EFFECTS OF DIET, ARTEMISIA, AND HELENIUM ON DISPOSITION AND BIOTRANSFORMATION OF DRUGS

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IN THE NAME OF ALLAHA, MOST GRACIOUS, MOST MERCIFUL

This thesis is dedicated to:

my parents

Zein Elabdin Eissa and Fawzia Osman

Who always taught me to excel

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Mammals are constantly exposed in their environment to a vast array of chemicals that are foreign to their bodies, the so-called xenobiotics. These foreign chemicals, or xenobiotics, can be of natural origin such as flavonoids, monoterpenes, diterpenes and sesquiterpene lactones which are prevalent in fruits, vegetables, and forage-plants or they can be man-made such as drugs, food additives, cosmetic ingredients, and environmental-pollutants.

Xenobiotics enter the mammalian body and represent a potential threat to the individual. Fortunately, mammalian cells have a variety of enzymatic processes capable of detoxifying foreign chemicals. Detoxification is achieved by converting xenobiotics into more water-soluble metabolites, thus restricting their ability to partition into biological membranes or to be reabsorbed; and consequently, facilitating their excretion and elimination from the body. The hydrophilicity conferred upon these xenobiotics is the sequel of an enzymatic process called biotransformation.

Biotransformation is the sum of two integrated phases by which a xenobiotic is subjected to chemical modification resulting in most cases in production of metabolites that are more polar and less lipotropic i.e. can be more readily excreted. However, it should be emphasized that in some

instances biotransformation can lead to generation of dangerous electrophiles that are even more toxic than the parent compound. This is particularly true for some chemical carcinogens and organo-phosphorous insecticides (Guengrich, 1991).

Phase I biotransformation reactions involve oxidation, reduction and hydrolysis. Many oxidation and reduction (Redox) reactions are catalyzed by cytochrome P-450 or flavin monooxygenase; a prime function of redox reactions is the addition of functional groups such as OH, SH, NH_2, and COOH to the original compound, thereby rendering it more water-soluble. Hydrolytic reactions are catalyzed by hydrolases, esterases, and amidases; a prime function of hydrolysis is the exposure of a pre-existing functional group.

Phase II biotransformation reactions involve the covalent linkage of xenobiotics or phase I-derived metabolites to an endogenous hydrophilic moiety producing a conjugate. In this case, the endogenous moieties are glucuronic acid, sulphate and amino acids. Conjugation of xenobiotics promotes their ionization at physiological pH, and thus facilitates their excretion through biliary and renal routes (Sipes and Gandolfi, 1991).

Having delineated the biphasic nature of biotransformation, it is worthy to note that there are numerous factors affecting these reactions, such as nutritional status, age, route of administration, dose, time of the day, sex, disease status, and enzyme induction or inhibition (Sipes and Gandolfi, 1991).

Dietary Influences on Biotransformation

Many dietary factors have the potential for markedly affecting

biotransformation. For instance, increased dietary protein enhances biotransformation of drugs as well as hydroxylation of endogenous steroids (Kappas et al., 1983). Meat eaters have more rapid antipyrine clearance as compared to vegetarians (Campbell and Hayes, 1974; Kappas et al., 1976, and Mucklow et al., 1982). Conversely, birds kept on a low-protein diet have manifested an impairment in biotransformation of malaoxon, a desulfurated metabolite of the insecticide malathion. Consequently, protein deficiency tends to increase malathion toxicity (Ehrich et al., 1984).

Vitamins are other essential dietary constituents that play a pivotal role in maintaining the activity of hepatic biotransformation reactions. For instance, deficiency of vitamins A,C, and E has been reported to lower the level of cytochrome P-450 and consequently compromise the biotransformation efficiency of the Mixed Function Oxidase system. (Colby et al., 1975; Zannoni et al., 1973; Horn et al., 1976). In contrast, administration of vitamin C (ascorbic acid) or E (α-tocopherol) has been reported to enhance hydroxylation reactions in phase I biotransformation (Zannoni et al., 1973; Horn et al., 1976).

Other examples illustrating the significant role of dietary chemicals and nutrients in modulating biotransformations are the effects of indoles, flavonoids, and various alkaloids present in plants. Indoles, prevalent in cruciferous vegetables such as brussels sprouts and cabbage, are potent inducing agents as attested to by the significant increase in antipyrine clearance in patients fed brussels sprouts and cabbage (Pantuck et al., 1979). Flavonoids, prevalent in citrus fruits, have been reported to increase the in vivo and in vitro metabolism of zoxazolamine to 6-hydroxyzoxazolamine in neonatal

rats. (Lasker et al., 1984). Similarly, the presence of methylxanthine and caffeine alkaloids in several plant families such as Aquifoliaceae, Rubiaceae, Sterculiaceae and Theaceae can lead to a remarkable increase in hepatic hydroxylation and O-demethylation (Mitoma et al., 1968; Fraser et al., 1977). Thus, ingestion of plants belonging to these families could be expected to hasten elimination of concurrently administered drugs.

Limited studies in rats indicate that pyrrolizidine alkaloids (PA) either in whole plant such as in Senecio jacobaea "Tansy ragwort" or purified such as seneciphylline, an extract from Senecio vulgaris, are capable of inducing hepatic epoxide hydrolase and glutathione-s-transferase when administered in the diet (Miranda et al., 1980; Kakrani and Kalyani, 1984).

Modulation of biotransformation produced by chemicals of plant origin may also be an important factor in exacerbation of toxicant effects. A prime example of interest in sheep is seen with ingestion of Tetradymia glabrata (horse brush). When it is preceded by ingestion of Artemisia nova (black sage brush), an overwhelming photosensitization occurs which is not otherwise a problem when Tetradymia is eaten alone. (Jennings et al., 1978; Johnson, 1978). This example underscores the role of Artemisia as a preconditioning plant and its ability to aggravate the hepatotoxicity exerted by Tetradymia ingestion. In fact, Artemisia nova was thought to be a potent inducer of the Mixed Function Oxidase (MFO) system. This hypothesis was extrapolated from an experiment done in mice where pretreatment with prototype inducers such as phenobarbital or 3-Methylcholanthrene caused death to ensue more quickly when the mice were challenged with tetradymol, a furanoeremophilane,

extracted from Tetradymia. The reverse was true when the mice were pretreated with classical inhibitors such as SKF-525A or piperonyl butoxide and then challenged with tetradymol. In the latter case, mice were able to survive longer as the death time increased significantly from a control of 7 hours to 17 hours (Jennings et al., 1978). These results from mice and those from a series of experiments conducted in sheep by Johnson (1978), in which he described the synergism between Artemisia and Tetradymia that facilitates development of the clinical syndrome "bighead," have reinforced the hypothesis that Artemisia exacerbates Tetradymia toxicity by inducing the Mixed Function Oxidase System.

Having discussed the stimulatory-type effect of several plants and plant chemicals, it would be of importance to consider plants and plant chemicals that exhibit an inhibitory-type effect such as piperine alkaloids, naringin, and sesquiterpene lactones from the pepper family, citrus fruits, and sunflower family, respectively.

Piperine, a major alkaloid found in green and red peppers, has been reported to inhibit UDP-glucuronyltransferase, an essential enzyme in Phase II biotransformation, leading to a decrease in the rate of glucuronidation (Sing et al., 1986). As a sequel of this inhibition, there is a delay in the metabolic conversion of compounds that require glucuronic acid conjugation. Consequently, piperine tends to prolong the residence time of drugs in the body. This phenomenon would be even more prominent in drugs that are directly glucuronidated without undergoing phase I biotransformation; for instance, phenylbutazone which is used in treatment of rheumatic diseases (Aarbakke et

al., 1977) and tripelennamine, an antihistaminic drug (Chaudhuri et al., 1976).

Naringin, an abundant natural flavonoid in grapefruit, was found to inhibit a specific isozyme within the cytochrome P-450 superfamily called (CYP3A4). Therefore, Naringin could be used in cancer chemoprevention in situations where the carcinogen requires bioactivation by the same isozyme. For instance, aflatoxins are not active per se but require bioactivation, mediated through the isozyme CYP3A4, in order to produce culprit epoxides that bind covalently to DNA forming adducts; thus, Naringin is used in blocking bioactivation of aflatoxins into reactive intermediates (Guengrich and Kim, 1990).

Sesquiterpene lactones Sesquiterpene lactones are characteristic components of the ubiquitous plant family Compositae (sunflower). Helenalin, hymenoxon and flexuosin are structurally related sesquiterpene lactones which have been isolated from Helenium microcephalum "small head sneeze weed", Hymenoxys odorata "western bitter weed", and Helenium flexuosum, respectively. (Herz, 1973; Herz, 1978; Kim et al., 1987). Sesquiterpene lactones are defined chemically as 15 carbon atom cyclic esters. These sesquiterpene lactones owe their toxicity to the presence of a highly reactive α-methylene-γ-lactone moiety, which acts as an electrophile that can alkylate thiol groups of biological nucleophiles through a Michael-type reaction (Kupchan, 1970; Merrill et al., 1986). Therefore, the chemical reactivity of these sesquiterpene lactones can potentially disrupt a number of metabolic pathways necessary for homeostasis. For example, phosphofructokinase, an essential allosteric enzyme that controls

the pace of the glycolytic pathway, was found to be inhibited by sesquiterpene lactones (Hanson et al., 1970).

Elissalde and Ivie in (1987) have reported that sesquiterpene lactones alkylate thiol groups of adenylate cyclase rendering it incapable of hydrolyzing pyrophosphate from an ATP molecule; thus, disrupting generation of cyclic AMP. Cyclic AMP is a known intracellular second messenger for many hormones and disruption of its production, because of deactivation of adenylate cyclase by sesquiterpene lactones, could negatively alter cellular communication.

Sesquiterpene lactones are also capable of depleting hepatic glutathione (Merrill et al., 1988) and by so doing they deprive animal cells of the beneficial effects of reduced glutathione (GSH), which acts as a sulfhydryl buffer against hydrogen peroxide and organic peroxides.

Numerous sesquiterpene lactones bearing an α-methylene-γ-lactone group have been isolated and characterized as having an antitumor activity. For instance, helenalin and tenulin have been reported by Lee et al. (1977) to act as suppressors of Ehrlich ascites tumors in CF_1 male mice. This suppression is achieved by inhibition of DNA synthesis and DNA polymerase enzymatic activities. Another example of interest would be Damsin, the major sesquiterpene lactone isolated from Ambrosia maritima, the antitumor activity of which has been assayed under the auspices of the Cancer Chemotherapy National Service Center (CCNSC) and which has been used as a reference standard for antitumor activities (Doskotch and Hufford, 1969).

Several plants belonging to the genus Artemisia, a genus in the family

Compositae, have also been reported to contain sesquiterpene lactones. For example, Artemisia genipi was reported by Appendino et al. (1982) as a source of sesquiterpene lactones such as artemorin, santamarine, and reynosin. This plant is used extensively by liqueur-producing industries in Italy to produce a liqueur called "Genipi." Another interesting species, Artemisia herba-alba, a widespread plant of the desert of Iraq known as Shih (Arabic name), has been reported as a panacea for treatment of diabetes mellitus (Al-waili, 1986) Interestingly, plant concoctions are not only capable of reducing elevated blood sugar but also capable of treating diabetics who are considered non-responsive to conventional oral antidiabetic therapy. The plant concoctions are apparently quite popular in Iraq, where they are sold in markets.

