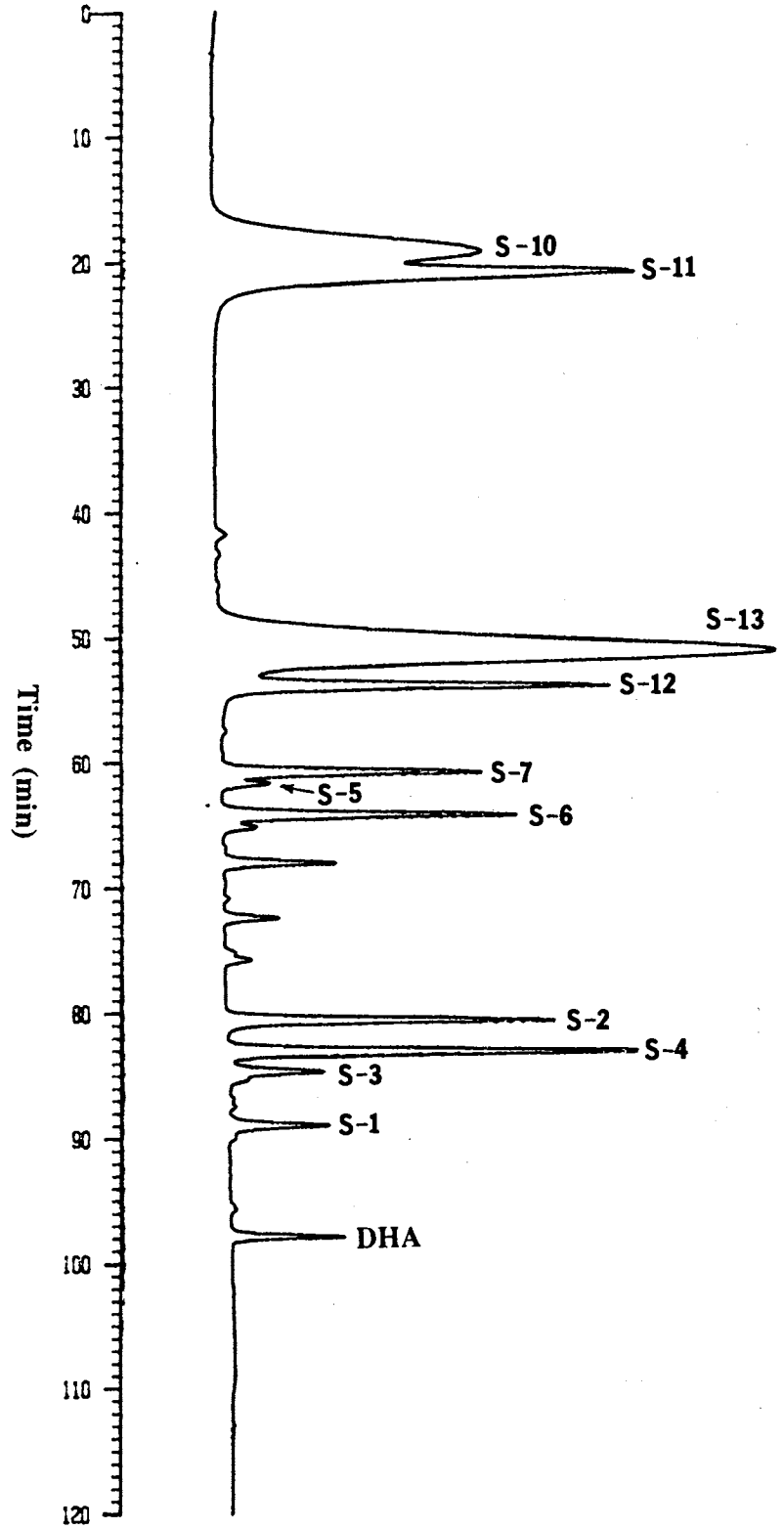
Flow Rate: 1.0 ml/min

Solvent Program: 35% HAC (0.01M)

65% CH_3OH Hold 10 min

↓ Linear, 60 min

100% CH_3OH Hold 5 min


Relative Absorbance
Time (min)
0
10
20
30
40
50
60
70
80
90
100
110
120
S-10
S-11
S-13
S-12
S-7
S-5
S-6
S-2
S-4
S-3
S-1
DHA

the same HPLC program. Peaks BM-1 to BM-6 were collected each time using a fraction collector and pooled.

Purification. In the initial analysis of the isolated metabolites by LSIMS, high content of Na^+ ions was found in all samples, and no metabolite ions could be detected (data not shown). This phenomenon was confirmed by analyzing the corresponding samples from the control bile. Because of the salt content in the bile, the LSIMS spectra of the metabolite and their controls appeared almost the same (data not shown). Therefore, all samples were desalted according to the procedure shown in Figure 66. After desalting, these metabolites were further purified by HPLC using various isocratic programs.

Figure 84 through Figure 88 show the UV spectra of the metabolite BM-2 through BM-6 along with the UV spectra of the perspective standards. No standard in this polarity region corresponds with BM-1. The UV spectrum of BM-1 is presented in Figure 89. As shown, not every UV spectrum of each of the unknown metabolites (BM-2 to BM-6) matches well with its respective standard. BM-2 [Figure 82(a)] has a maximum UV absorbance at 220 nm, which was lower than the maximum UV absorbance of S-2 [235nm, Figure 84(b)]. Nevertheless, both BM-2 and S-2 have a second-to-maximum absorbance around 290-300 nm. Metabolite BM-3 displays a maximum UV absorbance at 304 nm [Figure 85(a)], while that of S-4 is around 286nm [Figure 85(b)]. BM-4 has a maximum UV absorbance at 298 nm [Figure 86(a)], which well matches that of the standard S-8 [Figure 86(b)]. Likewise, the UV spectrum of BM-5 (Figure 87) and BM-6 (Figure 88) matches well with that of S-1 and DHA,, respectively. Besides the fact that different UV spectra between the metabolites and the possibly matching-standards, such as BM-2 and S-2, BM-3 and S-4, might indicate the differences in their structures, the impurity of the metabolites was probably the major problem in obtaining accurate UV data.

LSIMS Analysis. Results obtained from the HPLC analysis and UV spectroscopy had narrowed down the possibility that metabolites represented by peak BM-2, BM-3, BM-4, BM-5, and BM-6 may be structurally similar, or even the same in some cases, to that of

Figure 84. UV spectra of the Metabolite BM-2 (a), and the Standard S-2 (b).

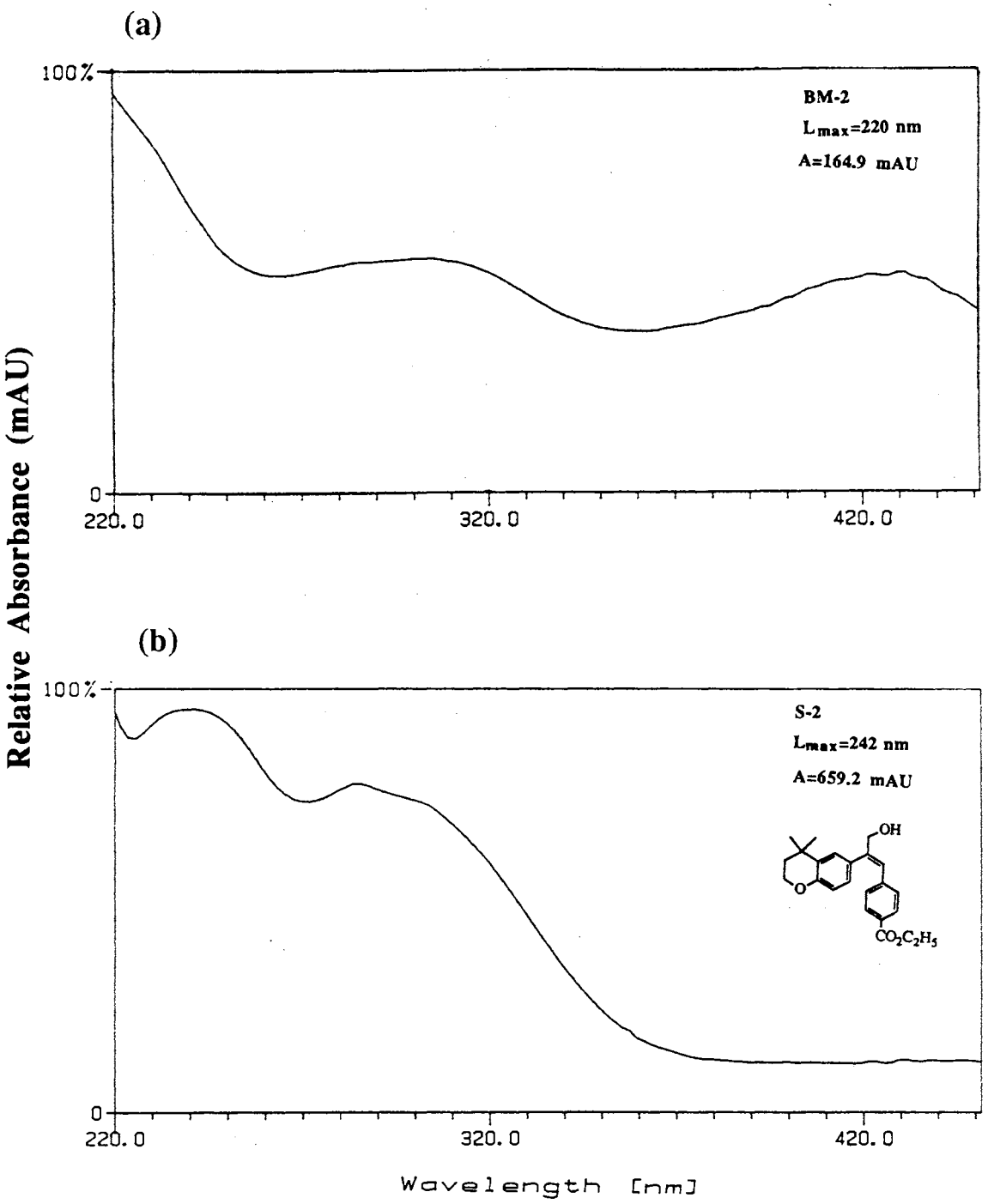

(a)
100%
0
BM-2
Lmax=220 nm
A=164.9 mAU
220.0
320.0
420.0
(b)
100%
0
S-2
Lmax=242 nm
A=659.2 mAU
OH
O
CO2C2H5
220.0
320.0
420.0
Relative Absorbance (mAU)
Wavelength [nm]

