A Thesis for the Degree of Doctor of Philosophy

In vitro Metabolism of Herbicide Flucetosulfuron by Human Liver Microsomes and Artificial Gastrointestinal Juices

제초제 Flucetosulfuron 의 인체간마이크로좀과 인공소화액에 의한 *in vitro* 대사

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農 學 博 士 學 位 論 文

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In vitro Metabolism of Herbicide Flucetosulfuron by Human Liver Microsomes and Artificial Gastrointestinal Juices

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ABSTRACT

***In vitro* Metabolism of Herbicide Flucetosulfuron by Human Liver Microsomes and Artificial Gastrointestinal Juices**

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To investigate *in vitro* metabolism of herbicide, flucetosulfuron, with human liver microsomes (HLMs) and artificial gastrointestinal juices (GIs), *threo*- and *erythro*-isomers of flucetosulfuron were prepared as 99.7% and 99.8% purity, respectively. *In vitro* metabolism of *threo*- and *erythro*- flucetosulfuron by HLMs produced M1, *threo*-metabolite (TM1; (1S,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate) from *threo*-flucetosulfuron and *erythro*-metabolite (EM1; (1R,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-lcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate) from *erythro*-flucetosulfuron, respectively. TM1 and EM1 were identified by LC-MS/MS and NMR and unambiguously confirmed by cochromatographic method by comparison with the synthesized authentic TM1 and EM1, having 99.7% and 96.5% purity, respectively. Hydrolytic metabolism of flucetosulfuron was proved to be mediated not by P450s or FMOs but by esterases. After optimization of reaction time, HLMs

concentration, and flucetosulfuron concentration, kinetics parameters of each flucetosulfuron isomers were obtained from Michaelis-Menten plot. The estimated V_{max} (nmol/min/mg HLMs) and K_m (μM) values were calculated 134.38 and 2798.53 for *threo*-flucetosulfuron and 151.41 and 3957.37 for *erythro*-flucetosulfuron, respectively. The intrinsic clearance ($CL_{int} = V_{max} / K_m$ (μL/min/mg HLMs)) values of the formation of TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron were 51.20 and 48.02, respectively. The CL_{int} values from flucetosulfuron isomers by HLMs did not show any significant difference. Inhibition test with selective esterases inhibitors indicated that the metabolizing esterases in hydrolysis of flucetosulfuron are carboxylesterases and cholinesterases. Esterases kinetics with human recombinant carboxylesterases (CES1b, CES1c and CES2), human acetylcholinesterase (AChE), and human butyrylcholinesterase (BChE) demonstrated that the metabolism of flucetosulfuron isomers is mediated by these esterases with the same results from the specific esterase inhibition. The CL_{int} values of CES2 for the formation of TM1 and EM1 showing the highest activity among the tested esterases were 16.98 and 17.43. The CL_{int} values of CES1b and CES1C were 3.99 and 4.5 for *threo*-flucetosulfuron and 2.59 and 3.07 for *erythro*-flucetosulfuron. CL_{int} value of AChE for *threo*-flucetosulfuron was 2.02, also showing not much of a difference to 2.32 for *erythro*-flucetosulfuron. However, CL_{int} value of BChE for *threo*-flucetosulfuron was 4.43, showing 5-fold higher than 0.87 for *erythro*-

flucetosulfuron, with the 3.8-fold higher V_{max} values of BChE for *threo*-flucetosulfuron than *erythro*-flucetosulfuron. The differences of the kinetic parameters between flucetosulfuron isomers indicated that there is a possibility of stereoselcetive metabolism between *threo*- and *erythro*-flucetosulfuron by esterase such as BChE. The investigation on *in vitro* metabolism of flucetosulfuron by artificial GIs (saliva, gastric juice, intestinal juice) showed that there are not significant differences in the degradation patterns between flucetosulfuron isomers in GIs. Flucetosulfuron was observed to be stable in saliva, while about 85% of flucetosulfuron in the reaction with gastric juice was rapidly degraded and produced metabolites, 2-(2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide (M2), 4,6-dimethoxypyrimidin-2-amine (M3), and 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl 2-methoxyacetate (M4), indicating the occurrence of the hydrolysis of sulfonylurea-bridge and ester bond of flucetosulfuron. In the reaction with intestinal juice, about 18% of flucetosulfuron was degraded and M1 was observed as only metabolite of flucetosulfuron, suggesting the breakdown of ester-bond of flucetosulfuron. During these studies, there was not any chiral conversion between flucetosulfuron isomers or metabolite isomers. On the basis of these results, the metabolic pathway of flucetosulfuron in HLMs and artificial gastric juices is proposed.

KEY WORDS: flucetosulfuron, human liver microsomes, esterase, gastrointestinal juices, metabolism

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LIST OF ABBREVIATIONS

AChE	Human acetylcholinesterase
AHAS	Acetohydroxyacid synthase
ALS	Acetolactate synthase
APP	Aryloxyphenoxypropionate
BChE	Human butyrylcholinesterase
BNPP	Bisnitrophenyl phosphate
CES	Human carboxylesterase
CHI	Closed head injury
CL_{int}	Intrinsic clearance value
CVA	Cerebro-vascular accident
EDTA	Ethylenediaminetriacetic acid
EM	*Erythro*-metabolite
ESI	Electrospray ionization
FAD	Flavin adenine dinucleotide
FD	Fluorescein diacetate
FMN	Flavin mononucleotide
FMO	Flavin-containing monooxygenase
GI	Gastrointestinal juice
hCE	Human carboxylesterase
HLM	human liver microsome
HPLC	High performance liquid chromatography
HRAC	Herbicide Resistance Action Committee
hrs	Hours
ICH	Intracerebal hemorrhage
K_m	Michaelis constant

LC-MS/MS	Liquid chromatography-tandem Mass Spectrometry
Lrp	Leucine-responsive protein
m/z	Mass/charge ration
M^+	Molecular ion
M1	N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)-2-(-2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide
M2	2-(2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide
M3	4,6-dimethoxypyrimidin-2-amine
M4	2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl 2-methoxyacetate
min	Minute
MS	Mass spectrometry
MVA	Motor vehicle accident
NAD(P)	Nicotinamide adenine dinucleotide(phosphate)
NAD(P)H	Nicotinamide adenine dinucleotide(phosphate), reduced form
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear overhauser effect
4-NPA	4-nitrophenyl acetate
OR	Oxido-reductase
PAPS	3`-Phosphoadenosine-5`-phosphosulfate
Prep-HPLC	Preparative high performance liquid chromatography
TM	*Threo*-metabolite
UDP	Uridine diphosphate
UGT	Uridine 5'-diphospho-glucuronosyltransferase
Uk	Unknown
V_{max}	Maximum velocity

LITERATURE REVIEW

1. Metabolism

Pesticides are a class of xenobiotics meant for preventing, destroying, or mitigating any pest, which can include all diverse chemicals such as agrochemicals, pollutants, drugs and pyrolysis products in food, alkaloids, secondary plant metabolites and toxins produced by molds, plants, and animals. The lipophilic properties of these pesticides enable them to be absorbed into human body and at the same time make them difficult to be excreted them out of the body. To be eliminated from the body, pesticides should go through conversion from lipophilic to hydrophilic chemicals by a process known as biotransformation including catalysis of enzymes in liver and other tissues. Xenobiotics have diverse effects on the biological systems, interestingly as being beneficial for drugs and deleterious for poison. Usually biotransformation terminates the beneficial effects of drugs and weakens the toxicity of xenobiotics. The intensity and duration of action of efficacy of drugs are determined by the enzymes catalyzing biotransformation. The enzymes for biotransformation play an important role in determining the intrinsic toxicity, detoxification and tumorigenesis of xenobiotics. The biotransformation of xenobiotics is the principal mechanism for keeping on the homeostasis of living organisms during in response to exposure to foreign molecules. A limited number of enzymes have been known to participate in the xenobiotic biotransformation with substrate specificities. Generally the enzymes are expressed constitutively,

but the xenobiotics also can trigger the expression of these enzymes (Parkinson 2001).

The enzymes for the xenobiotic transformation are widely distributed throughout the body. In vertebrates, these enzymes are located in liver, lung, gastrointestinal tract, kidney, pancreas, plasma and erythrocytes (Minder *et al.* 1971, Anders 1985, Krishna and Klotz 1994).

Liver is the major organ in the xenobiotics metabolism, with intestines playing secondary yet potentially important role in the systemic elimination of xenobiotics in the body. Studies on the biochemical properties and molecular genetics of metabolic enzymes for xenobiotics have been used for understanding the clinical aspects of xenobiotic metabolism such as kinetic variability, toxicity and interactions of xenobiotics (Venkatakrishnan *et al.* 2001).

The enzymes for xenobiotic biotransformation reaction are located primarily in microsomes or the soluble fraction of the cytosol within the liver and relatively little are in mitochondria, microflora, blood and lysosomes (Table 1). Once xenobiotics enter into body, they can be biotransformed in the gastrointestinal tract, firstly. After absorption from gastrointestinal tract, biotransformed xenobiotics are transported to the liver in which the *first-pass elimination* takes place, and the availability of xenobiotics is limited. Microsomes contains most enzymes such as cytochrome P450s (CYP450s), esterases, reductases and flavin-containing monooxygenases (FMOs) (Parkinson 2001).

Until the excretion of pesticide completed, it is metabolized by the biotransformation pathways classified into two groups:

Table 1. General pathways of xenobiotic metabolism and their location*

Reaction		Enzymes	Localization
Phase I	Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, and blood
		Peptidase	Blood, lysosomes
		Epoxide hydrolase	Microsomes, cytosol
	Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
		Carbonyl reduction	Cytosol, blood, microsomes
		Disulfide reduction	Cytosol
		Sulfoxide reduction	Cytosol
		Quinione reduction	Cytosol, microsomes
		Reductive dehalogenation	Microsomes
	Oxidation	Alcohol dehydrogenase	Cytosol
		Aldehyde dehydrogenase	Mitocondria, cytosol
		Aldehyde oxidase	Cytosol
		Xanthine oxidase	Cytosol
		Monoamine oxidase	Mitochondria
		Diamine oxidase	Cytosol
		Prostaglandin H synthase	Microsomes
		Flavin-monooxygenase	Microsomes
		Cytochrome P450	Microsomes
Phase II	Conjugation	Glucuronide conjugation	Microsomes
		Sulfate conjugation	Cytosol
		Glutathione conjugation	Cytosol, microsomes
		Amino acid conjugation	Mitocondria, microsomes
		Acylation	Mitochondria, cytosol
		Methylation	Cytosol, microsomes, blood

* Parkinson, 2001

- Phase I reactions involving the biotransformation of xenobiotics to yield more polar and more hydrophilic forms than the parent xenobiotics by hydrolysis, reduction or oxidation

- Phase II including acetylation, methylation and the conjugations with endogenous hydrophilic moieties such as glucuronic acid, sulfate, glutathione and amino acid to yield the metabolites that are readily excreted out of the body

In most cases Phase I reactions increase the hydrophilicity of xenobiotics with presenting a functional groups such as alcohols, amines, thiols and carboxylic acids to xenobiotics through hydrolysis, reduction and oxidation.

1.1 Phase I reactions

1.1.1 Reduction in Phase I reactions

Reduction mechanisms include the transformation of xenobiotics containing an aldehyde, ketone, disulfide, sulfoxide, quinone, N-oxide, alkene, azo, or nitro groups, though it is difficult to decide whether the reactions are catalyzed enzymatically or nonenzymatically by interaction with reducing agents such as the reduced forms of glutathione, FAD, FMN and NAD(P). The functional groups such as aldehydes can be either reduced to an alcohol or oxidized to carboxylic acid, whereas sulfoxides can be reduced to a sulfide or oxidized to a sulfone. Many of the reduction reactions can be catalyzed by cytochrome P450, NADPH-quinone oxidoreductase or aldehyde oxidase (Parkinson 2001). Prontosil undergoes azo-reduction and chloramphenicol goes through nitro-reduction. Prontosil, used as a clinical trial for the specific antibacterial chemotherapy, was finally discovered that the active drug was not prontosil but sulfanilamide, an metabolite

transformed by azo-reduction (Hewick 1982).

As an example of carbonyl reduction, xenobiotics including haloperidol, pentoxifylline, acetohexamide, daunorubicin, ethacrynic acid, warfarin, menadione and 4-nitroacetophenone are catalyzed by alcohol dehydrogenase and a family of carbonyl reductases to produce primary or secondary alcohols. Carbonyl reductases are monomeric and NADPH-dependent enzymes present in blood and the cytosolic fraction of the liver, kidney, brain, and other tissue (Inaba and Kovacs 1989, Estonius *et al.* 1996, Choi *et al.* 2002).

The reductions of disulfide and sulfoxide were shown in the reduction of disulfiram. Disulfiram is reduced and cleaved to their sulfhydryl components by glutathione reductase, whereas thioredoxin-dependent enzymes in liver and kidney cytosol have been reported to deduce sulfoxides, which themselves may be formed by cytochrome P450 or flavin monooxygenases. It is interesting that there are recycling route that these counteracting enzymes drive xenobiotics reversible reaction to prolong the half-life of certain xenobiotics in body (Anders *et al.* 1980, Anders *et al.* 1981).

1.1.2 Oxidation in Phase I reactions

A number of enzymes such as dehydrogenase for alcohol or aldehyde, carbonyl reductase, oxidases for aldehyde or amines, peroxidase, flavin-containing monooxygenases (FMOs) and cytochrome P450s (CYP450s) have an important role in oxidation of xenobiotics in Phase I reactions of xenobiotic transformations. Alcohols are oxidized to aldehydes by alcohol

dehydrogenase, and then further converted to carboxylic acids by aldehyde dehydrogenase with the cofactor NAD. Alcohols also can be oxidized to aldehydes by non-alcohol dehydrogenase enzymes in microsomes (CYP2E1) and peroxisomes (catalase) (Lieber 1987, Werner Goedde and Agarwal 1989).

1.1.2.1 FMO

FMOs, distributed mainly in liver, kidney and lungs, are oxidizing diverse xenobiotics by N-oxidation of amines, acrylamines or

hydrazines, sulfoxidation of sulfur-containing chemicals (thiols, thioether and thiocarbamates) and P-oxidation of phosphines and others (Ziegler 1988, Ziegler 1990, Hines *et al.* 1994, Lawton *et al.* 1994, Cashman 1995, Overby *et al.* 1997, Hodgson *et al.* 1998, Krueger and Williams 2005).

