농학석사학위논문

Comparative metabolism of Bensulide by Soil fungus *Cunninghamella elegans* and Human liver microsome

토양 곰팡이 *Cunninghamella elegans* 와 사람 간 microsome에 의한 Bensulide의 비교 대사

2014년 02월

서울대학교 대학원

농생명공학부 응용생명화학 전공

황 연 진

A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Comparative metabolism of Bensulide by Soil Fungus *Cunninghamella elegans* and Human liver microsome

Advisor : Jeong Han Kim

By

Yon-Jin Hwang

Major in Applied Life Chemistry

Department of Agricultural and Life Science

Seoul National University Graduate School

A thesis submitted to the faculty of the Seoul National University Graduate School in partial fulfillment of the requirement for the degree of Master of Science in the Department of Agricultural and Life Science.

Seoul, Korea

Feburary 2014

Approved by Major Advisor

Comparative metabolism of Bensulide by Soil fungus *Cunninghamella elegans* and Human liver microsome

토양 곰팡이 *Cunninghamella elegans* 와 사람 간 microsome에 의한 Bensulide의 비교 대사

지도교수 김 정 한

이 논문을 농학 석사학위논문으로 제출함.

2014년 2월

서울대학교 대학원
농생명공학부 응용생명화학 전공
황 연 진

황연진의 논문을 석사학위논문으로 인준함.

2014년 2월

위 원 장	배 의 영 (인)
부위원장	김 정 한 (인)
위 원	노 희 명 (인)

ABSTRACT

Comparative metabolism of Bensulide by Soil fungus *Cunninghamella elegans* and Human liver microsome

Yon-Jin Hwang

Major in Applied Life Chemistry

Department of Agricultural and Life Science

The Graduate School

Seoul National University

Bensulide [O-O-diisopropyl S-2-phenylsulfonylaminoethyl phosphoro - dithioate] is an organophosphorous herbicide, acting as a inhibitor of cell division in meristematic root tissues and seedling growth by conjugation of acetyl co-enzyme A. This study was performed to investigate in vitro metabolism of bensulide with soil fungi, *Cunninghamella elegans*(Cunn. elegans), human liver microsomes(HLMs) and characterize the specific isoforms of cytochrome P450 involved in metabolic reaction.

In the presence of *Cunn. elegans*, a well-known fungal species with its strong resemblance of the xenobiotic metabolism of the mammalian system, bensulide was biodegraded to its oxygen analog, bensulide oxon (N-[(2-(diisopropoxyphosphinoylthio-)-1-ethyl)]-benzenesulonamiade).

In sterilized *Cunn. elegans,* the metabolite did not form, indicating that *Cunn. elegans* metabolized bensulide. Bensulide degradation pattern showed that day 1 showed 5% of degradation, day 3, 89%, day 5, 99% and by day 7 bensulide in all three replicates degraded completely. By day 3 metabolite was detected.

With presense of NADPH, bensulide was metabolized by HLMs to give identical metabolite as microbial degradation, its oxygen analog, bensulide oxon. With boiled-denatured microsomes the metabolite did not form but with heat-denatured microsomes, the metabolite did form, indicating that metabolic enzymes are cytochrome P450s. In enzyme kinetic studies, V_{max}(counts/min/mg protein) of 18.5, K_m of 11.7 were obtained. A screen of 9 human cDNA-expressed CYP isoforms for metabolic ability with respect to the production of bensulide oxon demonstrated that 2 (CYP 3A4, CYP 2C19) CYP isoforms which are responsible for bensulide metabolism. Enzyme kinetics of those two CYP isoforms also demonstrated that CYP 3A4 has the highest affinity to bensulide.

Key words: Bensulide, *in vitro* metabolism, *Cunn. elegans*, human liver mirosomes, cytochrome P450, P450 isoforms, enzyme kinetics

***Student number*: 2012-21195**

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Ⅰ. INTRODUCTION

Pesticides are intentionally applied to many components of the environment and they undergo many chemical and biochemical processes to degrade and be transformed into related metabolites that may have a significant toxicological influence on environment and human. Thus, the investigation on the metabolism of pesticides in environment and human is very important in view if elucidation their fate and evaluating the risk for human and environment.

Pesticides undergo metabolic transformation in living organisms through various metabolic reactions. The term metabolite is used to deote derivative of the parent molecule, and bioalteratin reaction that leads to the metabolites will be devided into two categories: Phase Ⅰ(primary) metabolism and phaseⅡ metabolism, referred to as secondary or conjugated metabolism (Matsumura 1982).

Phase Ⅰ metabolism involves the production of a free metabolite through biotrasformation reactions such as dehydrohalogenation, dehalogenation, desulfuration, eopxidation, hydrolysis, hydroxylation, oxidation, and reduction. The addition of a functional group makes the pesticides more polar and therefore more likely to be excreted (Matsumura 1982). Mirosomal mixed function oxidase is the primary enzyme for phase Ⅰ reactions which converts pesticides into more soluble products

(Korzekwa et al 1985).

Phase Ⅱ metabolism involves the formation of conjugates through glycoside formation, sulfoconjugation, glutathione conjugation, amino acid conjugation, acetylation, and methylation. Thus, conjugated metabolites are derivates of the pesticide that have reacted with natural component of organism to form a new material. Generally, this type of reaction involves the formation of a free metabolite, followed by a second step converting the metabolite to a conjugate. These conjugates are usually extractable from the substrate with polar solvents but do not partition from water into organic solvents (Matsumara 1982).

1. Metabolism of pesticides by soil fungi

The principle of microbial infallibility is that all natural compounds are metabolized under the given favorable environmental conditions. The term “Xenobiotics” refers to artificial compounds that are foreign to biological systems. Xenobiotics may be polymers, gases, polychlorinated or polybrominated compounds, and pesticide, especially (Coyne 1999). It is important to recognize that pesticides can be degraded by abiotic as well as biotic reactions. The biotic (microbial) activities are generally more environmentally significant because their systems greatly accelerate degradation rates and may results in complete destruction, or mineralization,

of organic pollutants.

Microbial communities are thus important in the natural dissipation of xenobiotics in the environment and in contaminant cleanup operations, the latter by providing the potential for bioremediation (Hickey, W. J 1998). Even though microorganisms are capable of executing a wide array of chemical transformations, there are three types of reaction that microorganisms frequently use to initiate the breakdown of chemical compound. These are (1) oxidation using an electrophilic form of oxygen, (2) reduction by nucleophilic form of hydrogen or by direct electron delivery, and (3) hydrolysis via an enzymatically mediated nucleophile attack. In the first two approaches, organisms invest biochemical energy to form very reactive species that can interact with the organic compounds via mechanisms not operating in abiotic dark environments. The third approach entails catalysis of hydrolytic pathways. The goal of these initial reactions is to transform the xenobiotic compounds into a products that are structurally more similar to chemicals with which microorganisms are used to metabolizing (Schwarzenbach et al 1993).

One of the moieties most commonly present in pesticides is the ester linkage. This includes all of the organophosphates and carbamate insecticides as well as pyrethroids. Hydrolysis of ester, halide, ether, and amide bonds usually gives rise to nontoxic products. In the case of organophosphates, which are largely degraded via hydrolytic processes, this

general trend is clearly observable.

The major microbial degradation product of diazinon [O,O-diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate] is 2-isopropyl-6-methyl-4-hydroxypyrimidine, a product of hydrolysis at the P-O-bond, and not the hydroxylation products or the glutathione S-aryltransferase products that are prevalent in animal species. Other types of pesticides having hydrolysable bonds are subject to metabolic attack via esterases. These include pheoxyalkanoates and chlorinated pesticides, patriculary aliphatics, phenyamides, phenylureas, triazines, and thiopehenate (Matsumura 1982).

Another class of important microbial reaction on pesticidal chemicals is the reductive processes. One of the most common types of reductive reaction is dehalogenation. The importance of this type of reaction is that many environmentally problematic chemicals are halogenated chemcials and, moreover, in many instances dehalogenation is a rate-limiting reaction. The reaction proceeds by replacing a halogen atom such as chlorine in a nonaromatic carbon with hydrogen; the most well known case is the converstion of DDT to TDE(=DDD) (Matsumura 1982).

While the extent of reports on oxidative metabolism in the mircrobial world is somewhat less than may be found in other biological systems, many oxidative reactions occur widely among microorganisms. They are (1) epoxidation of cyclodienes such as aldrin and heptachlor to corresponding epoxide (e.g., dieldrin and heptachlor epoxide), (2) oxidation of thioethers

to sulfoxides and sulfones (e.g., phorate), (3) oxidative dealkylation of alkylamines (e.g., mexacarbate), (4) ring opening (e.g., 2,4-D), and (5) decarboxylation. One very important reaction that takes place only in the microbial world is the aromatic ring-opening process. The system is operated by a series of oxidative ring hydroxylation (including epoxidation) reactions. The ring hydroxylation can occur even at the chlorine-attached aromatic carbon, in contrast to reductive dechlorination reactions on chlorinated hydrocarbons (Matsumura 1982).

It was generally assumed by many pesticide scientists that the pattern of microbial metabolism were in general very similar to the nes already found in animals, particularly the mammalian species, since studies on microbial metabolism of pesticides were lagging far behind the comparable studies in mammalian species. However, as knowledge on microbial degradation has advanced, it has become apparent that in many cases the patterns of metabolism in these two different groups of organisms are often very different (Matsumura 1982). First of all, the purpose of all metabolic reactions on xenobiotics in higher animals is to eventually convert them into polar and therefore excreatable forms. Secondly, in higher animals the processes of primary metabolism of xenobiotics are centralized in a few specialized organs. In the case of the liver, its metabolic pattern is largely determined by the activity of an oxidative detoxification system, generally termed mixed-function oxidase (Matsumura 1982).

On the contrary, the predominant metabolic activities in the microbial world are meant for production of energy. In this respect, it is not even possible to define xenobiotics here, since most organic materials can serve as the source of energy to at least some microorganisms to changing environments through mutation and induction, particularly toward chemicals that are initially toxic to them (Matsumura 1982).

1.1 Soil fungi

Eukaryotic fungi have been important have been important role of metabolism of xenobiotic compounds in soil environmental community. Microbial degradation provides the major means of detoxification of many classes of xenobiotic, especially pesticides. Several soil fungi have been studied for their ability to metabolizing pesticides. For example, Rhizoctonia solani (Smith et al 1975), Chaetomium globosum (Tiedje et al 1975), and Phanerochaete chrysosporium (Ferrery et al 1994) etc were able to metabolize the aromatic ring carbon of alachlor that is herbicide used vastly. Moreover, microbial metabolism studies have been used successfully as model systems to predict metabolic pathways in humans or to increase the efficiency of drugs by metabolic activation (El Sayed 2000). Good example is the study of metabolism of naphthalene by Cunn. elegans. The metabolism of naphthalene by crude microsomal prepatation from Cunn.

elegans is very similar to the results obtained with hepatic microsomes and reconstituted enzyme systems that contain partially purified preparations of cytochrome P450 (Cerniglia et al 1978).

1.2 Cunninghamella elegans

Cunninghamella elegans, is zygomycete, was isolated for its ability to grow on crude oil as a sole source of carbon and energy. The organism was observed to utilize the alkane fraction for growth. Other alkane-utilizing filamentous fugi and yeasts have been identified. However, Cunn. elegans distinguishes itself from the others by its remarkable ability to metabolize numerous structurally diverse compounds, such as polycyclic aromatic hydrocarbons (PAHs), drugs, and N-, S-, O- heterocyclic aromatic compound by both phase Ⅰ and Ⅱ metabolism (Cerniglia et al 1995, Faber et al 2001, Moody et al 2000, Pothuluri et al 1992, Pothuluri et al 1996, Pothuluri et al 1999, Schlenk et al 1994, Sutherland et al 1998, Sutherland et al 1999, van den Brink et al 1998, Wackett 2001, Zhang et all 1996). So that reason, Cunn. elegans is a fungus, which has been used as a microbial model of mammalian metabolism (Moody et all 2000, Mountfield et al 1998, Wackett et al 1982, Wang et all 2000, Zhang et all 1996). The presence of a mammalian type microsomal P450 enzyme system in this fungus for xenobiotic metabolism has been recognized since the 1970s

(Yadav et all 2000).

Table 1. Metabolism studies of pesticide by microbial degradation

Chemicals	Microsome	Reference
Ethaboxam	Cunninghamella elegans	M.K. Park et al, 2003
Tributyltin chloride	Cunninghamella elegans	P. Bernat et al, 2007
Adrenosterone	Cunninghamella elegans	M.I. Choudhary et al, 2007
Isoproturon	Cunninghamella elegans	M. Hangler et al, 2007
Phenanthrene	Sinorhizobium sp. C4	Y.S. Keum et al, 2008
Nitrodiphenyl Ether	Sphingomonas wittichii RW1	Y.S. Keum et al, 2008
Methoxychlor	Cunninghamella elegans	Y.S Keum et al, 2009
Bisphenol A	Cunninghamella elegans	Y.S. Keum et al, 2010
Mepanipyrim	Cunninghamella elegans	Y.Z. Zhu et al, 2010

2. Metabolism of xenobiotics (Biotransformation)

Xenobitic is a chemical compound foreign to a given biological system. Agrochemicals and other xenobiotics are metabolized by xenobiotic-metabolizing enzymes to products that may be more or less toxic than the parent chemical (Ernest Hodgson et al., 2008). The mahority of xenobiotics that enter the body tissues are lipophilic, a property that enables them to penetrate lipid membranes and to be transported by lipoproteins in body fluids. The metabolism of xenobiotics, carried out by a number of relatively nonspecific enzymes, usually consists of two phases (Joyce A. Goldstein et al., 2008). During Phase I, Some polar functional group such as alcohols,

phenols, carboxylic acids which could make xenobiotic increase hydrophilicity are introduced into the xenobiotic molecule. The most important effent of Phase I is to make the xenobiotic be a suitable substrate for phase II reaction. Phase I reactions usually involve oxidation, which occurs when the toxicant loses electrons, reduction, when the toxicant gains electrons, or hydrolysis, a process that cleaves the toxicant into two or more simpler molecules, each of which then combines with a part of water at the site of cleavage (William Hughes, 1996).

In Phase II reactions, toxicants can also undergo metabolic conjugation, either directly or subsequent to phase I biotransformation. The conjugation process and phase II detoxification involves coupling of the toxicant to small, endogenous molecules that are present within the cell. Conjugation typically reduces the reactivity of the toxicant and generally facilitates the elimination of toxicants from the body through aqueous routes by increasing the aqueous solubility of the xenobiotic (Gerald A. Leblanc et al. 2008)

2.1. Cytochrome P450

The cytochrome P450s are hemoproteins that play critical roles in the bioactivation and detoxication of a wide variety of xenobiotic substrates. The term ‘cytochrome P450’ originates from the observation that the

reduced state of the proteins form complexes with carbon monoxide that exhibit absorbance maima at 450nm (Curtis J. Omiecinski et al., 1999)

Few enzymes are more striking in both versatility and in sheer number of substrates than the cytochrome P450 enzyme system. The cytochrome P450 detoxify harmful xenobiotics or, in some instances, bioactivate them to reactive species, through biotransformation (Parkinson,2001). In animals, CYP enzymes are found predominantly in the liver, as well as in the majority of extrahepatic tissue (Parkinson, 2001).

The CYP enzymes mediate phase I biotransformations, primarily acting as a monooxygenase, where an oxygen atom is incorporated into substrates (Nelson and Cox, 2005). These reactions usually convert hydrophobic xenobiotics into polar forms, making them more water soluble and thus more readily excreted. Unfortunately, this process can also activate certain substrates, such as potential carcinogens, via transformation into electrophilic species capable of interacting irreversibly with biological molecules, such as DNA, causing mutation and sometimes ineluctable carcinogenesis (Guengerich and Shimada, 1991). Nonoxidative reactions, such as reductions, rearrangements, and dehydrations catalyzed by CYPs, have also been documented (Mansuy, 1998). As both reversible and irreversible inhibition of CYP isozymes by particular compounds can lead to harmful drug-drug and drug-food interactions (Parkinson, 2001), research into this phenomenon is a burgeoning field in pharmacology and toxicology.