Figure 85. UV spectra of the Metabolite BM-3 (a), and the Standard S-4 (b).

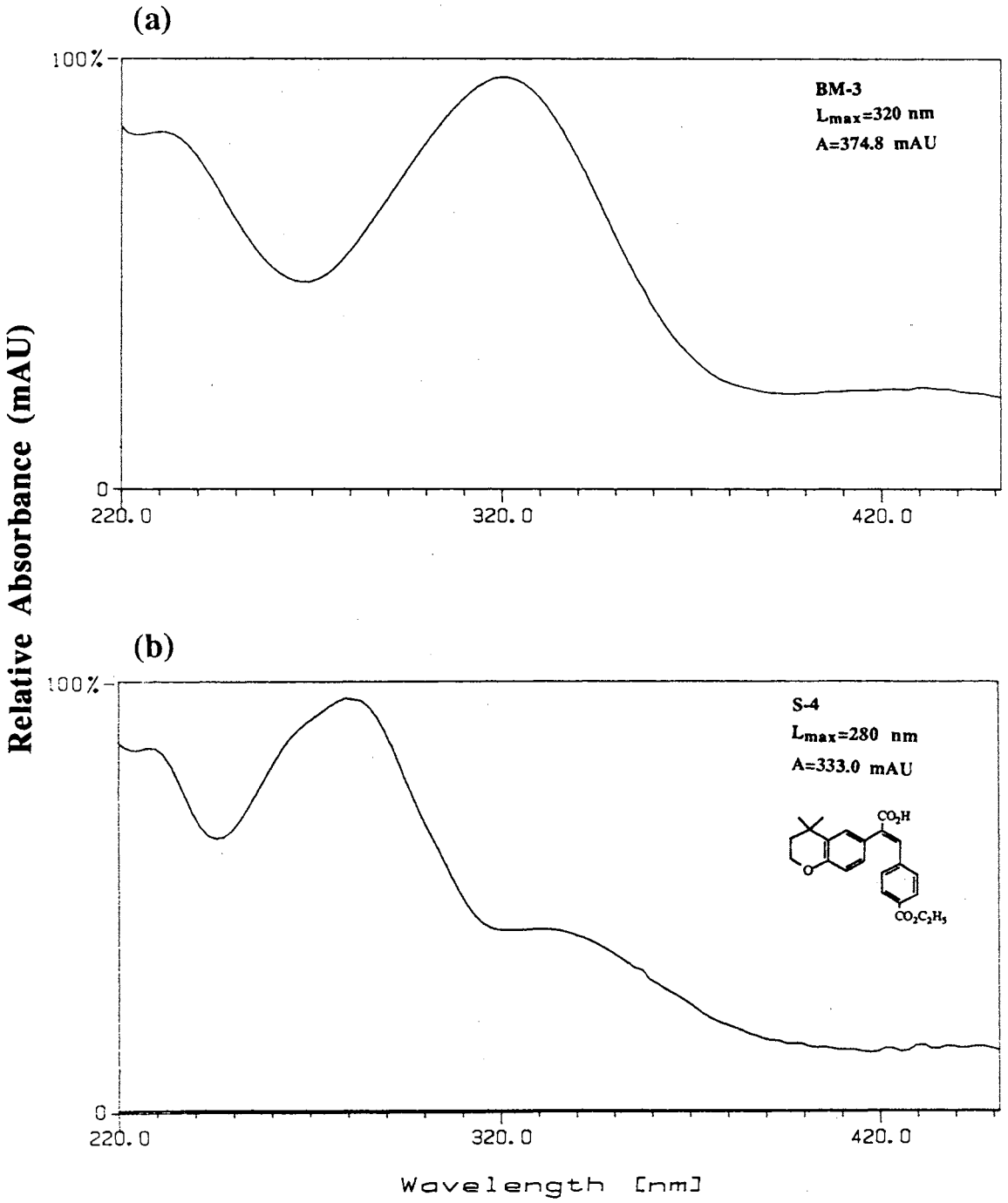

(a)
100%
0
BM-3
Lmax=320 nm
A=374.8 mAU
220.0
320.0
420.0
(b)
100%
0
S-4
Lmax=280 nm
A=333.0 mAU
CO2H
CO2C2H5
220.0
320.0
420.0
Relative Absorbance (mAU)
Wavelength [nm]

Figure 86. UV spectra of the Metabolite BM-4 (a), and the Standard S-8 (b).

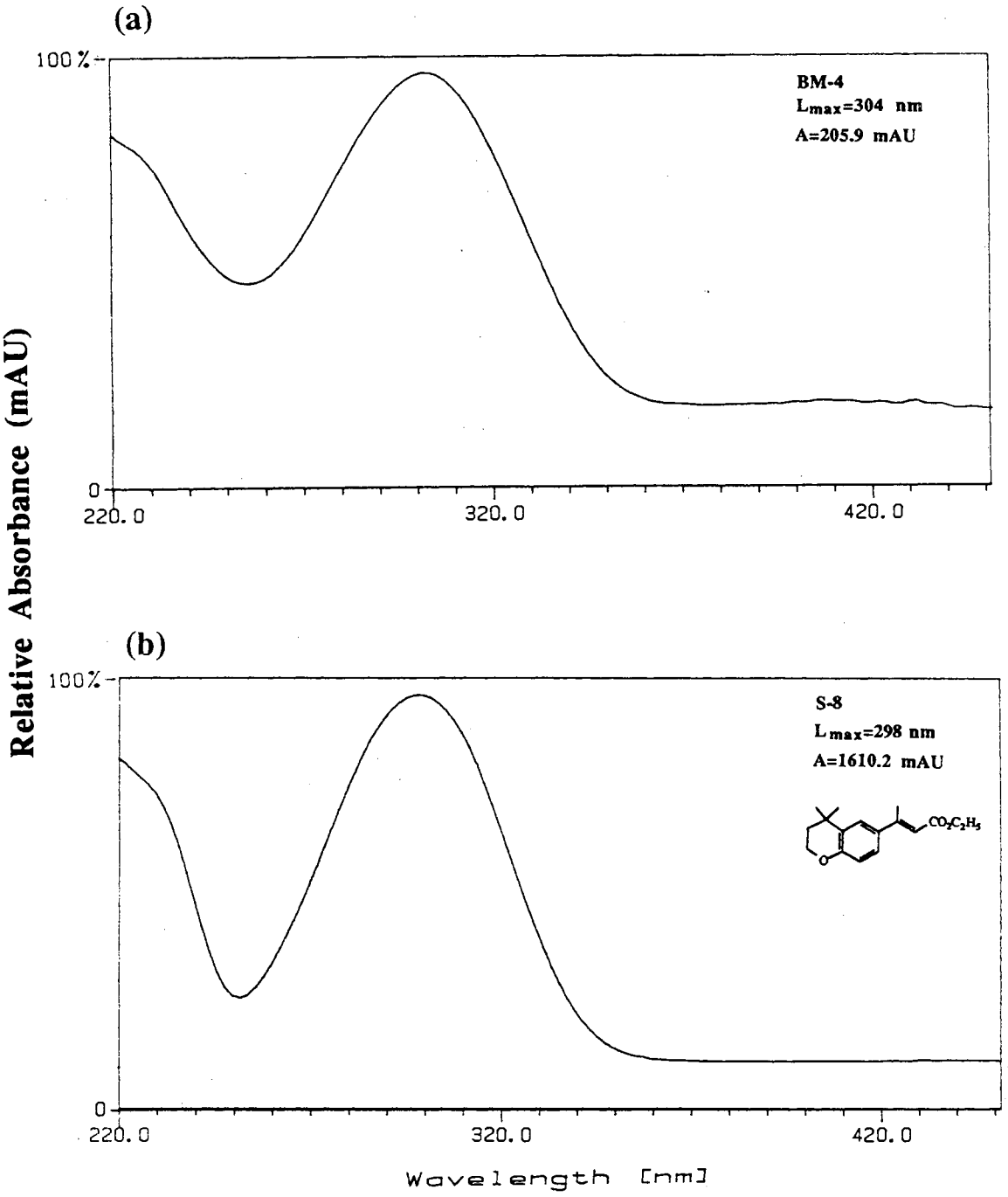

(a)
100 %
0
BM-4
Lmax=304 nm
A=205.9 mAU
220. 0
320. 0
420. 0
(b)
100 %
0
S-8
Lmax=298 nm
A=1610.2 mAU
220. 0
320. 0
420. 0
Relative Absorbance (mAU)
Wavelength [nm]

Figure 87. UV spectra of the Metabolite BM-5 (a), and the Standard S-1 (b).