A unique antimalarial sesquiterpene lactone, Artemisinin, has been isolated from the leafy portions of the chinese herb Artemisia annua. Artemisinin, known in Chinese language as Qinghaosu, has been used successfully in treatment of cerebral malaria, a pernicious malaria that leads to coma when more than 5% of the erythrocytes are parasitized by Plasmodium falciparum (Klayman, 1985).

Cytochrome P-450

The most important and predominant enzyme system involved in Phase I biotransformation is cytochrome P-450, a major component of the Mixed Function Oxidase (MFO) system, which acquires its name from the fact that when the reduced cytochrome P-450 forms a ligand with carbon monoxide, the maximal absorbance of light occurs at 450 nm and hence the name

cytochrome P-450 (Sipes and Gandolfi, 1991).

In cytochrome P-450 catalyzed reactions, the substrate combines with the oxidized form of cytochrome P-450 (Fe^{3+}) to form a substrate-cytochrome P-450 Complex. This complex then accepts an electron from NADPH (via NADPH-cytochrome P-450 reductase) and by so doing the iron in the cytochrome P-450 heme moiety is reduced (Fe^{2+}). The reduced substrate-cytochrome P-450 complex subsequently combines with molecular oxygen, which then accepts another electron from NADPH. The two electrons donated by NADPH are transferred to molecular oxygen resulting in the creation of a highly reactive oxygen species. One atom of this reactive oxygen is inserted into the substrate to yield an oxygenated substrate, while the other atom is reduced to water. The oxygenated substrate then dissociates from the complex to facilitate regeneration of the oxidized form of cytochrome P-450 and thus perpetuates its catalytic cycle (Guengrich and MacDonald, 1990).

In some cases, the second electron is donated by NADH instead of NADPH (via NADH-cytochrome b_5 reductase). For example, the catalysis of the O-demethylation of P-nitroanisole requires cytochrome b_5 as an obligatory hemoprotein which facilitates donation of the second electron (Miki et al., 1980).

Nomenclature of P-450 Superfamily

Numerous nomenclatures have been used to identify the different isozymes of cytochrome p-450. Originally, it was assumed that catalytic activities would help researchers in the assignment of the orthologous isozyme

genes across species; orthologous gene in two species refers to an ancestral gene that existed before the evolutionary divergence of the two species (Nebert et al., 1989). This assumption is no longer valid since it has been shown that a single amino acid difference in a cytochrome P-450 peptide of about 500 amino acids can be critical in changing the catalytic activity (Nebert et al., 1991). For this reason, old nomenclatures of cytochrome P-450 based on catalytic activities were discouraged and a new nomenclature based on amino acid similarities and gene mapping was adopted (Nebert et al., 1991)

Currently, the cytochrome P-450 superfamily is believed to be composed of 10 families that comprise 18 subfamilies; each subfamily appears to represent a cluster of tightly linked genes. The adopted nomenclature recommended the use of an italicized root symbol "CYP" for human and "cyp" for mice denoting cytochrome P-450. This root symbol is to be followed by an Arabic number denoting the family, then a letter designating the subfamily (if two or more genes exist within the subfamily, then again an Arabic numeral should follow the preceding letter to represent the individual gene). For example, on the basis of amino acid similarities, the orthologous genes CYP1A1 and CYP1A2 were found to exist in human, mouse, rat, rabbit, hamster, dog and monkeys and they were inducible by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene. These two orthologous genes are conserved across species lines, except in trout. Trout appear to lack the CYP1A2 gene (Nebert et al., 1991). It is worthy to mention that these two genes were referred to in the older literature as cytochrome P-448. On the other hand, certain subfamilies of cytochrome P-450 enzyme are inducible by phenobarbital

such as CYP2B, CYP2C, and CYP3A (Nebert et al., 1991).

Statement of Dissertation Objectives

The existing problematical issue in veterinary toxicology is the variety of chemical compounds present in plants which represent the sole source of food for large animals. These chemical compounds may act as inducers or inhibitors of drug biotransformation. Thus, leading to attenuation or amplification of biological responses to concurrently administered drugs.

The purpose of the first part of this study was to assess the impact of feeding Artemisia on the disposition of concurrently administered drugs in sheep. Artemisia was selected because it is a common range plant in western Oklahoma. Moreover, in addition to its purported value as a forage plant, there is some evidence suggesting that ingestion of Artemisia tends to enhance the expression of the hepatotoxicant effects exerted by Tetradymia ingestion. (Johnson, 1978; Jennings et al., 1978). This phenomenon indicates that Artemisia plays a synergistic role in the development of the clinical photosensitization syndrome "bighead". From that standpoint, Artemisia is intriguing as a possible modulator of biotransformation of concurrently administered drugs. To assess that possibility, sheep were administered antipyrine and erythromycin under two dietary regimens, one under a control-dietary regimen and the other under a regimen containing Artemisia. Subsequently, the disposition of drugs under the two dietary regimens were compared.

The purpose of the second part of this study was to determine the effect

of adding Artemisia or Helenium to the diet on the disposition of antipyrine in rabbits. The results of these studies will shed light on the role of dietary constituents as an important determinant of drug biotransformation and disposition.

The purpose of the third part of this study was to determine the effect of feeding Artemisia or Helenium on the activity of hepatic cytochrome P-450, an essential enzyme component of the Mixed Function Oxidase System (MFO) that mediates catalysis of xenobiotics in phase I biotransformation. In addition, the O-dealkylation of a series of alkoxyresorufin ethers by hepatic microsomes prepared from control-fed rabbits and plant-fed rabbits were investigated in order to gain further insights up to the level of isozymes within cytochrome P-450. Known inducers or inhibitors of cytochrome P-450 isozymes were used to provide a wide facet for comparison between dealkylation conferred by hepatic microsomes prepared from plant-fed rabbits versus dealkylation by microsomes prepared from other xenobiotic-treated rabbits.

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CHAPTER II

EFFECTS OF FEEDING ARTEMISIA SPP. ON DISPOSITION OF ANTIPYRINE AND ERYTHROMYCIN IN SHEEP

INTRODUCTION

There are many dietary components including proteins, minerals, and vitamins that can modulate xenobiotic or drug biotransformation. Not the least of these components are the naturally occurring chemicals present in food plants which act as inducers or inhibitors of the Mixed Function Oxidase System (MFO), a major enzymatic system that mediates biotransformation, and as such can lead to attenuation or amplification of biological responses to concurrently administered drugs. For example, indoles of the cabbage family (Cruciferae) have been reported to act as inducers of MFO; therefore, people consuming brussels sprouts and cabbage demonstrated a significant decrease in the half-life of concurrently administered antipyrine (Pantuck et al., 1979 and 1984). In contrast, rats and mice fed trans-anethole or myristicin of anise seeds and nutmeg, respectively, exhibited a remarkable prolongation of pentobarbital sleeping time indicating an inhibitory-type effect on MFO (Marcus and Lichtenstein, 1982).

A prime example of considerable interest in sheep is the apparent

biotransformation effect observed with ingestion of Tetradymia (horse brush) when it is preceded by ingestion of Artemisia (sage brush). In such instances, an overwhelming hepatic photosensitization and a remarkable serum enzyme elevation (GOT and LDH) occur which are not otherwise a problem when Tetradymia is eaten alone. This phenomenon indicates that Artemisia acts as a preconditioning or predisposing factor that tends to aggravate the hepatotoxicity exerted by Tetradymia ingestion (Jennings et al., 1978; Johnson 1978). From that standpoint, Artemisia is intriguing as a possible modulator of biotransformation of concurrently administered drugs.

In the present experiment the impact of feeding Artemisia filifolia (sand sagebrush), Artemisia ludoviciana (white sage), and Medicago sativa (alfalfa) on disposition of antipyrine and erythromycin were investigated. Alfalfa was added as a positive control since it has been reported to induce aryl hydrocarbon hydroxylase activity (Hendrich and Bjeldane, 1983). Antipyrine was selected because of its general acceptance as a model drug for indirect assessment of Mixed Function Oxidase (MFO) activity. (Vesell, 1979; Danhof and Teunissen, 1984). Antipyrine gained this popularity due to its many favorable characteristics including minimal plasma protein binding, and total dependence on hepatic MFO activity for biotransformation and elimination. Therefore, an alteration in antipyrine half-life is considered to be an indirect index for assessment of MFO activity. Typically, compounds known to induce MFO in the liver tend to shorten the plasma half-life of antipyrine whereas compounds known to inhibit MFO tend to prolong the plasma half-life of antipyrine (Baggot, 1977). Erythromycin was selected because it is a

commonly used antibiotic in veterinary practice; in addition, to its requirement for N-dealkylation and biotransformation. (Burrows, 1980)

MATERIALS AND METHODS

Experimental Protocol

Six white-face ewes, ranging in weight from 43-73 kg, were divided into 3 pairs. Each pair were housed in a separate pen. All animals were in apparent good health, maintained on a control diet of grass hay and concentrates, and allowed free access to water.

The experiment was carried out in three periods, each period composed of two phases (control-diet phase and test-diet phase). An interval of one month was allowed between experimental periods in order to avoid the possibility of residual or carry-over effect of a treatment into the following period. The purpose of the three periods was to permit each pair's diet to be changed to include one of the three test plants; thus each animal was tested under the influence of each of the three test diets.

In the first week of each period of the experiment (Control-diet phase), all six ewes were acclimated and maintained on the control diet. On days 5 and 7 of the first week, antipyrine and erythromycin were administered in a crossover-type design at a dose rate of 15 mg/kg i.v. On day 5, one animal from each pair received antipyrine while the other received erythromycin. On day 7, the two test drugs were reversed for the pair. Blood samples for drug assay were collected at 1,2,4,6,8,10, and 12 hours after drug administration. Serum was harvested and stored at -20°C until assayed.

In the second week of each period of the experiment (test-diet phase), each pair were fed a test plant (either Artemisia filifolia, Artemisia ludoviciana or alfalfa) in combination with grass hay and concentrates. In a similar fashion to the control-diet phase, antipyrine and erythromycin were administered on days 5 and 7. Blood samples for drug assay were collected, serum was harvested and kept frozen as described previously.