Thus, consideration of these important enzymes is essential when planning drug trials, analyzing drug interactions, and studying environmental risk factors such as exposure to pollutants.

There are well in excess of 2000 identified cytochrome P450 genomic and cDNA sequences that have been divided into a total of 265 different families. Multiple cytochrome P450 genes can be expressed simultaneously and the number of genes per species is highly variable with a tendency for higher eukaryotes to possess large numbers of paralogously-related sequences (Danielson, 2002).

The multiple CYP enzymes are classified into families, subfamilies, and isoforms (Nelson, 1996). The first number designates the “family” (> 40% sequence identity within family members), the letter that follows designates the “subfamily” (> 59% sequence identity), and the final number indicates a particular CYP isoform.

The cytochrome P450 is heme-thiolate proteins that utilize molecular oxygen (Danielson, 2002; Parkinson, 2001), and the reducing agent NADH or NADPH (which donates two electrons to the reaction), in the monooxygenation of its substrate and the formation of water (Mansuy, 1998)

The basic reaction mechanism of P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate (RH), and the other is reduced to water with reducing eqivalents derived from NADPH as follows:

$$RH + O^2 + 2H^+ + 2e^- \text{(from NAD[P]H)} \rightarrow ROH + H_2O$$

P450 and NADPH-P450 reductase are embedded in the phospholipids bilayer of the endoplasmic reticulum which facilitates their interactions. The catalytic cycle of P450 associated with monooxygenation reaction is explained in Figure 12. During catalysis, P450 binds directly to the substrate and molecular oxygen, but it does not interact directly with NADPH or NADH. The mechanism of electron transfer from NADPH to P450 depends on the subcellular localization of P450 with a high-spin form and this transfer is achieved by flavoprotein called NADPH-P450 reductase via FMN and FAD. In mitochondria, which contains many of P450s involved in steroid hormone biosynthesis and vitamin D metabolism, electrons are transferred from NADPH to P450 via two proteins, an iron-sulfer protein called adrendoxin known as ferredoxin and a FMN-containing flavoprotein called adrendoxin reductase also known as ferredoxin reductase.

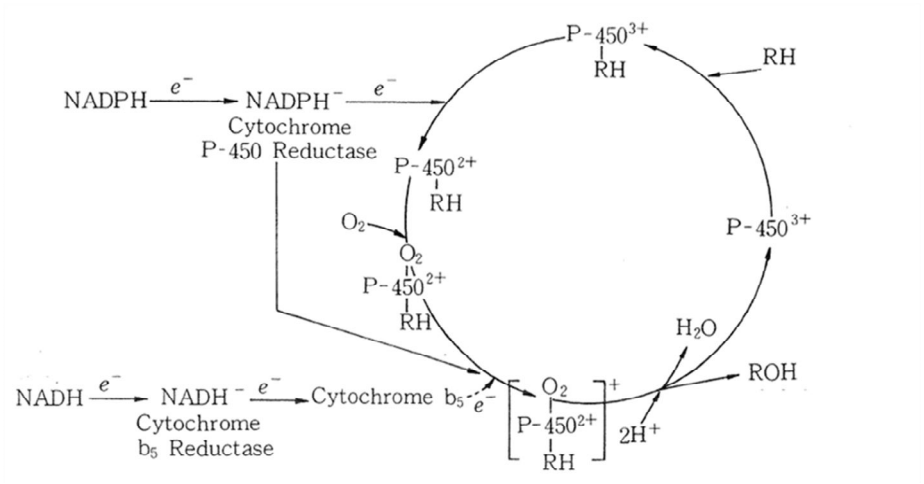


Figure 1. Catalytic cycle of P450

2.2. Human liver microsomal CYP450

The CYP superfamily consisted of more than 7,000 named sequences in animals, plants, bacteria and fungi. The human genome has 57 CYP genes, and the function for most of the corresponding enzymes is known at least to some degree. Fifteen individual CYP enzymes in families 1, 2 and 3 metabolize xenobiotics, including the majority of small molecule drugs currently in use. A typical feature of these CYPs is broad and overlapping substrate specificity (Guengerich et al. 2005).

Cytochrome P450 enzymes are found in practically all tissues, with highest abundance and largest number of individual CYP forms present in the liver. CYPs reside also in the intestine, lung, kidney, brain, adrenal gland, gonads, heart, nasal and tracheal mucosa, and skin. In human liver CYP enzymes comprise approximately 2% of total microsomal protein (0.3–0.6 nmol of total CYP per mg of microsomal protein). The content of drug-metabolizing CYPs is much lower in other tissues. While extrahepatic metabolism may have clinically significant local effects, systemic metabolic clearance of drugs occurs in the liver with a significant contribution by the gut wall in special cases (Pelkonen et al., 2008).

Ten individual CYP forms in the adult human liver carry out virtually the whole CYP-mediated metabolism. CYP3A4 is the highest abundance form and it metabolizes the greatest number of drugs and a very large

number of other xenobiotics. Together CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are responsible for more than 90% of known oxidative drug metabolism reactions (Pelkonen et al., 2008). Below figure illustrates the relative abundance of individual CYP forms in the liver, and lists some examples of substrates, inhibitorsand inducers (figure 2).

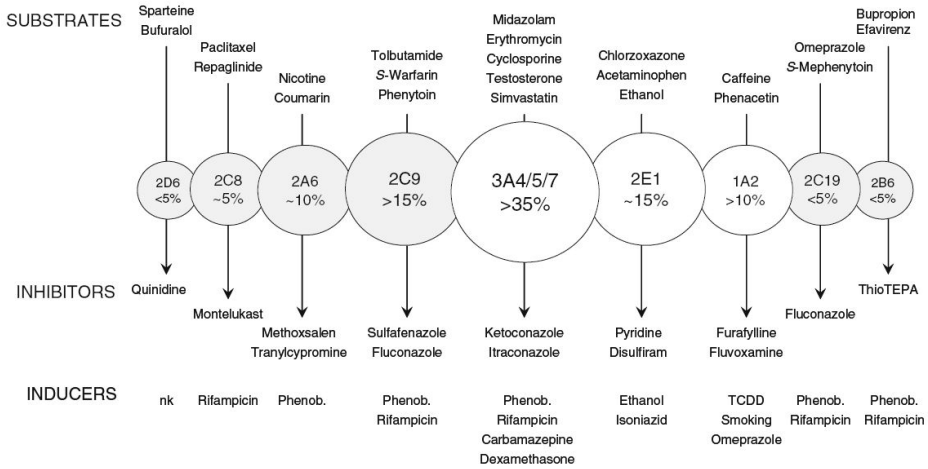


Figure 2. Major human hepatic P450 enzymes incolced in drug metabolism(Pelkonen et al., 2008)

CYP1 Family of microsomal cytochrome P450s consists of three members, CYP1A1, CYP1A2 and CYP1B1. The two members of the CYP1A family (CYP1A1 and CYP1A2) show greater than 70% amino acid sequence identity but display very different patterns of tissue expression.

CYP1A1 is expressed primarily in extrahepatic tissues such as the lungs, lymphocytes and placenta while only low-level expression has been reported in liver tissue. In contrast, CYP1A2 is expressed primarily in the liver with little if any detectable expression in the extrahepatic tissues (Danielson, 2002).

The CYP1A enzymes bioactivate several procarcinogens. CYP 1A1 activate benzo pyrene and other polyaromatic hydro carbons. CYP 1A2 activate aromatic amines, such as 2-acetylaminofluorene, heterocyclic amines, and aflatoxin B1 (Omiecinski et al, 1999).

CYP 1B1 is a more recently characterized member of the CYP1 family. CYP1B1 is constitutively expressed in most tissues but alse inducible through the Ah receptor pathway. CYP1B1 is involved in the metabolism of endogenous estrogens, as well as active in the biotransformation of heterocyclic amines found in charcoal broiled meats (Crofts et al., 1997).

The third member of the CYP1 family, CYP1B1 is constitutively expressed at low levels in a broad range of tissues including brain, colon, heart, kidney, leukocytes, liver, lung, ovary, placenta, prostate, skeletal muscle, small intestine, spleen, and thymus CYP 1A2 is universally expressed in human liver (Danielson, 2002)

CYP2 family is the largest family of cytochrome P450s in humans comprising approximately one third of human cytochrome P450s sequences. This family of microsomal cytochrome P450s is classified into 13

subfamilies that consist of 16 functional genes (CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1) and 13 confirmed pseudogenes (CYP2A7PT (telomeric), CYP2A7PC (centromeric), CYP2A18P, CYP2B7P1, CYP2B7P2, CYP2B7P3, CYP2D7AP, CYP2D8P, CYP2F1P, CYP2G1P, CYP2G2P, CYP2T2P, CYP2T3P). Members of the CYP2 family of cytochrome P450s play a significant role in drug metabolism although only CYP2A6 appears to be expressed to any significant degree in hepatic tissue. Other members of the CYP2 family are expressed in a sex-specific manner and thus it is not unexpected that these enzymes support the hydroxylation of steroids including sex-specific steroids. The CYP2A proteins, in particular are expressed at much higher levels in the olfactory mucosa of fetal tissues at gestational days 91- 125 than in corresponding hepatic tissues. It has been suggested that such prenatal expression of xenobioticmetabolizing cytochrome P450s indicates that the human fetal olfactory mucosa may be an important target site for chemical toxicity during early development (Gu et al., 2000).

CYP 2A6 may participate in procarcinogen activation but has a relatively minor role in drug biotransformation and account for only approximately 4% of total immunoquantified human hepatic CYP. CYP 2A6 participates in nicotine metabolism.

CYP 2B6 was previously described as a low abundance isoform in human liver, large interindividual variations are observed in expression, with hepatic microsomal content varying from as low as 0.3 pmole/mg protein to as high as 82 pmole/mg protein (Code *et al*., 1997).

CYP 2C subfamily consists of 2C8, 2C9, 2C18 and 2C19. CYP 2C8 is only known to play a major role in the metabolism of the anticancer agent taxol and it contributes to carbamazepine ephydroxylation. CYP 2C9 is the primary enzyme responsible for the metabolism of tolbutamide, S-warfarin, phenytoin, losartan, ibuprofen, and celecoxib.

CYP 3A subfamily consists of 3A4, 3A5, 3A7 and 3A43. CYP 3A isoforms are the most abundant in both human liver and small intestine, accounting for an average of 28.8% of total human hepatic CYP. The majority of oxidatively bio-transformed therapeutic agents are metabolized at least in part by this subfamily of enzymes. CYP 3A4 is the major adult isoform and is universally expressed both in human liver and in the small intestine. CYP 3A5 is polymorphically expressed in approximately 20% of the population, while CYP 3A7 is expressed only in fetal liver. CYP 3A43 has only been recently identified, and its importance in human drug metabolism remains to be established. Expression and activity of CYP 3A isoforms in humans show wide individual variability (Venkatakrishnan *et al*., 2001).

2.3. Recombinant human CYP450

The recombinant human CYP450 is the recombinant NADPH-cytochrome P450 reductase which is produced from insect cells infected with baculovirus containing the cDNA for human P450 reductase. This product is purified and retains high activity (20-60 μmol cytochrome reduced/min/mg). Recombinant human CYP450 replaces older discontinued rat materials. Applications of recombinant human CYP450 are for use with *in vitro* P450-dependent biotransformation reactions and *in vitro* drug metabolism and toxicology research. NADPH-P450 oxidoreducatase transfers electrons form NADPH to the various isoforms of CYP450s; it is typically used at a 1:1 or 2:1 ratio to P450 for *in vitro* reconstitution assays. It is a membrane-bound flavoprotein, containing one mol each of FAD and FMN. The product is human oxidoreductase produced from a recombinant baculovirus and recombinant (insect cells infected with a baculovirus).

The difficulty and tediousness of isolating and reconstituting the native enzymes from human tissue has hampered their use for drug metabolism studies with well-defined *in vitro* systems. Because of recent development of methods for high level expression of active, mammalian P450s with baculovirus and E. coli recombinant human P450 isoforms can be produced in three different formats: Soluble purified enzymes, purified enzymes in a reconstituted format, and recombinant microsomes.

Soluble, purified, recombinant human P450s are produced in E.coli. All have been modified to some degree at the *N*-terminal to allow expression in E.coli, but these changes have not caused any discernible changes in substrate specificity or catalytic properties. The purified enzymes are available either as separate components or in a reconstituted format with oxidoreductase, cytochrome b5, and the appropriate lipids, which offers the advantages of using highly purified enzymes. These microsomes are produced from insect cells co-infected with a baculovirus vector expressing both a P450 isoform and rabbit NADPH-P450 oxidoreductase. This format is very simple to use and requires no additional protein components for full enzymatic activity. The turnover numbers for P450s in both formats are similar to that seen using human liver microsomes.

3. Metabolism of pesticide in human liver microsomes

In recent years, a number of metabolism studies of pesticide in human liver microsomes have been conducted and published (Table 22). Major metabolizing P450 isoforms were CYP 1A2, CYP 2B6, and CYP 3A4 in human. (Tang *et al*., 2001; Buratti *et al*., 2003; Usmani *et al*., 2004; Buratti *et al*., 2005)

Table 2. Metabolism studies of pesticide in human liver microsomes

Pesticide	Chemical class	Usage	Reference
2,4,-D	Phenoxy	Herbicide	Ohkawa, 1998
Acetachlor	Chloroacetanilide	Herbicide	Coleman *et al*., 2000
Alachlor	Chloroacetanilide	Herbicide	Coleman *et al*., 1999
Aldrin	Organochlorine	Insecticide	Newlands, 1992
Ametryne	Triazine	Herbicide	Cresteil *et al*., 1979
Atrizine	Triazine	Herbicide	Cresteil *et al*., 1979
Azinphos-methyl	Organophosphate	Insecticide	Buratti *et al*., 2003
Bifenthrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Bioresmethrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Butachlor	Chloroacetanilide	Herbicide	Coleman *et al*., 2000
Carbaryl	Carbamate	Insecticide	Kurata *et al*., 1998
〃	〃	〃	Tang *et al*., 2002
Carbufuran	Carbamate	Insecticide	Usmani *et al*., 2004
Chlorfenvinphos	Organophosphate	Insecticide	Huston and Logan, 1986
Chlorpyrifos	Organophosphate	Insecticide	Kurata *et al*., 1998
〃	〃	〃	Sams *et al*., 2004
〃	〃	〃	Tang *et al*., 2001
〃	〃	〃	Buratti *et al*., 2003
〃	〃	〃	Mutch., 2006
〃	〃	〃	Foxenberg *et al*., 2007
Cyfluthrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Cyhalothrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Deltamethrin	Pyrethroid	Insecticide	goldin *et al*., 2006
〃	〃	〃	goldin *et al*., 2007
Demethoate	organophosphorothionate	Insecticide	Buratti *et al*., 2007
Diazinon	Organophosphate	Insecticide	Sams *et al*., 2004
〃	〃	〃	Kappers *et al*., 2001
〃	〃	〃	Buratti *et al*., 2003
〃	〃	〃	Sams *et al*., 2004
〃	〃	〃	Mutch *et al*., 2006
Disulfoton	Organophosphate	Insecticide	Usmani *et al*., 2004
Diuron	Phenylurea	Herbicide	Abass *et al*., 2007
Endosulfan	Chlorinated cyclodien	Insecticide	Lee *et al*., 2006

Esbensulide	Pyrethroid	Insecticide	goldin *et al*., 2006
〃	〃	〃	goldin *et al*., 2007
Fenthion	Organophosphate	Insecticide	Furnes and Schlenk, 2005
Fipronil	Phenylpyrazole	Insecticide	Kurata *et al*., 1998
〃	〃	〃	Joo *et al*., 2007
Furametpyr	Anilide	Fungicide	Nagahori *et al*., 2000
Inidacloprid	Neonicotinoid	Insecticide	Schulz-Jander *et al*., 2002
Malathion	Organophosphate	Insecticide	Buratti *et al*., 2005
Methiocarb	Carbanate	Insecticide	Usmani *et al*., 2004
Methoxychlor	Organochlorine	Insecticide	Stresser *et al*., 1998
			Hu and Kupfer, 2002
Metolachlor	Chloroacetanilide	Herbicide	Coleman *et al*., 2000
Molinate	Thiocarbamate	Herbicide	Jewell and Miller, 1999
Myclobutanil	Triazole	Fungicide	Barton *et al*., 2006
Parathion	Organophosphate	Insecticide	Butler and Murray, 1997
〃	〃	〃	Sams *et al*., 2004
〃	〃	〃	Buratti *et al*., 2003
〃	〃	〃	Mutch *et al*. ,2006
〃	〃	〃	Foxenberg *et al*., 2007
Permethrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Phorate	Organophosphate	Insecticide	Usmani *et al*., 2004
Phorate	Organophosphate	Insecticide	Hodgson E, 2003
S-Bioallethrin	Pyrethroid	Insecticide	Scollon *et al*., 2009
Sulprofos	Organophosphate	Insecticide	Usmani *et al*., 2004
Terbuthylazine	Triazine	Herbicide	Cresteil *et al*., 1979
Terbutryne	Triazine	Herbicide	Cresteil *et al*., 1979
Triadimefon	Triazole	Fungicide	Barton *et al*., 2006

4. Bensulide

Bensulide (C14H24NO4PS3), [O, O-diisopropyl S-2-phenylsulfonylamino- ethyl phosphorodithioate] is one of the few herbicides from the organophosphate group used for control of weeds that threaten numerous crops (Antonious, 2009). Bensulide is a pre-emergent organophosphate herbicide that inhibits cell division in meristematic root tissues and seedling growth by conjugation of acetyl co-enzyme A. Bensulide is registered for use in agricultural crops such as Cole crops, cucurbits, leafy vegetables, legumes, onion and garlic (Coolong et al, 2009). It is used to control a variety of grasses and weeds in food crops (60 to 65 % of all use) including carrots, fruiting vegetables, leafy vegetables (mostly head lettuce), dry bulb vegetables (onions), cucurbits (mostly melons), and cole crops (cauliflower, cabbage, broccolini and broccoflower). Bensulide products may be used outdoors by homeowners on lawns and ornamentals, and by professional lawn care operators. Bensulide may be used on turf (primarily golf course greens and tees), on ornamentals, and for greenhouse and outdoor uses in commercial nurseries (EPA 738-F-00-001, 2000).