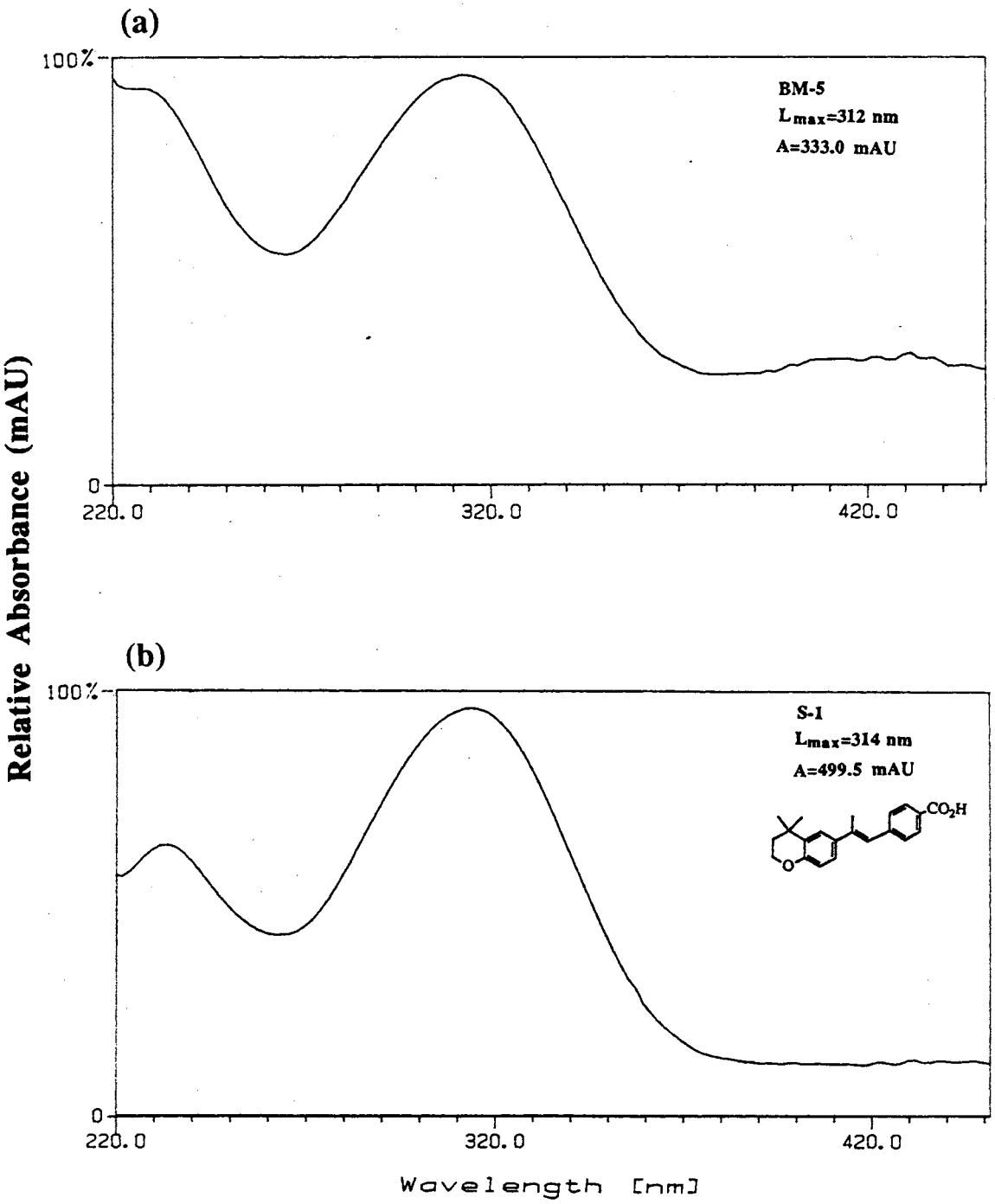
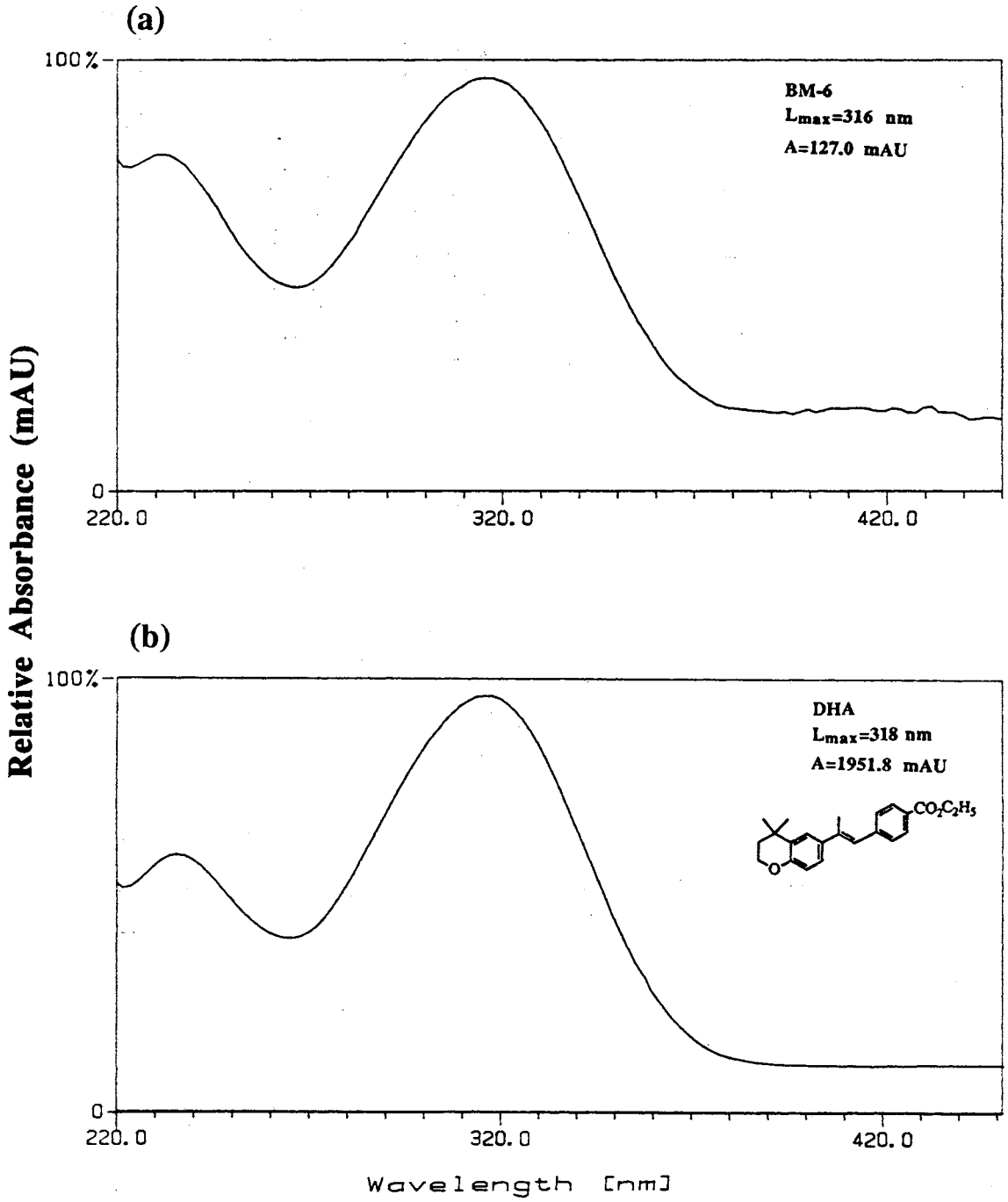

(a)
100%
0
220.0
320.0
420.0
BM-5
Lmax=312 nm
A=333.0 mAU
(b)
100%
0
220.0
320.0
420.0
S-1
Lmax=314 nm
A=499.5 mAU
CO2H
O
Relative Absorbance (mAU)
Wavelength [nm]

Figure 88. UV spectra of the Metabolite BM-6 (a), and the Standard DHA (b).