Drugs Assay

(i) Antipyrine

Antipyrine concentrations in serum were quantitated using a gas chromatographic method reported by Clarke et al. (1989 and 1992). Forty microliter of 5N NaOH and 2 ml of ethyl acetate (containing 2.5 μg/ml of phenacetin as an internal standard) were added in sequence to 250 μL of the sample. The sample was then vortexed for 30 seconds to assure extraction uniformity. The combination was centrifuged at 600 g for 15 minutes. Aliquots of the supernatant (1.5 ml) were transferred to clean test tubes and evaporated to dryness under nitrogen. The resultant precipitate was redissolved in 250 μL of hexane. Antipyrine standards (1, 2.5, 5, 10, 25, and 50 ppm) were prepared by dissolving appropriate amounts of antipyrine in 250 μL of blank sheep serum. These standards were subjected to the same extraction and analytical procedures as the test sera. Antipyrine concentrations were quantitated by injecting 4 μL aliquots of the extracted sample, dissolved in hexane, into a gas chromatograph (Model 565, Tracor Instruments Austin Inc., Austin, Tx, U.S.A.) with a 6-foot, 2-mm ID, 1/4-inch OD glass column

packed with GP 3%, SP-2250-DB on 100/120 supelcoport (Supelco Inc., Bellefonte, PA, U.S.A.). The temperature of the column, injection port, and nitrogen-phosphorous detector (Model 702, Tracor Instruments Austin Inc.) were adjusted to 180, 230, and 250°C, respectively. Flow rate for helium carrier gas was 25 ml/min; for detector hydrogen, 2.5 ml/min; and for air, 120 ml/min. Peak heights were measured and recorded using an integrator (Model SP4290, Spectra-physics, San Jose, CA, U.S.A.). Ratios of the standard peak heights were correlated with their corresponding known concentrations in order to establish the linear regression equation or the so-called deterministic equation ($Y = mX + c$). Unknown concentrations of the sample sera (Y's) were calculated using the pre-established deterministic equation.

(ii) Erythromycin

Erythromycin serum samples were bioassayed by an agar-well diffusion method, employing Micrococcus luteus ATCC 9341 as the test organism (Bennett et al., 1966). Standard solutions were prepared in sheep serum by appropriate serial dilutions of erythromycin. Inhibition-zone widths were measured and correlated with known standard concentrations to establish the standard curve. Widths of inhibition-zones from unknown test sera were measured and converted to serum concentrations using standard curves developed for each 81-well agar plate.

Pharmacokinetic methods and statistics

Coefficients and exponents were calculated using an iterative least squares non-linear regression analysis computerized program, ESTRIP (Brown

and Manno, 1978). The data were best described by a monoexponential equation: $CP = Be^{-\beta t}$ due to paucity of blood samples collected during the first hour post-injection that limited our ability to discern the distribution phase.

The following pharmacokinetic parameters were used for comparisons: (1) The overall elimination rate constant (β) = log-linear slope of disposition curve; (2) elimination half-life ($t_{½\beta}$) = $0.693/\beta$; (3) apparent volume of distribution (V_d) = Dose/B (where B represents the extrapolated zero time serum drug concentration); (4) body clearance (Cl_B) = $\beta x V_d$

Testing of statistical significance was accomplished using the paired comparisons between test diets and corresponding control values. The effect of test-diets on the slope of drug elimination (β) was tested using paired t-test. The non-parametric test, Wilcoxon's rank sum, was used to compare the effect of test-diets on $t_{½\beta}$, V_d, and Cl_B since a normal distribution cannot be assumed for those parameters (Powers et al., 1978; Powers, 1990).

RESULTS

This experiment was prematurely terminated due to environmental factors. Results reported herein include only the first and second periods of the experimental design, the third period was excluded. Therefore, pharmacokinetic variables were calculated for only four sheep per dietary-test plant instead of six sheep. Thus, the same four sheep are not represented for each diet. Pharmacokinetic variables of controls during the first and second periods were not statistically different.

The effects of various diets on antipyrine and erythromycin pharmacokinetic variables are shown in Table 1 and Table 2, respectively. For antipyrine, a significant increase in the slope of elimination (β) and a marked decrease in half-life ($t_{½\beta}$) were observed when the sheep diet was changed from grass hay and concentrates to any one of the three test plants in combination with grass hay and concentrates. Antipyrine volume of distribution (V_d) remained unchanged during the different dietary regimens. The relevant increase in the slope of elimination (β) in the presence of a constant volume of distribution (V_d) resulted in an increase in clearance ($Cl_B = \beta \times V_d$). Clearance increased approximately two fold during diets containing Artemisia species or alfalfa as compared to control diet.

With regard to erythromycin, there were no significant changes among pharmacokinetic variables in response to dietary supplementation with either Artemisia filifolia, Artemisia ludoviciana or alfalfa.

Table 1.

MEAN OR MEDIAN PHARMACOKINETIC VALUES FOR ANTIPYRINE (15 mg/kg) IN SHEEP FED WITH DIFFERENT SPECIES OF ARTEMISIA.

Diet	Pharmacokinetic Value			
	β*min^{-1}	$t_{½\beta}$†min	V_d†ml/kg	Cl_B†ml/min/kg
Control diet	0.0051 ± 0.0015^A	137 ± 36^A	1010 ± 130^A	5.2 ± 1.1^A
Artemisia filifolia	0.0096 ± 0.0023^B	78 ± 15^B	1090 ± 132^A	9.8 ± 1.3^B
Control diet	0.0050 ± 0.0018^A	132 ± 32^A	1018 ± 125^A	5.1 ± 1.1^A
Artemisia ludoviciana	0.0106 ± 0.0019^B	67 ± 9^B	1160 ± 45^A	10.3 ± 0.9^B
Control diet	0.0049 ± 0.0013^A	138 ± 34^A	1012 ± 122^A	4.8 ± 1.2^A
Alfalfa diet	0.0115 ± 0.0031^B	60 ± 9^B	970 ± 68^A	10.7 ± 1.7^B

*Mean ± SD

†Median ± Median deviation (Median deviation $= \frac{\Sigma|x_i - x_m|}{n}$)

In comparison of different diets to the control diet means or medians within a column with different superscripts^A or ^{B} are significantly different from each other ($P \leq 0.05$) using paired-t-test or Wilcoxon's rank sum test.

Table 2.

MEAN OR MEDIAN PHARMACOKINETIC VALUES FOR ERYTHROMYCIN (15 mg/kg) IN SHEEP FED WITH DIFFERENT SPECIES OF ARTEMISIA

Diet	Pharmacokinetic Value			
	β*min^{-1}	$t_{½\beta}$†min	V_d†ml/kg	Cl_B†ml/min/kg
Control diet	0.0064 ± 0.0015[A]	105 ± 18[A]	1427 ± 240[A]	10.4 ± 1.5[A]
Artemisia filifolia	0.0058 ± 0.0013[A]	113 ± 20[A]	1375 ± 208[A]	10.2 ± 2.7[A]
Control diet	0.0063 ± 0.0018[A]	100 ± 12[A]	1410 ± 215[A]	10.3 ± 1.4[A]
Artemisia ludoviciana	0.0067 ± 0.0020[A]	113 ± 15[A]	1334 ± 253[A]	10.1 ± 1.4[A]
Control diet	0.0065 ± 0.0012[A]	103 ± 15[A]	1352 ± 196[A]	9.8 ± 1.7[A]
Alfalfa diet	0.0066 ± 0.0017[A]	108 ± 21[A]	1286 ± 162[A]	10.7 ± 1.8[A]

*Mean ± SD

†Median ± Median deviation (Median deviation = $\frac{\Sigma|X_i - X_m|}{n}$)

In comparison of different diets to the control diet means or medians within a column with different superscripts [A] or [B] are significantly different from each other ($P \leq 0.05$) using paired t-test or Wilcoxon's rank sum test.

DISCUSSION

The present studies demonstrated that ingestion of a diet containing approximately 2% Artemisia for a week resulted in statistically significant changes for antipyrine pharmacokinetic variables. Thus, indicating the potential for such a diet to increase the metabolism of antipyrine. Conversely, there was little or no change in the metabolism of erythromycin. Thus, it appears that the requisite biotransformation pathways for antipyrine and erythromycin differ. The pathway for antipyrine biotransformation is mainly via hydroxylation and carboxylation (Tufenkji et al., 1988; Witkamp et al., 1991) which are catalyzed by the isozyme CYP2B, an isozyme within cytochrome P-450 superfamily (Bachmann et al., 1991). In the case of erythromycin, the biotransformation pathway is mainly via N-demethylation which is catalyzed by the isozyme CYP3A (Pineau et al., 1990). Therefore, it could be deduced from the present studies that Artemisia is capable of exerting an inducing-type effect on the isozyme CYP2B but not CYP3A as reflected by the increased metabolism of antipyrine while erythromycin metabolism remained unchanged.

This experiment showed that both Artemisia species as well as alfalfa decreased antipyrine half-life ($t_{½\beta}$) and increased its clearance and elimination, presumably as a result of inducing Mixed Function Oxidase System (MFO) or at least inducing some isozymes within MFO system. This finding may explain the increased toxicity and photosensitization seen when Tetradymia ingestion is preceded by Artemisia ingestion. In essence, Tetradymia contains tetradymol which is further metabolized by the MFO system into culprit intermediates that are even more toxic than the parent compound i.e. lethal synthesis or

bioactivation (Jennings et al., 1978). Therefore, since Artemisia induces MFO, its ingestion would expedite the process of lethal synthesis or hasten the conversion of tetradymol into more deleterious intermediates.

The results of this study indicate that ingestion of plants such as Artemisia may have a marked effect on disposition of concurrently administered drugs as exemplified by antipyrine. This would be even more important when chemicals of greater toxicological impact are used concomitantly. For example, insecticides such as Diazinon and parathion are converted in the animal body by MFO into harmful metabolites, namely, diazoxon and paraoxon, respectively. (Abdelsalam and Ford, 1986; Dikshith, 1991). However, in some situations, a diet containing Artemisia or alfalfa might augment or increase the pharmacological effects of drugs that are not active per se but require biotransformation to yield the active metabolites; such as prontosil and fenbendazole which are metabolized in the animal body by MFO to yield sulfanilamide and fenbendazole sulfoxide, respectively (Yang and Lu, 1987).

In conclusion, the effects of dietary constituents on the disposition of drugs will only be fully appreciated when the underlying mechanisms of biotransformation and interactions conferred by these dietary constituents are scrutinized.

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CHAPTER III

THE EFFECTS OF *ARTEMISIA FILIFOLIA* AND *HELENIUM FLEXUOSUM* ON DISPOSITION OF ANTIPYRINE IN RABBITS

INTRODUCTION

Humans and animals are exposed on a daily basis to a myriad of chemicals present in their food. These chemicals may affect the host directly or indirectly through a variety of biological interactions and may act to the benefit or detriment of the host. Examples of chemicals in food plants which may interact in such a manner are sesquiterpene lactones, sesquiterpene alcohols and oxidized matricarins (Geissman and Irwin, 1973) of *Artemisia and Helenium* (Asteraceae). In addition, these plant chemicals may interact with those from other ingested plants. For example, *Artemisia nova* is reported to act as a preconditioning plant for toxicity caused by ingestion of *Tetradymia glabrata*. When ingestion of *Tetradymia glabrata* is preceded by ingestion of *Artemisia nova*, an overwhelming hepatic photosensitization occurs; which is not otherwise a problem when each plant is eaten alone (Johnson, 1978). Sesquiterpene lactones in *Helenium* species are well documented livestock toxicants (U.S. Department of Agriculture, 1968). In this case, however, both direct and indirect effects are apparent.