FMOs play an important role in the biotransformation, not only in humans but also in mammals. In a species-specific and tissue-specific manner of the gene family, the mammalian FMO comprises of five enzymes, designated as FMO1 to FMO5 (Parkinson 2001). The major FMO expressed in human and mouse liver microsomes is FMO3, whereas FMO1 is the major FMO expressed in rabbit and pig liver microsomes and human kidney. The various forms of FMOs are distinct gene products with different physiological properties and substrate specificities. FMOs contain two highly conserved glycine-rich regions binding noncovalently FAD near active site and binding NADPH, respectively. FMOs share similarity with CYP450 in that both are microsomal enzymes that require NADPH and O2, and many substrates can be catalyzed by both FMOs and CYP450s. However, FMOs are heat-labile

and can be inactivated in the absence of NADPH by warming microsomes to 50℃ for 1min, whereas, CYP450s can be inactivated with nonionic detergent such as 1% of Emulgen 911 having a minimal effect on FMO activity. The chemical inhibitors such as methimazole are used to characterize the relative contribution of FMOs to microsomal reactions. Cimetidine and sulindac sulfide are examples of sulfur-containing chemicals that converted to sulfoxides by FMOs. Only FMO can reduce or oxidize between sulindac and sulindac sulfide though it is a futile cycle.

1.1.2.2 Cytochrome P450s (CYP450 enzymes)

Cytochrome P450s (CYP450 enzymes) are the most important enzymes among the phase I reactions due to their catalytic diversity, the number of known CYP450 enzymes (exceeding 1000), and the number of xenobiotics which are reported to be detoxified or activated to intermediates by this family of enzymes (Shimada *et al.* 1996, Anzenbacher and Anzenbacherova 2001, Venkatakrishnan *et al.* 2001, Ding and Kaminsky 2003, Guengerich 2005). Since the first report on cytochrome P450 was published in 1958 (Klingenberg 1958, Omura and Sato 1962), intensive studies of CYP450 enzymes have been performed for 40 years (Omura 1999).

CYP450 enzymes are present not only in microsomes but also in mitochondria, and widely distributed as various forms in animals, plants and microorganisms. Studies on the function of CYP450 enzymes in metabolism of drugs and the role of CYP450 enzymes in the metabolic activation of various carcinogenic chemicals were done till 1970s.

CYP450 enzymes are present in all tissues and especially at the highest concentration in liver. CYP450 enzymes in liver microsomes play a very important role in determining intensity, and duration of drug effects, and detoxification of xenobiotics. CYP450 enzymes also play a key role in the oxidation of a variety of drugs, carcinogens, steroids, pesticides and other chemicals in all kind of living organisms (Guengerich and Shimada 1991, Shimada *et al.* 1996). CYP450 enzymes act in the biosynthesis or catabolism of steroid hormones, bile acids, fat-soluble vitamins, fatty acids and eicosanoids. Figure 1 demonstrates the catalytic cycle of CYP450 enzymes is associated with monooxygenase reaction.

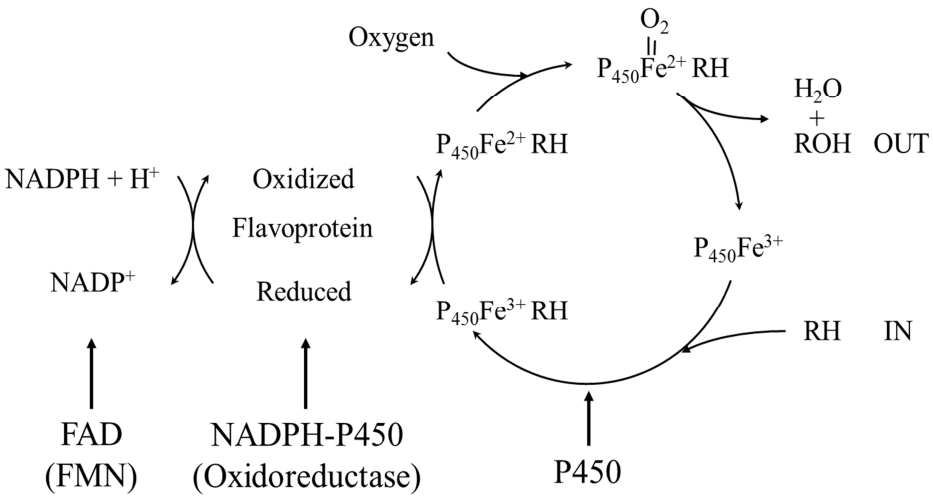
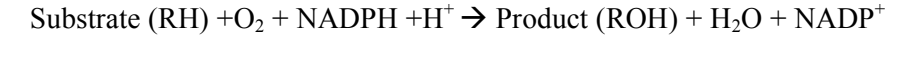


Figure 1. Catalytic cycle of CYP450

The basic mechanism of CYP450 enzymes is monooxygenation process as follows:



$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product (ROH)} + H_2O + NADP^+$$

In the monooxygenation process, one atom of oxygen molecule is transferred to a substrate (RH), and the other binds hydrogen to form water with reducing equivalents derived from NADPH. Although CYP450 enzymes act as a monooxygenase, the products are not limited to alcohols and phenols due to rearrangement reactions (Guengerich and Shimada 1991).

During the catalysis of CYP450 enzymes, electrons relayed from NADPH to CYP450 via two proteins such as ferredoxin and ferredoxin reductase (Namely FMN-containing flavoprotein). CYP450 enzyme and ferredoxin reductase are embedded in the phospholipid bilayer of the microsome, which facilitates their interaction. The oxidative reactions catalyzed by cytochrome P450 are hydroxylation of aliphatics or aromatics, dealkylation from N, S and O, deamination, desulfuration, epoxidation, peroxidation and dehalogenation. An overview of CYPs which may play a key role in metabolisms of xenobiotics is presented in Table 2 (Anzenbacher and Anzenbacherova 2001). Human drug-metabolizing CYPs are present in most of organs in the body. Human liver microsomes (HLMs) have been the most widely used *in vitro* metabolic models, and the major human drug-metabolizing CYPs belong to families 1, 2, and 3. CYP 3A4, CYP 2D6, CYP 2C9, CYP 2C19, CYP 1A2 among the first three families have been found to

metabolize most of the commercialized drugs (Parkinson 2001, Peng *et al.* 2003, Testino and Patonay 2003).

In the past decade, it was not easy to isolate and reconstitute the native enzymes from human tissue for drug metabolism studies to build a well-defined *in vitro* system.

As several human P450 genes have been identified and information of their expression and catalytic mechanism are available (Guengerich 1994), the methods for high level expression of mammalian P450s with baculovirus and recombinant human P450 isoforms from *E. coli* enabled to produce in the different enzymes, such as soluble-purified enzymes, purified enzymes in a reconstituted format, and recombinant microsomes. The human P450s are modified slightly at N-terminal to be produced form *E.coli*, whereas they offer the advantages of using highly purified enzymes without limitation. There are not any differences between native and recombinant enzymes in the *in vitro* metabolism of xenobiotics in that the turnover numbers for recombinant human P450 in both formats are similar to that seen using HLMs.

Table 2. Human P450 enzymes metabolizing xenobiotics*

CYP	Location	Substrate	Typical inducer
1A1	Lung, liver, brain, GIT, lymphocytes, heart	PAH (Polycyc. Arom. Hydrocarbons), pyrethroids	PAH, dioxins
1A2	Liver	Aromatic amines, PAH, caffeine, deltamethrin	PAH, smoking, β-Naphthoflavone,
1B1	Skin, brain, heart, lung, placenta, liver, kidney, spleen	PAH	Dioxin
2A6	Liver	Coumarin, steroids	Barbiturates, dexamethasone
2B1/2	Brain	Morphine	Nicotine
2B6	Liver, heart	Nicotine, α-endosulfan	Barbiturates
2C8	Liver, kidney	Retinoids, taxol	-
2C9/10	Liver	Tolbutamide, diclofenac	Barbiturates, rifampicin
2C19	Liver, heart	(S)-mephenytoin, omeprazole, diazepam, pyrethroids	Barbiturates, rifampicin
2D6	Liver, brain, heart	Antidepressives, β-blockers	-
2E1	Liver, lung, brain, endothelium, heart, bone marrow	Ethanol, nitrosamines, acetaminophen	Ethanol, starvation
2F	Lung	Coumarins	-
3A4/5	Liver, GIT, kidney, lung, brain, endothelium, placenta, lymphocytes	Different–Ca channel blockers, cyclosporine, acetaminophen, taxol, steroids, β-endosulfan	Steroids, barbiturates
3A7	Fetus, placenta, (liver)	Various, similar to 3A4	Steroids, barbiturates
4A9/11	Kidney	Fatty acids	Clofibrate
4B1	Lung, plasma	-	-
4F2/3	Kidney	Arachidonic acid derivatives	-

* Cytochrome P450 database and Guengerich, 1994.

1.1.3 Hydrolysis in Phase I reactions

Mammals have diverse hydrolytic enzymes that hydrolyze endogenous compounds containing functional group, such as ester, amide, thioester, phosphoric ester, diacylglycerol, palmitoyl-CoA, esterified lipids and acid anhydride to the catalyzed metabolites (Satoh and Hosokawa 1998).

In 1953, Aldridge classified esterases in three groups (A-esterases, B-esterases and C-esterases) based on the nature of their interaction with organophosphates. Esterases such as organophosphatase hydrolyzing organophosphate were classified as A-esterase having cysteine group as reaction moiety. Esterases such as carboxylesterases and cholinesterases, inhibited by organophosphates are classified as B-esterases having serine group as reaction moiety, whereas other enzymes having no interaction with organophosphate are classified as C-esterases (Aldridge 1953, Augustinsson *et al.* 1973).

Although A-esterases are not inhibited by organophosphates, A-esterases are inhibited by mercurial compounds that react with free thiol groups. Esterases have an important effect on the toxicity of organophosphate pesticides. The toxicity mechanism of organophosphates pesticides and carbamate insecticides having a similar structure like acetylcholine results from inhibition of acetylcholinesterase, a serine-containing esterase by blocking and terminating the action of the neurotransmitter, acetylcholine. Acetylcholines are hydrolyzed by some esterases such as A-esterase and bind stoichiometrically, and then most of them bind irreversibly to carboxylesterases and cholinesterases. Many studies have shown the

relationship between esterase hydrolytic activity and susceptibility to the toxicity of organophosphates. Namely, the decrease of the esterase activity elevates the potential of the toxicity of organophosphates whereas the increase of the carboxylesterase results in a protective effect against organophosphates (Parkinson 2001, Worek *et al.* 2004). In case of the K_m values for methyl paraoxon and other substrates, esterases were reported to have an important role in the detoxification of undesirable compounds formed in the body. And the detoxifying mechanism was supported by the study on the sensitivity of esterase isozyme to cations as well. Shishido and Fukami elucidated that metal ions such as Ce^{3+}, Cu^{2+}, Cd^{2+} and Hg^{2+} almost completely inhibited the diazoxon hydrolase, a kind of A-esterase, present in rat and Ba^{2+}, Ni^{2+}, Ag^{2+}, Co^{2+}, $Pb2^{2+}$, Mn^{2+} and Zn^{2+} also inhibited from 20 to 70% at concentration of 1×10^{-4} M (Shishido and Fukami 1972). Among these metal ions Hg^{2+}, Ag^{2+} and Cd^{2+} ions showed strong inhibition of the methyl esterase isozyme of the *H. armigera*. Furthermore, they could know that the insect enzyme had a lower sensitivity to metal ions than that the mammalian enzyme from the fact that Cd^{2+} and Cu^{2+} inhibited the enzyme slightly and other ions had no effect on the mammalian enzyme (Srinivas *et al.* 2006).

Pyrethroids, widely used today in agriculture, possess acid and alcohol moieties in their structure. The presence of chiral centers in the cyclopropane ring of the acid moiety and the α-carbon of the alcohol moiety results in diverse enantiomers. Each enantiomer may show differences in insecticidal potency, mammalian toxicity, and metabolic pathway. These pathways

include NADPH-dependent oxidation and non-NADPH-dependent hydrolysis catalyzed by cytochromes P450 (P450s) and esterases, respectively (Crow *et al.* 2007, Godin *et al.* 2007, Scollon *et al.* 2009, Lavado *et al.* 2014).

The possibility exists that enantiomers or diastereomers of a pyrethroid may show the dissimilar interactions in metabolism or dynamics. The metabolism of pyrethroids is believed to be a detoxification process. The studies on the metabolism of pyrethroids by esterases were thoroughly examined in rat and human hepatic microsomes. Evidence of streoselective metabolism of some pyrethroids has been reported in rats and mice with rates of conversion of trans-permethrin to hydrolytic metabolites being greater than cis-permethrin (Gaughan *et al.* 1977, Soderlund and Casida 1977).

The pyrethroids bifenthrin, S-bioallethrin, bioresmethrin, β-cyfluthrin, cypermethrin, cis-permethrin, and trans-permethrin were studied in rat and human hepatic microsomes (Scollon *et al.* 2009). The intrinsic clearance value (CL_{int}) of the pyrethroids for rat microsomes was 5- to 15-fold greater in rat relative to human microsomes except for trans-permethrin. The metabolisms of bifenthrin, S-bioallethrin, and cis-permethrin in rat and human hepatic microsomes were solely the result of oxidative processes, whereas the metabolisms of bioresmethrin and cypermethrin in human hepatic microsomes were solely the result of hydrolytic processes. Bioresmethrin and cypermethrin in rat hepatic microsomes and β-cyfluthrin and trans-permethrin in microsomes from both species were metabolized by

both oxidative and hydrolytic pathways. The metabolism of trans-permethrin was suppressed when incubated with its diastereomer, cis-permethrin, in both rat and human hepatic microsomes. Species-specific differences and isomer interactions in metabolism can result in variable detoxification of pyrethroids (Scollon *et al.* 2009).

Hepatic and extrahepatic carboxylesterases metabolize pyrethroids (Soderlund and Casida 1977, Soderlund *et al.* 2002, Crow *et al.* 2007, Scollon *et al.* 2009). It is known that the *in vitro* hydrolysis rate of pyrethroids is greater for rat hydrolase A than hCES1, two abundant hepatic carboxylesterases, without the exceptional case for trans-permethrin and deltamethrin (Nishi *et al.* 2006, Godin *et al.* 2007, Yan and Lyubimov 2011).

There is a species-specific difference in the two major enzymatic pathways of *in vitro* pyrethroid metabolism. Metabolism of bioresmethrin and cypermethrin in human hepatic microsomes did not require NADPH, suggesting that it was solely by hydrolysis, whereas metabolism of these two pyrethroids in rat hepatic microsomes was a combination of oxidation and hydrolysis. Similar results have been reported with deltamethrin in human and rat microsomes (Heidari *et al.* 2005, Ross *et al.* 2006, Godin *et al.* 2007). These literatures show a general relationship between pyrethroid structure and its pathway, and rate of metabolism as Phase I reaction.

1.2 Phase II reactions

Glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis) and conjugation with amino acids

such as glycine, taurine and glutamic acid are the reactions included in Phase II reactions (Caldwell 1986, Burchell and Coughtrie 1997, Weinshilboum *et al.* 1997). The cofactors for the phase II reactions react with functional groups that are originally present in the xenobiotics or introduced by the Phase I reactions. Phase II biotransformation reactions with the exception of methylation and acetylation mostly result in an increase of the hydrophilicity of xenobiotics to promote the excretion. Phase II reactions proceed much faster than Phase I reactions, so the rate of elimination of xenobiotics is generally determined by the phase I reactions because the excretion depends on biotransformation by Phase I enzymes followed by phase II conjugation (Parkinson 2001).