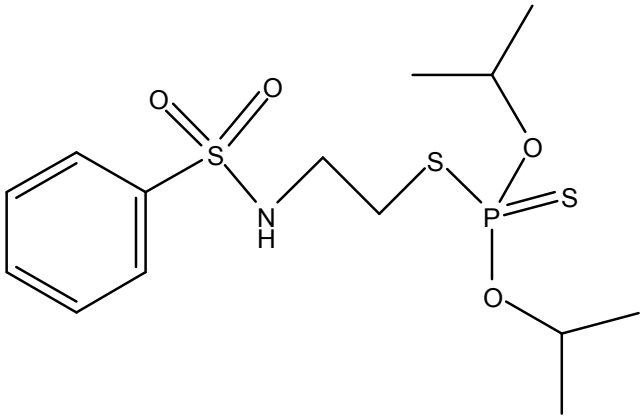


Figure 3. Structure of bensulide

5. Purpose of the present study

Metabolic pattern of bensulide has not been investigated for metabolism by microbial degradation and human liver microsome. The purpose of the present study is to elucidate the metabolic pattern of organophosphate herbicide bensulide by *Cunn. elegans* and human liver microsomes.

The study consists of (1) comparative analysis of identifying the metabolite of bensulide, (2) optimizing metabolic condition of bensulide by human liver microsomes, (3) determining which CYP450 isoforms contribute for metabolism of bensulide : incubation with cDNA-expressed human CYP450 isoforms.

Ⅱ. MATERIALS AND METHODS

1. Materials Equipments

1.1 Chemicals and reagents

Bensulide (Purity : 99.3%) was purchased from Sigma-Aldrich. Potato dextrose agar and potato dextrose broth were purchased from Difco Laboratories (Detroit, MI, USA). β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-P-D), potassium phosphate monobasic / dibasic and 2-(4-chlorophenyl)-3-methylbutyric acid were purchased from Sigma-Aldrich (St. Louis, MO). Solvents (acetonitrile, ethyl acetate) were HPLC grade (Fisher Scientific CO., Pittsburgh, PA, USA) and the other chemicals were of the highest quality available.

1.2 *Cunninghamella elegans* and incubation culture

Cunninghamella elegans ATCC 36112 were contributed from national center for toxicology research, U.S. Food and Drug Administration.

Cunn. elegans were grown in a potato dextrose broth. All cultures were maintained on potato dextrose agar and stored at 4℃.

Table 3. The composition of culture media

Medium	Composition (g/L)	
PDA	Potato Dextrose Agar	39
PDB	Potato Dextrose Broth	24

1.3 Human liver microsomes and cDNA-expressed CYPs

Pooled human liver microsomes (HLMs) were purchased from BD Gentest Co. (Woburn, MA) and stored at -80 ℃ prior to use. Assay result and enzyme activity information of Purchased human liver microsomes were described at Table 23. Also, the details of the liver microsomes donors are given in Table 24.

cDNA-expressed CYP isoforms (Supersomes®) (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4), were also purchased from BD Gentest Co. (Woburn, MA). All human P450 isoforms in Supersome® are co-expressed with human P450 reductase. All cDNA-expressed CYP isoforms were stored at -80℃. Boiled human liver microsomes were prepared at 100℃ for 10 minutes.

Table 4. Assay results of pooled human liver microsomes

Enzyme Measured	Assay	Enzyme Activity [in pmol/(mg×min]]
Total P450	Omura and sato	250 pmol/mg
OR	Cytochrom c Reductase	330
Cyt. b_5	Spectrophotometric	460 pmol/mg
CYP1A2	Phenacetin O-deethylase	820
CYP2A6	Coumarin 7-hydroxylase	1200
CYP2B5	(S)-Mephenytoin N-demethylase	28
CYP2C8	Paclitaxel 6α-hydroxylase	190
CYP2C9	Diclofenac-4'-hydroxylase	3000
CYP2C19	(S)-Mephenytoin N-hydroxylase	53
CYP2D6	Bufuralol 1'-hydroxylase (The amount of activity inhibited by 1 μM quinidine)	99
CYP2E1	Chlorozoxazone 6-hydroxylase	1900
CYP3A4	Testosterone 6β-hydroxylase	4900
CYP4A11	Lauric acid 12-hydroxylase	3500
FMO	Methyl p-Tolyl Sulfide Oxicase	680
UGT1A1	Estradiol 3-Glucuronidation	920
UGT1A4	Trifluoperazine Glucuronidation	890
UGT1A9	Propofol Glucuronidation	2400

(Package contents : 0.5 mL; protein content : 20 mg/mL in 250 mM sucrose)

1.4. Analytical instrument and analytical conditions

1.4.1 HPLC

For microbial degradation the bensulide and bensulide oxon were measured by reverse-phase high performance liquid chromatography method (Lasker et al., 1980). The HPLC system was consisted of Shiseido Nanospace SI-2 with UV detector (220 nm). The samples were separated on a 100 mm X 2.1 mm, 2.6 -µm particle; Phenomenex) at 40 ºC. The mobile phase for pump A was water whereas pump B was acetonitrile. A gradient system was employed over 50 minutes at a flow rate of 0.2 mL/min with A : B as follows: initial, 90:10; 30min, 10:90; 35 min 10:90; 39 min 90:10; 45 min 90:10. Using this gradient, bensulide and bensulide oxon were well separated. The time of retentions obtained for bensulide and bensulide oxon were 23.9 min and 17.5 min respectively.

For Human liver microsome, and the specific isoforms of cytochrome P450 the bensulide and bensulide oxon were measured by reverse-phase high performance liquid chromatography method (Lasker et al., 1980). The HPLC system was consisted of Agilent 1100 series HPLC (Agilent, Wilmongton, CA) with UV detector (220 nm). The samples were separated on a Luna C18 column (250 mm X 4.6 mm, 5-µm particle; Phenomenex) at

40 ºC. The Human liver microsome samples were separated on C18 column (250 mm X 4.6 mm, 5-µm particle; Phenomenex) at 40 ºC. The mobile phase for pump A was water whereas that for pump B was acetonitrile. A gradient system was employed over 30 minutes at a flow rate of 1.0 mL/min with A:B as follows: initial, 50:50; 0 min, 20:80; 18min, 20:80; 21min, 50:50; 24min, 50:50; 30min. The time retention obtained for Bensulide and Bensulide oxon were 8.1 min and 15.1 min respectively.

1.4.2 LC-MS

LC/MS was carried out by coupling Varian HPLC system (Agilent 1100 series HPLC, Agilent, Wilmongton, CA) to a tandem mass spectrometer (Varian 500-MS, USA) with electrospray ionization (ESI+) mode. The capillary voltage, RF voltage, Needle voltage and CID voltage were kept at 53.94 V, 145.80 V, 4000 V, and 1.43 V, respectively. An electron multiplier voltage of 640 V was used. The nebulizer and desolvation gas were ultrapure nitrogen set at 80 and 397 L/h, respectively. Turbo DDS was carried out using nitrogen as the collision gas. The collision energy was kept at 50.0 eV. The cone voltage and capillary voltage were adjusted to 35V and 3.43 kV, respectively.

2. Method

2.1. Preparation of standard and calibration curves.

Bensulide standard solutions were prepared in acetonitrile at the concentration of 0.05, 0.1, 0.5, 1, and 2 μM. The calibration curves were fitted with high linearity and R^2 value were 0.999. For metabolic reaction, in all experiments the final concentration of the organic solvent did not exceed 0.5 % (Rodrigues, 1999; Kim *et al*., 2006).

2.2 Metabolism of Bensulide by soil fungi, Cunninghamella elegans, 36112.

For metabolic study of bensulide, cultures were allowed to grow for 3 days at 28℃. The mycelia from a plate was transferred to sterile potato dextrose broth (PDB, Difco Laboratories, Detroit, MI) and incubated in shaking incubator for 3 days at 28℃, 200 rpm. Approximately 2 mL portion of blended mycelia suspension were used to inoculate in 250 mL Erlenmeyer flasks containing 100mL potato dextrose broth. The cultures were incubated at 28℃ in a shaking incubator operating 200 rpm. 4000 ppm (ul/ml) 100 ul was added to each flask. In control experiment, a culture without bensulide and sterile flasks containing only media and bensulide

was incubated. Each culture was triplicated. After 0, 1, 3, 5, 7, 10 days of incubation, 10mL fungi cultures were extracted with 40 mL acetonitrile and water was dried through Na2SO4. The solvent was evaporated in vacuo. The residue was dissolved in acetonitrile (1ml) and analyzed by HPLC.

2.3 Fractionation of metabolite of bensulide

Fraction collector, Gilson FC-205 was used to collect the metabolite of bensulide.

2.4. LC-MS

2.4.1. Optimization of 500-MS

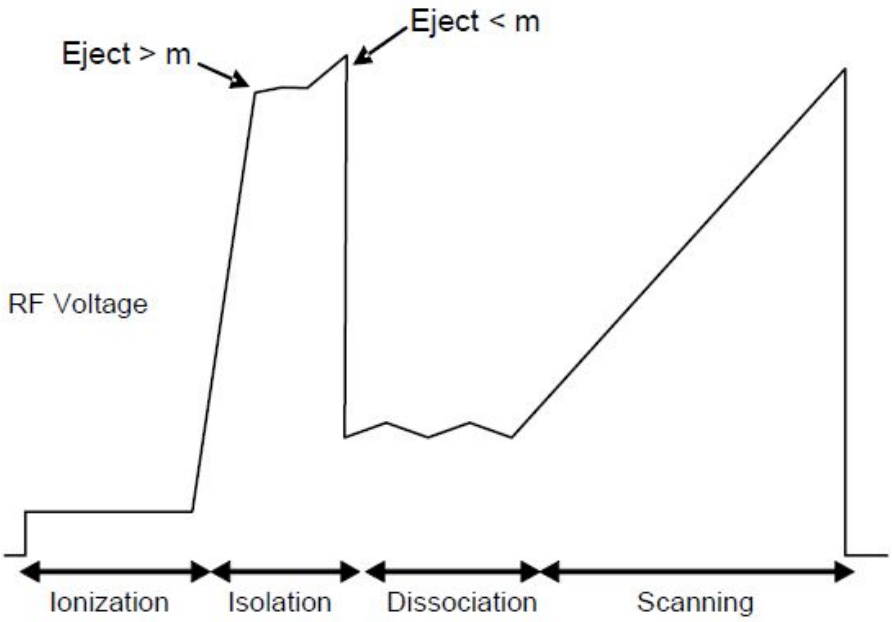


Figure 4. The MS/MS scan function

The ion trap MS/MS process is first fills the ion trap with the selected precursor ion, forming the product ion spectrum to obtain qualitative structural information to obtain qualitative structure information; and the increases the signal-to-noise ratio by eliminating interfering matrix ions in the product ion spectrum through isolation and dissociation. Capillary voltage, RF loadings, needle voltage and CID voltage are needed to be optimized to obtain best mass spectroms. Capillary voltage effects the

focusing of ions into the capillary as well as the energy imparted to them as they exit the capillary. RF loading (%) optimizes the amount of energy that an ion acquires after injection into the ion trap. Lower values may minimize fragmentation for a given ion. Higher values provide a deeper potential well for trapping, which also imparts more energy to the ions to overcome the deeper potential well. Precursor ions are transformed into product ions by collision-induced dissociation (CID). The precursor ions constantly collide with helium gas in the ion trap. Normally these collisions involve relatively small energies, but if the translational energy of the precursor ion is increased, the collisions may convert the translational kinetic energy into internal vibrational energy. If the precursor ion acquires enough vibrational energy, one or more chemical bonds in the ion may be broken, and in the case of singly charged ions, forming ions of lower m/z than the original precursor ion. If the precursor ion is multiply charged, then the product ions may be observed throughout the mass range. The ion distribution that results from the CID process depends on the characteristics of the precursor ion and the amount of energy that has been converted into internal vibrational energy (500-MS IT Mass Spectrometer MS workstation, version 6, 2010).

2.4.2. Identification of fractionated metabolite of bensulide

Fractionated metabolite was identified with Agilent 1100 series HPLC

and Varian 500-MS ion trap mass spectrometer.

2.4.3. Turbo DDS

Turbo Data Dependent Scanning (TurboDDS) was performed to do automated MS^n scans on metabolite peak as it eluted from the column. In a typical TurboDDS run, a survey scan was done followed by a high resolution scan and then multiple MS^n sequences were done.

2.5. *In vitro* metabolism reaction of bensulide with human liver microsomes (HLMs) and identification of major metabolite

For metabolic reaction of bensulide, 50 mM phosphate buffer (pH 7.4) and 250 mM $MgCl_2$, bensulide 1 mM, 0.4 mg protein/ml were prepared in 1.5 mL microcentrifuge tubes. Then, NADPH-generating system (1 mM NADPH, 1 mM $NADP^+$, 5 mM glucose-6-phosphate, and 2U/mL glucose-6-phosphate-dehydrogenase) was added to initiate reaction. The final volume was 200 uL. Reaction was carried out by time interval and terminated with 200 uL cold acetonitrile, followed by pulse-vortexing. Samples were centrifuged at 15,000 rpm for 5 minutes and supernatants were analyzed in HPLC, as described in HPLC analysis ahead. All samples were done

triplicate.

2.6. Optimization of microsomal reaction conditions

2.6.1. Optimization of incubation time

To determine the optimal incubation time for the formation of metabolite with HLMs, the reaction mixtures were incubated for 0, 5, 15, 30, 60, 90, 120, 180, 240, 360 min with 0.4 mg/ml of HLMs concentration and 10 μM of bensulide. The incubation and analysis of reaction mixtures were the same as described in section 2.5.