(a)
100%
0
220.0
320.0
420.0
BM-6
Lmax=316 nm
A=127.0 mAU
(b)
100%
0
220.0
320.0
420.0
DHA
Lmax=318 nm
A=1951.8 mAU
CO2C2H5
Relative Absorbance (mAU)
Wavelength [nm]

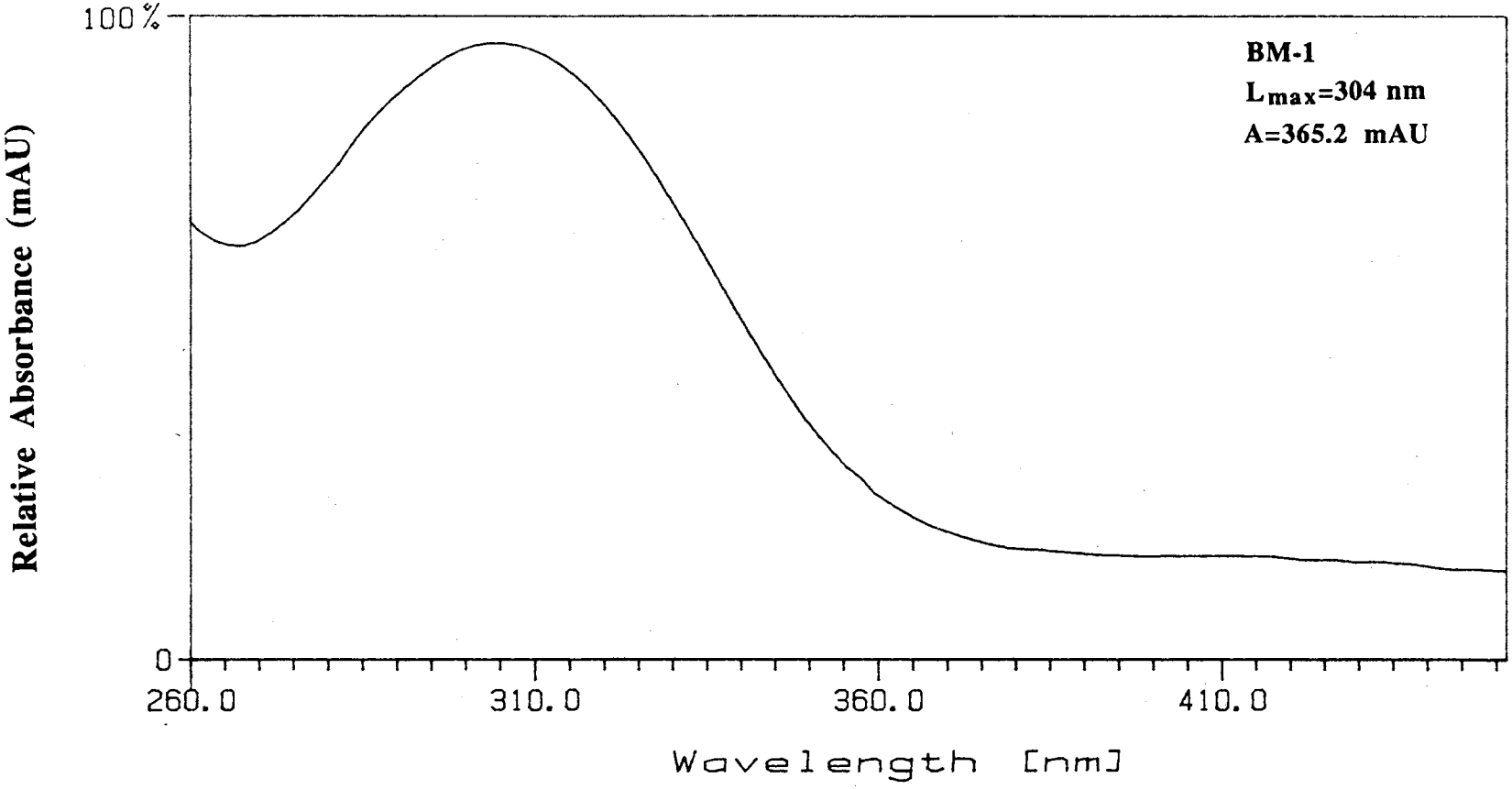


Figure 89. UV spectrum of the Metabolite BM-1.

the standards S-2, S-4, S-8, S-1, and DHA, respectively. To confirm the findings discussed above, they were subjected to LSIMS analysis, which was successfully applied to the mass spectrometric analysis of the standards (see Results and discussions in Chapter VI). Unfortunately, no satisfactory LSIMS spectra of the metabolites were obtained except for that of BM-3. Not enough sample material might be the reason for the overall unsatisfactory results from the LSIMS analysis.

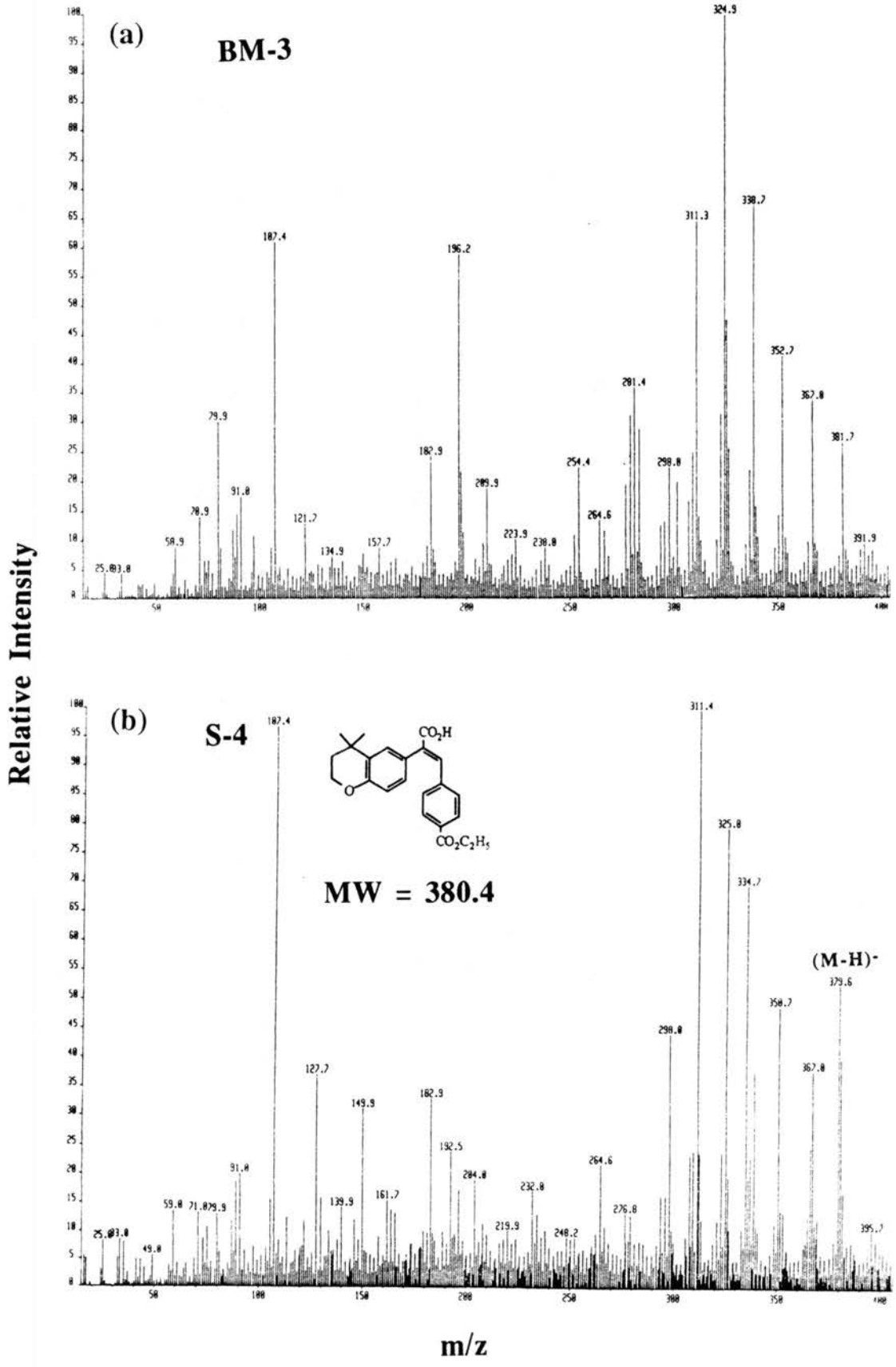
A straight negative LSIMS spectrum of BM-3 is shown in Figure 90(a). It can be seen that the general pattern of the $LSIMS^-$ spectrum of BM-3 is very similar to that of S-4 [Figure 90(b)]. Although, the molecular ion and one major fragment ion, m/z = 381.7, m/z = 352.7, in the spectrum of BM-3 are 2 mass units more than their corresponding peak at m/z = 379.6 and m/z = 350.7, respectively, in the spectrum of S-4. Other major fragment ions of BM-3, m/z = 338.7, m/z = 324.9, m/z = 311.3, and m/z =298.0, correspond well with those peaks at m/z = 338.0, m/z = 325.0, m/z = 311.4, and m/z =298.0 in the spectrum of S-4. m/z = 367.0 is a major matrix peak as indicated in the $LSIMS^-$ spectrum of the matrix (spectrum not shown). Although both the UV and mass spectrometric information indicated that BM-3 and S-4 are structurally similar, more study is definitely needed to elucidate the actual structure of BM-3.