Glucuronidation is the most important pathway of xenobiotic biotransformation in the most mammalian species (Miners and Mackenzie 1991, Tukey and Strassburg 2000). Glucuronidation requires the cofactor, uridine diphosphate-glucuronic acid (UDP-Glucruronic acid), and the reaction is catalyzed by UDP-glucuronosyltransferases, which are located in the endoplasmic reticulum of liver and other tissue. Glucuronide conjugates of xenobiotics are polar, water-soluble and eliminated from the body through urine or bile, depending on the size of the parent xenobiotics or phase I metabolites (Byrd *et al.* 1992, Liu *et al.* 2005, Zhou *et al.* 2007). Though glucuronidation commonly detoxifies xenobiotics as beneficial process in mammal, in a case of involving glucuronidated steroid hormones, the reaction causes cholestasis, implicating as an epigenetic mechanism of thyroid tumor formation (Barter and Klaassen 1992).

Sulfate conjugation, a reaction of the sulfonate transfer from PAPS to xenobiotics, usually produces a highly water-soluble sulfuric acid ester. The reaction is catalyzed by sulfotransferases, found in the liver and other tissues. The cofactor for the reaction is 3`-phosphoadenosine-5`-phosphosulfate (PAPS). In addition to alcohols and phenols produced after Phase I reactions, aromatic amine, hydroxylamine and hydroxylamide can be sulfated to the corresponding sulfate conjugation. Nagata and Yamazoe summarized the nomenclature, which is similar to the system developed for P450 enzymes and esterases. These enzymes were classified into five families based on amino acid sequences, whereas the names of the individual forms were not determined yet. A study of cyprodinil in rat by Muller et al. showed that sulfotransferases in a sex-specific manner catalyzed the sulfation of hydroxyl metabolite which was processed by phase I reaction and the sulfate conjugates were rapidly eliminated through urine (Müller *et al.* 1999).

Tripeptide glutathione is composed of glycine, cysteine, and glutamic acid. Glutathione conjugation by glutathione s-transferases is basically different from the conjugation with other amino acids and peptides in that their substrates include an enormous array of electrophilic compounds, or xenobiotics that can be transformed to electrophiles (Parkinson 2001). Glutathione also can conjugate with electrophilic heteroatoms such as O, N, and S. Glutathione conjugates such as organophosphorous pesticides formed in the liver also can be excreted rapidly in bile, or they can be converted to mercapturic acids in the kidney and excreted through urine (Menn 1978, Lucas *et al.* 1993, Fujioka and Casida 2007).

Because metabolisms of xenobiotics determine the adverse effective concentration of the pesticide in target organ or human body, which is measures for the human safety of the pesticide, numerous researches are still going on.

1.3 *In vitro* metabolism of pesticides by HLMs

When living organisms are exposed to xenobiotics, especially pesticides, against which they don't have metabolic mechanism, they could not survive through their intrinsically given life time and the quality of their life also may go bad and eventually will not be recovered to the live status. *In vitro* human metabolism studies of pesticides are very important and useful for prediction and management of the hazard and risk arising from introduction of pesticides in that understanding metabolism of pesticides. The main reasons for the necessity of the human metabolism study were summarized in Table 3. Fortunately, all living organisms have the following detoxifying process for the metabolism of xenobiotics to protect themselves. Oxidation, reduction, hydrolysis, and conjugation are the reactions in the degradation or detoxification pathways for pesticide. The chemical structure of pesticides, environmental conditions, species of the living organisms, metabolic factors, and the genetic regulation of these biochemical pathways are the factors influencing metabolic pathway. Studies on these enzymatic processes related with mechanism of action, resistance, selectivity, tolerance, and environmental fate is important for understanding of pesticide science, and

of plant and microbial biochemistry and physiology (Van Eerd, Hoagland et al. 2003).

Table 3. Human metabolism studies relevant to human health risk analysis*

1. Defining exposure**
2. Showing associations between exposure and toxic endpoints**
3. Defining human variation**
4. Determining the most appropriate animal model
5. Providing insight into uncertainty factors
6. Demonstrating the potential for human-specific interactions**
7. Defining human populations or individuals at increased or decreased risk**

* Hodgson and Rose 2008.
** Cannot be determined using surrogate animals.

Liver is the major organ of xenobiotic metabolism including pesticides, with intestines playing secondary yet potentially important role in the systemic elimination of pesticide in the body. Studies of the biochemical and enzymatic properties of these enzymes and their molecular genetics and regulation of gene expression and activity was used for understanding of several aspects of clinical view such as kinetic variability, toxicity and interactions of pesticides (Venkatakrishnan *et al.* 2001).

Especially the enzymes for biotransformation reactions are located primarily in microsomes or the soluble fraction of the cytosol within the liver and relatively little are in mitochondria, microflora, blood and lysosomes.

Once pesticides having hydrophobic moieties are entered into body, they can be biotransformed in the gastrointestinal tract, firstly. After being absorbed from gastrointestinal tract, the pesticides are transformed by the *first-pass elimination* in the liver, through which the availability of the pesticides is reduced. Microsomes contains most enzymes such as cytochrome P450s (CYP450s), esterases, reductases and flavin-monooxygenases (FMOs) as the enzymes for Phase I reactions.

The *in vitro* studies with microsomal reactions provide specific details of the chemical identity of metabolites and intermediates, the pattern of their formation, and the metabolic pathway (Kim *et al.* 2003d, Liu and Kim 2003, Lee *et al.* 2006). A number of papers on the activity of CYP450s, MFOs and esterases in HLMs involved in the metabolism of some pesticides have been published recently. It has been demonstrated that HLMs contain major metabolizing enzymes responsible for pesticide metabolism (Shono *et al.* 1979, Tang *et al.* 2002, Tang *et al.* 2004, Usmani *et al.* 2004, Buratti *et al.* 2005, Lee *et al.* 2006).

Over the last decades hundreds of literatures on the subject of the *in vitro* metabolism of pesticides and other chemicals used in agriculture and public health have been published. Most of them have been utilizing experimental animals, usually rodents, whereas small parts of studies have been dealing with *in vitro* human metabolism. Recently, researches on *in vitro* metabolism of pesticide have been carried out with the increased emphasis on the role of the studies for deepening the scientific basis of human health risk assessment and facilitating the development of new, effective and safer pesticides. The

development of methods for the expression of recombinant HLMs and their isomers has made possible the investigation of pesticide by HLMs.

In vitro metabolism study of pesticide with HLMs has been required because most knowledge of pesticide toxicity in human has been indirectly deduced from the metabolism study of other animals with the limitation of the difference factors such as intra or extra species of animal, body size, sex and ages. Thus, to overcome the limitation of animal study and get more correct information on human toxicity from pesticides, a number of *in vitro* metabolism studies of pesticides in HLMs have been conducted and published in recent years (Table 4).

Table 4. Metabolism studies of pesticide in HLMs

Pesticide	Chemical class	Usage	Reference
2,4,-D	Phenoxy	Herbicide	Ohkawa *et al.* 1998
Acetochlor	Chloroacetanilide	Herbicide	Coleman *et al.* 2000
〃	〃	〃	Kale *et al.* 2008
Alachlor	Chloroacetanilide	Herbicide	Coleman *et al.* 1999
〃	〃	〃	Kale *et al*. 2008
Ametryne	Triazine	Herbicide	Cresteil *et al.* 1979
Atrizine	Triazine	Herbicide	Cresteil *et al*. 1979
〃	〃	〃	Joo *et al.* 2010
Azinphos-methyl	Organophosphate	Insecticide	Buratti *et al*. 2003
Benfuracarb	Carbamate	〃	Abass *et al.* 2014a
〃	〃	〃	Abass *et al.* 2014b
Bifenthrin	Pyrethroid	〃	Scollon *et al.* 2009
Bioresmethrin	Pyrethroid	Insecticide	Scollon et al., 2009
Butachlor	Chloroacetanilide	Herbicide	Coleman *et al*. 2000
Carbaryl	Carbamate	Insecticide	Tang *et al.* 2002
Carbofuran	Carbamate	Insecticide	Usmani *et al.* 2004
Carbosulfan	〃	〃	Abass *et al.* 2009
Chlorfenvinphos	Organophosphate	Insecticide	Hutson and Logan 1986

Chlorpyrifos	Organophosphate	Insecticide	Tang *et al.* 2001
〃	〃	〃	Buratti *et al.* 2003
〃	〃	〃	Sams *et al.* 2004
〃	〃	〃	Mutch and Williams 2006
Chlorpyrifos	Organophosphate	Insecticide	Choi *et al.* 2006
〃	〃	〃	Foxenberg *et al.* 2007
〃	〃	〃	Croom *et al.* 2010
〃	〃	〃	Smith *et al.* 2011
β-cyfluthrin	Pyrethroid	Insecticide	Scollon et al., 2009
λ-cyhalothrin	Pyrethroid	Insecticide	Scollon et al., 2009
cypermethrin	Pyrethroid	Insecticide	Scollon et al., 2009
Deltamethrin	Pyrethroid	Insecticide	Godin *et al.* 2006
〃	〃	〃	Godin *et al.* 2007
Diazinon	Organophosphate	Insecticide	Kappers *et al.* 2001
〃	〃	〃	Buratti *et al*. 2003
〃	〃	〃	Sams *et al*. 2004
〃	〃	〃	Mutch *et al*. 2006
Dimethoate	organophosphorothionate	Insecticide	Buratti and Testai 2007
Disulfoton	Organophosphate	Insecticide	Usmani *et al*. 2004
Diuron	Phenylurea	Herbicide	Abass *et al.* 2007
Endosulfan	Chlorinated cyclodien	Insecticide	Lee *et al*. 2006
Esfenvalerate	Pyrethroid	Insecticide	Godin *et al*. 2006
〃	〃	〃	Godin *et al*. 2007
Fenthion	Organophosphate	Insecticide	Furnes and Schlenk 2005
〃	〃	〃	Leoni *et al.* 2008
Fipronil	Phenylpyrazole	Insecticide	Tang *et al.* 2004
〃	〃	〃	Joo *et al.* 2007
Furametpyr	Anilide	Fungicide	Nagahori *et al.* 2000
Imidacloprid	Organophosphate	Insecticide	Schulz-Jander *et al.* 2002
Isocarbofos	Organophosphate	Insecticide	Zhuang *et al.* 2014
Malathion	Organophosphate	Insecticide	Buratti *et al.* 2005
Methiocarb	Carbamate	Insecticide	Usmani *et al*. 2004
Methoxychlor	Organochlorine	Insecticide	Stresser and Kupfer 1998
〃	〃	〃	Hu and Kupfer 2002
Metolachlor	Chloroacetanilide	Herbicide	Coleman *et al*. 2000
〃	〃	〃	Kale et al. 2008
Molinate	Thiocarbamate	Herbicide	Jewell and Miller 1999
〃	〃	〃	Campbell 2009
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
〃	〃	〃	Lavado *et al*. 2014
Phorate	Organophosphate	Insecticide	Usmani *et al*. 2004
〃	〃	〃	Hodgson 2003
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

During the recent years, several papers have been published on the activity of human livermicrosomes for pesticides such as organophosphorus compounds, carbamates, nicotinoids and pyrethroids as insecticides, chloroacetamide and thiazines as herbicide. In these studies, the major metabolizing enzymes of pesticides were CYP450 isoforms such as CYP 1A1, CYP 1A2, CYP 2A6, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2C19, CYP 2D6, CYP 2E1, CYP 3A4, CYP 3A5, and CYP 3A7 (Hodgson 2001, Hodgson 2003, Abass *et al.* 2012).

1.3. 1 Esterases in human body

Liver microsomes in all mammalian species contain at least one type of carboxylesterase, but the exact number of carboxylesterases expressed in tissue or species is not known because the variations in carbohydrate content can give rise to multiple forms of the same enzyme (Parkinson 2001).

Carboxylesterases (CES; EC 3.1.1.1) in various tissue and serum, acetylcholinesterases (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8; pseudocholinesterase) in blood can catalyze the hydrolysis reactions of esters, amides and thioesters. Oranophosphatases (EC 3.1.8.1), serum enzymes (known as Paraoxonase, aryldialkylphosphatase, or arylesterase), hydrolyze phosphoric acid esters. Didiopropylfluorophosphatases (EC 3.1.8.2) play a role in hydrolyzing phosphoric acid anhydrides such as Diisopropylfluorophosphate.

Satoh and Hosokawa proposed the nomenclature system for classifying carboxylesterases, based on the difference in the amino acid sequence of a human carboxylesterases (Satoh and Hosokawa 1998, Satoh and Hosokawa 2006).

Genes encoding carboxylesterases are divided in four gene families with three subdivisions in the first gene family (namely, CES 1A, CES 1B, CES 1C, CES 2, CES 3 and CES 4) and CES 1A is subdivided to CES1A1, CES 1A2 and CES1A3.

The highest activity of carboxylesterase is observed in liver, where the two major carboxylesterases, the Carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2), are expressed. CES1 or CES2 are the major hydrolytic enzymes responsible for the metabolism of numerous therapeutic agents as well as endogenous and exogenous compounds. It is known that the substrate specificities of CES1 and CES2 and tissue distribution are significantly different (Zhu *et al.* 2009). For example, the CES1 isozyme mainly hydrolyzes a substrate with a small alcohol group and large acyl

group, but its wide active pocket sometimes allows it to act on structurally distinct compounds of either a large or small alcohol moiety. In contrast, the CES2 isozyme recognizes a substrate with a large alcohol group and small acyl group, and its substrate specificity may be restricted by the capability of acyl-enzyme conjugate formation due to the presence of conformational interference in the active pocket (Hosokawa 2008)

Human carboxylesterase 1 (hCES1), a serine esterase playing a role in both drug metabolism and activation, as well as other biological processes, is known as a broad-spectrum bioscavenger that catalyzes the hydrolysis of heroin, the transesterification of cocaine and the detoxification of organophosphate chemicals, such as sarin, soman and tabun. hCES1's active site contains rigid and flexible pockets, explaining the enzyme's ability to act both specifically and promiscuously. hCES1 has also been reported to demonstrate the activities of cholesteryl ester hydrolase, fatty acyl-CoA hydrolase and acyl-CoA : cholesterol acyltransferase, and thus appears to be involved in cholesterol metabolism. Redinbo et al. revealed the molecular structure of hCE1 (Bencharit *et al.* 2003, Redinbo *et al.* 2003)

Regarding differences of CESs in species and tissue, the hydrolytic kinetics using the CES1 substrates with esterified drugs such as imidapril, derapril, and oxybutynin were performed to clarify these differences in humans and rats. The kinetics of the metabolite formation were different depending on species, tissue and preparations in HLM and cytosol (HLC) and exhibited Michaelis-Menten or sigmoidal patterns. These results showed that the kinetics were different between individuals (Takahashi *et al.* 2008).