2.6.2. Optimization of protein concentration

To determine the optimal protein (microsmes) concentration for the formation of metabolite with HLMs, the reaction mixtures were incubated for 5 minutes with various concentrations of human liver microsomes (0, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 mg/ml) and 10 μM of bensulide. The incubation and analysis of reaction mixtures were the same as described in section 2.5.

2.6.3 Optimization of bensulide concentration

To determine the optimal bensulide concentration for the metabolism of bensulide with HLMs, the reaction mixtures were incubated for 5 minutes with HLMs (0.4 mg/ml) and various concentrations of bensulide (0.1, 1, 2, 5, 10, 20, 50, 100 μM). The incubation and analysis of reaction mixtures were the same as described in section 2.5.

2.7. Metabolism with human cDNA-expressed CYP isoforms.

For the enzyme kinetic studies, an increasing concentration of bensulide (0.1, 1, 2, 5, 10, 20, 50, 100 μM) was incubated with human cDNA-expressed 2C19, 3A4 (4 pmole / 200 μl) and with an NADPH-generating system for 5 min at 37°C. The incubation and analysis of reaction mixtures were carried out as described in section 2.5.

2.8. Metabolism of bensulide with cDNA-expressed CYP isoforms.

The cDNA-expressed CYP isoforms (1A2, 2A6, 2B6, 2D6, 2C8, 2C9, 2C19, 3A4, and 2E1 for 20 pmol) were incubated with bensulide (10 μM; a optimized drug concetration) for 5 min at 37℃ (final volume of 200 μl). The concentrations of the proteins were determined by activities in native

HLMs which were recommended by supplier, BD Gentest Co. (Woburn, MA). The incubation and analysis of reaction mixtures were carried out as described in section 2.5..

2.9. Calculation of TNR (Total Normalized Rate).

The normalized rates for each cDNA-expressed CYP isoform were summed, yielding a "total normalized rate (TNR $=\sum f_i \cdot V_i$)" and the normalized rate for each P450 isoform (= $f_i \cdot V_i$) was expressed as a percentage of the net reaction rate (= 100 x $f_i \cdot V_i/\sum f_i \cdot V_i$, where f_i indicates the fraction of each P450 isoform content in the human liver, and $V_i = V_{maxi} \cdot [S_i]/K_{mi} + [S_i]$). The patterns of TNR of each CYP in various concentrations of bensulide were also investigated.

Ⅲ. RESULTS and DISCUSSION

1. Metabolism of bensulide with HLMs.

1.1 Calibration curve of bensulide standard

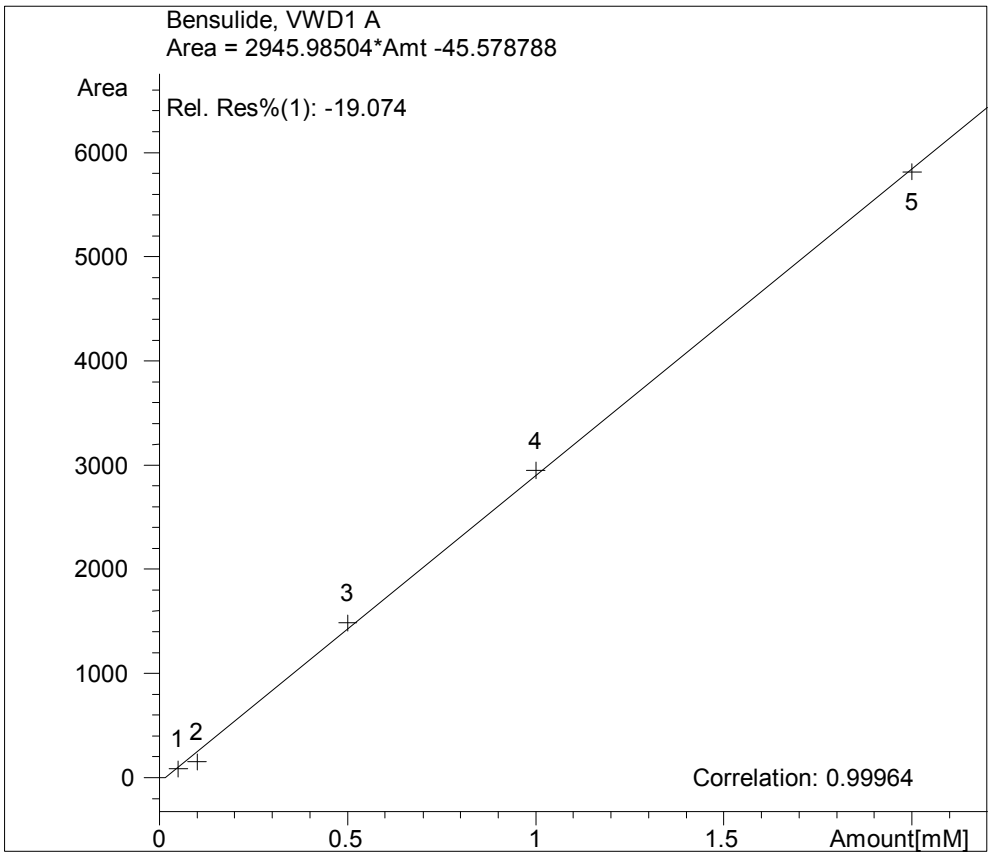


Figure 5. Calibration curve of bensulide standard (0.05 ppm, 0.1 ppm, 0.5 ppm, 1 ppm, 2 ppm)

2. Metabolism of bensulide in reaction mixture

2.1. Analysis of metabolite of bensulide in reaction mixture

This experiment was conducted ahead of HLM metabolism because Cunn. elegans is well known for it's similarity of mammalian metabolism. By using HPLC gradient elution, bensulide and its metabolite were well separated. Microbial incubation of bensulide in the presence of *Cunn. elegans* resulted in the formation of unknown metabolite, M1. In the sterilized *Cunn. elegans* media, the metabolite did not form, indicating that metabolic degradation was from *Cunn. elegans*. Time retention of bensulide and M1 were obtained for 23.9 and 17.5 min. Figure 5 shows the chromatogram of bensulide and M1 after microbial incubation. The chromatogram on the top is sample obtained on the day of incubation (day 0), chromatogram in the middle is sample obtained after 3 days of incubation (day 3), and the chromatogram on the bottom is sample obtained from sterilized *Cunn. elegans,* which was used as a control group. Day 3 sample shows a little trace of bensulide left which means bensulide was degraded almost completely. There was not any metabolite peak or interfering peak in the control metabolic incubations.

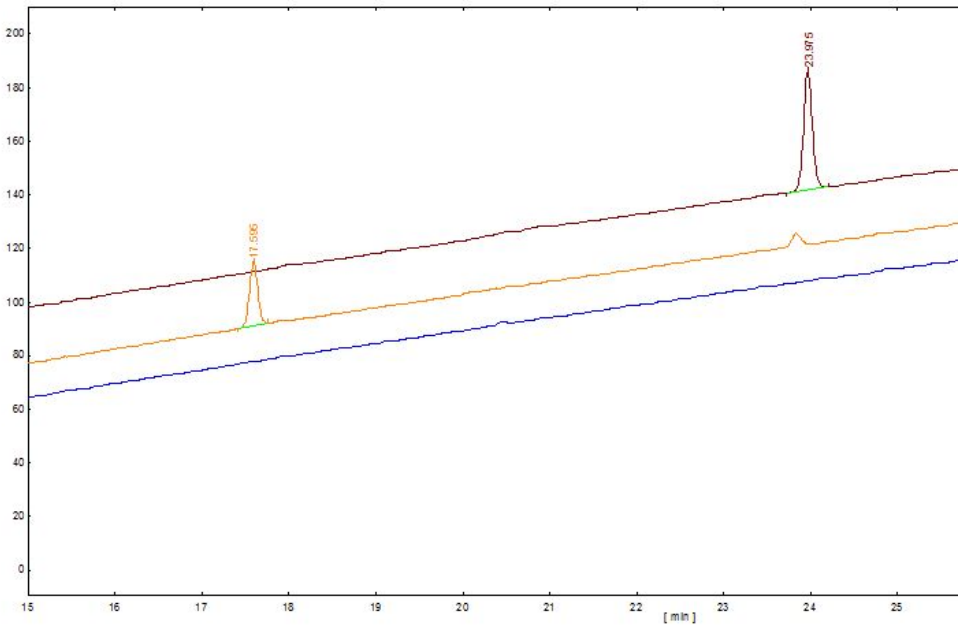


Figure 6. The HPLC chromatograms of bensuide and its metabolite from microbial degradation.

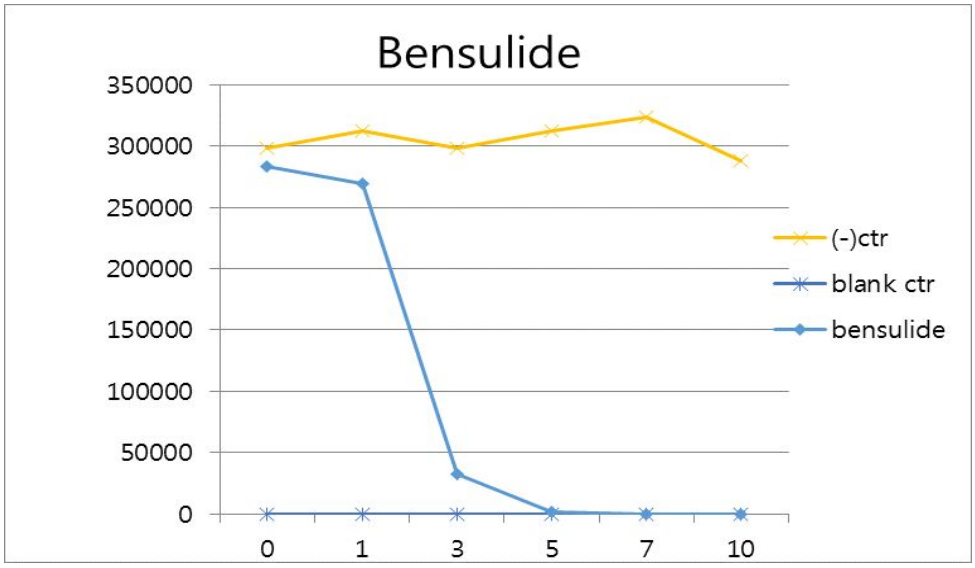


Figure 7. Bensulide degradation pattern of microbial degradation by *Cunn. elegans.*

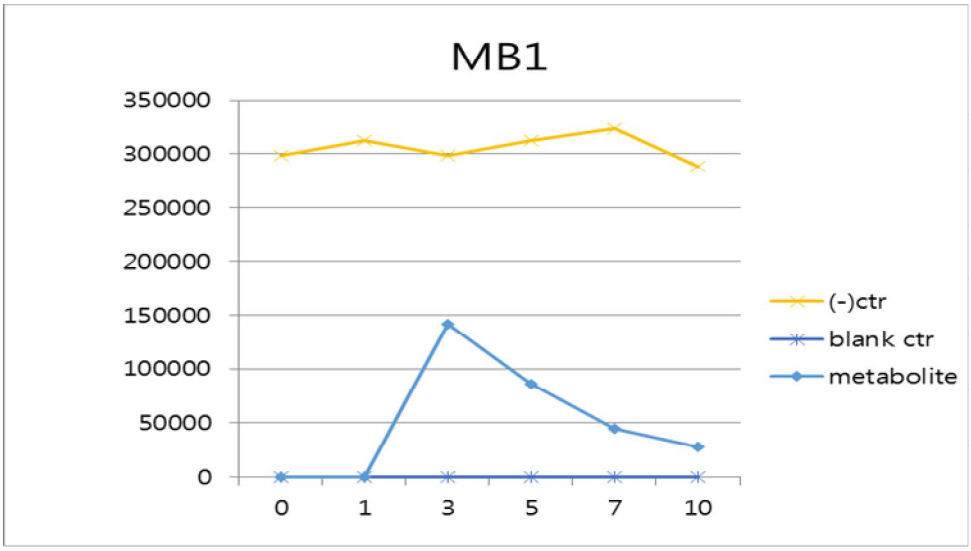


Figure 8. Metabolite of bensulide formation pattern of microbial degradation by *Cunn. elegans.*

2.2. Identification of metabolite of bensulide in reation mixtutre

Fractionated metabolite was analyzed with Varian 500-MS in optimized condition. Optimized parameters and values are shown in Fig. 9.

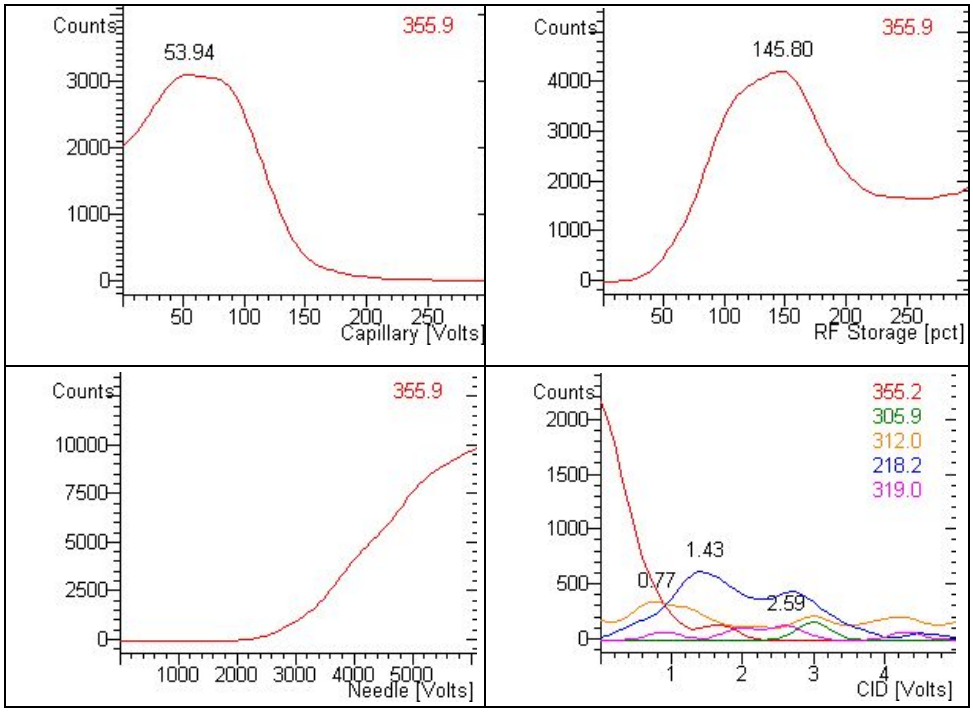


Figure 9. Optimized LC-MS condition- capillary voltage, RF voltage, Needle voltage, and CID voltage.