Purification of ^{14}C-Labelled Parent Compound DHA*

Prior to the purification, possible radioactive contamination from the injector, as well as from other parts of the HPLC system, was tested by injecting 400 µl of pure CH_3OH into the system, and running the HPLC with the same program that would be used for the purification. 1.0-ml Fractions were collected for 10 min, and all fractions were assayed for radioactivity. Only background level of radioactivity was detected (data not shown). Hence, no radioactive contamination existed in the HPLC system.

As described earlier, purification of the crude ^{14}C-labelled DHA (total amount of 22 mg with a total radioactivity of 16.20 µci) was carried out on HPLC using a C_8 reversed

Figure 90. LSIMS Spectra of the Metabolite BM-3 (a), and the Standard S-4 (b).
Instrument: VG-ZAB-2SE
Mode: Straight, Negative Ionization


(a)
BM-3
(b)
S-4
MW = 380.4
(M-H)⁻
Relative Intensity
m/z

phase column, and an isocratic program. The HPLC chromatogram of the first purification is shown in Figure 91. Verified by the unlabeled parent compound (DHA), the radioactive DHA* and its isomer were eluted at about 24 and 16 min, respectively. Both isomers were collected in the first-time purification. The crude sample contained a large amount of contaminants, most of which were more polar compounds and eluted earlier. The radioactive trace of the crude material (see Figure 91 insert) however, indicated that very little of the contaminants were ^{14}C-labelled. The only two peaks that contained radioactive material are DHA* and its isomer.

Figure 92 shows the HPLC profile of the second purification. The radioactive trace of the same HPLC profile is shown in the insertion of Figure 92. It is clear that the non-radioactive and radioactive profiles are the same. This result indicated that, after a double purification procedure, the sample contained very little contamination, either chemical or radioactive. Only DHA* was collected from this purification, and the isomer was discarded. The average yield of purification was 52.5%.

Light-sensitive Isomerization of DHA*

A small amount of double-purified DHA* dissolved in 80% CH_3OH:20% HAC (0.01M) was exposed to fluorescent light for 3 min. The sample was then analyzed by HPLC using the same eluting program as that used for the purifications. Fifty 1-ml fractions were collected and counted. The result is shown in Figure 93. The relative percentages of DHA* and its isomer were estimated by their DPM counts and by measuring their relative peak areas from Figure 92 and 93, respectively. The results were summarized in Table VII. The values obtained from the two methods were very similar. The average percentage of DHA* to its isomer was 85.8%:14.2% after the light exposure compared to 86.6%:13.4% prior to the light exposure. Therefore, only 0.9% of DHA* was isomerized to its isomer during the 3-min light exposure.

Figure 91. HPLC Profile of the First Purification of DHA*.

DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

Instrument: Waters-6000 HPLC System
Fisher Recordall UV Recorder
UV Detector Wavelength Setting: 254 nm
Column: Whatman C_8 ODS-3, Spherical 5 μm, 0.46 x 12.5 cm
Flow Rate: 1.0 ml/min
Solvent Program: 70% CH_3OH:30% 0.01M HAC Isocratic

Insert: ^{14}C Radioactive Trace of the First Purification of DHA*

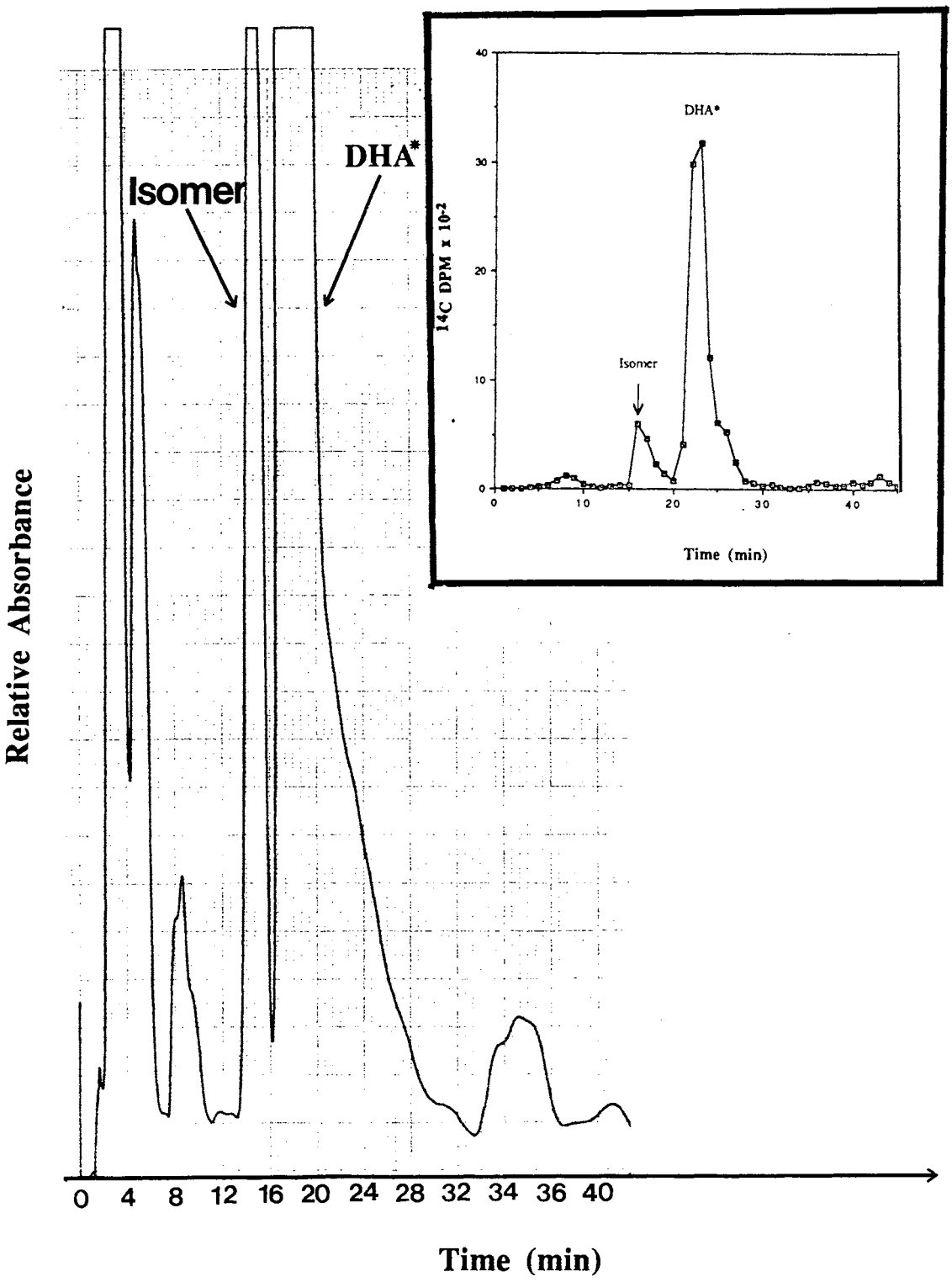

Isomer
DHA*
Relative Absorbance
0 4 8 12 16 20 24 28 32 34 36 40
Time (min)
14C DPM x 10-2
DHA*
Isomer
0 10 20 30 40
Time (min)

Figure 92. HPLC Profile of the Second Purification of DHA*.

DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

Instrument: Waters-6000 HPLC System
Fisher Recordall UV Recorder
UV Detector Wavelength Setting: 254 nm
Column: Whatman C_8 ODS-3, Spherical 5 μm, 0.46 x 12.5 cm
Flow Rate: 1.0 ml/min
Solvent Program: 70% CH_3OH:30% 0.01M HAC Isocratic