The sesquiterpene lactones possess an exocyclic methylene group

($=CH_2$) which is believed to react with thiol-containing compounds by a Michael-type addition leading to depletion of reduced glutathione (GSH) (Kupchan et al., 1970; Merrill et al., 1988). Reduced glutathione (GSH) is formed by reduction of oxidized glutathione (GSSG), a reaction that utilizes NADPH as an electron donor (Stryer, 1988). Similarly, cytochrome P-450, a major component of Mixed Function Oxidase system, requires NADPH as an electron donor to catalyze oxidation of various xenobiotics (Guengrich and MacDonald, 1990). Thus, it appears that both GSH and cytochrome P-450 are dependent on NADPH as a cofactor. From that standpoint, we hypothesized that plants containing sesquiterpene lactones will not only deplete GSH but will also affect Mixed Function Oxidase system (MFO) indirectly.

To evaluate the potential for indirect effects, model drugs for assessing hepatic oxidative enzyme activity are useful. Antipyrine has become popular for this purpose (Danhof & Teunissen, 1984) because it is distributed evenly in total body water with minimal plasma protein binding, and is almost exclusively metabolized in the liver by MFO (Vesell, 1979). Therefore, compounds known to inhibit MFO in the liver typically prolong the plasma half-life of antipyrine, and those that induce MFO frequently shorten the plasma half-life of antipyrine (Baggot, 1977).

The purposes of this study were: (1) to determine the effects of adding *Artemisia filifolia* or *Helenium flexuosum* to the diet on antipyrine pharmacokinetic parameters as a possible probe for assessment of alterations in Mixed Function Oxidase system (MFO)activity, and (2) to evaluate the rabbit as an animal model for assessing the impact of xenobiotics in food.

MATERIALS AND METHODS

Two groups of rabbits were used, each group composed of six adult New Zealand white male rabbits weighing between 3-4 Kg. Rabbits were caged individually in a controlled environment (12:12 lighting schedule & temperature maintained at 70°F) and allowed to acclimate for a week before treatment commenced. Standard rabbit pellets (ACCO) and water were available *ad libitum*.

The experiments were carried out in series to permit each animal to serve as its own control. In the first phase (Control diet) on day 1, all 12 rabbits were administered antipyrine into the marginal ear vein at a dose rate of 25 mg/kg. Blood samples were collected from the contralateral vein at 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 min after dosing. Serum was harvested and kept frozen at -20 C until assayed using a gas chromatographic method (Clarke et al., 1992).

The second phase (test-diet) commenced on day 14, when the six rabbits of group 1 were given *Artemisia filifolia* and the six rabbits of group 2 were given *Helenium flexuosum,* in addition to the standard rabbit pellets. The air-dried plants were finely ground and given in gelatin capsules at a dose rate of 250 mg/kg daily for 5 days. On day 19, each rabbit was again administered antipyrine (25 mg/kg) and blood samples were collected using the same intervals as those after the first administration during the control diet phase.

Pharmacokinetic parameters were calculated using mono- or biexponential equations by means of an iterative least squares regression analysis computer program (Brown and Manno, 1978). The data were best

represented by a biexponential equation: $Cp = Ae^{-\alpha t} + Be^{-\beta t}$, where Cp is the concentration of antipyrine in serum at time t, A and B are intercept terms, and α and β are hybrid rate constants related to distribution and elimination phases, respectively.

The pharmacokinetic parameters: slope of elimination (β) = terminal slope of the semilogarithmic plot of serum antipyrine concentration versus time, half-life of elimination ($t_{½\beta}$) = $0.693/\beta$, elimination from central compartment (K_{el}) = $\alpha\beta/K_{21}$, volume of distribution (V_d) = Dose/AUC.β, and total body clearance (Cl_B) = Dose/$AUC_{0-\infty}$ were determined for an open-two compartment model with elimination from the central compartment (Gibaldi and Perrier, 1982).

Statistical analysis

The variances were determined to be homogenous by the F-test; therefore, the student t-test with df($N_1 + N_2$-2) was used to determine the effect of the test-diet on the slope of antipyrine elimination (β). The non-parametric test, Wilcoxon's rank sum, was used to compare $t_{½\beta}$, V_d, K_{el} and Cl_B since a normal distribution cannot be assumed for those parameters (Powers et al., 1978; Powers, 1990).

RESULTS

There was no significant change in antipyrine pharmacokinetic parameters in response to dietary supplementation with *Artemisia filifolia* as shown in table 1.

Feeding *Helenium flexuosum* to rabbits resulted in a significant decrease in the slope of antipyrine elimination (β), from a mean of 0.0067min^{-1} during the control diet to a mean of 0.0036 min^{-1} after feeding the *Helenium* diet (Table 1). The sequel of this decrease in the slope of elimination is a marked lengthening of half-life ($t_{½\beta}$) from a median of 99.5 min (control diet) to a median of 215.5 min (*Helenium* diet). The decay of antipyrine in the serum of a representative rabbit during control diet versus a diet containing *Helenium* is shown in figure 1. This rabbit was selected as a representative of the group fed *Helenium flexuosum* because antipyrine pharmacokinetic variables were closest to the median for all rabbits in this group.

Median elimination rate from the central compartment (K_{el}) decreased significantly from a median of 0.0245 min^{-1} (Control diet) to a median of 0.0084 min^{-1} (*Helenium* diet) as shown in table 1. This decrease in the rate of elimination from central compartment was not accompanied by a significant decrease in total body clearance since there was a significant increase in the volume of distribution after feeding *Helenium flexuosum*.

Table 1.

MEAN ± SD OR MEDIAN ± MEDIAN DEVIATION VALUES FOR ANTIPYRINE PHARMACOKINETIC PARAMETERS OBTAINED BEFORE AND AFTER FEEDING TEST-DIETS CONTAINING EITHER *ARTEMISIA* OR *HELENIUM*.

	Antipyrine		Antipyrine	
	Control diet	Artemisia diet	Control diet	Helenium diet
β,min^{-1}	0.0065 ± 0.0017	0.0076 ± 0.016	0.0067 ± 0.0011	0.0036* ± 0.0008
$t_{½\beta}$,min†	111 ± 21.5	93.5 ± 13.83	99.5 ± 15	215.5** ± 28
K_{el},min^{-1}†	0.0137 ± 0.0019	0.021 ± 0.006	0.0245 ± 0.005	0.0084** ± 0.0007
V_d, ml/kg†	1716.5 ± 218.8	1458.5 ± 283.16	1119.5 ± 226.8	1966.5** ± 458.7
cl_B, ml/min. Kg†	10.19 ± 1.19	11.06 ± 0.968	7.86 ± 1.5	7.29 ± 1.11

†Values are expressed as median ± median deviation (Median Deviation = $\frac{\Sigma |X_i - X_m|)}{n}$

* significant at $P \leq 0.05$ (using student t-test)

** significant at $P \leq 0.05$ (using Wilcoxon's Rank sum test)

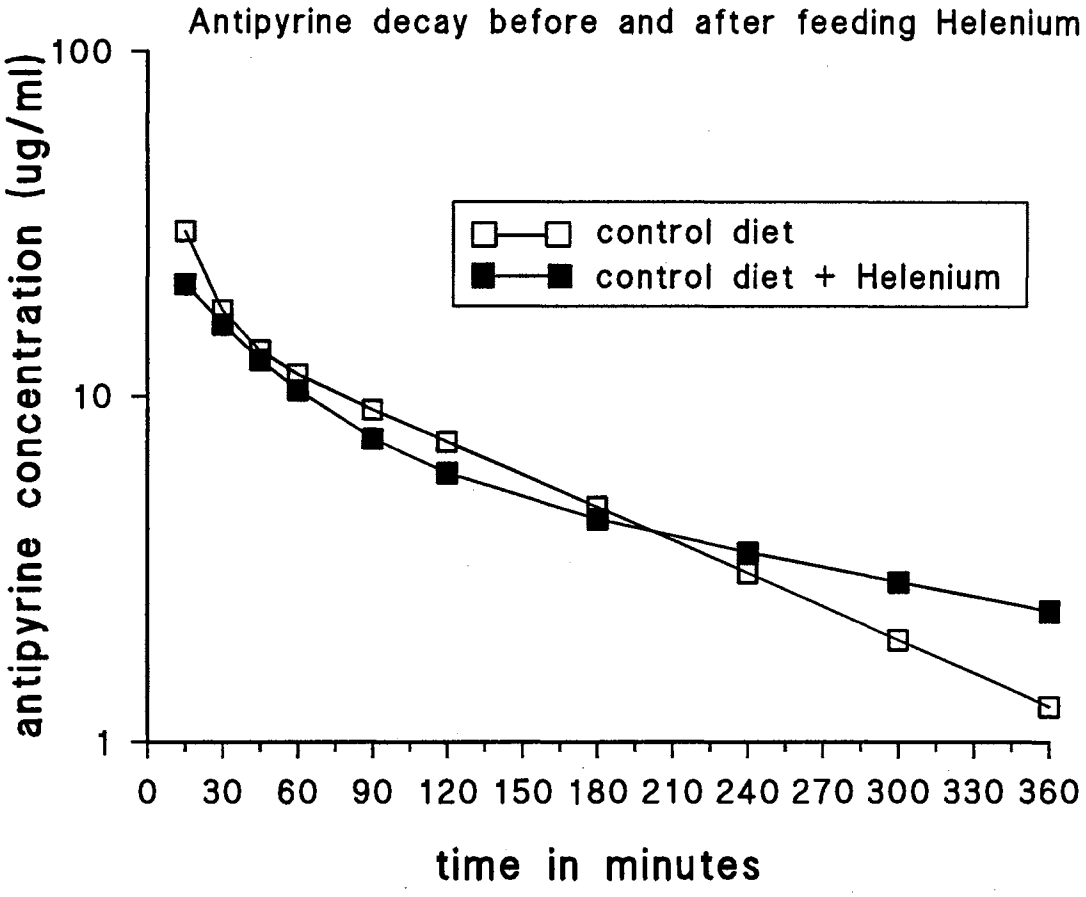

FIG 1. Antipyrine disposition in one rabbit taken as a representative of the group fed Helenium flexuosum; the decay is less steeper during (control diet + Helenium) indicating a longer half-life of antipyrine elimination.

DISCUSSION

Antipyrine disposition is commonly employed as an indirect index for measurement of MFO system activity. Changes in the half-life of elimination and clearance of antipyrine are considered to provide an overall assessment of inhibition or induction of the MFO system (Vesell, 1979; Danhof & Teunissen, 1984).

In the present study, *Helenium flexuosum* prolonged markedly the half-life of antipyrine elimination ($t_{½\beta}$) from a median of 99.5 min (control diet) to a median of 215.5 min (*Helenium* diet). The lengthening of $t_{½\beta}$ and the concomitant decrease in β from a mean of 0.0067 min^{-1} to a mean of 0.0036 min^{-1} is strongly indicative that *Helenium flexuosum* inhibits the MFO system. These findings corroborate the finding of Chapman et al. (1988) who reported that helenalin, a sesquiterpene lactone isolated from *Helenium microcephalum*, acts as an inhibitor of cytochrome P-450 and cytochrome b_5, key enzymes in the MFO system. Furthermore, these *in vivo* results are in agreement with *in vitro* findings of Dalvi and McGowan (1982), who reported that incubation of helenin with microsomal proteins produced a profound loss of cytochrome P-450 activity. These results are indicative of the potential for *Helenium* species to influence the disposition and biological effects of other chemicals to which the animal may be concurrently exposed.