Human liver microsomal incubation of bensulide in the presence of NADPH resulted in the formation of unknown metabolite, M1, as same as microbial degradation experiment. I did 2 control group experiments, which was boiled microsome and heated microsome. 1) Microsome was boiled at

100℃ for 10 mins to make microsome lose its cytochrom P450 activity. 2) Microsome was heated at 45℃ for 1 min to deactivate its FMO activity. In boiled control groupe the metabolite did not form, indicating that metabolic enzymes are cytochrome P450s. Secondly, in heated control group, metabolite did form, indicating that metabolic enzymes are not associated in FMO group. Time retention of bensulide and M1 were obtained for 15.1 and 8.1 min

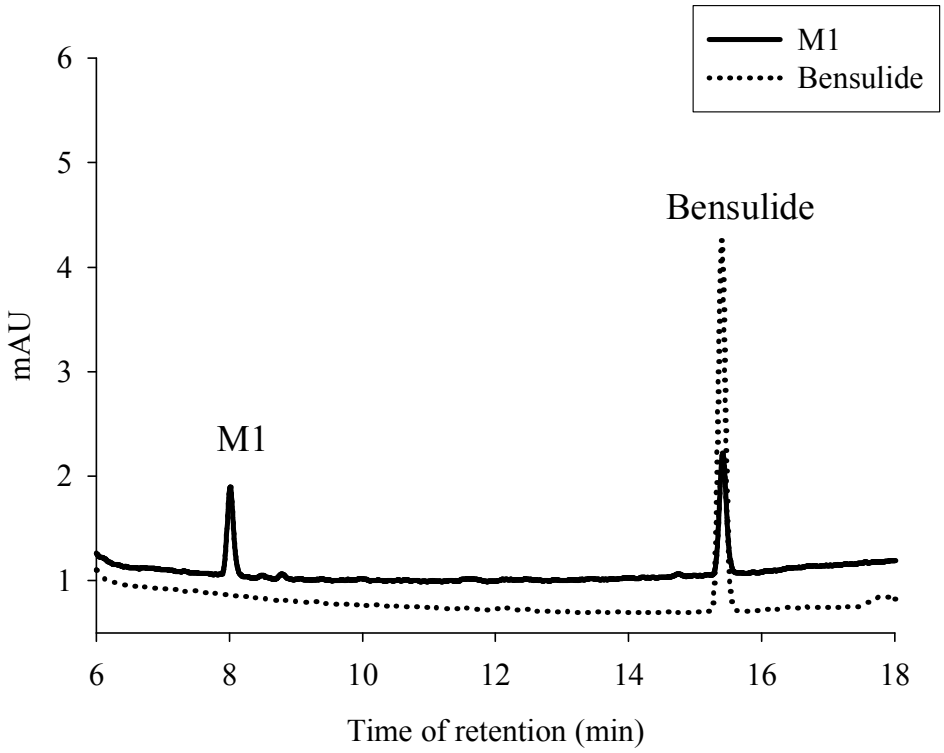
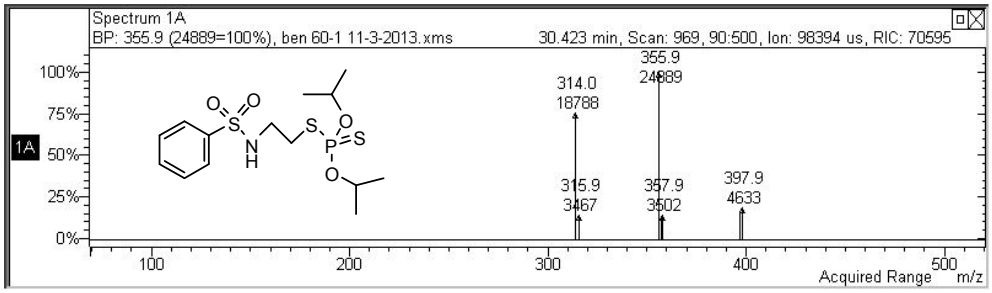


Figure 10. The HPLC chromatograms of bensulide and metabolite of bensulide from *in vitro* incubation with human liver microsomes

2.3. Identification of metabolites of bensulide and bensulide oxon by LC-MS

After screening the reaction mixture and finding metabolite from metabolism experiment, identifying work was done. To identify the M1, the metabolic reaction mixtures were investigated by LC-MS. The samples were separated on a 100 mm X 2.1 mm, 2.6 -μm particle; Phenomenex at 40 ºC. The mobile phase for pump A was water whereas that for pump B was acetonitrile. A gradient system was employed over 30 minutes at a flow rate of 1.0 mL/min with A:B as follows: initial, 50:50; 0 min, 20:80; 18min, 20:80; 21min, 50:50; 24min, 50:50; 30min. Positive-ion ESI mass spectrum indicated a peak at m/z = 356.0 was a fractionated and protonated molecular ion $[MH]^+$ of parent compound, bensulide, and a peak at m/z = 340.0 was a fractionated and protonated molecular ion $[MH]^+$ of bensulide oxon. M1 was identified as bensulide oxon (Figure 11 and 14). Figure 12 and 13 is EIC (extracted ion chromatogram) of bensulide and bensulide oxon. All EIC peaks are shown in same RT, which confirms that all peaks are from one molecule. Figure 15, 16 is fragmented mass spectrum result from Turbo DDS mode. Predicted molecules are shown in each mass spectrum using Mass frontier program.



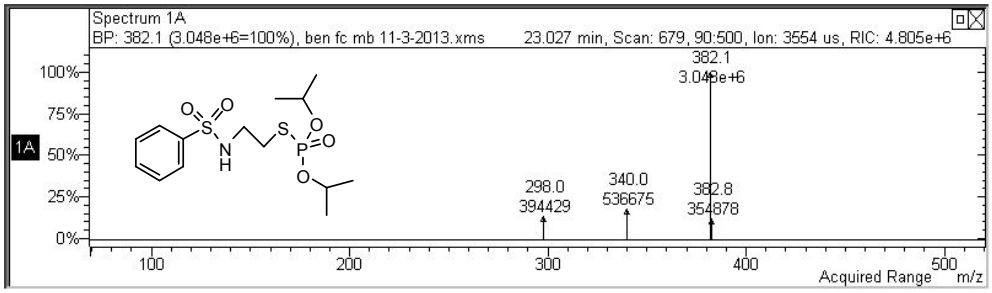


Figure 11. LC-MS spectrum of bensulide standard and fractionated bensulide oxon formed from bensulide reaction mixture with *Cunn. elegans*.

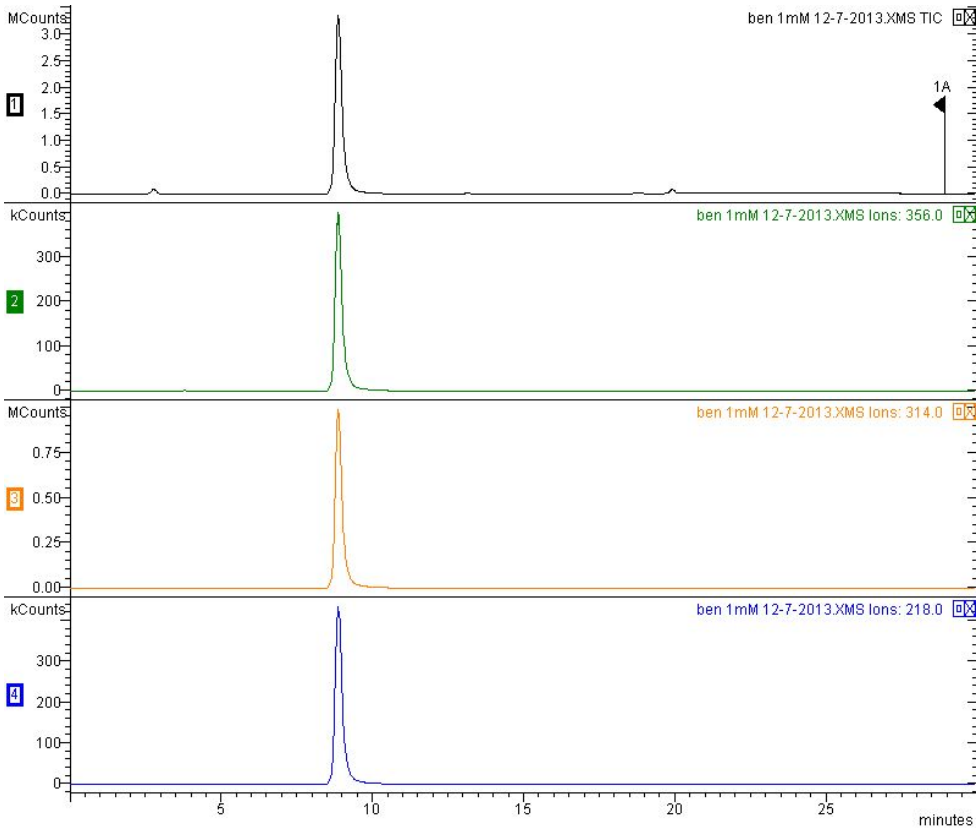


Figure 12. The LC-MS chromatograms (EIC) of bensulide standard.

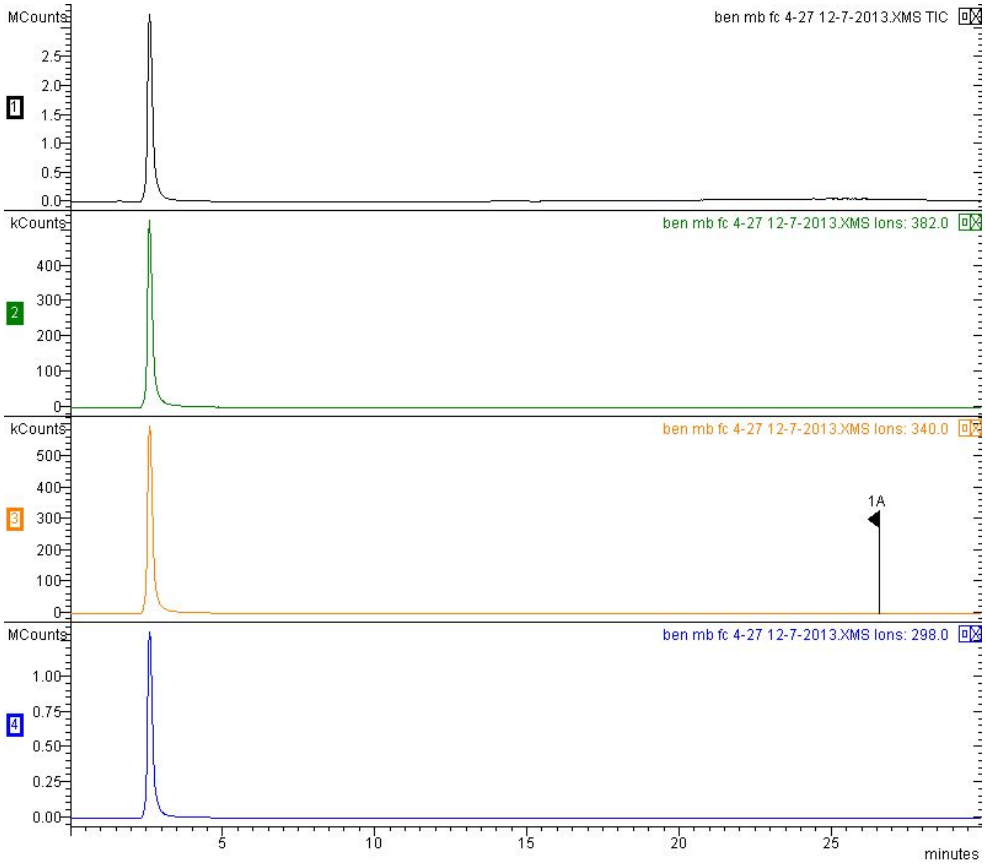
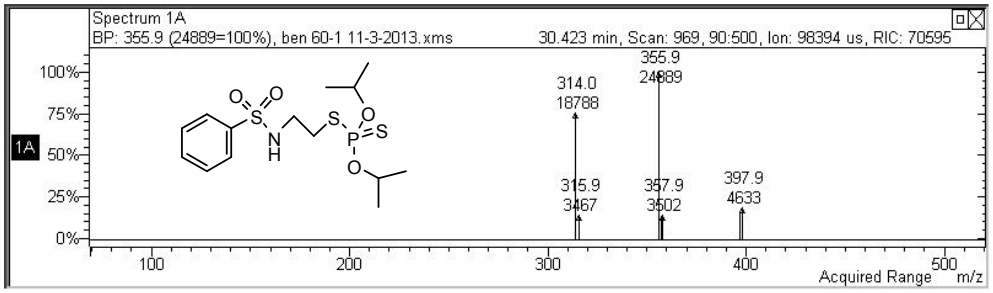


Figure 13. The LC-MS chromatograms (EIC) of bensulide oxon collected by fraction collector.



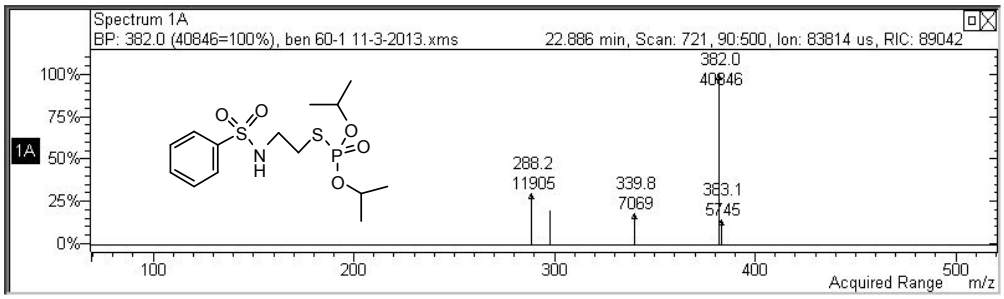


Figure 14. LC-MS spectrum of bensulide and bensulide oxon formed from bensulide reaction mixture with human liver microsomes.

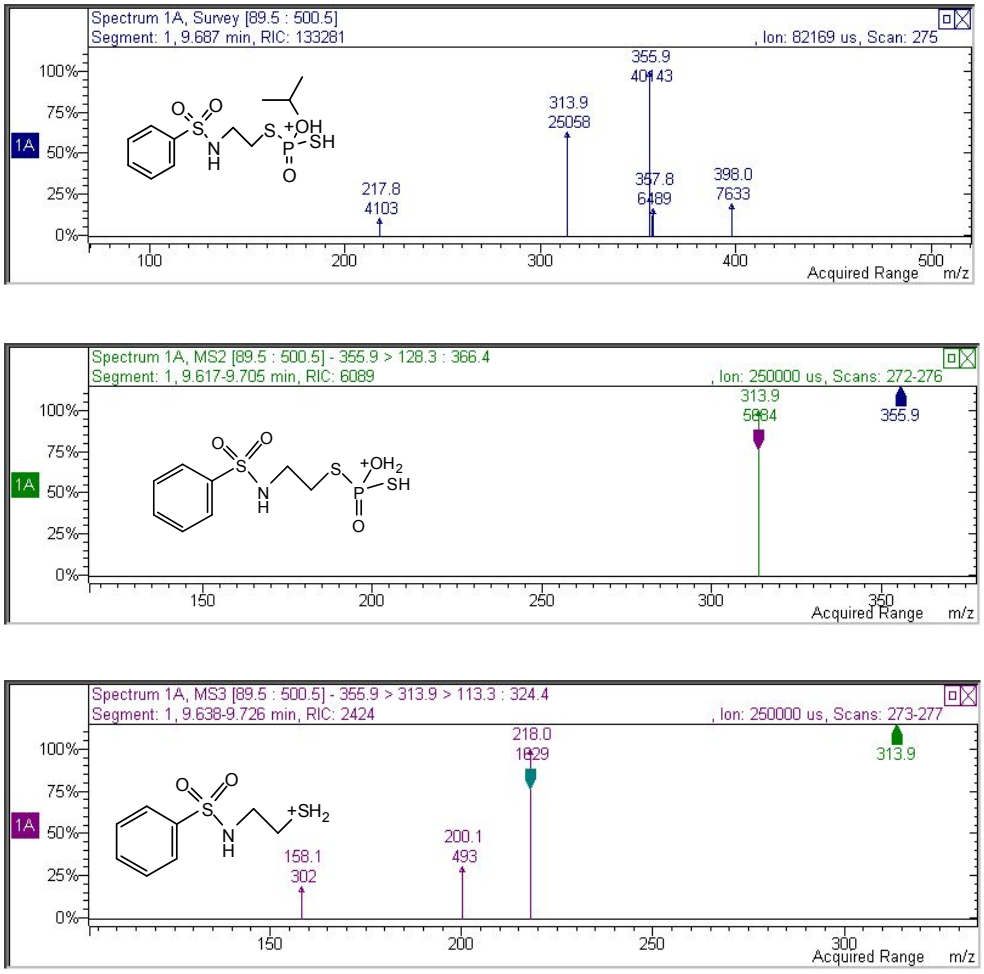


Figure 15. Fragmented bensulide with Turbo DDS mode.