Insert: ^{14}C Radioactive Trace of the Second Purification of DHA*

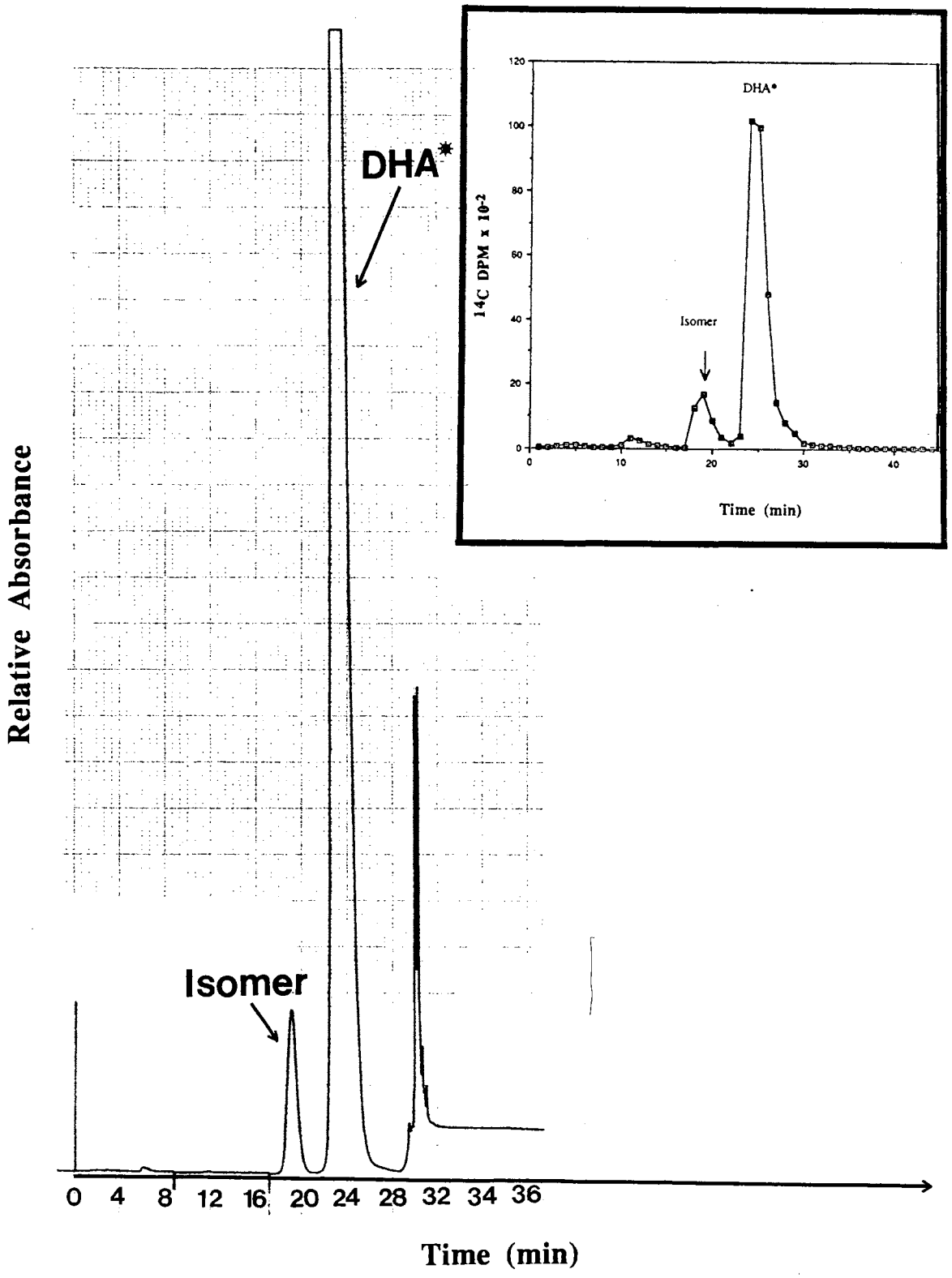

DHA*
Isomer
Relative Absorbance
0 4 8 12 16 20 24 28 32 34 36
Time (min)
DHA*
Isomer
^{14}C DPM x 10^{-2}
0 20 40 60 80 100 120
0 10 20 30 40
Time (min)

Figure 93. ^{14}C Radioactive Trace of the Purified DHA* After 3-min Light Exposure.
DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

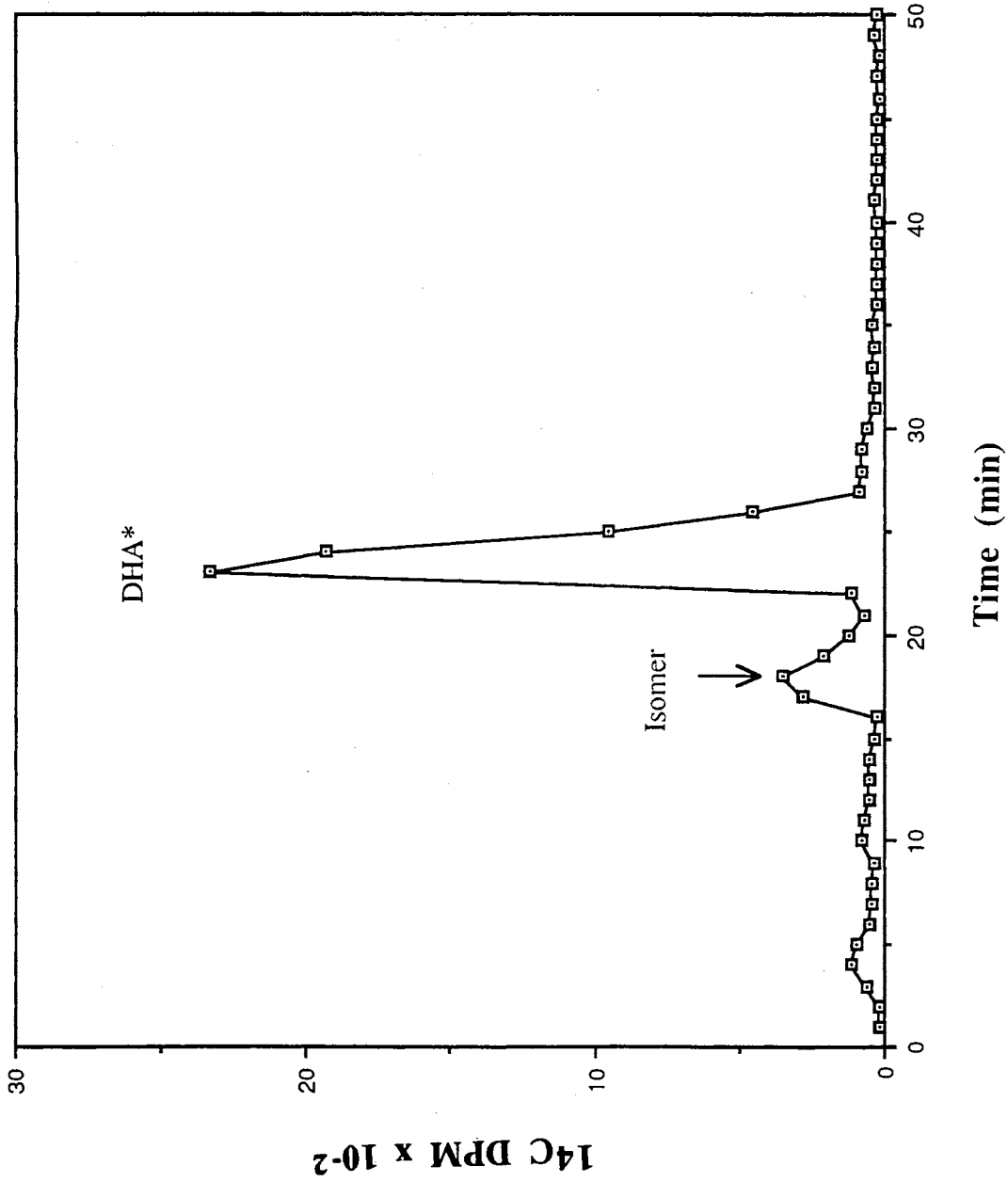

DHA*
Isomer
14C DPM x 10-2
Time (min)
0
10
20
30
40
50

TABLE VII

RELATIVE PERCENTAGE OF DHA* AND ITS ISOMER BEFORE AND AFTER A LIGHT EXPOSURE OF THREE MINUTES

	Before Light Exposure (%)			After Light Exposure (%)		
	by area	by DPMs	average	by area	by DPMs	average
DHA*	86.1	87.1	86.6	85.1	86.5	85.8
ISOMER	13.9	12.9	13.4	14.9	13.5	14.2

Metabolites of DHA* in Bile

The radioactive trace of the parent compound DHA* before its administration into the chicken is shown in Figure 94. The single radioactive peak of DHA* was eluted at about 140 min. This result indicated that the purification of DHA* from the crude radioactive material was effective. Only a dose of highly purified, radioactive parent compound was injected into the animal. The bile sample was collected, processed, and then analyzed by the HPLC system. Figure 95 shows the total radioactive trace of the biliary metabolites of DHA*. Despite the low specific activity of DHA* injected, the chromatogram revealed a number of metabolites within the range of 55-140 min with a major one eluting at 110 min. The parent compound was recovered in the bile, and was detected at 140 min. In addition, 2 or 3 metabolites that were eluted after the parent compound could also be observed although their intensities were very low.

Metabolites of DHA* in Liver

Liver extract was analyzed by HPLC using an isocratic program. The radioactive trace of the liver metabolites is shown in Figure 96. It can be seen that there were at least two metabolites in the liver. They are more polar than the parent compound, which was detected at about 24 min. Overall, the DPM values were very low for the liver metabolites. Therefore, after 6 hr of metabolism, very little of the metabolites remained in the liver. This indicated that the 6-hour bile collection is sufficient to obtain most metabolites secreted into the bile.

Owing to the low specific radioactivity of the starting material (0.74 μci/mg of crude DHA*) and still lower radioactivities of the metabolites detected, this preliminary study was only intended to verify the feasibility of the experimental procedures designed to carry out metabolism studies with radioactive materials. No further identification was intended.