There were no significant changes in antipyrine pharmacokinetic parameters when rabbits were given *Artemisia filifolia*. However, in all six rabbits, there was a consistent shortening of antipyrine half-life of elimination. This consistent change was small and statistically insignificant but may have

been significant under different experimental conditions, such as a longer period of gavaging and/or more animals.

In the present study, in order to avoid pharmacokinetic intraspecies variations reported in rabbits by Chambers and Jefferson (1982) and by Paxton (1983), each rabbit was used to serve as its own control. Rabbits appear to be good animal models to assess the impact of xenobiotics in food, this could be attributed to the absence of hepatic vein sphincters, a criterion shared by Humans (Orszulak-Michalak and Kaszubski, 1987). The lack of hepatic vein sphincters would enable rabbits to have a consistent blood flow to the organ of biotransformation and elimination.

The significant increase in V_d experienced after feeding *Helenium flexuosum* compared to the control diet was an unexpected finding. A similar change in volume of distribution of antipyrine in rabbits has been reported by Taylor and Houston (1985) following promethazine pretreatment. The increase in V_d had counterpoised the decrease in β and thus prevented a change in Cl_B which would have been evident had the V_d not changed ($Cl_B = \beta x V_d$). There is no ready explanation for this phenomenon but perhaps a reasonable conjecture would be that the toxic effects of *Helenium flexuosum* delayed attainment of the distribution equilibrium.

In conclusion, the results of our study showed that *Helenium flexuosum* exerted a marked influence on disposition of antipyrine presumably as a result of inhibition of the MFO system. Other plants available as food for range animals may also modify metabolism and pharmacokinetic parameters of drugs administered during consumption of these plants. Therefore, the type of food

available for animals should be taken into consideration when drug doses are calculated.

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CHAPTER IV

EFFECTS OF *ARTEMISIA FILIFOLIA* AND *HELENIUM FLEXUOSUM* COMPARED TO PHENOBARBITAL, 3 METHYLCHOLANTHRENE, AND SKF-525A ON ALKOXYRESORUFIN O-DEALKYLATION BY RABBIT HEPATIC MICROSOMES

INTRODUCTION

It is well established that drugs and other xenobiotics, such as pesticides, food additives, cosmetic ingredients, environmental pollutants, and natural plant products enter the body and represent a potential threat to the health of the individual. These xenobiotics may be biotransformed to less toxic metabolites or activated to dangerous electrophiles that are detrimental to the host. In most instances, this is accomplished by a superfamily enzyme system called cytochrome P-450 (Guengrich, 1991).

The cytochrome P-450 superfamily, the so-called "CYP" for human and "cyp" for mouse, exists in multiple forms of isoenzymes: at least 23 and 38 isoenzymes have been characterized by genetic mapping in rabbits and rats, respectively (Nebert et al., 1991). Each P-450 isoenzyme possesses different but possibly overlapping catalytic activities. In the previous decade, O-dealkylation of alkoxyresorufin ethers has become accepted as an exceptionally

useful tool to differentiate isoenzymes within the cytochrome P-450 superfamily. For instance, ethoxyresorufin has been reported to be a highly specific substrate for 3-methylcholanthrene (3MC)-inducible forms of cytochrome P-450, such as CYP1A1 and CYP1A2; whereas pentoxyresorufin has been shown to be the substrate of choice for phenobarbital (PB)-inducible forms of cytochrome P-450, such as CYP2B1 and CYP2B2 (Burke and Mayer, 1983; Lubet et al., 1985; Yang et al., 1988).

Two plants, *Artemisia filifolia* and *Helenium flexuosum*, were evaluated in this experiment as they have been reported to contain sesquiterpene lactones with an exocyclic methylene group, a highly reactive moiety that can readily alkylate thiol-containing compounds by Michael-type addition (Herz, 1978; Appendino et al., 1982; Merrill et al., 1988). This property prompted us to hypothesize that metabolism of these plants might be mediated through cytochrome P-450, and thereby lead to alterations in its ability to catalyze O-dealkylation of alkoxyresorufin ethers.

In the present experiments, we studied the O-dealkylation of a series of alkoxyresorufin ethers by hepatic microsomes prepared from control and xenobiotic-treated rabbits in order to:

1. determine the ability of prototype P-450 inducers PB and 3MC and the prototype P-450 inhibitor proadifen hydrochloride (SKF-525A) to affect the level of hepatic cytochrome P-450 and consequently modulate the O-dealkylation of alkoxyresorufin ethers.

2. determine the effect of incorporating xenobiotics of plant origin in the diet such as *Artemisia filifolia* and *Helenium flexuosum* on hepatic cytochrome P-

450 and on the ability of hepatic microsomes to dealkylate alkoxyresorufin ethers.

3. Compare the specificity of alkoxyresorufin ethers for various prototype and non-prototype xenobiotics.

MATERIALS AND METHODS

Chemicals

Phenobarbital (PB) was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ 08003-4099). 3-methylcholanthrene (3MC) and NADPH were purchased from Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO 63178). Proadifen hydrochloride (SKF-525A) was a generous gift from SmithKline Beecham Pharmaceuticals (P.O. Box 1539, King of Prussia, PA 19406).

Methyl-, ethyl-, pentyl- and benzyloxyresorufin and standard resorufin were obtained from Molecular Probe Inc. (Junction City, OR 97448). Propoxyresorufin was synthesized in our laboratory according to the methods described by Elangbam et al. (1991).

Plants of *Artemisia filifolia* and *Helenium flexuosum* were collected from sites in western and eastern Oklahoma, respectively. They were air-dried, finely ground, and stored in plastic bags.

Experimental Protocol

Adult male New Zealand albino rabbits (n = 24) were caged individually in a controlled environment room under 12:12 lighting schedule with rabbit chow (Acco) and water available *ad libitum*. Rabbits were allowed to acclimate

for a week before treatments were commenced.

The experimental protocol was carried out in a quadrinomial manner. In each term, six rabbits were done at a time. Rabbit No. 1 was untreated and served as a control. Rabbit No. 2 was administered phenobarbital (PB) daily at a dose rate of 60 mg/kg i.p. for 5 days. Rabbit No. 3 was administered 3-methylcholanthrene (3MC) daily at a dose rate of 25 mg/kg i.p. for 5 days. Rabbit No. 4 was administered SKF-525A at a dose rate of 100 mg/kg i.p. just 1 hour before the animal was sacrificed. Rabbit No. 5 and Rabbit No. 6 were gavaged with *Artemisia filifolia* and *Helenium flexuosum*, respectively. The finely ground plants were packed in gelatin capsules and gavaged using a balling gun at a dose rate of 250 mg/kg daily for 5 consecutive days.

Preparation of Liver Microsomes

Microsomes were prepared from four individual animals per treatment. Animals were fasted overnight and euthanatized in the 6th day by CO_2 asphyxiation. Ten grams of each animal liver were immediately washed twice in cold 1.15% (w/v) KCl, minced, and homogenized in Tris-KCl buffer. Microsomes were harvested and total cytochrome P-450 was assayed using the method of Omura and Sato (1964). Microsomal protein was estimated using the BCA protein assay (Smith et al., 1985). The resulting microsomal pellets were suspended in SET buffer (pH = 7.4) and stored in 0.2 ml aliquots at -135°C.

Analysis of Alkoxyresorufin Metabolism

Assay of the O-dealkylation of various alkoxyresorufin ethers to the common metabolite, resorufin, was carried out essentially as described by Burke et al. (1985). Incubations were performed directly in a 4.0 ml fluorometric cuvette at 37°C with the use of a Spex-FluroMax™[1], and formation of the fluorescent metabolite, resorufin, was detected at wavelength settings of 540 nm and 585 nm for excitation and emission, respectively.

Thirty microliter of 1 mM solution of the substrate, alkoxyresorufin, was mixed with 2700 μl of 0.1 M Na/K phosphate buffer, pH 7.6 and microsomal protein was added in a volume of 140 μl. Total protein concentration was between 25 μg/ml and 103 μg/ml (i.e., protein concentration inside the cuvette was between 1.20 μg/ml and 4.95 μg/ml) depending on the inducing or inhibiting agent used in the experimental protocol. The baseline was recorded for 20 seconds, then dealkylation was initiated by the addition of 10 μl of 60 mM NADPH (Sigma), and the reaction was allowed to proceed for 2 minutes to measure the slope generated by the increase in fluorescence due to formation of resorufin as a sequela of dealkylation. Calibration was then achieved by the addition of 30 μl 0.1 mM standard resorufin.

Statistical Analysis

The decision tree method was used to select the appropriate hypothesis-testing procedure (Gad and Weil, 1988). Data were first subjected to Bartlett's

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[1a] Spex-FluroMax Industries, Inc., Edison, NJ USA.

test to detect homogeneity of variances; since the variances were homogenous ANOVA was used. As a result of a significant F-value, Dunnett's-test was carried out as a post-hoc to ANOVA to provide further intracomparison of groups vs. control without increasing Type I error. Results were considered significant at $P < 0.05$.

RESULTS

Phenobarbital (PB) and 3-methylcholanthrene (3MC) increased the total hepatic microsomal cytochrome P-450 content by 2 - 3 fold as compared to the control (table 1).

PB-induced microsomes significantly increased the rate of O-dealkylation of benzyloxyresorufin and pentoxyresorufin as compared to the control (table 2). Benzyloxyresorufin was the substrate of choice showing the highest rate of metabolism (3537 pmole/min/mg) and the greatest degree of induction over control, approximately 47 fold (table 2 and figure 1).

In contrast, 3MC-induced microsomes significantly increased the rate of O-dealkylation of all alkoxyresorufin substrates as compared to the control (table 2). Methoxyresorufin substrate showed the highest rate of metabolism (5633 pmole/min/mg) but not the greatest degree of induction over the control. The greatest degree of induction over the control in 3MC-induced microsomes was shown by pentoxyresorufin, approximately 8 fold, but its rate of metabolism was only 339 pmole/min/mg which means that the differential factor (DF) between methoxyresorufin and pentoxyresorufin in case of 3MC-

induced microsomes was 16.6 times in favor of methoxyresorufin and the DF between methoxyresorufin and benzyloxyresorufin was 18.4 times in favor of methoxyresorufin (DF = substrate showing the highest rate of metabolism ÷ substrate showing the lesser rate of metabolism).

The 3MC-induced microsomes showed higher activity for dealkylation of short-alkyl chain (C1 to C3) and as the number of carbon atoms in the alkyl chain increased, the propensity to dealkylate decreased. Therefore, the capability of 3MC-induced microsomes to dealkylate alkoxyresorufin ethers can be arranged in the following order: methoxy > ethoxy > propoxy > pentoxy > benzyloxy (table 2).