Zhu et al. revealed that the expression and activity of CES1 and CES2 in the liver were markedly weaker in newborns than adults and increased gradually with age regardless of growth hormone level and sex (Zhu *et al.* 2009). Yang et al also demonstrated by analyzing liver samples from104 individuals that the expression level of hCE1 and hCE2 of the adult group is significantly higher than those of the child group. HCE1 and HCE2 of the child group also expressed significantly higher than the fetal group, in hydrolyzing aspirin, deltamethrin and permethrin (Yang *et al.* 2009).

Nishi et al identified and characterized hCES-1 and hCES-2 as human pyrethroid-hydrolyzing esterases. Differences of hydrolysis activities in hCES for stereoisomers of pyrethroids (enantio/diastereoselectivities) may lead to differences in human toxicity. Furthermore, individual-to-individual differences in the abundance of these esterases may result in differences in sensitivity to pyrethroid. Most of hydrolytic reactions by carboxylesterases were reported with not only pyrethroids but also organophosphates.

Cholinesterases are enzymes that belong to the superfamily of α/β-hydrolase fold proteins. Acetylcholinesterases (AChE) are located both as a dimeric enzyme on erythrocyte membranes and as a tetrameric form in nervous tissue, whereas butyrylcholinesterases (BChE) are found in serum. AChE terminates the action of acetylcholine at the post-synaptic membrane in the neuromuscular junction. On the other hand, BChE hydrolyses acetlcholine as well as many other esters. But has no known physiological function. BChE is studied by pharmacologists because it is responsible for the hydrolysis of succinylcholine, a drug used in surgery as a short-acting

blocker of the acetylcholine receptor (Chatonnet and Lockridge 1989). While two different enzymes can hydrolyse acetylcholine and share many characteristics, they also possess many important differences. Whereas they have about 54% animo acid sequence homology, their specificity towars various substrates and inhibitors differ. The reason is that the active site porge of AChE is considerably smaller than that of BChE. Both have been sown to display simple and complex kinetic mechanisms, depending on the particular substrate, the substrate concentration, and incubation conditions (Shenouda *et al.* 2009). The hydrolysis of substrate by both enzymes proceeds through a transacylation step. However, active site gorge of AChE is lined with 14 aromatic amino acid residues rather than the 6 found in BChE. The phenylalanine residues, Phe-295 and Phe-297 of the acyl pocket in AChE are substituted with Lys-286 and Val-228 in BChE, leading to a larger acyl pocket that can accommodate larger substrates (Vellom *et al.* 1993, Nicolet *et al.* 2003). Fore example, whereas the inhibition of BChE by chlorpyrifos oxon was fitted by simple Michaelis-Menten kinetics, the inhibition of AChE by Chlorpyrifos oxon followed concentration-dependant kinetics, indicating that the much larger active site gorge of BChE kilely contributes to its much greater reactivity towards chlorpyrifos oxon, compared to AChE (Shenouda *et al.* 2009). BChE can be studied as bioscavengers or antidotes for the poisoning by organophosphorus compounds (OPs) and carbamates, because the BChe can catalytically hydrolyze OPs and stoichiometrically bind to OPs without any damage form OPs (Doctor and Saxena 2005).

1.4 Absorption through gastrointestinal tracts

Digestion is a series of mechanical and chemical catabolism of food into smaller components that are more easily taken up into a blood stream. In the human digestive system, food undergoes mechanical digestion with saliva in the mouth. Saliva secreted by the salivary glands in mouth contains amylase having a role of digesting starch in food. After salivary digestion, the food moves down into stomach by the action of peristalsis. Gastric juice in the stomach enables to digest protein. Gastric juice mainly contains hydrochloric acid, pepsin, and trypsin. As acidic condition of these chemicals may damage the stomach wall, mucus is secreted to protect the stomach by making a shielding layer against the damaging effects of the acidic chemicals. It is known that it takes 1–2 hours in humans to change a chewed food to chime by gastric juice in stomach (Kong and Singh 2008). After digestion of stomach, chyme enters a series of digestive process in small intestine, where the food is dissolved into the juices from the pancreas, liver, and intestine. When the chyme is fully digested, All of the digested nutrients are absorbed through the intestinal walls and minerals are absorbed back into the blood in large intestine (Kong and Singh 2008).

The whole digestive system in human is more than 5 meters long. In an adult human this process can take between more than 24 hours. Food digestion physiology varies between individuals and upon other factors such as the characteristics of the food and size of the meal (Kong and Singh 2008).

1.4.1 Saliva

Saliva is secreted in large amounts (1-1.5 liters/day) by three pairs of salivary glands (parotid, submandibular, and sublingual) in the mouth, and is mixed with mechanistic chewing. Saliva sanitizes the oral cavity, moistens the food, and contains digestive enzymes such as amylase for the chemical breakdown of starch into disaccharides such as maltose. (Dawes 1972, Young and Schneyer 1981).

Human saliva is 99.5% water, while the other 0.5% of saliva is composed of a variety of electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates. Immunoglobulins, proteins, enzymes, mucins, and nitrogenous products, such as urea and ammonia are also found in saliva. The role of these components were known as following functions: *(1) bicarbonates, phosphates, and urea act to modulate pH and the buffering capacity of saliva; (2) mucins serve to cleanse, aggregate, and/or attach oral microorganisms and contribute to dental plaque metabolism; (3) calcium, phosphate, and proteins work together as an anti-solubility factor and modulate demineralization and remineralization; and (4) immunoglobulins, proteins, and enzymes provide antibacterial action*. The pH in salivary flow can range from 5.3 (low flow) to 7.8 (peak flow) (Young and Schneyer 1981, Humphrey and Williamson 2001, Adenugba *et al.* 2008).

1.4.2 Gastric juice

Food in stomach is thoroughly mixed with gastric acid, pepsin and other digestive enzymes to break down proteins. The acidic condition provides an

optimum pH for the reaction of the enzyme pepsin and kills most of the ingested bacteria and can also denature proteins. The gastric glands of the stomach secrete mucus, which plays an important role in lubrication and protection of the stomach wall against the highly concentrated hydrochloric acid. Small molecules such as alcohol are absorbed in the stomach by passing through the membrane of the stomach directly. Gastric acid is a digestive fluid, formed in the stomach. It has a pH of 1-2 and is composed of hydrochloric acid (HCl) (around 0.5%) as high as 0.1 M, and large quantities of potassium chloride (KCl) and sodium chloride (NaCl). The acid plays a key role in digestion of proteins, by activating digestive enzymes, and making ingested proteins unravel so that digestive enzymes break down the long chains of amino acids. To help digestion of protein, the stomach secretes inactive enzyme, pepsinogen, which can be activated to pepsin by hydrochloric acid (Ganong and Barrett 2005). The pH of gastric acid is 1.5 to 3.5 in the human stomach lumen, the acidity being maintained by the proton pump H^+/K^+ ATPase (Gray and Bucher 1941, Lian *et al.* 2003, Adenugba *et al.* 2008).

1.4.3 Intestinal juice

After digestion in stomach, the chyme is passed to the small intestine, in which most of digestion and absorption occurs and digested chime is further mixed with three different liquids: 1) Pancreatin and trypsin, which is activated from trypsinogen by intestinal enterokinase in intestinal juice, are secreted by the pancreas. 2) Bile is produced by the liver and released

through the bile duct and has a role in neutralizing the chyme and enables to excrete waste products such as bile acids. 3) other components in intestinal juice is secreted by the intestinal glands in the small intestine and contains enzymes such as enteropeptidase, trypsin, and chymotrypsin (Borgström *et al.* 1957).

The pH level is medium to slightly alkaline in the small intestine as all three fluids have optimal activity in alkaline condition. A more basic environment causes more helpful for enzymes to activate and begin the breakdown of molecules such as fats (Rao *et al.* 1989). After the absorption by increasing the surface area of the intestine and enhancing speed, the absorbed nutrients is transferred away from the small intestine to liver through blood stream (Borgström *et al.* 1957).

Compared to *in vivo* studies, *in vitro* techniques can save labor and time, reduce cost, and improve accuracy and reproducibility while avoiding the ethical problems of human studies. A number of *in vitro* GI tract models are currently available for nutrition, toxicology, pharmacology, and safety assessments. A well-designed *in vitro* model may provide an accurate estimate of what is occurring *in vivo*; however, the correlation between *in vitro* and *in vivo* studies is highly dependent on model design and the physical and chemical properties of the materials tested. Therefore, before the *in vitro* model is applied, a quantitative validation of the model is recommended to estimate the *in vivo* situation (Versantvoort and others 2004).

GI metabolism includes digestion by saliva, gastric juice, and intestinal juice. When secreted into the stomach, gastric juices have a pH = 2, but the acidity may be decreased by dilution with food. Studies on the relationship between drug absorption and food digestive conditions have shown that food intake influences the bioavailability of some drugs (Melander 1978, Weitschies *et al.* 2005). It was also reported that food could significantly delay drug tablet disintegration in the stomach by formation of a film around the tablet (Abrahamsson *et al.* 2004). Adenugba et al accurately described the composition of artificial GIs (Adenugba *et al.* 2008). The compositions of GI juices can be modified according to the purpose of the study, such as digestion pattern of chemicals, stability of coating material for yogurt bacteria, availability of bound residual pesticides, or absorption of drugs (Rao *et al.* 1989, Gorbach *et al.* 1992, Yang *et al.* 1993, Adenugba *et al.* 2008, Virkel *et al.* 2009, Hur *et al.* 2011).

Study of gastrointestinal digestion will aid the general understanding of the disintegration and absorption of pesticides after ingestion and show the stability of the pesticide in each digestive organ of the human body.

2. Acetolactate Synthase and sulfonylureas

More than 400 herbicides have been registered, or are under development process. These are categroized according to their target sites, modes of action, similarity of induced symptoms or chemical classes by the Herbicide Resistance Action Committee (HRAC) in cooperation with the Weed Science Society of America. To date, ten enzymatic herbicide targets have been charaterized in details, some more may be clearly determined by mode of action studies. ALS inhibitors are categorized as 'B group' according to their target sites (modes of action) and induced symptoms or chemical classes by HRAC (Table 5). In 1980s sulfonylureas were introduced as the acetolactate synthase (ALS) inhibitors, represented by imidazolinones, pyrimidinyloxybenzoates and so on. Since the first sulfonylurea herbicide, chlorsulfuron at 1982, several sulfonylureas have been commerized and still developed actively (Shimizu *et al.* 2002).

2.1 Acetolactate synthase

Since 1980s, the global pesticide companies have tried to develop the more efficient and higher sensitivity to the target organism, and the safer compounds to environment and invertebrates than the former decades. ALS (EC 4.6.3.8, also known as acetohydroxy acid synthase, or AHAS; EC 4.1.3.18) was the main target enzyme for the herbicidal action to satisfy their purposes for more than 20years.

Table 5. Classification of herbicide by their site of action*

	Herbicide Group	Site of Action	HRAC	Example Herbicide
1	ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	Diclofop-methyl
2	Thiocarbamates and others	Inhibition of lipid synthesis - not ACCase inhibition	N	Triallate
3	Chloroacetamides and others	Inhibition of cell division (Inhibition of very long chain fatty acids)	K3	Butachlor
4	Glutamine synthase inhibitors	Inhibition of glutamine synthetase	H	Glufosinate-ammonium
5	Arylaminopropionic acids	Unknown	Z	Flamprop-methyl
7	Mitosis inhibitors	Inhibition of mitosis / microtubule polymerization inhibitor	K2	Propham
8	Cellulose inhibitors	Inhibition of cell wall (cellulose) synthesis	L	Dichlobenil
9	Triazoles, ureas, isoxazolidiones	Inhibition of carotenoid biosynthesis (unknown target)	F3	Amitrole
10	Organoarsenicals	Unknown	Z	MSMA
11	Dinitroanilines and others	Microtubule assembly inhibition	K1	Trifluralin
12	Carotenoid biosyn. inhibitors	Inhibition of carotenoid biosynthesis at phytoene desaturase (PDS)	F1	Flurtamone
13	4-HPPD inhibitors	Inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	F2	Isoxaflutole
14	Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	Bromoxynil
15	PPO inhibitors	Inhibition of protoporphyrinogen oxidase (PPO)	E	Oxyfluorfen
16	Ureas and amides	Inhibition of photosynthesis at photosystem II	C2	Chlorotoluron
17	Glycines	Inhibition of EPSP synthase	G	Glyphosate
18	Bipyridiliums	Photosystem-I-electron diversion	D	Paraquat
19	Synthetic Auxins	Synthetic auxins (action like indoleacetic acid)	O	2,4-D
20	Photosystem II inhibitors	Inhibition of photosynthesis at photosystem II	C1	Atrazine
21	**ALS inhibitors**	Inhibition of acetolactate synthase(ALS or AHAS)	B	Chlorsulfuron

* Weed Science org, http://www.weedscience.org.

ALS is a protein found in plants and micro-organisms such as bacteria, fungi and algae. ALS catalyzes the first step (the formation of acetolactate from two molecules of pyruvate and the formation of acetohydroxybutylate from pyruvate and ketobutylate) in the biosynthesis of the branched-chain amino acids such as valine, leucine and isoleucine, which are essential amino acids only produced by microorganisms and plants and eventually consequences to inhibition of protein synthesis.

The biosynthesis pathway of branched-chain amino acids from threonine and pyruvate through a common series of reactions has been well studied in bacteria and fungi (Miflin and Lea 1977, Bryan 1980, Miflin and Lea 1982). The details of the biosynthesis of Val, Leu and Ile were well explained in biosynthesis of amino acid (Ray 1989, Devine *et al.* 1992, Stryer 1995). The ALS biological pathway exists only in plants, not animals, which makes the ALS-inhibitors the safest herbicides among the any other herbicides.

There are four specific residues (Val (Position: 485), Met (Position: 513), His (Position: 643), and Gly (Position: 511)) that are responsible for catalytic activity of this enzyme. They are listed here with cofactors required written after. The specific residue that is responsible for this is a glycine at position 511 in the protein. This is the one that requires a cofactor of TPP for its function (Kim *et al.* 2004).

ALS in microorganisms consists of three pairs of subunits. Each pair includes a large subunit, which is thought to be responsible for catalysis, and a small subunit for feedback inhibition. Each subunit pair is located on its own operon which involved in branched-chain amino acid biosynthesis. The *ilvGMEDA* operon (encoding ALS II, branched-chain-amino-acid

transaminase, dihydroxy-acid dehydratase, and threonine ammonia-lyase) is regulated by feedback inhibition in the form of transcriptional attenuation. Transcription of the operon is reduced in the presence of the pathway's end-products, the branched-chain amino acids. The *ilvBNC* operon, which encodes ALS I and a ketol-acid reductoisomerase, is regulated similarly, to the *ilvGMEDA* operon, However, regulation of the *ilvBNC* operon is specific to isoleucine and leucine only,; valine does not affect the transcription of the operon directly. Both the *ilvGMEDA* and *ilvBNC* operons are derepressed during shortages of the branched-chain amino acids by the same mechanism that represses them. Both of these operons as well as the third, *ilvIH*, are regulated by leucine-responsive protein (Lrp) (Hines *et al.* 1994, Joo and Kim 1999) .