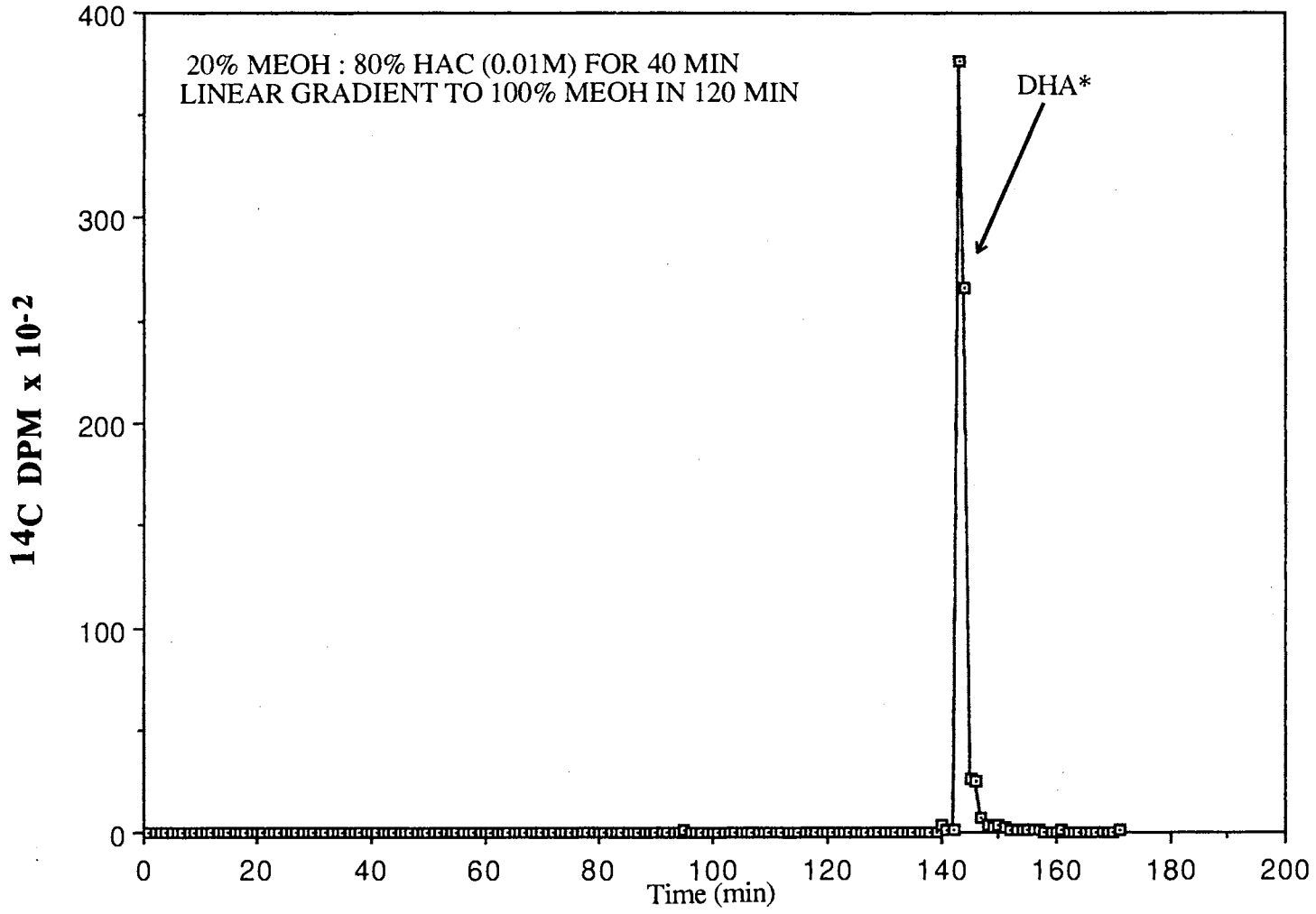


Figure 94. Radioactive Trace of the Purified DHA* Prior to the Drug Administration.

DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

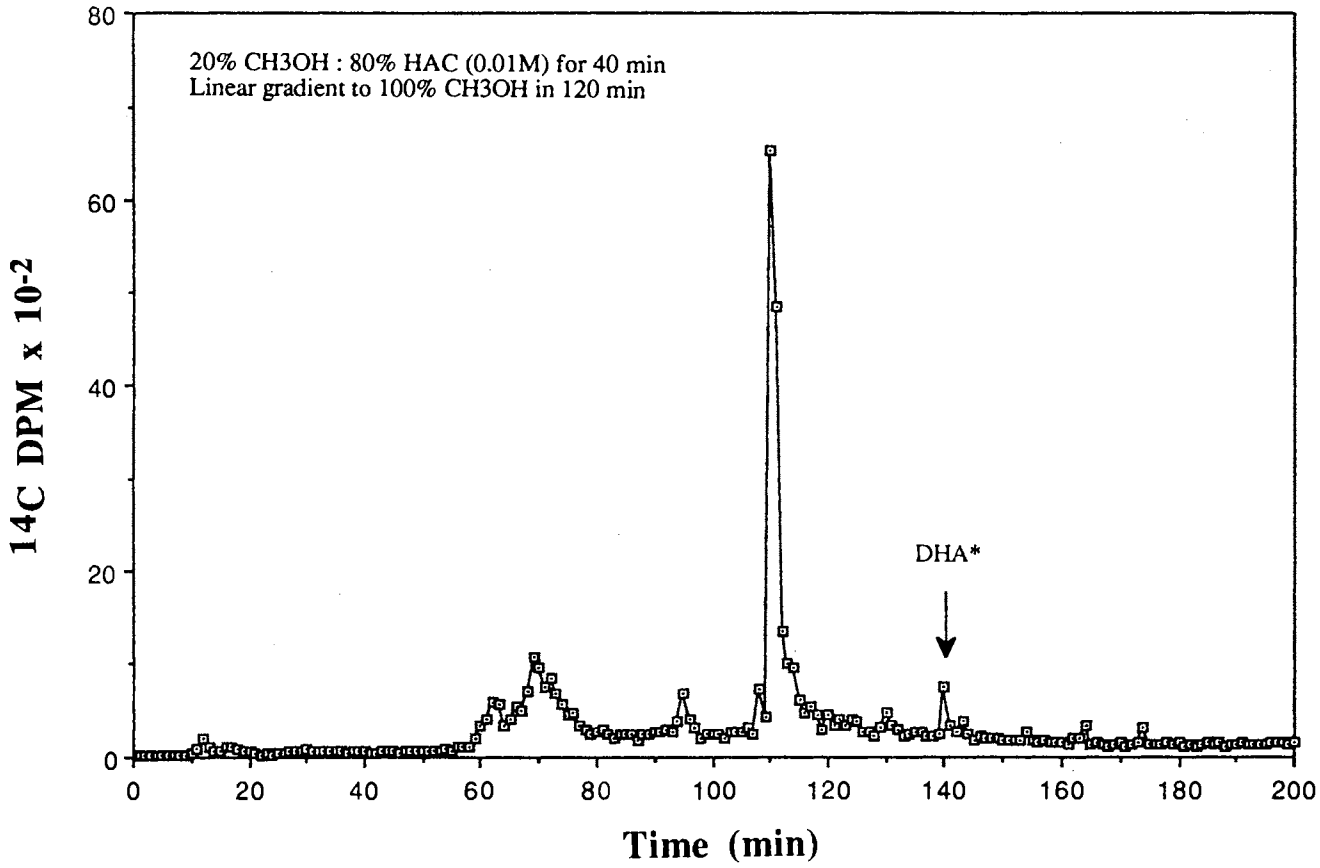


Figure 95. Radioactive Trace of a Chick Biliary Metabolites of DHA*.

DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

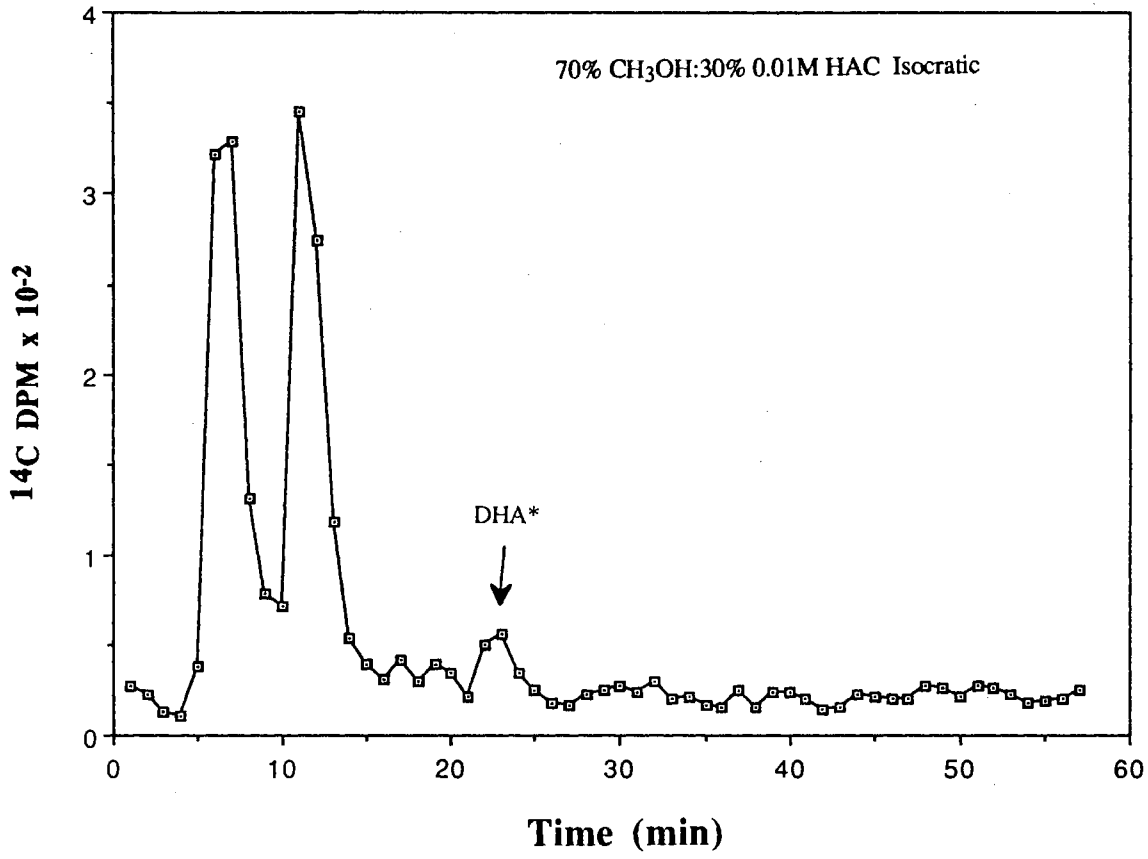


Figure 96. ^{14}C Radioactive Trace of a Chick Liver Metabolites of DHA*.