SKF-525A had no affect on the total cytochrome P-450 content in the rabbit nor did it affect the rate of O-dealkylation of alkoxyresorufin ethers (table 1, table 2, and figure 3).

Microsomes prepared from rabbits that were fed *Artemisia filifolia* or *Helenium flexuosum* caused a dramatic decrease, approximately 50%, in the rate of O-dealkylation of short-alkyl chain alkoxyresorufin ethers (table 2, figure 4 and figure 5).

Table 1.

EFFECT OF VARIOUS XENOBIOTICS (INCLUDING KNOWN INDUCERS OR INHIBITORS) ON THE LEVEL OF HEPATIC CYTOCHROME P-450 IN THE RABBIT

	Treatment	Cytochrome P-450 (nmole/mg of protein)
1.	Control (no treatment)	1.50 ± 0.24[a]
2.	Phenobarbital (PB)	3.85 ± 0.06[b]
3.	3-Methycholanthrene (3MC)	3.94 ± 0.07[b]
4.	Proadifen hydrochloride (SKF-525A)	1.34 ± 0.05[a]
5.	*Artemisia filifolia*	1.37 ± 0.33[a]
6.	*Helenium flexuosum*	1.34 ± 0.32[a]

Values are expressed as means ± standard deviation.
[a,b]Means with the different superscripts differ ($p < 0.05$).

Table 2.

ALKOXYRESORUFIN O-DEALKYLATION ACTIVITY IN RABBITS FOLLOWING ADMINISTRATION OF VARIOUS XENOBIOTICS.

Treatment (n = 4)	Dose	O-dealkylation of alkoxyresorufin activity (pmole resorufin/min/mg protein) Methoxy	Ethoxy	Propoxy	Pentoxy	Benzyloxy
Control	---	2001 ± 319	3055 ± 176	1073 ± 74	41 ± 10	75 ± 8
Phenobarbital	60 mg/kg i.p. for 5 days	1617 ± 174	2630 ± 422	832 ± 194	688**±127 (~ 17)	3537**±516 (~47)
3 MC	25 mg/kg i.p. for 5 days	5633**±697 (~2.8)	4754**±265 (~1.55)	1658**±62 (~1.54)	339** ± 70 (~8.0)	305** ±28 (~4.0)
SKF-525A	100 mg/kg i.p. 1 hour before animal sacrifice	1874 ±300	2791 ±371	1147 ±221	40 ± 9	70 ± 8
Artemisia filifolia	250 mg/kg gavaged for 5 days	824 **±160	1638** ± 79	526**± 41	35 ± 8	75 ± 8
Helenium flexuosum	250 mg/kg gavaged for 5 days	1153**± 56	1856** 254	642**±140	45 ± 10	65 ± 6

**Significantly different at $p < 0.05$ using Dunnett's-test.
Values are expressed as means ± standard error of the mean for four animals per treatment.
(~) = Approximate folds of induction = (reaction rate with induced microsomes divided by reaction rate with control microsomes)
___ = Underlined substrates depict the highest rate of O-dealkylation for the specific treatment.

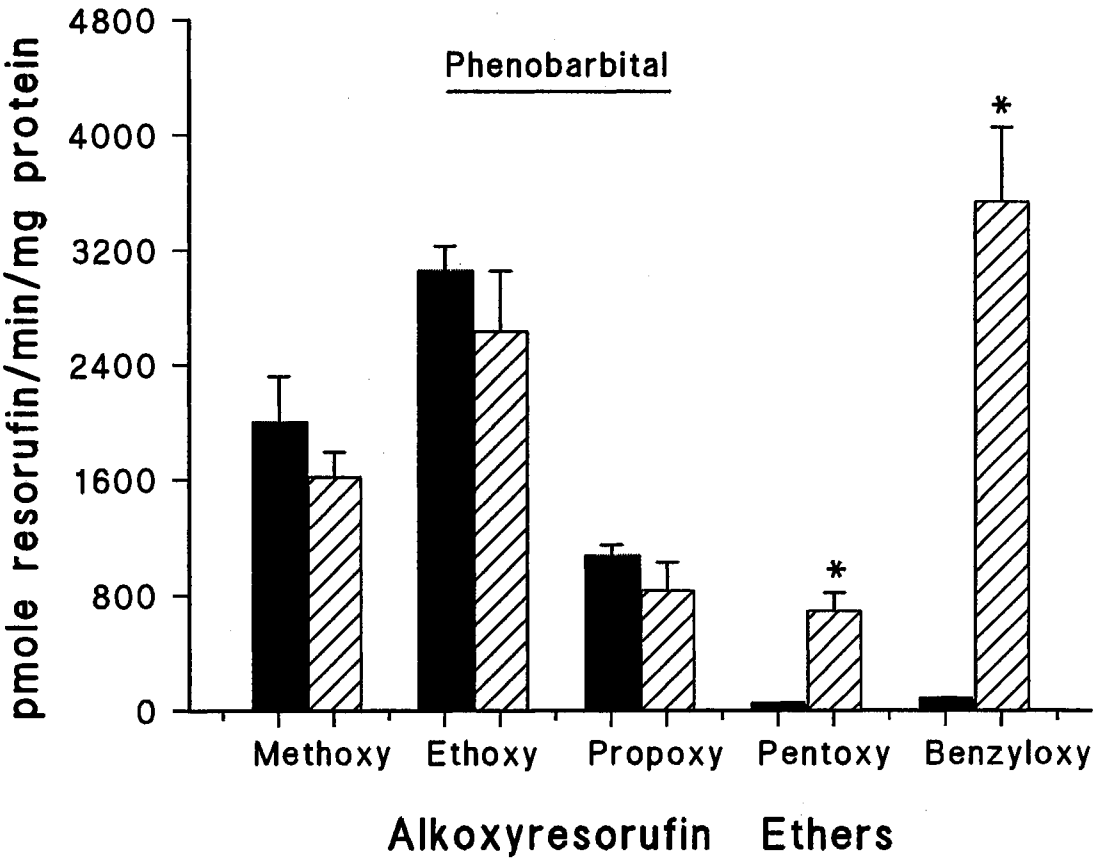

Figure 1. Mean ± SEM of hepatic alkoxyresorufin O-dealkylation activity in control rabbits ■ and after 5 days of phenobarbital (PB) pretreatment ▨ (n=4). *, significantly different from control.

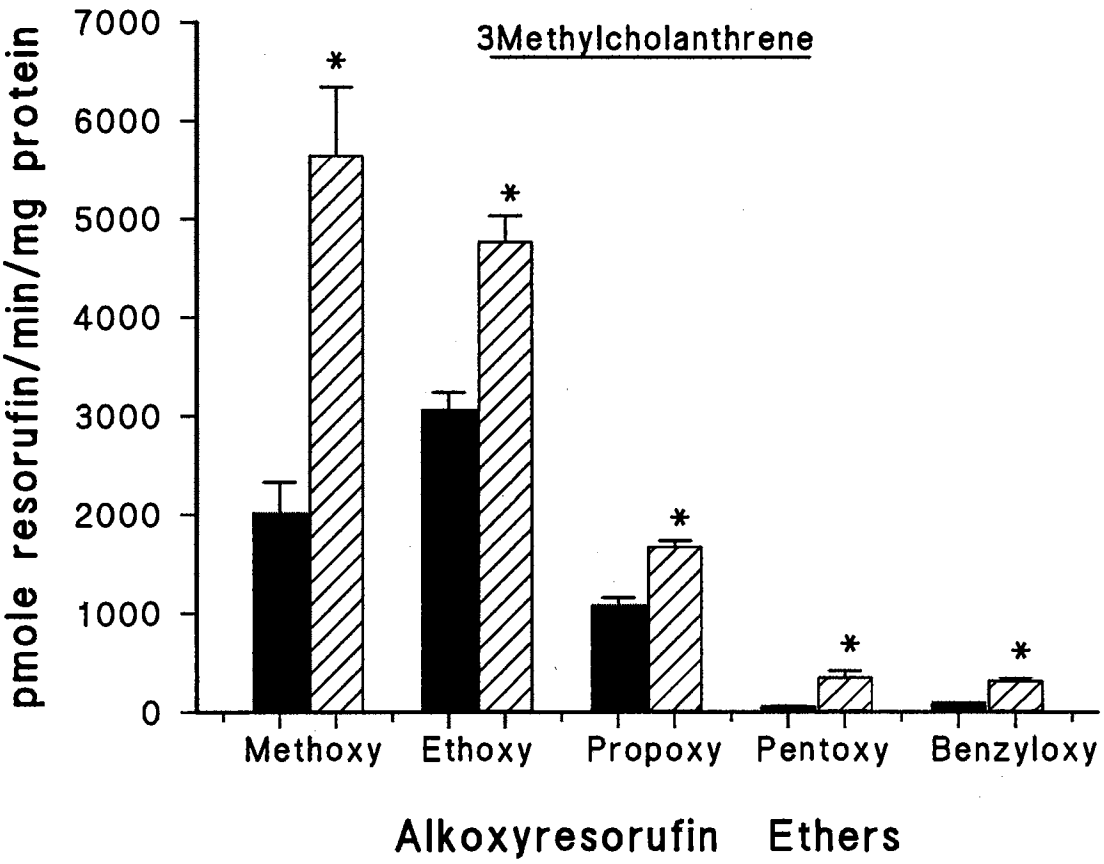

Figure 2. Mean ± SEM of hepatic alkoxyresorufin O-dealkylation activity in control rabbits ■ and after 5 days of 3-methylcholanthrene (3MC) pretreatment ▨ (n=4). *, significantly different from control.