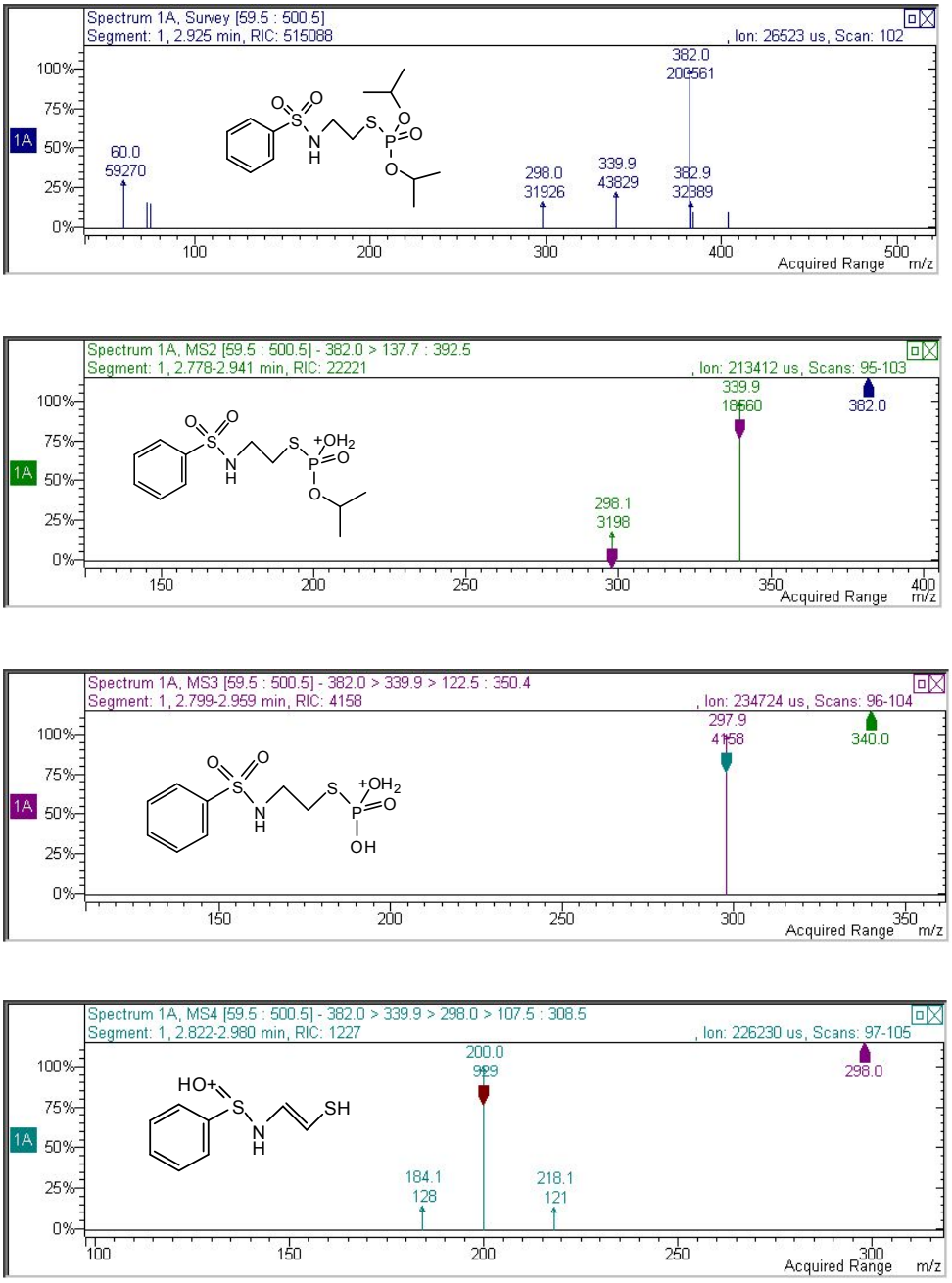


Figure 16. Fragmented bensulide oxon with Turbo DDS mode.

2.4 Optimization of metabolic reactions

Metabolic conditions of HLM experiment were optimized to get accurated kinetics parameters. Kinetic parameters explain relation between drug and CYP. Optimizing parameters were incubation time, protein concentration, and drug concentration.

2.4.1. Optimization of incubation time

To obtain optimal metabolic results, incubation time, protein (microsomes) concentration, and substrate (bensulide) concentration should be in the linear range for the formation of metabolite. Optimization of metabolic reaction conditions were tested in various combinations of factors, including reaction times, protein and bensulide concentrations. Figure 17 shows the pattern of metabolite formation from bensulide dependence on the incubation time. The rate of formation of metabolite was proportional to incubation times up to 240 min.

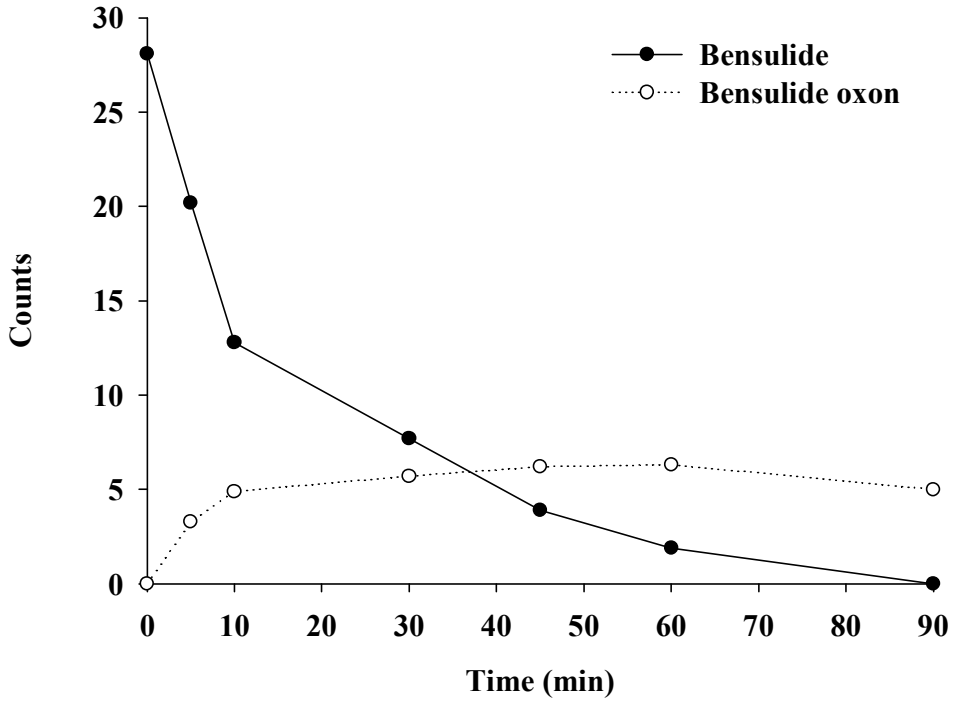


Figure 17. Formation of bensulide oxon from bensulide depending on the incubation time with human liver microsomes

2.4.2 Optimization of protein concentration

Figure 18 shows the pattern of metabolite formation from bensulide depending on the protein concentrations. The rate of formation of metabolite was proportional protein concentration up to 0.5 mg/ml. So the optimal protein concentration chose as 0.25 mg/ml.

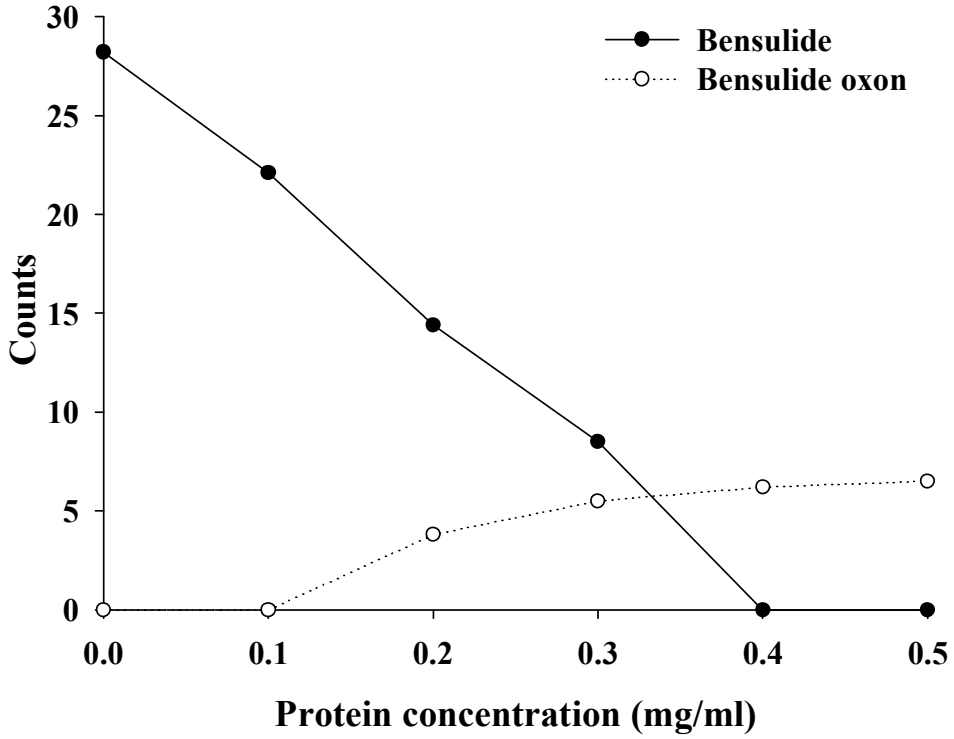


Figure 18. Formation of bensulide oxon from bensulide depending on the protein concentration of human liver microsomes

2.4.3 Optimization of bensulide concentration

Figure 19 shows the pattern of metabolite formation from bensulide dependence on the bensulide concentrations. At 100 μM of bensulide, formation of bensulide oxon was saturated.

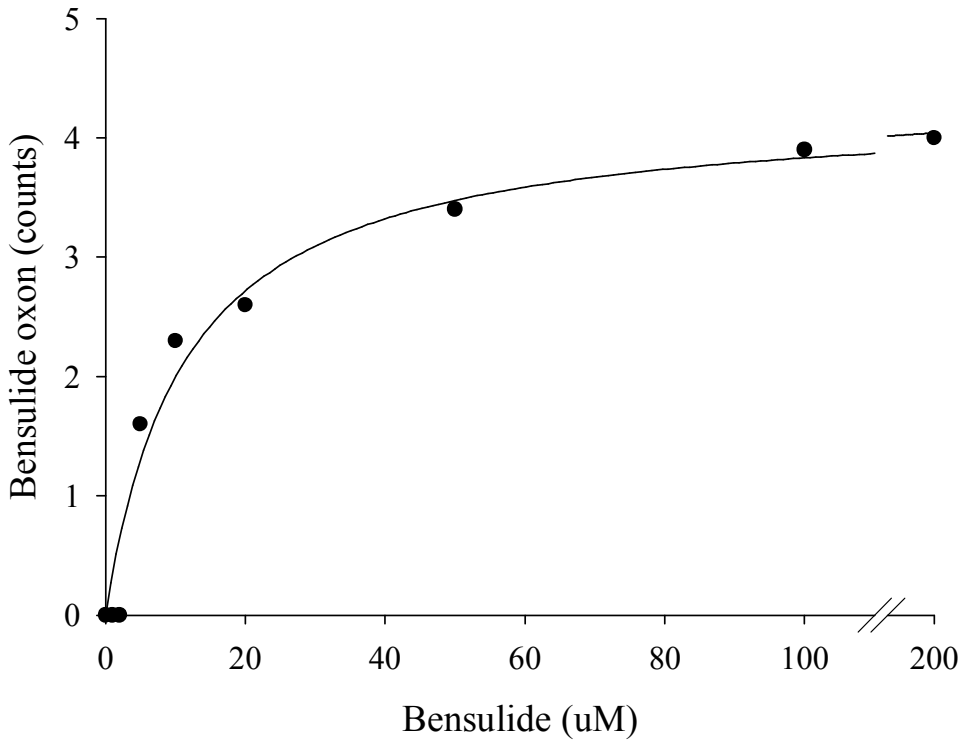


Figure 19. Formation of bensulide oxon from bensulide depending on the concentration of bensulide in human liver microsomes

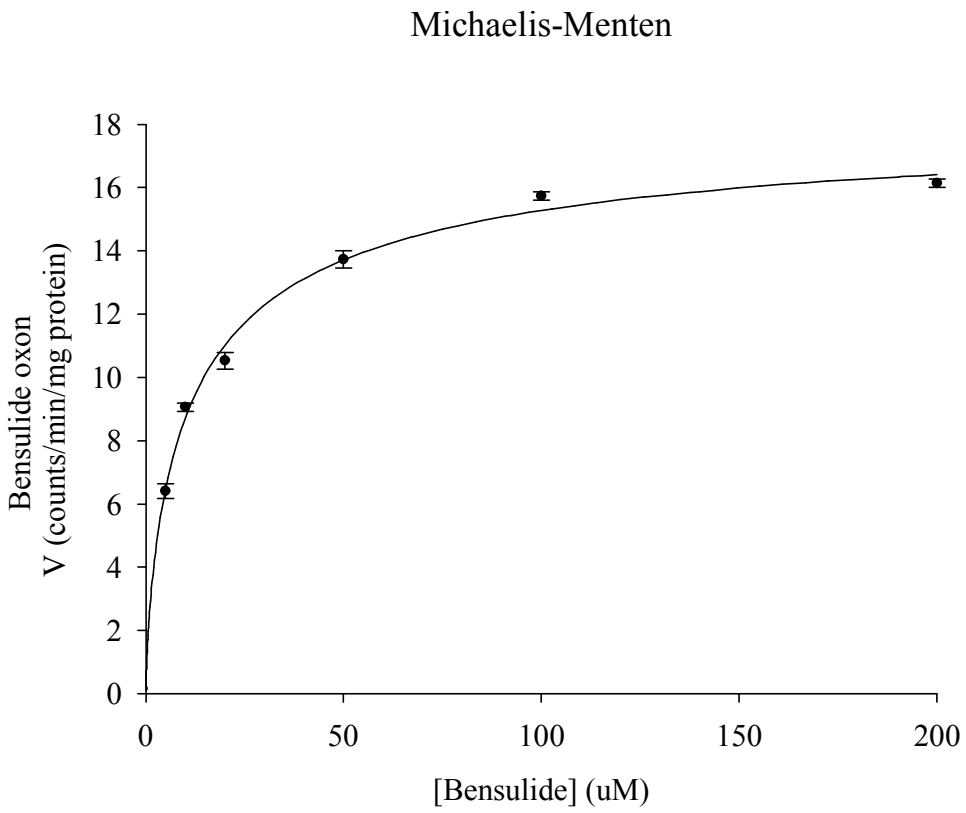


Figure 20. Kinetics for the formation rate of bensulide oxon from bensulide with human liver microsomes ; Michaelis-Menten plot.

The important terms maximum velocity V_{max}, and Michaelis constant K_m. The Michaelis constant is equal to the substrate concentration that sufficient to give half the maximum velocity for the enzyme. One should get an intuitive feeling for the magnitude of K_m. A small concentrations of substrates are sufficient to saturate the enzyme and to reach the maximum catalytic efficiency of the enzyme. These K_m and V_{max} values were then used to calculate the intrinsic clearance value ($Cl_{\text{int}} = V_{max} / K_m$), the functional ability of the enzyme.