2.2 Sulfonylureas as ALS inhibitors

Sulfonylureas are known to inhibit ALS, as do other ALS-inhibitors such as imidazolinones (Shaner *et al.* 1984, Muhitch *et al.* 1987), pyrimidinyloxybenzoates (Koo and CASELEY 2008), sulfonylamino carbonyltriazolinone (Zhou *et al.* 2007), and triazolopyrimidines (Table 6).

Table 6. Acetolactate synthase inhibitors*

ALS inhibitors	Active ingredients
Imidazolinones	imazamethabenz-methyl, imazamox, imazapic, imazapyr, imazaquin, imazethapyr
Pyrimidinylthiobenzoates	bispyribac-sodium, pyribenzoxim, pyriftalid, pyriminobac-methyl, pyrithiobac-sodium
Sulfonylaminocarbonyltriazolinones	flucarbazone-sodium, propoxycarbazone-sodium
Sulfonylureas	Amidosulfuron, azimsulfuron, bensulfuron-methyl, chlorimuron-ethyl, chlorsulfuron, cinosulfuron cyclosulfamuron ethametsulfuron-methyl, ethoxysulfuron, flazasulfuron, flucetosulfuron, flupyrsulfuron-methyl-sodium, foramsulfuron halosulfuron-methyl, imazosulfuron, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, nicosulfuron, orthosulfamuron, oxasulfuron, primisulfuron-methyl, prosulfuron, pyrazosulfuron-ethyl, rimsulfuron, sulfometuron-methyl, sulfosulfuron, thifensulfuron-methyl, triasulfuron, tribenuron-methyl, trifloxysulfuron-sodium, triflusulfuron-methyl, tritosulfuron
Triazolopyrimidines	cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, penoxsulam, pyroxsulam

* Weed Science org, http://www.weedscience.org.

Pyrimidinylsulfonyl urea including azimsulfuron, imazosulfuron, nicosulfuron, pyrazosulfuron-ethyl, rimsulfuron, and triazinylsulfonylurea including chlorsulfuron, cinosulfuron and metsulfuron-methyl are sub-

groups of sulfonylureas. Flucetosulfuron belongs to sulfonylureas, especially pyrimidinylsulfonylurea group (Chaleff and Mauvais 1984, Brown 1990). The ALS-inhibiting mechanism of the sulfonylurea herbicides has been revealed by the herbicide scientists. LaRossa et al suggested that the interference with the branched-chain amino acid biosynthesis, mediation of inhibition of ALS and slow-binding inhibition of ALS isozyme II are the mode of action of sulfonylurea by the study of metsulfuron methyl and mutation method (LaRossa and Schloss 1984). They also elucidated that metsulfuron methyl have ALS inhibitory activity that is insensitive to sulfometuron methyl because of mutations in or near *ilvG*, the structural gene for ALS isozyme II.

2.2.1 Herbicidal selectivity of sulfonylureas

Basically, physico-chemical properties of the active ingredients, such as adsorption/desorption and, hydrolytic properties can determine the selectivity of herbicides before incorporation into plant by affecting possibility to contact. In the study on sorption of metsulfuron-methyl and sulfosulfuron, the poorly sorbed sulfosulfuron may be lowered the possibility to contact because of higher leaching tendency than metsulfuron (Singh and Singh 2012).

The selectivity of herbicides can be explained by the difference of uptake, translocation and metabolism in plant. Metabolism of herbicides in plants including crops and weeds is comprised of a three-phase process, which is similar to that of mammalian species (Table 7).

Table 7. Summary of the three phases of pesticide metabolism*

Characteristics	Initial properties	Phase I	Phase II	Phase III
Reactions	Parent compound	Oxidation, hydrolysis, reduction	Conjugation	Secondary conjugation or incorporation into biopolymers
Solubility	Lipophilic	Amphophilic	Hydrophilic	Hydrophilic or insoluble
Phytotoxicity	Toxic	Modified or less toxic	Greatly reduced or Non-toxic	Nontoxic
Mobility	Selective	Modified or reduced	Limited or immobile	Immobile
Bioavailability[a]	**	**	***	**** or unvailable

* Van Eerd, Hoagland et al. 2003
** Readily absorbed in GI tract of animals; *** less absorption; **** limited absorption.

In Phase I reaction, pesticides are biotransformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product comparing to the parent. The conjugation processes of pesticides or their metabolites with sugar, amino acid, or glutathione follow Phase I reaction, called as Phase II reaction. Phase II reaction reduces lipophilicity and toxicity of the parent pesticide. Metabolites biotransformed by Phase II reaction usually have little or no phytotoxicity and may be stored in cellular organelles. There can be also the Phase III reaction, such as conversion of Phase II metabolites into secondary conjugates after Phase II reaction (Van Eerd *et al.* 2003).

In plants and microorganisms, the original properties of parent compounds are transformed through oxidation, hydrolysis or reduction to generally

produce a more water-soluble and usually a less toxic product than the parent compounds through Phase I metabolism. Phase II metabolism, including conjugation of pesticide or herbicide metabolites with sugar, amino acid, or glutathione, increases the water solubility and reduces phytotoxicity of the parent herbicide metabolites from Phase I metabolism. The third phase, not yet elucidated in mammalian species, transforms the Phase II conjugates into secondary conjugates, which are also non-toxic (Hatzios 1991).

Lipophilic herbicides that are absorbed or penetrate into plants are usually subsequently subjected to Phase I reactions, such as hydrolysis, oxidation and/or reduction by which polar groups are introduced to the molecules. The transformed polar groups are conjugated with cell components such as glucose, amino acid, glutathione, etc. (Phase II reaction). These processes are a series of detoxification of herbicides. Metabolites of the herbicides are usually further incorporated and separated into macro-molecules such as cellulose and lignin, and then transported and accumulated in vacuoles, or decomposed finally to CO_2.

To show herbicidal activities, all absorbed or post-emerged herbicides through root or foliar must move to the site of action in plant. Absorption and translocation of the herbicides can primarily contribute to the differential tolerance in plants. Because of its rapid translocation properties and relatively slow detoxification rate, smooth pigweed is sensitive to trifloxysulfuron, while cotton having limited translocation properties showed tolerance to the herbicide (Richardson *et al.* 2003, McElroy *et al.* 2009).

Some studies suggested that microorganisms play roles in degradation of sulfonylureas before their reaching to the target plant. The isolated bacterium, Serratia marcescens, showing a highly efficient degradation of nicosulfuron by breaking the sulfonylurea bridge also degraded some other sulfonylurea herbicides, including ethametsulfuron, tribenuron-methyl, metsulfuron-methyl, chlorimuron-ethyl, and rimsulfuron(Zhang *et al.* 2012). Pyrazosulfuron ethyl is a short-lived molecule, and it is dissipated rapidly in soil and water. The faster degradation of pyrazosulfuron in non-sterile soil than in sterile soil indicated microbial involvement in the degradation of this herbicide (Singh *et al.* 2012).

Once absorbed, the selectivity of herbicides is determined by the balance between herbicidal detoxification mechanism and activity of herbicide on the target sites in plant. Tolerant species can rapidly detoxify sulfonylureas to inactive products, while herbicidal metabolism is much slower and less extensive in susceptible species than tolerant species.

Tolerant species such as maize against rimsulfuron and resistant weeds such as barnyard grass against triflusulfruon-methyl possess capability of a highly efficient detoxification metabolism in their systems, whereas sensitive species or non-resistant weeds such as *Brassica napus*, *Matricaria inodora*, and *Veronica persica* have low detoxification ability against herbicides (Wittenbach *et al.* 1994, Koeppe *et al.* 2000).

2.2.2 Hydrolysis of sulfonylureas

In common, sulfonylureas have structures composed of sulfonylurea bridge, R1 moiety having either aliphatic, aromatic, or heterocyclic groups,

and R2 moiety. The R2 moiety is either a substituted triazine or pyrimidine ring (Figure 2). Sulfonylureas are weak acids and have pKa values generally ranging from 3 to 5. The herbicides in aqueous solution exist primarily in the neutral form at pH values below pKa, and in the anionic form at pH levels above the pKa. Therefore, the herbicides are predominantly anionic in most agricultural soils (Sarmah and Sabadie 2002).

Sulfonylureas are subject to pH-dependent hydrolysis of the sulfonylurea linkage. The two primary hydrolytic mechanisms are acid-catalyzed cleavage and base-catalyzed contraction/rearrangement of the sulfonylurea linkage. This bridge or the linkage is susceptible to attack by water on the carbonyl carbon of the bridge, and thus produces CO_2 and the corresponding aryl sulfonamide and amino-heterocyclic portions of the molecule. The rate of this reaction can often be hundreds of times faster under acidic condition (Beyer *et al.* 1987, Strek 1998, Sarmah and Sabadie 2002).

A literature on the degradation and metabolism of sulfonylurea herbicides in soil and water showed that the common degradation pathways are the cleavage of the sulfonylurea bridge, O- and N-dealkylation, aryl and aliphatic hydroxylation, ester hydrolysis, and conjugation reactions with glutathione and carbohydrates (Beyer *et al.* 1987, Brown 1990). Cleavage of the sulfonylurea bridge can also occur through the base-catalyzed reaction of the linkage. For instance, pyridine-2-sulfonylureas such as flazasulfuron, rimsulfuron, and flupyrsulfuron methyl have been reported to undergo an interesting base-catalyzed contraction rearrangement of the sulfonylurea bridge, and their hydrolysis rates are faster in alkaline conditions than acidic conditions (Schneiders *et al.* 1993).

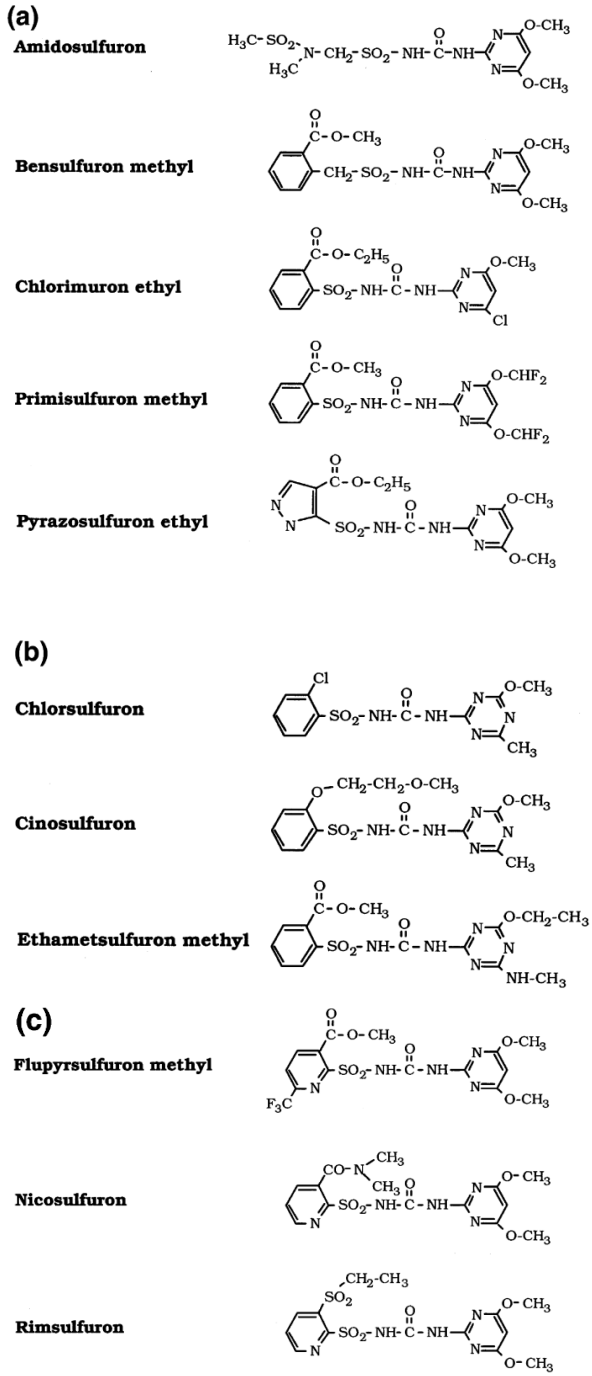


Figure 2. Sulfonylureas (a) triazine without pyridine (b) triazine (c) pyrimidine with pyridine (sarmah and sabadie 2002)

Degradation of chlorimuron-ethyl in soil was reported to be pH dependent and the identified degradation products (phenylsulfonamide; aminopyrimidine; saccharin) suggest that typical hydrolysis of the sulfonylurea bridge is the primary mode of degradation in soil (Sharma *et al.* 2012). Two degradation pathways were proposed; one is a saponification of ethyl ester-bond under alkaline conditions (> pH 10) and the other is a hydrolytic breakdown of sulfonylurea bridge at <pH 8 (Figure 3).

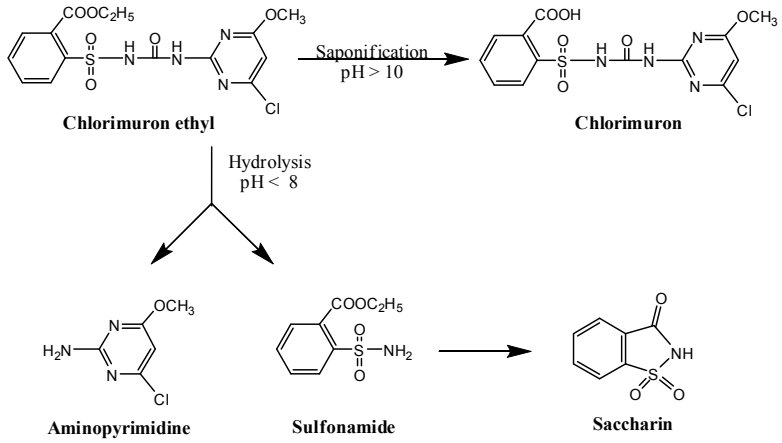


Figure 3. Hydrolytic pathway of chlorimuron-ethyl

Several factors such as temperature, pH, moisture content, and the biological activity of the soils can affect the degradation rate for sulfonylurea herbicides.

The investigation on the degradation of azimsulfuron in different pH also suggested that the hydrolysis rate was pH dependent and was much faster in acidic than in neutral or weakly basic conditions. The two main products from hydrolysis in mild acidic solution were identified as 2-amino-4,6-dimethoxy-pyrimidine and sulfonamide, both produced as a result of the

sulfonylurea bridge cleavage (Boschin *et al.* 2007).

Most of literature suggested that the main metabolic route of sulfonylureas is degradation to sulfonamide and amine by the breakdown of sulfonylureabridge, whereas the kinetics for the degradation were in a pH-dependent manner and was affected by the temperature (Sarmah *et al.* 1999, Sarmah *et al.* 2000, Vega *et al.* 2000, Morrica *et al.* 2001, Saha and Kulshrestha 2008, Zheng *et al.* 2008, Singh and Singh 2013).