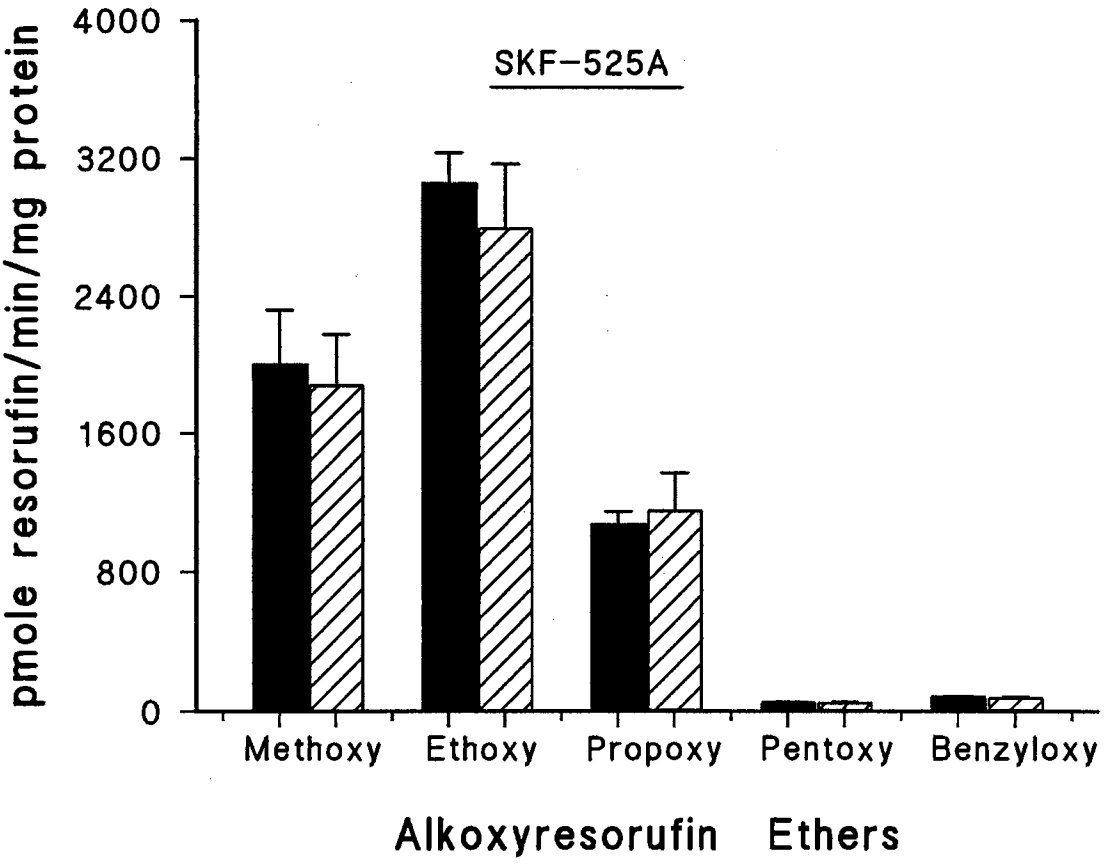

Figure 3. Mean ± SEM of hepatic alkoxyresorufin O-dealkylation activity in control rabbits ■ and after 1 hour of proadifen hydrochloride (SKF-525A) pretreatment ▨ (n = 4).

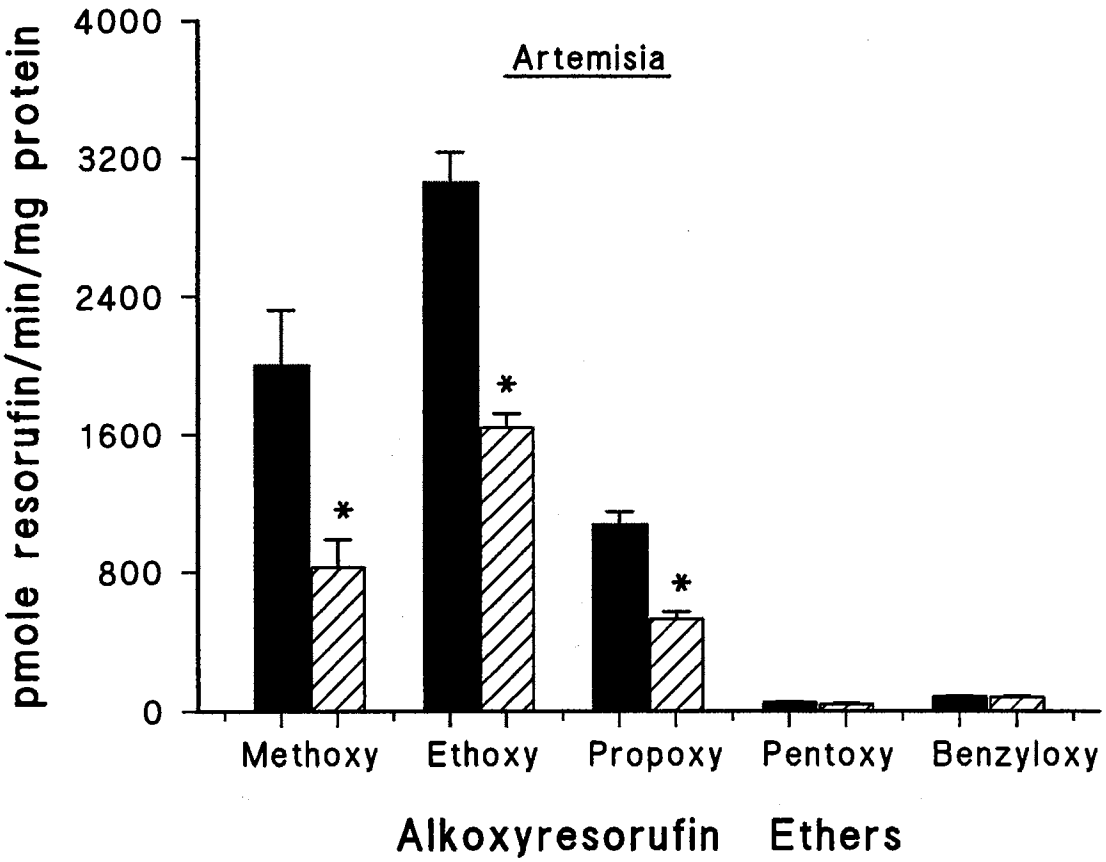

Figure 4. Mean ± SEM of hepatic alkoxyresorufin O-dealkylation activity in control rabbits ■ and following 5 days of dietary exposure to *Artemisia filifolia* ▨ (n=4) . *, significantly different from control.

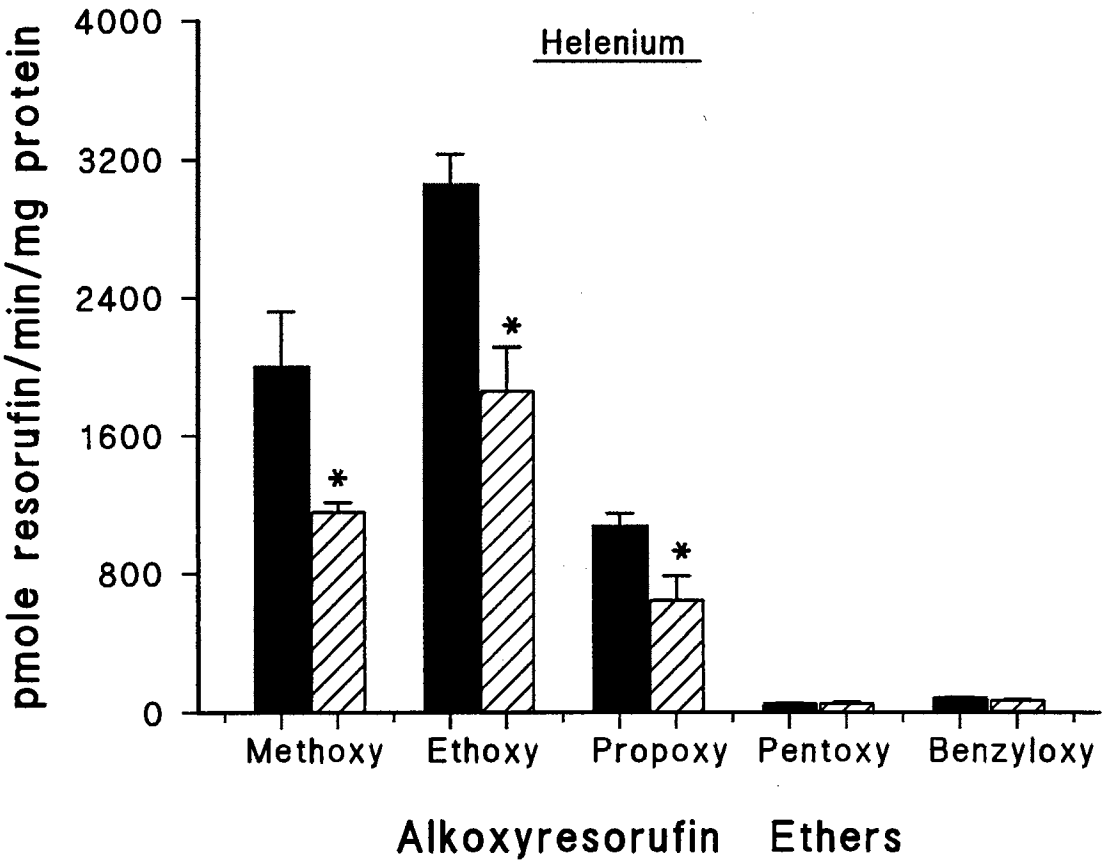

Figure 5. Mean ± SEM of hepatic alkoxyresorufin O-dealkylation activity in control rabbits ■ and following 5 days of dietary exposure to *Helenium flexuosum* ▨ (n=4). *, significantly different from control.

DISCUSSION

In PB-pretreated rabbits there was a profound induction of benzyloxyresorufin O-dealkylation, approximately 47 fold as compared to the control. In contrast, the degree of induction of pentoxyresorufin O-dealkylation conferred by PB-pretreatment was moderate, approximately 17 fold as compared to the control. This finding indicates that benzyloxyresorufin is the best substrate to distinguish between PB-induced microsomes and other xenobiotic-induced microsomes in the rabbit. Furthermore, this finding is in good agreement with the recent study by Lubet et al. (1990) who reported that the rate of O-dealkylation of benzyloxyresorufin in PB-induced rabbit liver is consistently higher than the rate of O-dealkylation of pentoxyresorufin.

It is of interest to note that there are obvious species and interspecies variations in catalytic preferences of O-dealkylation of alkoxyresorufin ethers. For example, pentoxyresorufin is typically used as the substrate of choice to differentiate PB-inducible forms of cytochrome P-450 in C57/BL10 mice (Burke and Mayer, 1983) as well as in Sprague-Dawley rats (Burke et al., 1985), Fischer rats (Lubet et al., 1985), and cotton rats (Novak and Qualls, 1989). Whereas, benzyloxyresorufin was reported to be the most appropriate substrate to distinguish PB-inducible forms of cytochrome P-450 in Wistar rats (Godden et al., 1987; Mayer et al., 1990).

In the present study, the 3MC-induced microsomes produced a significant increase in the rate of O-dealkylation of all alkoxyresorufin substrates tested. The folds of increase as compared to the control was in the following order: pentoxy = 8 fold, benzyloxy = 4 fold, methoxy = 2.8 fold, ethoxy =

1.55 fold, and propoxy = 1.54 fold. However, this does not necessarily indicate that pentoxy is the substrate of choice to differentiate 3MC-inducible microsomes since its rate of O-dealkylation was only 339 pmole/min/mg whereas the rate of O-dealkylation of methoxy was 5633 pmole/min/mg, i.e., 16.6 times greater than pentoxy. Thus, based on having the highest O-dealkylation activity and consequently formation of the largest amount of resorufin as the substrates undergo O-dealkylation, methoxyresorufin was selected as the substrate of choice to evaluate 3MC-inducible forms of cytochrome P-450 in the rabbit. In fact, all alkoxyresorufin ethers with short-alkyl chain such as methoxy (C1), ethoxy (C2), and propoxy (C3) showed higher and faster rates of O-dealkylation as opposed to those with long-alkyl chain such as pentoxy (C5) and benzyloxy (C7). This indicates that alkoxyresorufin ethers with short-alkyl chain are preferred as differential probes for 3MC-type of induction in spite of the fact that they did not show the greatest fold of induction as compared to the control.