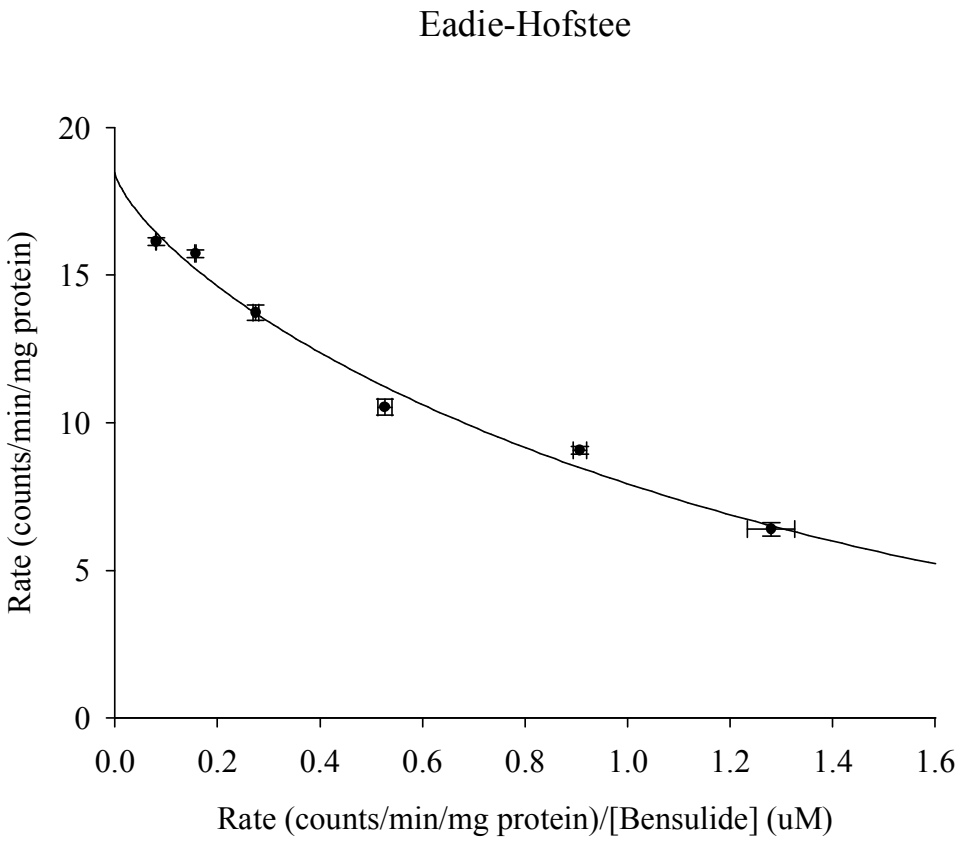


Figure 21. Kinetics for the formation rate of bensulide oxon from bensulide with human liver microsomes; Eddie-Hofstee plot.

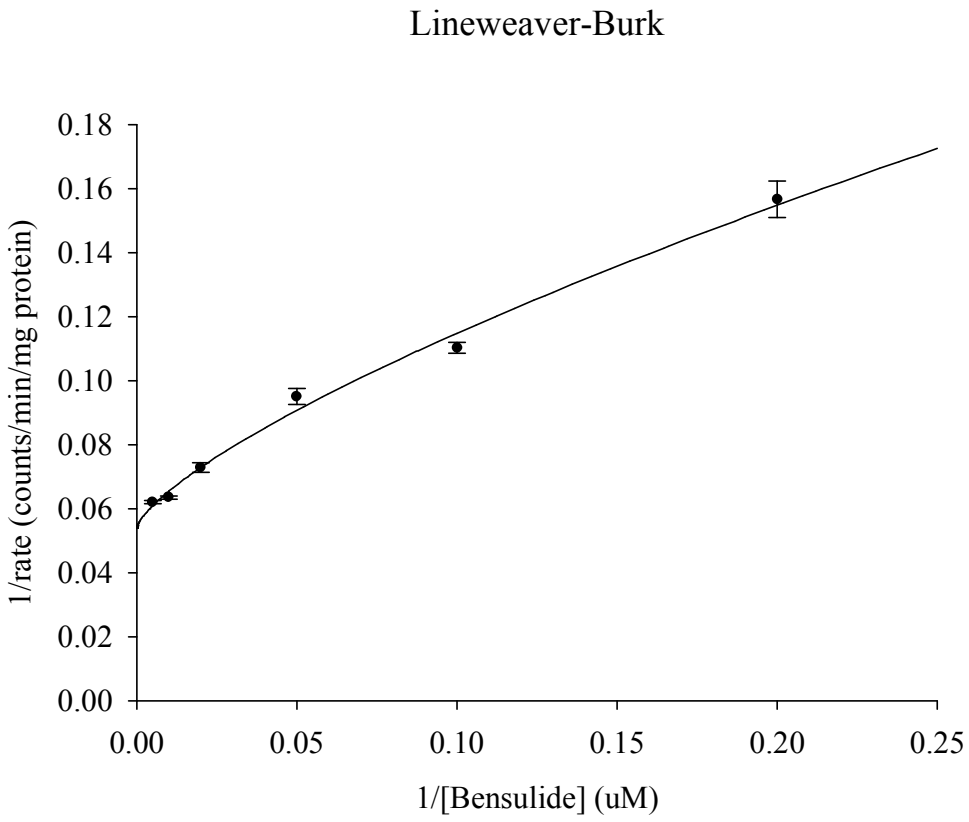


Figure 22. Kinetics for the formation rate of bensulide oxon from bensulide with human liver microsomes ; Lineweaver - Burk plot.

The Lineweaver–Burk plot, double reciprocal plot was obtained to get accurate values of Km and Vmax.

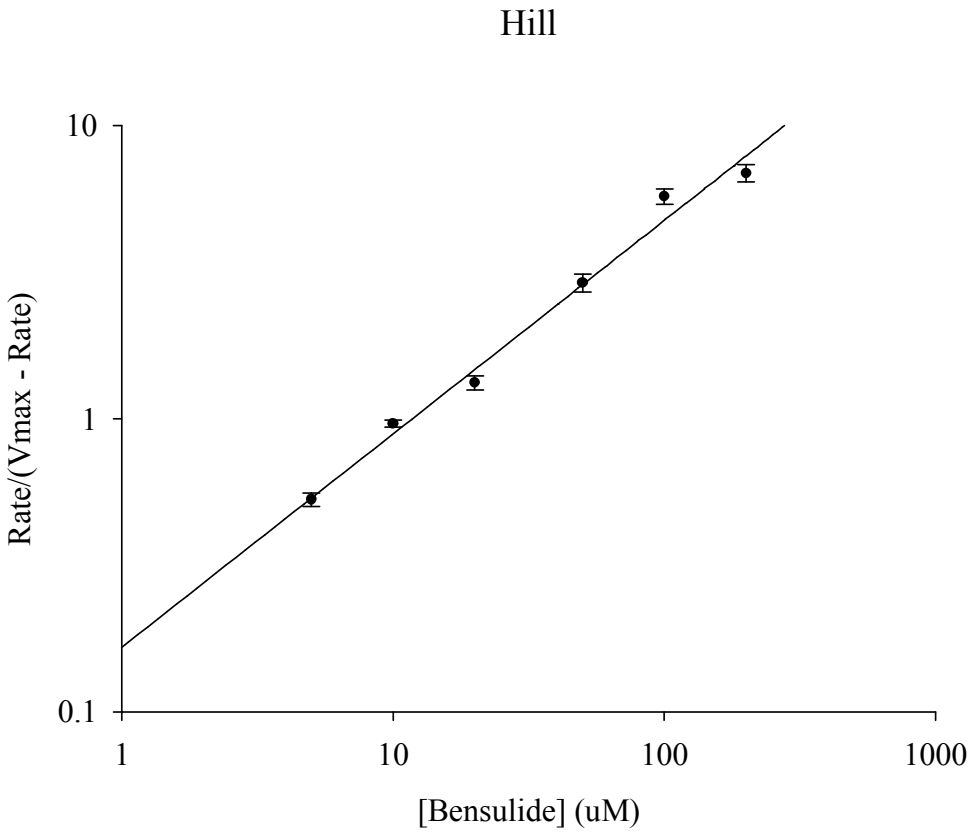


Figure 23. Kinetics for the formation rate of bensulide oxon from bensulide with human liver microsomes ; Hill plot.

In optimization experiments, 10 μM of bensulide concentration, 0.25 mg/ml of microsomes protein content, and 5 min of incubation time were obtained as the optimized conditions.

Table 5. Formation of metabolite from bensulide under optimized reaction condition.

a. Optimized reaction condition of formation of bensulide oxon from bensulide.

Human liver Microsomes (mg/ml)	bensulide (μM)	Reaction time (min)
0.25	11.7	5

b. Formation of metabolite from 10 uM of bensulide under optimized reaction condition.

Human liver Microsomes (mg/ml)	bensulide (counts)	Bensulide oxon (counts)	Bensulide oxon Formation (%)
0.25	31.15	2.4	7.02

3. Identification of the P450 isoforms involved in bensulide metabolism with human cDNA-expressed CYP isoforms

Bensulide oxon formation from bensulide was studied with 9 different human cDNA-expressed CYP isoforms (CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4). The cCNA-expressed CYP isoforms were pre-incubated for 5 minutes at 37℃ in the presence of the NADPH-generating system. At a single fixed concentration for each substrate, bensulide (10 μM) was utilized in all samples.

Bensulide oxon were appeared in CYP 2C19 and 3A4. It seems that these 2 CYP isoforms play a role in metabolism of bensulide. It showed higher rates of formation in CYP 3A4 than CYP 2C19. It was interesting that formation rate of CYP 2C19 continiously increased along with the increase of concentaration of bensulide. Because in most of cases, it shows saturation curve like in case of CYP 3A4.

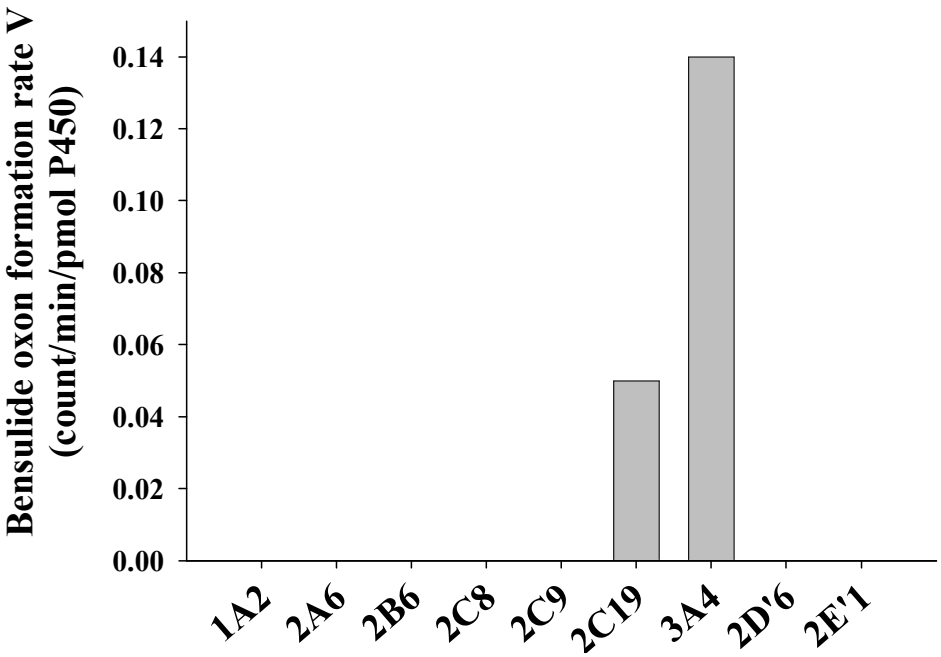


Figure 24. Formation rates of bensulide oxon from bensulide with various cDNA-expressed CYP isoforms

4. Drug concentration dependent different formation of bensulide oxon

The composition of bensulide oxon by CYP isoforms changed in process of reaction time. We could see the increasing rate of bensulide oxon with increasing concentration of bensulide with CYP 2C19 and CYP 3A4. CYP 2C19 shows higher formation of bensulide oxon with higher concentration of bensulide. But as concentration of bensulide reaches 10 uM in CYP 3A4 concentraion of bensulide oxon does not change but shows a steady slope line.

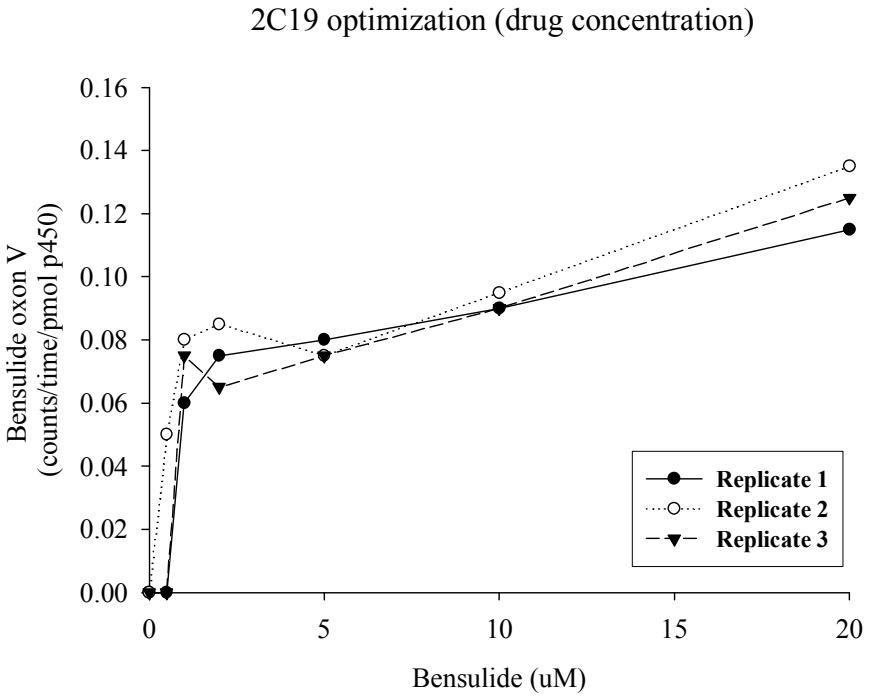


Figure 25. Formation of bensulide oxon with CYP 2C19 from bensulide depending on the concentration of bensulide

Table 6. Obtained Kintetics parameters

V_{max}	K_m	Cl_{int}
0.11	7.40	0.01

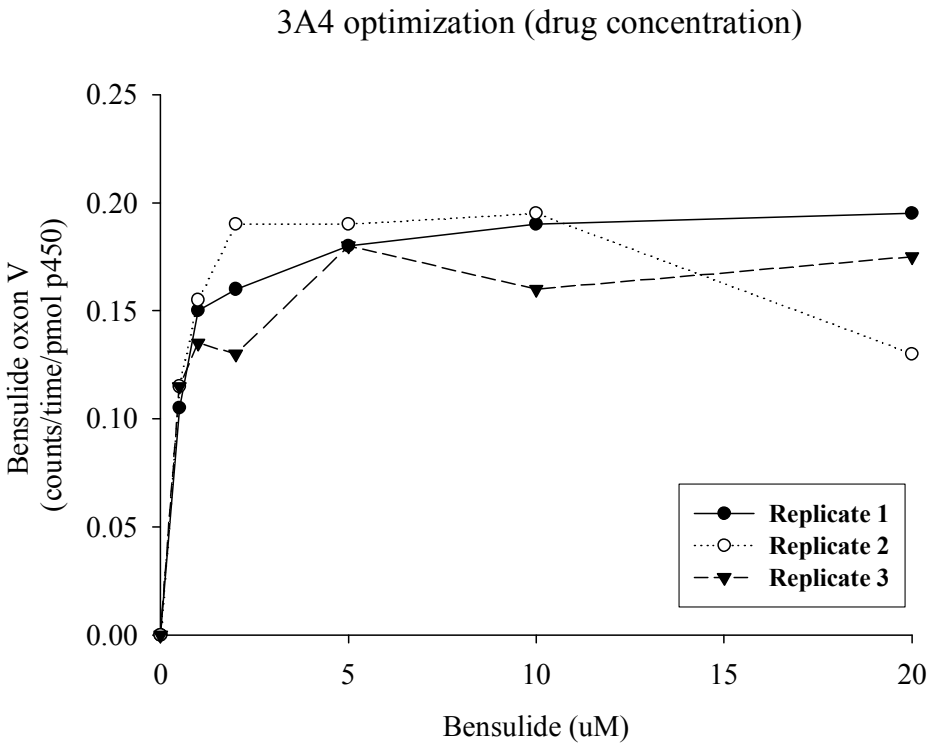


Figure 26. Formation of bensulide oxon with CYP 3A4 from bensulide depending on the concentration of bensulide.

Table 7. Obtained Kintetics parameters

V_{max}	K_m	Cl_{int}
0.18	0.26	0.69

5. TNR in *in vitro* metabolism

The percentages of total normalized rates (% TNR) of CYPs were calculated to investigate the contribution of CYP isoforms in the metabolism.