3. Chirality in chemicals

Chirality is one of the most critical concepts in pharmaceuticals and agrochemicals such as pesticides, insect hormones, and plant growth regulators and the significance of chiral properties has long been recognized since discovery of the racemate (optical isomers) of tartarate by Pasteur and the establishment of tetrahedral theory on the stereochemistry of chiral compounds. Optical purity, enantiopurity, meaning the purity of each chiral molecule is important in pure chemistry and applied chemistry (Crosby 1996). Until now, various efficient and stereo-specific synthetic processes and purification methods have been improved by development of enantio-selective catalysts and enabled the commercialization of optically active pesticides (Kurihara and Miyamoto 1998).

In pharmaceutical, it is imperative to prepare the chiral compounds and to examine their bioactivities, pharmacokinetics and pharmacodynamics of each enantiopure compounds. Historically, the tragedy of thalidomide makes scientists to realize the importance of chirality of chemicals. Fablo et al. reported that thalidomide producing congenital malformations, namely

teratogenicity, in man is the optically inactive form in (±)-Thalidomide, having chiral center (Fabro *et al.* 1967).

Powell et al. proposed a basic classification of chiral isomers as four group with regard to pharmaceuticals and it also can be applied in pesticide isomers(Powell *et al.* 1988, Hirai *et al.* 2002). *(1) Only one isomer shows biological activity, the other might be regarded as an isomeric impurity (2) The isomers don't have the same qualitative and quantitative biological activity (3) though the isomers have qualitatively similar biological activity, the quantitative potency is different (4) the isomers have qualitatively different biological activity.*

Chirality is also essential to agrochemistry, the same as the importance of chirality in pharmaceuticals. Though the discovery and development of enantiopure pesticides are relatively late, various enantiopure pesticides are commercialized and a lot of information of chirality in pesticides was accumulated. In 1980s, the significance of chirality of pesticides was recognized by pesticide scientists during they were confront with problems such as the growth inhibition by chirality in triazoles against both fungi and plants (Kramer *et al.* 1982, Köller 1987) and the chiral inversion of S-aryloxyphenoxypropionates herbicides such as fluazifop-butyl (Dicks *et al.* 1985, Bewick 1986). Those problems were solved and enable to accumulate information on agrochemicals as follows: (1) Aryloxypropionate herbicides show that a single isomer has more biological activity than its racemic counterpart. (2) In case of the delayed neuropathy effect by EPN, S-isomer having lower insecticidal activity than R-isomer shows an adverse effect that is not or less observed for R-isomer. (3) The fact that thiazoles generally has

fungicidal activity, but some of their less fungicidal isomers act as an much greater plant growth regulators indicates that enantiomeric pairs sometimes may have quite different type of activity (Kramer *et al.* 1982, Köller 1987, Omokawa 2002).

Chirality has become increasingly important in that enatioselectivity on herbicidal or insecticidal activity and metabolism by microorganisms, plants, and animals occur diversely as a biological response. Generally, one enantiopure isomer is the most effective, because bioactive form shows enantioselective responses to target organisms, in which the target site constitutes a special chiral circumstance. The relationships between chirality and biological activity have been reported diversely for insecticides, fungicides, herbicides and plant growth hormones. As the molecular chirality has been an fundamental concept to design a pesticides with increased activity and decreased side effects to human and environment, various pesticides including pyrethroids, organophosphates, triazole-related fungicides, insect juvenile hormones, aryloxyphenoxypropionates herbicides, and so on were developed and commercialized to date.

Pyrethroids, representative insecticides having chiral isomers, have been elucidated by a number of studies on the chemistry and biological activity of them since 1920s.

The absolute stereochemistry of the pyrethroids was not completely established until the early 1970s (Begley *et al.* 1972). Most of the developed pyrethroids contains a stereospecific enantiopure isomer and shows diverse stereoselective insecticidal properties for them (Kurihara and Miyamoto 1998).

Metalaxyl, registered as a fungicide, originally can control enantioselectively the growth of fungi by *R*-isomer and plant by *S*-isomer, respectively. Metalaxyl-M, a separated *R*-enantiomer of metalxyl, replaced the racemic mixture, metalaxyl.

Aryloxyphenoxypropionate (APP), acyl alanine, and carbamate herbicides were developed with an enantiopure compound.

R-isomer of APPs such as cyhaofop-butyl, diclofop-methyl, fenoxaprop-p-ethyl, fluazifop, and quizalorop-P shows herbicidal activity, enantioselectively (Sakata *et al.* 1985, Uchiyama *et al.* 1986, Shimabukuro and Hoffer 1995). *S*-isomer of dimethenamid, a chloroacetamide, was known to be strong herbicide of algal growth by inhibition of fatty acid elongation (Couderchet *et al.* 1997). A chiral diphenylether peroxidizing herbicides inhibits plant growth by blocking of protoporphyrinogen oxidase (Hallahan *et al.* 1992, Nandihalli *et al.* 1994).

These chiral pesticides show enantioselectively different degradation patterns in soil and microbial conditions. *R*-isomer of Dichlorprop and mecoprop was degraded significantly slower than *S*-isomer in soil by the enantioselective action of microorganisms (Garrison *et al.* 1996, Zipper *et al.* 1996, Müller and Buser 1997).

The chiral inversion reaction can be occurred in chiral position of pesticides. The differential chiral conversion of aryloxyphenoxypropionates such as fluazifop-butyl, dichfop-methyl, mecoprop, and fenoxaprop-ethyl was reported in incubation in soil (Dicks *et al.* 1985, Wink and Luley 1988, Blaser and Spindler 1997, Harrison *et al.* 2003, Cai *et al.* 2008). The enantiomerization proceeds in both directions (*R*-from $\Leftrightarrow$ *S*-form), favoring

the herbicidal active R-enantiomer. Ibuprofen, an anti-inflammatory drug, also undergoes configurational changes with unidirectional metabolic inversion via the stereoselective formation of the acyl-COA thioesters and enzymatic or nonenzymatic inversion of the thioesters of the propionates (Müller and Buser 1997).

To reduce environmental contamination and control resistant insects, weeds or fungi against commercialized pesticides, enantioselective pesticides having ideal physiological properties and high performance relating to selective activity are required increasingly to be developed. With newly developed enantioselective pesticides, the extensive studies on them should be executed continuously.

3.1 Stereoselective metabolism of pesticides in human

A lot of researches on the chiral substances have been carried out to explain different biological activities of drugs and other pharmaceuticals but, unfortunately, there is little information known on the enantioselective toxicities of the chiral pesticides.

A study on enantioselective toxicity of cyclodiene (chlordiene, chlordiene epoxide and heptachlor *exo*-epoxide) pesticides on male adults of German cockroach (*Blattella germanica*) was conducted and resulted that (+)-chlordiene, (–)-chlordiene epoxide and (+)-heptachlor *exo*-epoxide enantiomers showed higher toxicity in comparison

to their corresponding mirror images (Miyazaki *et al.* 1979).

Buronfosse et al. studied on the sulfoxidation of methiocarb (an N-methylcarbamate insecticide) by rat liver microsomes. And they reported

that Stereoselective formation of methiocarb sulfoxide from the methiocarb sulfide by flavin-containing monooxygenases (FMO) and by cytochromes P450, as known that the enantioselectivity of sulfoxidation can vary with the form of P450s involved in the metabolism (Buronfosse *et al.* 1995).

Lee et al. reported that endosulfan sulfate formation catalyzed by CYP3A4 and CYP3A5 were consistently higher for β-endosulfan than for the α-form, resulting in the stereoselective metabolism of α-endosulfan and β-endosulfan by CYP2B6 and CYP3A of P450 family (Lee *et al.* 2006).

Pyrethroids are ester-containing compounds containing various acid and alcohol moieties. Pyrethroids have esters of primary alcohols or secondary alcohols that contain a cyano group at the α-carbon of the alcohol moiety. Chiral centers can exist in both the acid and alcohol moieties, leading to the existence of several stereoisomers for each pyrethroid. The hydrolysis rate of trans-isomers of cypermethrin and permethrin with murine carboxylesterase *in vitro* was at least 4-fold faster than that of the cis-counterparts., suggesting that pyrethroid-hydrolyzing carboxylesterases from mammals in general have similar stereopreferences for cis/trans-configurations of cypermethrin and permethrin

Nishi et al identified and characterized hCE-1 and hCE-2 as human pyrethroid-hydrolyzing esterases. Differences of hydrolysis activities in hCEs for stereoisomers of pyrethroids (enantio/diastereoselectivities) may lead to differences in human toxicity. Furthermore, individual-to-individual differences in the abundance of these esterases may result in differences in sensitivity to pyrethroid (Nishi *et al.* 2006).

4. Flucetosulfuron

Flucetosulfuron (LGC-42153; N-[[(4,6-dimethoxy-2-pyrimidinyl)amino] carbonyl]-2-[2-fluoro-1-(methoxymethylcarbonyloxy)propyl]-3-pyridinesulfonamide), a relatively new herbicide developed by LG Life Sciences Ltd., Korea (formerly LG Chem. Ltd.) has been commercialized since 2003. This herbicide is inhibiting ALS, a key enzyme for the biosynthesis of the branched-chain amino acids (Leucine, isoleucine, and valine) in plants (Alister and Timothy 1988, Brown 1990, Janjic *et al.* 2002).

4.1 Synthetic scheme of sulfonylureas

A synthesis method of similar sulfonylureas to flucetosulfuron was introduced in European patent EP 0788486 B1 (Kim *et al.* 1994). They also provided the herbicidal effects of the synthesized compounds in post-emergence of weeds in the paddy condition. The derivatives of sulfonylurea show excellent efficacy against barnyard grass, bulrush, flat-sedge, monochoria, and arrow head, as a typical herbicidal effect of sulfonylureas.

On the basis of their patent, various pyrimidine sulfonylureas having chiral centers were synthesized as depicted in Figure 4. L of L-COCHF(CH_3) in the figure 4 means alkoxy, -N(CH_3)$_2$ or -NCH_3(OCH_3) and R is substituent such as acetyl, propyl, butyl, methoyacetyl, ethoxyacetyl groups.

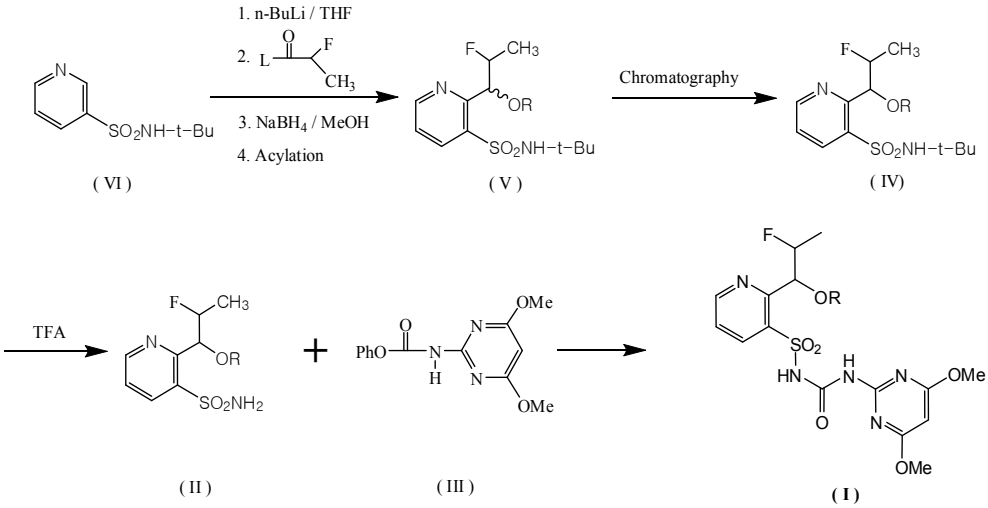


Figure 4. Synthetic process of sulfonylurea derivatives (Kim *et al.* 1994)

The patent demonstrated that flucetosulfuron can be synthesized by the following processes. The synthesis process was divided into three main steps.

The first step is a synthesis of 1-(3-(N-tert-butylsulfamoyl)pyridin-2-yl)-2-fluoropropyl 2-methoxyacetate (**V**) from N-tert-butylpyridine-3-sulfonamide (**VI**), as described in Figure 5.

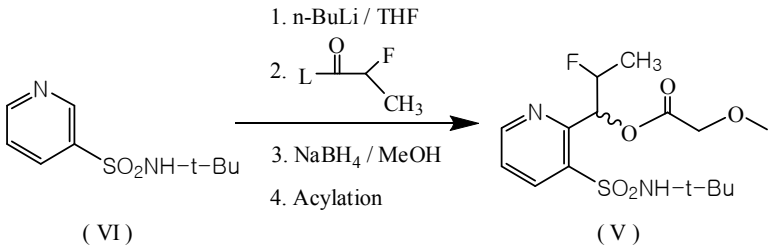


Figure 5. Synthesis of 1-(3-(N-tert-butylsulfamoyl)pyridin-2-yl)-2-fluoropropyl 2-methoxyacetate (**V**) from N-tert-butylpyridine-3-sulfonamide (**VI**) (Kim *et al.* 1994)

This reaction consists of four sub-steps including acylation of the methoxyacetyl group. To acquire *erythro*- or threo-isomer, additional purification process by column or prep-LC is required.

The second step is a synthesis process of 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl -2-methoxyacetate (**II**) from 1-(3(N-tert-butylsulfamoyl)pyridine-2-yl)-2-fluoropropyl-2-methoxyacetate (**IV**), as described in Figure 6. The sulfonamide can be synthesized by being treated with an acid such as trifluoroacetic acid (TFA).

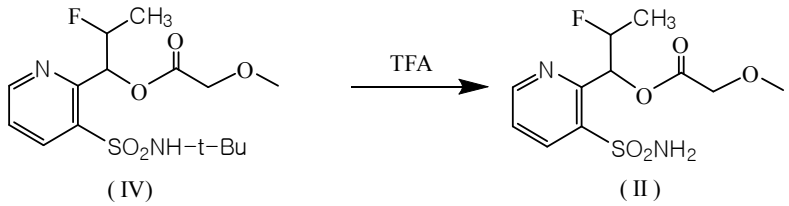


Figure 6. Synthesis of 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl -2-methoxyacetate (**II**) from 1-(3(N-tert-butylsulfamoyl)pyridine-2-yl)-2-fluoropropyl-2-methoxyacetate (**IV**) (Kim *et al.* 1994)

There are generally two coupling methods for preparing sulfonylurea (-$SO_2NHCONH$-) herbicides. One is shown in a coupling process of chlorsulfuron, which is synthesized by condensation of sulfonylisocyanate moiety with 2-amino-1,3,5-triazine moiety. The other method is a coupling of sulfonamide with phenyl acetamide, which is applied to prepare flucetosulfuron. The coupling process of 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl-2-methoxyacetate (**II**) with N-(4,6-dimethoxypyrimidin-2-yl)-2-phenylacetamide (**III**) is depicted as a final coupling process of

flucetosulfuron in Figure 7. This reaction should be carried out under strong base condition such as 1,4-diazabicyclo[2.2.2.]octane (DABCO) or 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU).