The reason for the relatively small folds of induction observed with 3MC pretreatment in rabbits may in some measure be attributed to the inherently high basal activity of control microsomes (untreated) in catalyzing dealkylation of short-alkyl chain alkoxyresorufin ethers. Dealkylation of short-alkyl chain alkoxyresorufin ethers by untreated rabbit microsomes is 10 times greater than dealkylation by untreated microsomes from other species. For example, in our laboratory, dealkylation of methoxyresorufin and ethoxyresorufin ethers by untreated rabbit microsomes was 2001 pmole and 3055 pmole, respectively. This is in contrast to dealkylation of methoxy and ethoxy by microsomes from

untreated cotton rats of 340 pmole and 290 pmole, respectively; or by microsomes from untreated Sprague-Dawley rats of 140 pmole and 130 pmole, respectively (Novak and Qualls, 1989). Similarly dealkylation of ethoxyresorufin in white-footed mice (*Peromyscus leucopus*) was 250 pmole (Simmons and McKee, 1992). Thus, rabbits appear to have greater propensity to dealkylate short-alkyl chain as compared to rats or mice.

In the present study, short-alkyl chain ethers such as methoxy, ethoxy, and propoxy appear to represent the most appropriate substrates for differentiation of 3MC-inducible forms of cytochrome P-450 in the rabbit. The selection of the preferred substrates O-dealkylated by 3MC-induced microsomes was based on the rate of O-dealkylation activity rather than on the number of folds of induction. According to order of magnitude: methoxy = 5633 pmole, ethoxy = 4754 pmole, and propoxy = 1658 pmole. Thus, methoxy is favored as a differential probe for 3MC type of induction in rabbits. Unfortunately, Lubet et al. (1990) did not use 3MC in rabbits; therefore, there was no facet for comparison. However, they reported similar methoxyresorufin preferential O-dealkylation in 3MC-pretreated hamsters. In this respect, it is worthy to note, ethoxyresorufin has been reported to be the preferential substrate for 3MC-inducible forms of cytochrome P-450 in rats and mice (Burke and Mayer, 1983; Lubet et al., 1985; Burke et al., 1985).

It is well documented that at least two forms of cytochrome P-450 are induced by 3MC; CYP1A1 and CYP1A2. In the present study, it could be deduced that 3MC pretreatment in the rabbit resulted in induction of CYP1A2 at a greater rate than CYP1A1 since CYP1A2 preferentially dealkylate

methoxyresorufin while CYP1A1 preferentially dealkylate ethoxyresorufin (Yang et al., 1988).

An interesting observation in the present study was the insignificant reduction in total cytochrome P-450 as well as the negligible inhibition of alkoxyresorufin O-dealkylation conferred by pretreatment of rabbits with SKF-525A, a known potent inhibitor of PB-inducible forms of cytochrome P-450 in rats (Rossi et al., 1987). Although this finding was unexpected, it is consistent with the findings of Kato and Takayangi (1966) who showed that the potency of SKF-525A as an inhibitor in the rabbit is far less than in the rat. Moreover, Chambers and Jefferson (1982) reported a lack of SKF-525A effect on antipyrine clearance in rabbits, unlike its effect in rats where a clear-cut impedance of antipyrine clearance took place. Changes in antipyrine half-life and clearance are considered to be an indirect index for the overall degree of inhibition or induction of cytochrome P-450 since the drug is totally metabolized by cytochrome P-450 (Vesell, 1979; Danhof and Teunissen, 1984).

The consecutive daily administration of *Artemisia filifolia* or *Helenium flexuosum* at a dose rate of 250 mg/kg for 5 days in the diet (i.e., 1% of the total diet) resulted in a significant decrease, approximately 50%, in hepatic microsomal O-dealkylation activity of short-alkyl chain alkoxyresorufin substrates, primarily methoxy, ethoxy, and propoxyresorufin (as shown in table 2, figure 4 and figure 5). This finding suggests that ingestion of these plants produces an inhibition of isozymes that are normally induced by 3MC.

This study is unique in using plant xenobiotics as modulators of alkoxyresorufin ethers O-dealkylation. The results suggest that natural plant

xenobiotics are capable of inhibiting specific isozymes of cytochrome P-450. Some of these isozymes may be responsible for activation of innocuous chemicals into harmful toxicants. In such instances, natural plant products could be useful in blocking bioactivation of these chemicals into deleterious metabolites.

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CHAPTER V

SUMMARY AND CONCLUSION

The literature is replete with evidence that dietary constituents represent an important determinant of the pharmacotoxicological activities of drugs and other xenobiotics. Animals continually ingest or are exposed to a variety of chemical compounds present in their food plants. These chemicals may act as inducers or inhibitors of the Mixed Function Oxidase System (MFO), a major enzymatic system that mediates biotransformation, and as such can lead to attenuation or amplification of biological responses to concurrently administered drugs.

In the first experiment of this study, six white-face female sheep were pair fed Artemisia filifolia, Artemisia ludoviciana, or alfalfa in combination with grass hay and concentrates. Drug disposition effects during these test-dietary regimens were determined by administration of antipyrine and erythromycin in a crossover-type design. Serum concentrations of drugs were determined and subjected to pharmacokinetic analysis. Both species of Artemisia as well as alfalfa produced an inducing-type effect on Mixed Function Oxidase System (MFO) as reflected by the significant increase in antipyrine clearance and elimination. Thus, indicating the potential for such diets to increase the

metabolism of antipyrine. Conversely, there was little or no change in metabolism of erythromycin. The result of this study indicated that dietary constituents are not only capable of altering drug residence time in sheep but also indicated that the requisite biotransformation pathways for antipyrine and erythromycin are different. The latter finding was later proved to be the case when it was shown that antipyrine metabolism is mediated by the isozyme CYP2B whereas erythromycin metabolism is mediated by the isozyme CYP3A.

In the second experiment of this study, twelve adult male New Zealand white rabbits were acclimated and maintained on commercial rabbit pellets (ACCO) for a week before treatment commenced. On day 1, all rabbits were given antipyrine 25 mg/kg i.v.. Beginning on day 14, six of the rabbits were given Artemisia filifolia at a daily dosage of 250 mg/kg for 5 days while the remaining six were given Helenium flexuosum at the same dosage schedule. Antipyrine administration was repeated in all rabbits on day 19. Serial blood samples were taken following each antipyrine administration and serum concentrations were determined and subjected to pharmacokinetic analysis. Feeding Helenium flexuosum in combination with the standard rabbit pellets resulted in a significant increase in antipyrine half-life of elimination, indicating that Helenium flexuosum ingestion exerts a typical inhibitory-type effect on MFO. Feeding Artemisia filifolia was not accompanied by a significant change in antipyrine pharmacokinetic parameters in rabbits.

It is very intriguing to find that ingestion of Artemisia filifolia exerted a pronounced inducing-type effect on MFO in sheep but not in rabbits, as reflected by changes in antipyrine metabolism. This phenomenon may be

explained within the realm of interspecies variation in metabolism which has been well documented in the literature. For example, the major metabolite of sulphadimidine in horses is 5-hydroxysulphadimidine while in camels the major metabolite is N^4-Acetylsulphadimidine. A further example illustrating inter-species variation in metabolism is the metabolism of coumarin which has been found to be metabolized into 7-hydroxycoumarin in man, whereas in rats the predominant metabolite is 3-hydroxycoumarin. This explains why coumarin is effective in preventing dimethylbenzanthracene-induced breast tumors in rats but not in humans; 7-hydroxycoumarin, the major metabolite in humans, is inactive against dimethylbenzanthracene-induced neoplasia.

In the third experiment of this study, the O-dealkylation of a series of alkoxyresorufin ethers by hepatic microsomes prepared from control-fed rabbits and plant-fed rabbits, with either Artemisia or Helenium, were compared. In addition, prototype P-450 inducers such as phenobarbital and 3-methyl-cholanthrene and the prototype P-450 inhibitor proadifen hydrochloride (SKF-525A) were used to provide a wider facet for comparison between dealkylation conferred by hepatic microsomes prepared from plant-fed rabbits and dealkylation conferred by hepatic microsomes prepared from other xenobiotic-pretreated rabbits.

Pretreatment of rabbits with prototype P-450 inducers phenobarbital and 3-methylcholanthrene increased the basal hepatic cytochrome P-450 content by 2-3 fold whereas pretreatment with either the prototype P-450 inhibitor proadifen hydrochloride (SKF-525A) or test-plants, Artemisia filifolia and Helenium flexuosum, had no effect on basal cytochrome P-450 content.

Phenobarbital-induced hepatic microsomes significantly increased the rate of O-dealkylation of benzyloxyresorufin and pentoxyresorufin, approximately 47 fold and 17 fold respectively. In contrast, 3-methylcholanthrene-induced hepatic microsomes increased the rate of O-dealkylation of all alkoxyresorufin substrates tested; preferentially dealkylating substrates with a short-alkyl chain. Dealkylation of the short-alkyl chain substrates conferred by 3-methylcholanthrene pretreatment was in the following order of magnitude: methoxyresorufin >ethoxyresorufin >propoxyresorufin.

The consecutive daily administration of Artemisia filifolia or Helenium flexuosum at a dose rate of 250 mg/kg for 5 days in the diet (i.e., 1% of the total diet) resulted in a remarkably significant decrease in hepatic microsomal O-dealkylation activity of short-alkyl chain substrates. In particular, dealkylation of methoxy-, ethoxy-, and propoxyresorufin was inhibited by approximately 50%. This finding suggests that ingestion of these plants produces an inhibition of the same isozymes that are induced by 3-methylcholanthrene.

In recapitulation of the second and third experiment, feeding Helenium flexuosum to rabbits prolonged the sojourn of antipyrine in the body indicating an inhibitory-type effect on Mixed Function Oxidase (MFO) System. Similarly, the O-dealkylation of short-alkylchain alkoxyresorufin ethers was inhibited by approximately 50%. With respect to the effects of feeding Artemisia filifolia, antipyrine disposition was not affected. However, the O-dealkylation of short-alkyl chain alkoxyresorufin ethers was remarkably inhibited.

In order to ratiocinate the apparently opposed metabolic effects exerted by ingestion of the two plants, one must bear in mind that the two substrates

used as indices to monitor modulation of biotransformation are metabolized by different isozymes. Antipyrine is metabolized by the isozyme CYP2B whereas short-alkyl chain alkoxyresorufin ethers are metabolized by the isozymes CYP1A1 and CYP1A2. Thus, it could be inferred that Artemisia filifolia exerted an inhibitory effect only on the two isozymes responsible from the O-dealkylation of short-alkyl chain alkoxyresorufin ethers whereas Helenium flexuosum exerted an inhibitory effect on all three isozymes, the one that is responsible from antipyrine metabolism as well as the two that are responsible from alkoxyresorufin O-dealkylation.

Isozyme substrate specificity may explain the discrepancy between the effects of the two plants on different substrates. It is also possible that the active principles in the two plants are different, albeit the fact that they belong to one family. This may warrant further research to isolate and characterize the active ingredients present in these two plants.

It has become increasingly clear that the challenge of understanding the effects of the different dietary regimens on metabolism of different drugs is an extremely difficult challenge but with the increase in knowledge and research pertinent to this area the ambiguity will be resolved.

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