5.1. TNR for bensulide metabolism

Briefly, the bensulide oxonformation rates measured with individual cDNA-expressed CYP isoforms were normalized with respect to the nominal specific contents of the corresponding CYP in native HLMs.

Immunologically determined CYP isoform liver contents reported previously by many investigators (Gentest Corp, 1997; Shimada T *et al*., 1994; Imaoka *et al*., 1997; Jung *et al*., 1997) (Table 7). However the percentages of each CYP isoforms are somehow different according to the data of them. In this study, I adapted the CYP isoforms liver content data reported by Gentest Corp. Because the data from Gentest Corp is clear and integrated well.

Table 8. Nominal specific content of individual CYP isoforms in native human liver microsomes

CYP form	pmol CYP / mg (% Total)*		
	(1)	(2)	(3)
CYP1A2	45 (8.0)	42 (13)	15
CYP2A6	68 (13)	42 (13)	12
CYP2B6	39 (7.0)	1.0 (0.2)	3.0
CYP2C8	64 (12)		
CYP2C9	96 (18)		
CYP2C18	<2.5		
CYP2C19	19 (4.0)		
CYP2D6	10 (2.0)	5.0 (2.0)	15
CYP2E1	49 (9.0)	22 (7.0)	
CYP3A4	108 (20)	98 (29)	40
CYP3A5	1.0 (0.2)		
Total‡	534	344	

*Levels of each CYP (pmol/mg microsomal protein) were determined by imnunoblotting procedures. Data in parentheses represent percent of total CYP. (1) Mean data obtained from the Gentest Corp (October 1997). Using a pool of liver microsomes from different organ donor subjects (N=12); (2) mean data from sixty different livers; (3) mean data from sixteen different human livers

‡ Total CYP was measured spectroscopically as a ferrous-carbon monoxide complex.

TNR is essential for the information of the contribution rate of CYP isoforms involved in metabolism. For example in this study, although cDNA-expressed CYP 2C19 well metabolize the bensulide into bensulide oxon*in vitro*, however it doesn't greatly contribute to oxidation of bensulide formation in actual human organism. Because it has a poor expression rate in HLMs (% TNR = 2%).

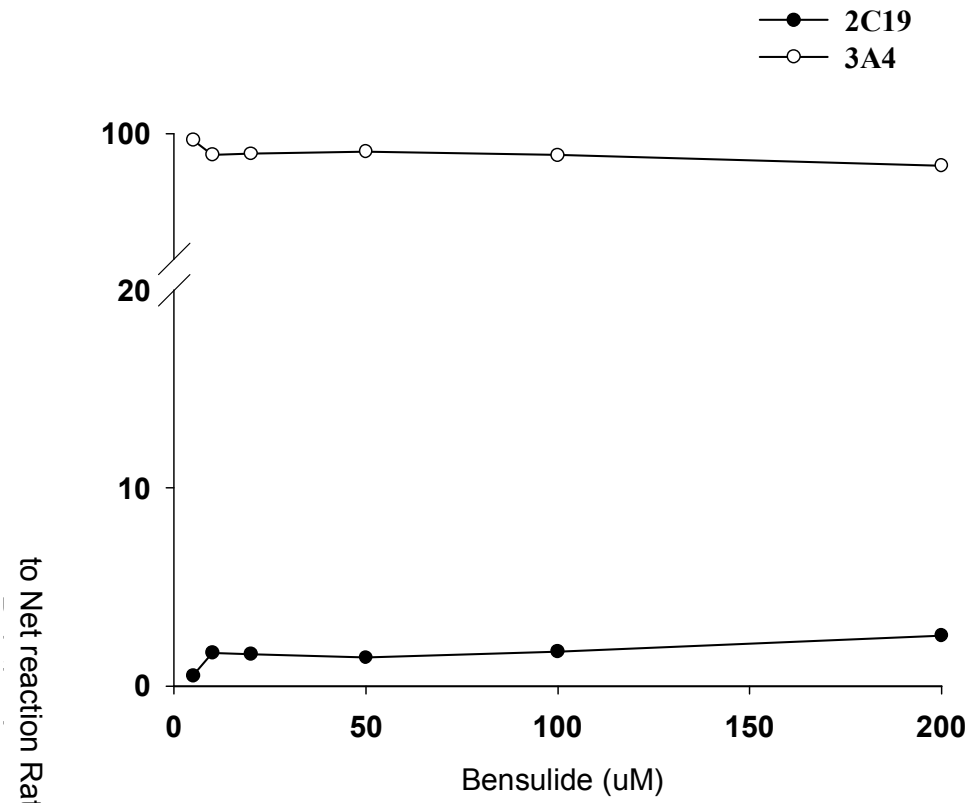


Figure 27. TNR of metabolism from bensulide

The results of TNR calculation suggest that two CYPs, CYP 2C19, 3A4, are involved in the bensulide metabolism. The contribution two CYPs were 2 %, 98% for bensulide oxon. It is noteworthy CYP 3A4 is the most drug metabolizing CYP isoforms in human (Tang *et al*., 2001; Buratti *et al*., 2003; Peng *et al*., 2003; Usmani *et al*., 2004; Buratti *et al*., 2005). CYP 3A4 which is usually main contributor did take major part in bensulide metabolism in this study.

Ⅳ. CONCLUTION

Comparative metabolism of herbicide bensulide by soil fungus *Cunn. elegans* and human liver microsomes was performed.

Cunninghamella elengans ATCC 36112 metabolized bensulide into one metabolite. The metabolite was tentatively identified by LC/MS as bensulide oxon with Turbo DDS program.

The in vitro metabolism of bensulide using human liver microsomes produced only one metabolie which is the identical compounds of bensulide by *Cunn. elegans*. Metabolite formation increased while bensulide decreased.

The metabolite was analyzed LC/MS and Turbo DDS to be assigned as bensulide oxon. The enzyme responsible for microsomal metabolism was found to be CYP 450 rather than FMO. To determine asnd characterized CYP isoforms which are involved in oxidation of bensulide. In metabolic reaction with 9 human cDNA-expressed CYP isoforms, 2(CYP 3A4, CYP 2C19) were responsible for bensulide metabolism.

The present in vitro metabolism study of bensulide with Cunninghamella elegans, human liver microsomes and human cDNA-expressed CYP isoforms will give significant information for risk assessment of bensulide for human.

REFERENCES

Archakov, A. I.; Karuzina, I. I; Petushkova, N. A; Lisitsa, A. V; Zgoda, V. G; Production of carbon monoxide by cytochrome P450 during iron-dependent lipid peroxidaition, Toxicology in Vitro, 16, pp 110 (2002).

Asha, S.; Vidyavathi, M. *Cunninghamella*- A microbial model for drug metabolism studies- A review. Biothechnol. Adv. 27, 16-29 (2009).

B. P. Leung E.M., G. R. Park,. The effect of propofol on midazolam metabolism in human liver microsome suspension. Anaesthesia 52: 945-948 (1997).

Brown C.M., Reisfeld B. and Mayeno A.N., Cytochromes P450: A Structure-Based Summary of Biotransformations Using Representative Substrates. Drug Metabolism Reviews 40: 1 – 100 (2008).

Cerniglia, C.E; Gibson, D. T; Metabolism of naphthalene by cell extracts of *Cunninghamella elegans,* Arichives of Biochemistry and Biophysics, 186(1), pp 121-127 (1978)

Coyne, M.S; Soil Microbiology: An exploratory Aproach, In Bioremidiation, ed. Bernham, J; Myers A. E; Delmar publisher: New York, pp 415-425 (1999)

Code E.L., Crespi C.L., Penman B.W., Gonzalez F.J., Chang T.K. and Waxman D.J., Human Cytochrome P4502B6. Interindividual Hepatic Expression, Substrate Specificity, and Role in Procarcinogen Activation. Drug Metab Dispos 25: 985-993 (1997).

Coles R. and Kharasch E., Stereoselective Metabolism of Bupropion by Cytochrome P4502B6 (CYP2B6) and Human Liver Microsomes. Pharmaceutical Research 25: 1405-1411 (2008).

Crofts F., Strickland P., Hayes C. and Sutter T., Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by human cytochrome P4501B1. Carcinogenesis 18: 1793-1798 (1997).

Danielson P.B., The Cytochrome P450 Superfamily: Biochemistry, Evolution and Drug Metabolism in Humans. Current Drug Metabolism. Bentham Science Publishers Ltd., pp 561-597(2002).

Davison, K. L.; Feil, V. J.; Lamoureus, C. H. Methoxychlor metabolism in

goats. Jour. Agriculture and Food chemistiry, 30, 130-137 (1982).

EI Sayed, K. A; Halim, A.F; Zaghloul, A. M; Dunbar, D. C; McChesney, J. D; Transformation of jervine by *Cunninghamella elegans* ATCC 9245, Phytochemistry, 55, pp 19-22 (2000)

Ernest Hodgson R.L.R., Metabolic interactions of agrochemicals in humans. Pest Management Science 64: 617-621 (2008).

Ferrey, M. L; Koskinen, W. C; Blanchette, R. A; Burnes, T. A; Mineralization of alachlor by lignin-degrading fungi, Canadian Journal of Microbiology, 40, pp 795-798 (1994)

Godin S.J., Scollon E.J., Hughes M.F., Potter P.M., DeVito M.J. and Ross M.K., Species Differences in the in Vitro Metabolism of Deltamethrin and Esbensulide: Differential Oxidative and Hydrolytic Metabolism by Humans and Rats. Drug Metab Dispos 34: 1764-1771 (2006).

Grifoll, M.; Hammel, K. E. Initial steops in the degradation of methoxychlor by the white rot fungus Phanerochaete chrysosporium. Appl. Environmental Microbiology, 63, 1175-1177, (1997).

Gu J., Su T., Chen Y., Zhang Q.-Y. and Ding X., Expression of Biotransformation Enzymes in Human Fetal Olfactory Mucosa: Potential Roles in Developmental Toxicity. Toxicology and Applied Pharmacology 165: 158-162 (2000).

Guengerich F.P. and Shimada T., Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chemical Research in Toxicology 4: 391-407 (1991).

Hickey, W. J; Principles and application of soil microbiology, In Biochemistry and metabolism of xenobiotic chemicals, ed. Sylvia, D. M; Fuhrmann, J. J; Hartel, P. G; Zuberer, D. A, Prentics Hall, New Jersey, pp 447-468.

Hodgson E. and Rose R.L. 2007. The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals. Pharmacology & Therapeutics 113: 420-428.

Keum Y.S, Lee YH, Kim JH, Metabolism of Methoxychlor by *Cunninghamella elegans* ATCC 36112, Jour. Agriculture and Food chemistry. 57, 7931-7937 (2009).

Kim K.-A,., Kim M.-J., Park J.-Y., Shon J.–H., Yoon Y.-R., Lee S.-S., Liu K.-H., Chun J.-H., Hyun M.-H. and Shin J.-G, Steroeoseletive Metabolism of Lansoprazole by human liver cytochrome P450 enzymes. Drug Metab Dispos 31; 1227-1234. (2003)

Korzekwa, K; Trager, W; Gouterman, M; Spangler, D; Loew, G. H; Cytochrome P450 mediated aromatic oxidation: A theoretical study, Jounal of the Amerian Chmeical Society, 107, pp 4273-4279 (1985)

Mansuy D. The great diversity of reactions catalyzed by cytochromes P450. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 121: 5-14. (1998).

Matsumura, F; Biodegradation of pesticides, In Degradation of pesticides in the environment by microorganisms and sunlight, ed. Matsumura, F; Krishna Murti, D. R; Plenum Press: New York ad London, pp 67-87 (1982).

Moody, J. D; Zhang. D; Heinze, T. R; Cerniglia, . E; Transformation of amoxapine by *Cunninghamella elegans,* Applied and Environmental Microbiology, 66(8), p 3646-3649 (2000).

Mountfield, R J; Hopper, D. J; The formation of 1-

hydroxymethylnaphthalene and 6-hydroxymethylquinoline by both oxidative and reductive routes in *Cunninghamella elegans*, Applied and Microbiology and Biotechnology, 50, pp 379-383 (1998)

Omiecinski C., Remmel R. and Hosagrahara V., Concise review of the cytochrome P450s and their roles in toxicology. Toxicol Sci 48: 151-156 (1999).

Parkinson, A. Biotransformation of xenobiotics. In C. D. Klaassen (ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons pp. 172–191 (2001).

Pelkonen O., Turpeinen M., Hakkola J., Honkakoski P., Hukkanen J. and Raunio H., Inhibition and induction of human cytochrome P450 enzymes: current status. Archives of Toxicology 82: 667-715 (2008).

Rodrigues AD, Integrated cytochrome P450 reaction phenotyping (Attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes) Biochem. Pharmacol. 57: 465-480 (1999).

Shewarzenbach, R. P, Gschwend, P. M, Imboden, D. M, Envirionmental

organic chemistry, In Biological transformation reaction, JOHN WILEY & SONS, INC; New York, pp 494-495 (1993).

Tiedje, J. M; Hagedorn, M. L; Degradation of alachlor by a soil fungus, *Chaetomium globosum*, Journal of Agricultural and Food Chemistry, 23(1), pp 77-81 (1975).

Tianwen Chen G.C. Identification and quantitation of pyrethroid pesticide residues in vegetables by solid-phase extraction and liquid chromatography/electrospray ionization ion trap mass spectrometry. Rapid Communications in Mass Spectrometry 21: 1848-1854 (2007).

U.S. Environmental Protection Agency, EPA 738-F-00-001. U.S. EPA, Washington, DC. (2000).

Venkatakrishnan K., Von Moltke L. and Greenblatt D., Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. J Clin Pharmacol 41: 1149-1179. (2000)

Wackett, L. P; Gibson, D. T; Metabolism of xenobiotic compunds by enzyme systems in fungi, Fungal Gentics and Biology, 23, pp 117-122 (1982).

Wang, R. F; Cao, W.W; Khan, A. A; Cerniglia, C. El Cloning sequencing and expression in *Escherichia coli* of cytochrome P450 gene from *Cunninghamella elegans*, FEMS Microbiology Letters, 188, pp 55-61 (2000).

Yadav, J. S; Loper, J, C; Cloning and characterization of the cytochrome P450 oxidoreductase gene from the zygomycete fugus *Cunninghamella*, Biochemical and Biophysical Research Communic, 268, pp 345-353 (2000).

국문 요약

토양 곰팡이 *Cunninghamella elegans* 와 사람 간 microsome에 의한 Bensulide의 비교 대사

황 연 진

본 연구는 토양곰팡이 *Cunninghamella elegans* ATCC 36112와 인체 간 마이크로좀을 이용하여 유기인계 제초제 bensulide의 대사 현상을 비교하였다. 포유류와 가장 비슷한 외래이물질 대사과정을 갖고 있는 Cunn, elegans를 통하여 bensulide를 대사시킨 결과, 하나의 대사체가 생성되었다. 인체 간 마이크로좀을 이용하여 bensulide를 대사시킨 결과 동일하게 하나의 대사체가 생성되었고 각각의 대사체를 LC-MS와 Turbo DDS mode를 통해 분석해본 결과 bensulide의 산화물인 bensulide oxon 임이 확인되어 대사과정 중 oxidation 현상이 발생하였음을 관찰하였다. 마이크로좀의 끓임과 열처리로 인한 불활성화의 결과 bensulide oxon의 생성에는 cytochrome P450이 관여하는 것으로 판단되었다. 효소동력학 시험을 실시하여 Vmax(counts/min/mg protein), K_m 값을 구하고, 재조합 인체 cytochrome P450을 이용하여 대사에 관여하는 CYP

450 isoform을 확인한 결과 CYP 3A4가 주로 관여하며 CYP 2C19도 관여하여 bensulide oxon이 생성됨을 나타내었다.

주요어: Bensulide, 토양곰팡이 *Cunninghamella elegans* ATCC 36112, 인체 간 마이크로좀, 시토크롬 P450, 효소동력학

학번: 2012-21195