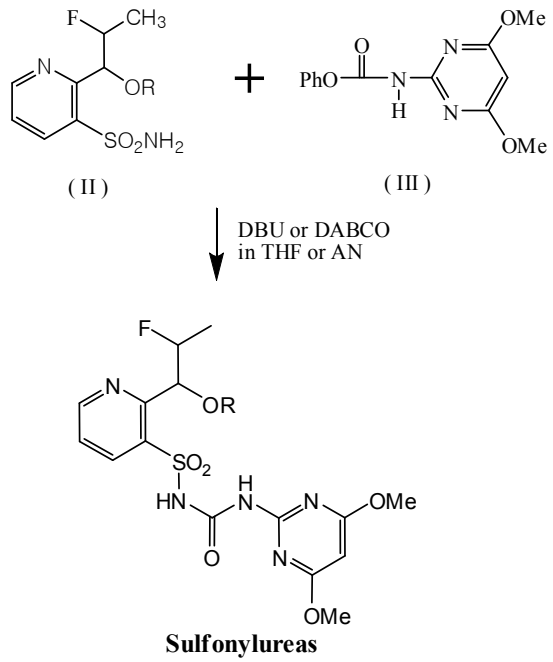


Figure 7. Synthesis of sulfonylureas such as flucetosulfuron by coupling process of 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl -2-methoxyacetate (**II**) and N-4,6-dimethoxypyrimidin-2-yl)-2-phenylacetamide (**III**) (Kim *et al.* 1994)

The final coupling process between sulfonamide and pyrimidine moiety is positively necessary. Although the intermediates for sulfonylurea synthesis can be manufactured by diverse synthetic routes from various raw materials, the last coupling step essential and identical to each intermediate to complete the synthesis.

Flucetosulfuron consists of two isomers, *threo*- and *erythro*-isomer. The results from the chromatographic analysis for flucetosulfuron by the

standardized method provided by Korea Pesticide Analytical Council, shows that flucetosulfuron consists of *erythro*- and *threo*-flucetosulfuron respectively to *R* or *S* configurations on carbon position containing the acyl group, which is acylated with a methoxy acetic acid (KOPAC 2009). Other patent also indicated *erythro*-flucetosulfuron as (1R,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate (Koo *et al.* 2000) and *threo*-flucetosulfuron as (1S, 2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate (Figure 8).

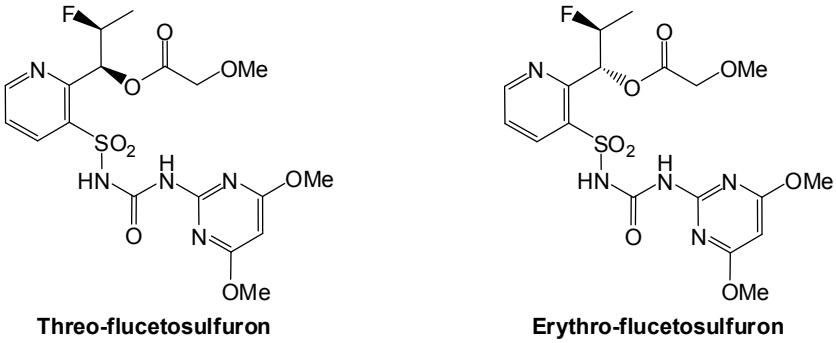


Figure 8. Structure of *threo*- and *erythro*-flucetosulfuron

4.2 Recent study on flucetosulfuron

It is known to be very effective in controlling broadleaf weeds, grass weeds and sedges. It was known to be very effective against broadleaf weeds, some grass weeds and sedges. In rice cultivar, flucetosulfuron provides excellent control of barnyard grass (*Echinochloa crus-galli*) at 15 to 30 g a.i./ha(Kim *et al.* 2003a). It was elucidated that flucetosulfuron was

absorbed via roots, stem, and leaf, and translocation of flucetosulfuron via leaf is faster than that of glyphosate and pyribenzoxim. It was also known that the LD_{50} for rice was 83.8 g/ha, higher than 4.5g/ha for *Echinochloa crus-galli.* The symptoms by herbicidal action of flucetosulfuron were a typical pattern of sulfonylureas, such as growth cessation, chlorosis, death of apical meristem, and subsequently whole plant death.

The study on metabolism of flucetosulfuron was, only in soils, conducted in both aerobic condition and flooded condition to propose the metabolic pathway, showing the cleavage of the ester linkage and the sulfonylurea linkage in both conditions (Kim *et al.* 2003b, Kim *et al.* 2003c). A number of papers commented that Such breakdowns in soil and water occur in other sulfonylureas having similar structure, which are chlorsulfuron (Sarmah *et al.* 1999), pyrazosulfuron-ethyl (Singh and Singh 2011, Singh and Singh 2013), imazosulfuron (Morrica *et al.* 2001), metsulfuron-methyl (Sarmah *et al.* 2000, Caselli 2005), and rimsulfuron (Rosenbom *et al.* 2010) .

Flucetosulfuron having two chiral centers is diastereomer and constituted of two isomers, *erythro*- and *threo*-flucetosulfuron. It has also been reported that the enantiomers may react with the achiral chemicals at different rates. There are greater chances of the chiral reaction at different rates in chiral pesticides such as flucetosulfuron.

THE PURPOSE AND SCOPE OF THE PRESENT STUDY

Metabolism of pesticides in biological or environmental systems is interesting because metabolites and their formation patterns are the important determinants in pesticide safety. In mammals, the liver plays a major role in the metabolism and systemic elimination of pesticides after exposure. For this reason, studies of *in vitro* metabolism by HLMs are generally conducted to investigate pesticide metabolic pathways, the formation pattern of metabolite and intermediate, and kinetics (Kim *et al.* 2003d, Liu and Kim 2003, Lee *et al.* 2006).

A number of papers have been published on HLM metabolism of pesticides such as phosphorothioates, pyrethroids, chloracetamides, and triazines (Shono *et al.* 1979, Kim *et al.* 2003d, Usmani *et al.* 2004, Buratti *et al.* 2005, Lee *et al.* 2006), indicating that cytochrome P450s (CYP450), flavin-containing monooxygenases (FMOs), and esterases are major metabolic enzymes for these pesticides. Mutch et al. showed that many human CYP450 isoforms catalyzed the transformation of parathion, diazinon, and chlorpyriphos to the oxon form, which can be detoxified via hydrolysis by A-esterases (paraoxonases/arylesterases), carboxylesterases, and cholinesterases (Mutch and Williams 2006). For pyrethroid metabolism, the major pathways involve CYP450-dependent oxidation and esterase-mediated

hydrolysis (Soderlund and Casida 1977, Nishi *et al.* 2006, Ross *et al.* 2006, Crow *et al.* 2007, Godin *et al.* 2007, Scollon *et al.* 2009).

Although sulfonylurea herbicides have been used globally, currently there is no published information describing their metabolism by HLMs. Investigation of flucetosulfuron metabolism by HLMs will provide a better understanding of the metabolism and detoxification routes for related sulfonylureas in the human body.

Hepatic metabolism of flucetosulfuron in humans has not been previously investigated *in vitro*; nor have the contributions of HLMs to metabolic pathways been elucidated. An understanding of the metabolic pathways and the varying contributions of HLMs involved will enable a better understanding of the differences in metabolism among individuals. The sulfonylurea herbicides were widely spread in the world, but the application volume was extremely low because of the relative high activity against weeds. So there is not much available information on the metabolism of sulfonylurea herbicide in humans, especially for risk assessment for exposed workers. As sulfonylurea herbicides, flucetosulfuron also has never been tested yet.

Flucetosulfuron developed by LG Life Sciences is a low toxic herbicide, applied in rice cultivar. There are little known on the comprehensive metabolism and kinetics for the substance so far. To understand the enzymatic reaction and toxicity of diastereomer such as flucetosulfuron, the extensive research on the metabolism of each isomer is required because each isomer of chiral pesticides be subjected to the different metabolism

even by the same enzymes.

The goal of this study is to investigate *in vitro* metabolism of the herbicide, flucetosulfuron, by the systems using HLMs and artificial GIs.

This dissertation is composed of two parts to better understand metabolism and degradation of flucetosulfuron in *in vitro* human systems:

1) *In vitro* metabolism of flucetosulfuron by HLMs

2) *In vitro* metabolism of flucetosulfuron by artificial GIs.

This study is the first report on the metabolism of flucetosulfuron by HLMs and GIs. The results from these studies can provide expansion of understanding and fundamental knowledge on the stereoselective metabolism on the pesticides or drugs containing a chiral center.

PART I

In vitro Metabolism of Flucetosulfuron by Human Liver Microsomes

Ⅰ. Introduction

Metabolism of pesticides in biological or environmental systems is interesting because metabolites and their formation patterns are important determinants of pesticide safety. In mammals, the liver plays a major role in the metabolism and systemic elimination of pesticides after exposure. For this reason, studies of *in vitro* metabolism by HLMs are generally conducted to investigate pesticide metabolic pathways, the pattern of metabolite and intermediate formation, and kinetics.

A number of papers have been published on HLM metabolism of pesticides such as phosphorothioates, pyrethroids, chloracetamides, and triazines, indicating that cytochrome P450s (CYP450), flavin-containing monooxygenases (FMOs), and esterases are major metabolic enzymes for these pesticides. Mutch *et al.* showed that many human CYP450 isoforms catalyzed the transformation of parathion, diazinon, and chlorpyriphos to the oxon form, which can be detoxified via hydrolysis by A-esterases (paraoxonases/arylesterases), carboxylesterases, and cholinesterases. For pyrethroid metabolism, the major pathways involve CYP450-dependent oxidation and esterase-mediated hydrolysis.

Although sulfonylurea herbicides have been used globally, currently there is no published information describing their metabolism by HLMs. Investigation of flucetosulfuron metabolism by HLMs will provide a better understanding of the metabolism and detoxification routes for related sulfonylureas in the human body.

Therefore, the purpose of the present study is to elucidate *in vitro* metabolism of flucetosulfuron by HLMs and artificial GIs. The main frame of the present study consists of as follows:

(1) preparation of *threo*-flucetosulfuron and *erythro*-flucetosulfuron

(2) synthesis and identification of flucetosulfuron metabolite(s)

(3) investigation on kinetics of flucetosulfuron biotransformation by HLMs

(4) characterization of the specific HLM enzymes responsible for flucetosulfuron metabolism

(5) investigation on kinetics of each enzyme responsible for the metabolism of flucetosulfuron

(6) proposal of relevant metabolic pathway of flucetosulfuron, based on the metabolism by HLMs.

This study is the first report to describe the *in vitro* metabolism of flucetosulfuron by HLMs and identify the metabolizing enzymes responsible for its hydrolysis.

Ⅱ. Materials and Methods

1. Reagents and Materials

1.1 Flucetosulfuron and metabolites

Flucetosulfuron technical (98.4%) and working standard (99.4%) were provided by LG Life Sciences. Ammonium acetate, acetic acid, formic acid, and $CDCl_3$ (above 99.9 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were purchased from Burdick and Jackson (MI, USA). Chloroform, dichloromethane, and hexane were purchased from J. T. Baker (Phillipsburg, NJ, USA). 1N HCl and sodium sulfate were purchased from Daejung chem. (Ansan, Korea). TLC, Silica gel (Kieselgel 60, 230-240 mesh) was purchased from Merck (Germany) and activated at 150℃ (2 h) before its use.

1.2 Chemicals and reagents

Pooled HLMs were purchased from BD Gentest (Woburn, MA). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide ($NADP^+$) were purchased from Sigma-Aldrich (St. Louis, MO). Various esterase inhibitors, such as bisnitrophenyl phosphate (BNPP), calcium chloride, cobalt chloride, dibucaine, ethylenediaminetriacetic acid (EDTA), eserine, magnesium chloride, mercuric chloride, and quinidine, were also purchased from Sigma-Aldrich (St. Louis, MO).

Potassium phosphate monobasic/dibasic and sodium hydroxide (NaOH) were purchased from Daejung Chem. (Ansan, Korea). The solvents were HPLC grade (Burdick and Jackson, MI), and the other chemicals were also HPLC grade.

1.3 Enzymes

1.3.1 HLMs

HLMs (BD Ultra Pool HLM 150) were purchased by BD Biosciences (San Jose, CA) and stored at -80 ℃ prior to use. The package contents were 0.5 mL and protein content was 20 mg/mL in 250 mM sucrose. Assay result and enzyme activity information of the purchased HLMs in Table 8 were provided from BD Biosciences. Also, the details of the liver microsomes donors are given in Table 9.

Table 8. Characteristics of BD Ultra Pool HLM 150*

Enzyme Measured	Assay	Enzyme Activity [in pmol/(mg×min)]
Total P450	Omura and sato	250
OR**	Cytochrome c Reductase	610
Cyt. b_5	Spectrophotometric	510
CYP1A2	Phenacetin O-deethylase	580
CYP2A6	Coumarin 7-hydroxylase	910
CYP2B5	(S)-Mephenytoin N-demethylase	26
CYP2C8	Paclitaxel 6α-hydroxylase	170
CYP2C9	Diclofenac-4'-hydroxylase	3000
CYP2C19	(S)-Mephenytoin N-hydroxylase	67
CYP2D6	Bufuralol 1'-hydroxylase***	62
CYP2E1	Chlorozoxazone 6-hydroxylase	2100
CYP3A4	Testosterone 6β-hydroxylase	4400
CYP4A11	Lauric acid 12-hydroxylase	1400
FMO	Methyl p-Tolyl Sulfide Oxidase	940
UGT1A1	Estradiol 3-Glucuronidation	730
UGT1A4	Trifluoperazine Glucuronidation	500
UGT1A9	Propofol Glucuronidation	2200

* Provided from BD Biosciences

** OR= Oxido-reductase.

*** The amount of activity inhibited by 1 μM quinidine.

Table 9. Available details for the human liver donors in the product, BD Ultra Pool HLM 150*

Specimen	HH42	HH56	HH75	HH85	HH95	HH123
Gender	Female	Female	Male	Male	Male	Male
Age	57	57	55	42	28	10
Race	Caucasian	Caucasian	Caucasian	Caucasian	Hispanic	African-American
Cause of death	CVA/stroke	Anoxia	Anoxia	Head Trauma	CHI/MVA	Anoxia
Social history	No history of alcohol, tobacco or drug use	Not available	Social alcohol use	Not available	Tobacco use	Not available

Specimen	HH166	HH167	HH173	HH505	HH515	HH524
Gender	Male	Male	Male	Female	Female	Male
Age	49	60	46	62	42	51
Race	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Cause of death	Head trauma	CVA	CVA	ICH	ICH	Subarachsanoid hemorrhage
Social history	Tobacco use	No alcohol or tobacco use	Tobacco (1/2 pack/day×25 years), alcohol use, and marijuana use (once per week)	Tobacco use	Tobacco use	Alcohol use

* Provided from BD Biosciences.