DHA*: 9,10,11-^{14}C Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

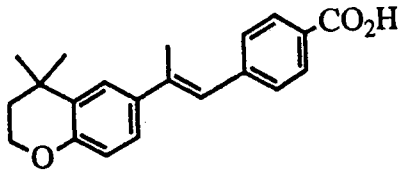
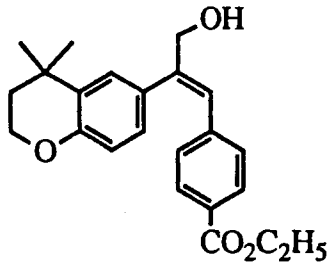
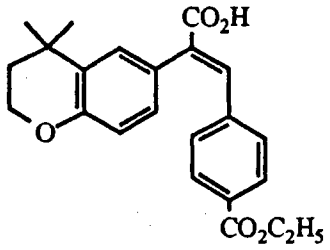
Conclusions

Methods have been developed to study the metabolism of heteroarotinoids *in vivo*. A successful rat bile cannulation procedure was established for convenient collection of biliary metabolites under normal or near normal physiological conditions, and for extended use of the animals. Methods for bile sample processing and purifications designed to eliminate artifacts generated from chromatographic procedures and various other sources of isomerization, oxidation, and deteriorations by controlling temperature, air and light exposure.

Three major biliary metabolites of a selected heteroarotinoid, ethyl (*E*)-4-[2-(3,4-dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate, were tentatively identified using combined techniques of HPLC, UV, Plasmaspray LC/MS and LSIMS. The structures of these metabolites are shown in Figure 97.

Studying drug metabolism in a biological fluid as complicated as bile without labeled compounds is very time-consuming and labor intensive. Isotope-labeling of some drugs, like the heteroarotinoids studied here, is difficult and costly, especially with expected specific activities. Nevertheless, results obtained from this study are encouraging. It is possible that a majority of the work in the identification of drug metabolites can be accomplished using non-radioactive compounds. With suitable application of modern techniques such as HPLC and other chromatographic methods, metabolites can be separated and isolated effectively. Plamaspray LC/MS is a powerful and an ideal technique in the analysis of drug metabolism without requiring excessive separation and isolation procedures. Should metabolites be isolated of sufficient quantity and purity, other spectroscopic techniques, such as LSIMS and ^{1}H- or ^{13}C-NMR, can be applied to further examine the structures. Isotope-labeled compounds may be required in the final confirmatory step(s).

Figure 97. The Structures of the Tentatively Identified Biliary Metabolites of DHA in Rats.
DHA: Ethyl (*E*)-4-[2-(3,4-Dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate.

STRUCTURE	DESIGNATION
	M-1
	M-2
	BM-3

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Heteroarotinoids are a family of newly developed retinoids. They retain the basic features of the retinoid skeleton with the incorporation of at least one heteroatom and an aryl ring. They have shown marked anticancer activities in the tracheal organ culture assay, the ornithine decarboxylase assay, and in the HL-60 cell line. Equally important is that some have shown reduced toxicity when compared to naturally occurring retinoids and other synthetic retinoids. Investigations of their biological activities and the metabolism of these heteroarotinoids will provide important information for pharmacological evaluation and future clinical utilization.

One of the most important biological functions of vitamin A is its essential role in promoting normal growth. A growth assay was developed and utilized to evaluate the growth-promoting activity of the heteroarotinoids by monitoring the weight-changes of vitamin A-deficient rats fed with or without supplementation of these heteroarotinoids. Vitamin A-deficiency was introduced synchronously in all the test animals. A diaryl heteroarotinoid (DHA), ethyl (*E*)-4-[2-(3,4-dihydro-4,4-dimethyl-2*H*-1-benzopyran-6-yl)-1-propenyl]benzoate, and a monoaryl heteroarotinoid (MHA), (2*E*,4*E*,6*E*)-3,7-dimethyl-7-(1,2,3,4-tetrahydro-4,4-dimethyl-6-chromanyl)-2,4,6-heptatrienoic acid or its ethyl ester, were tested. The drugs were given to rats, either by periodic supplementation and withdrawal of a fixed amount of the test compounds (periodic feeding approach), or by continuous supplementation of the compounds over an extended period of time (continuous feeding approach). The activity of heteroarotinoids was compared to that of all-*trans*-

retinoic acid (RA). The results demonstrated that DHA supported growth at a rate very similar to that of RA when both were fed at the level of 100 μg/day. At a higher dose level (200 μg/day), DHA was more potent in stimulating growth than RA. Neither DHA nor RA supplemented at 10 μg/day was sufficient to maintain normal growth in rats. In contrast, while MHA apparently sustained growth for a short time, it did not support growth effectively, even when it was supplemented at a level as high as 200 μg/day/rat.

The vitamin A-like activity of DHA and MHA, as well as all-*trans*-retinoic acid (RA), in terms of promoting the differentiation of epithelial cells was evaluated *in vivo* using a modified vaginal-smear assay performed on vitamin A-deficient ovariectomized rats. The vaginal epithelium of vitamin A-deficient rats was marked by persistently cornified epithelial cells. The cornified epithelium, in ovariectomized rats, responded solely to the total body vitamin A status, and as manipulated in this assay, to the topically administrated compounds exhibiting vitamin A-like activities. DHA, MHA and RA, each in the amount ranging from 10^{-14} mol to 10^{-8} mol, were applied intravaginally to the rats in 27 μl of a mixture of aldehyde-free alcohol : normal saline (2:15, v/v) immediately after the first vaginal smear was taken (at time 0). Vaginal smears were then taken 18 hours after drug application, and every 24 hours thereafter for 6-7 days. The anti-keratinization activity of the tested heteroarotinoids was clearly demonstrated by the reversal of the cornified epithelial cells after drug administration. In most cases, the responses first became significant at 42 hours, and reached a peak at about 66 hours. Cornified vaginal epithelium, as the result of vitamin A deficiency, was reversed by dose levels as low as 10^{-14} moles. The relative activity of DHA (ED_{50} = 1.0 x 10^{-11} mol/vagina) was slightly higher than that of RA (ED_{50} = 4.0 x 10^{-11} mol/vagina), while MHA (ED_{50} = 4.0 x 10^{-10} mol/vagina) was ten-fold less active than RA.

Rats that were used in the continuous feeding experiment were submitted to a postmortem examination. They had been supplemented daily with DHA, MHA and RA, each at various doses, for continuous 26-40 days at which time they were sacrificed.

Epithelial tissues were taken from various organs that were known to be most susceptible to vitamin A deficiency. The tissues were evaluated histopathologically and compared to those of the control rats. The control rats were age-paired rats of the same strain raised on a normal diet. Results demonstrated that both DHA and MHA, similar to RA, maintained the integrity of the epithlial tissues taken from the liver, trachea, lung, and all organs of the gastrointestinal tract. Lesions similar to those resulting from vitamin A-deficiency, including atrophy, edema, metaplastic keratinization and inflammation, were seen in the epithelial tissues of the prostate, epididymis, testes, and the urinary bladder of most of the drug-treated rats. The most severe lesions were observed in the epithelia of the three male genital organs, but no distinctive differences could be generalized on the effects of the different drugs (inter-compound comparisons), or of the same drug at different dose levels (intra-compound comparisons). While epithelial degeneration of the urinary bladder, manifested as desquamation of the epithelial cells, was more severe in RA-treated animals than heteroarotinoid-treated animals, the atrophy in the kidney was more profound in heteroarotinoid-treated rats, and was observed only occasionally in RA-treated rats. Intra-compound differences were not obvious overall. In conclusion, the effects of the heteroarotinoids on maintaining the integrity of epithelial tissues are similar to that of retinoic acid. They are tissue-specific in that they are able to promote the normal epithelial growth of some organs, but not of others. Among the epithelia that these compounds fail to preserve are the epithelial tissues of the genitourinary tract.

A metabolism study of DHA was conducted *in vivo* using bile-duct cannulated rats. Procedures for bile sample purification were established. Methods were developed to isolate and identify major biliary metabolites derived under normal physiological conditions using combined HPLC, UV, Plasmaspray LC/MS and LSIMS techniques. Three major oxidative metabolites of DHA were identified. Methods employing the utilization of radioactive tracers in confirmatory studies were also established.

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