1.3.2 Human Carboxylesterases

Human Recombinant Carboxylesterases (CES1b, CES1c and CES2 having the content of 5mg/ml protein of all of them) were purchased from BD Biosciences (San Jose, CA) stored at -80 ℃ prior to use. All of their protein concentrations were 5mg/ml. The Kinetic parameters for the purchased carboxyesterases were provided from BD Biosciences in Table 10 and Figure 9. The K_m value of CES1c for 4-nitrophenyl acetate (4-NPA) was higher than that of HLM suggesting the main hydrolyzing enzyme in HLM is CES1b, whereas CES2 is most potential activity for Fluorescein diacetate (FD). And, Figure 9 shows that the time course of product formation of 4-nitrophenyl acetate for three carboxylesterases is completely linear out to at least 30 min.

Table 10. Michaelis-Menten Kinetic parameters for each human recombinant carboxylesterases*

	4-NPA(Substrate)		**FD(Substrate)**	
Enzyme	K_m (μM)	V_{max}(nmol/mg/min)	K_m (μM)	V_{max}(nmol/mg/min)
CES1b	208 ± 41	1623 ± 92	1.6 ± 0.3	307 ± 11
CES1c	441 ± 67	1873 ± 108	4.6 ± 0.9	213 ± 11
CES2	173 ± 22	718 ± 24	4.8 ± 1.1	14646 ± 939
HLM 150	198 ± 17	3407 ± 83	4.9 ± 0.5	18492 ± 534

* Provided from BD Biosciences.

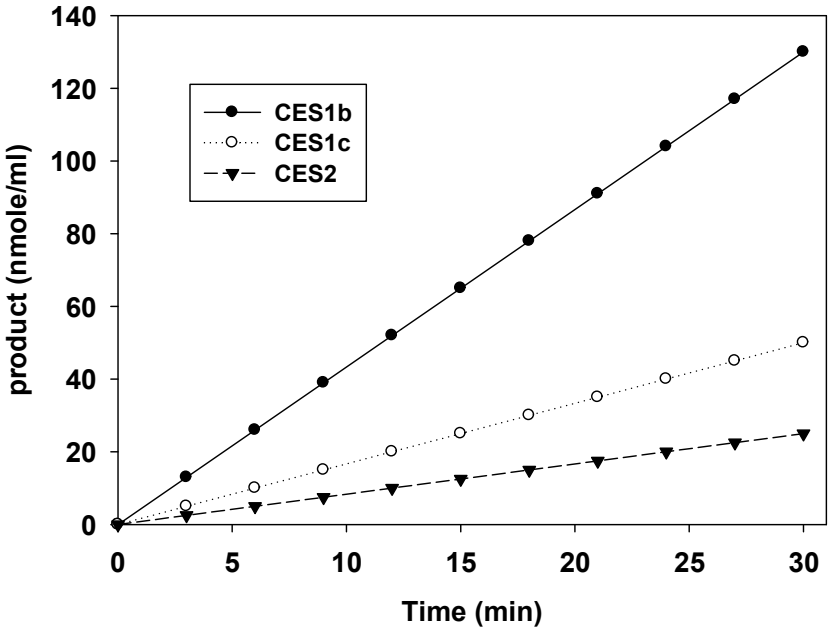


Figure 9. Time course of product formation of three CES for 4-nitophenyl acetate (BD Biosciences)

1.3.3 Human acetylcholinesterase

Human acetylcholinesterase (AChE), having the activity of 860 units/mg protein for acetylcholine having was purchased from Sigma-aldrich (St. Louis, MO) and stored at 2-8 ℃ prior to use. The specification was provided form sigma-aldrch in Table 11.

Table 11. Specification of human acetylcholinesterase*

Test	Specification	Result
Appearance (Form)	Clear Solution	Clear Solution
Appearance (Color)	Colorless	Colorless
Protein Content (Method)	BCA	BCA
Prot. Content (mg Protein/mL)	≥ 0.00	0.10
Enzym. Assay (Activity U/mL)	≥ 0	86
Enzym. Assay (Spec. Activity)	≥ 500.00 U/mgP	860.00 U/mgP
Enzym. Assay (Contam. Name)	Butyrylcholinesterase	Butyrylcholinesterase
Enzym. Assay (Activity U/mgP)	≤ 20.00 U/mgP	0.65 U/mgP
Vial Content (Size)	50 – 50	50
Vial Content (Unit Of Measure)	Units	Units
Vial Content (Actual Content)	50.00 – 75.00	55.00
Vial Content (Volume μL)	≥ 0 μL	640 μL

* Provided from Sigma-aldrich co.

1.3.4 Human butyrylcholinesterase

Human butyrylcholinesterase (BChE), having the activity of 170 units/mg protein for butyrylcholine was purchased from Sigma-aldrich (St. Louis, MO) and stored at 4 ℃. The specification was provided form sigma-aldrch in Table 12. This enzyme was reconstituted in 20 mM sodium phosphate buffer, pH 7.0, with 0.15 M sodium chloride, prior to use.

Table 12 Specification of human butyrylcholinesterase*

Test	Specification	Result
Vial Content (ug protein/vial)	≥ 20	528
Units/mg protein*	≥ 50	170
Units/vial	≥ 5	90

* Provided from Sigma-aldrich co.

** One unit will hydrolyze 1.0 μM of butyrylcholine per min at pH 8.0 at 37℃.

1.4 Instrument and analytical conditions

1.4.1 HPLC

The flucetosulfuron isomers, TM1, and EM1 were detected by HPLC (High Performance Liquid Chromatography), which composed of Waters alliance 2690 HPLC and UV 4890 detector(Waters, Milford, CA). The concentrations of flucetosulfuron isomers and its metabolites (TM1 and EM1) in *in vitro* metabolism were measured by Waters alliance 2690 HPLC equipped with a Capcell pak C18 UG120 column (4.6 mm i.d. x 150 mm, 3 μm; Shiseido, Tokyo, Japan) at 40 °C. The mobile phases consisted of 20 mM ammonium acetate buffer containing 0.1 M acetic acid (A) and acetonitrile (B). The gradient condition was as follows: 20% B at 0 min, 27% B at 4 min, 30% B at 25 min, and 20% B at 28-33 min. The injection volume was 10 μL and peaks were detected at 254 nm. 3minutes of post time was given to stabilize initial condition.

The completeness of hydrolytic reaction from each flucetosulfuron isomer was confirmed by HPLC equipped with a Capcell pak C18 UG120 column

(4.6 mm i.d. x 150 mm, 3 μm) at 40 °C. The mobile phases were same as above. An isocratic solvent system for HPLC analysis was A:B as 70%:30% at a flow rate of 1.0 mL/min for 10 minutes during the study. The injection volume was 10 μL.

Quantitative analysis was performed with an external standard calibration method with the absorption of peak at the wavelength 254 nm.

1.4.2 Preparative HPLC

To separate and collect each isomer of flucetosulfuron form flucetosulfuron technical, Waters Delta Prep-4000 Preparative Chromatography system coupled with UV 4890 detector (Waters, Milford, CA) was equipped with a waters deltapak column (50mm i.d. x 300mm, 15μm, 100A; Waters, Milford, CA). The mobile phases consisted of 20 mM ammonium acetate buffer containing 0.1 M acetic acid (A) and acetonitrile (B). An isocratic solvent system for Prep-HPLC analysis was A : B as 36% : 64% at a flow rate of 20.0 mL/min for 120 min.

The injection volume was 10ml contains 0.03g of flucetosulfuron technical in acetonitrile: buffer solution with 0.02M ammonium acetate and 0.1M acetic acid (6:4) and peaks were detected at 254 nm.

1.4.3 LC-MS/MS

For the identification of flucetosulfuron and its metabolites, a tandem mass quadrupole mass spectrometer (API2000 LC-MS/MS, Applied Biosystems, Foster City, CA) coupled with an Agilent 1100 series high-performance liquid chromatography (HPLC) system (Agilent, Wilmington,

DE), was used. The separation was performed on a Capcell pak C18 UG120 column (4.6 mm i.d. × 150 mm, 3 μm; Shiseido, Tokyo, Japan) using the mobile phase that consisted of 10 mM ammonium acetate containing 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 1.0 ml/min. The gradient condition was as follows: 20% B at 0 min, 27% B at 4 min, 30% B at 25 min, and 20% B at 28-33 min.

Mass spectra were recorded by electrospray ionization (ESI) with a positive mode. The turbo ion spray interface was operated at 4500 V and 550°C. The operating conditions were optimized by flow injection of analytes and were determined as follows: nebulizer gas flow, 50 psi; curtain gas flow, 10 psi; and collision energy, 30 eV. Quadrupole Q1 and Q3 were set on unit resolution.

1.4.4 ^{1}H and ^{13}C NMR Analyses

^{1}H and ^{13}C NMR spectra were recorded on 400 MHz NMR spectrometer (Jeol JNM-LA400 with LFG, JEOL, Japan) in $CDCl_3$ (99.8%, Merck) at 297 K. TMS was used as reference (δ=0).

1.4.5 Centrifuge

Hanil Micro17TR centrifuge having temperature control system was manufactured by Hanil Ltd. (Incheon, Korea).

2. Methods

2.1 Preparation of flucetosulfuron isomers

2.1.1 Purification of *threo*-flucetosulfuron

Ten ml of flucetosulfuron technical in acetonitrile (0.03g of compound) was injected into Waters Delta Prep-4000 Preparative Chromatography system equipped with a waters deltapak column (50mm i.d. x 300mm, 15μm, 100 Å). An isocratic solvent system was used such as A:B = 64%:36% at a flow rate of 20.0 mL/min for 120 min (A: buffer solution with 0.02M ammonium acetate and 0.1M acetic acid, B: acetonitrile).

First fraction, 150~170ml having retention time of 80~90min was collected and partitioned twice with each 70ml of dichloromethane. Dichloromethane layer was washed off with 100ml of distilled water, dried over 30g of sodium sulfate and was evaporated under high vacuum at 30℃ to obtain 2.6 ~ 4.0 mg of *threo*-flucetosulfuron from one cycle of the fractionation/purification. After several repeat of the same processes, about 0.3g of *threo*-isomer was obtained.

2.1.2 Purification of *erythro*-flucetosulfuron

Second fraction, about 250ml having retention time of 94 ~ 110min was collected from Prep HPLC system and processed with same procedure as for *threo*-flucetosulfuron, producing 23 ~ 26 mg of *erythro*-flucetosulfuron from one cycle of the fractionation/purification process. After repeating the same processes many times, about 2.0 g of *erythro*-isomer was obtained. To confirm the identity of *erythro*-isomer, LC-MS analysis and ^{1}H and ^{13}C

NMR were also carried out. The purity of *erythro*-isomer was measured by quantitative analysis with working standard of flucetosulfuron.

2.2 *In vitro* metabolism of flucetosulfuron by HLMs

As a basic incubation, HLMs (0.5 mg protein/ml) were pre-incubated in 50mM potassium phosphate buffer (pH 7.4) in the presence of NADPH-generating system containing NADP (0.8 mM), glucose-6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (1 unit) for 5 minutes at 37℃ in shaking water bath (80 cycle/min). The reaction was started by adding 100 μM of each flucetosulfuron isomer (final volume of 250 μL). After 30 minutes, the reaction was terminated by adding of 250 μL of Acetonitrile and vortexing and immediately extracted by vortexing for 1min, and centrifuged at 10,770 × *g* for 5 minutes. The supernatant was collected to quantify the concentration flucetosulfuron. The 10 μL of the collected sample was analyzed by HPLC.

To determine which enzymes among CYP450s, FMO or esterases containing carboxylesterase in the presence of an NADPH generating system play a key acting role in the metabolism of flucetosulfuron, the flowing test using the heated or denatured HLMs was carried out.

The incubation mixtures containing 32 μL of 0.5 mg of pooled HLMs (H161, Gentest) were reconstituted in 50 mM phosphate buffer (pH 7.4) and pre-incubated for 5 min at 37°C. The reaction was initiated by adding the *threo*- or *erythro*-flucetosulfuron isomer (100 μM) and the NADPH-generating system (0.8 mM $NADP^+$, 10.0 mM glucose-6-phosphate, 1.3 mM $MgCl_2$, and 1.0 unit/mL glucose-6-phosphate dehydrogenase) and further

incubated for 30 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 250 μL acetonitrile, and the samples were centrifuged at 10,770 × *g* for 5 min at 4°C.

The control incubations were conducted with denatured HLMs for 5 min at 100°C and NADPH generating system. To confirm whether metabolite formation is NADPH-dependent (activity of CYP450), NADPH-free incubations were also performed. To confirm whether metabolite formation is cause of the activity of FMOs, NADPH-free incubations were also performed with heated HLMs for 1 min at 50 °C.

2.3 Synthesis and identification of flucetosulfuron metabolites

2.3.1 Analysis of the formed metabolites by microsomal incubation

The incubation mixtures containing 32 *μ*L of 0.5 mg of pooled HLMs (H161, Gentest) were reconstituted in 50 mM phosphate buffer (pH 7.4) and pre-incubated for 5 min at 37°C. The reaction was initiated by adding the *threo*- or *erythro*-flucetosulfuron isomer (100 μM) and the NADPH-generating system (0.8 mM $NADP^+$, 10.0 mM glucose-6-phosphate, 1.3 mM $MgCl_2$, and 1.0 unit/mL glucose-6-phosphate dehydrogenase) and further incubated for 30 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 250 μL acetonitrile, and the samples were centrifuged at 10,770 × *g* for 5 min at 4°C. The supernatant was analyzed by HPLC and LC-MS/MS to identify the formed metabolites.

2.3.2 Synthesis of metabolites, TM1 and EM1

2.3.2.1 Synthesis of TM1 (*threo*-N-(4,6-dimethoxypyrimidin-2-

ylcarbomoyl)-2-(2-fluoro-1-hydroxypropyl)pyrimidine-3-sulfonamid)

Threo-flucetosulfuron (149mg, 1 eq.) was dissolved in 30 ml of acetonitrile in a round bottom receiver (100 ml) and 7.5 ml of 0.1 M NaOH solution (30mg, ~2.5 eq.) was added drop-wise and then the reaction mixture was stirred for 1.5 hrs. After the reaction, the reaction mixture was transferred to a Sep. funnel (250ml) and 20 ml of distilled water was added to the reaction mixture. The reaction mixture was washed off twice by 25 ml dichloromethane. After washing off by dichloromethane, the water layer was adjusted to pH 3.0 with 0.1N HCl solution to obtain TM1 as a white solid, which was filtered, washed off with distilled water and dried under nitrogen purging for 10 hrs (90 mg, yield 70%).

2.3.2.1 Synthesis of EM1 (*erythro*-N-(4,6-dimethoxypyrimidin-2-ylcarbomoyl)-2-(2-fluoro-1-hydroxypropyl)pyrimidine-3-sulfonamid)

Erythro-flucetosulfuron (487mg, 1 eq.) was dissolved in 100 ml of acetonitrile in a round bottom receiver (250 ml) and 25 ml of 0.1 M NaOH solution (100mg, ~2.5 eq.) was added dropwise and then the reaction mixture was stirred for 1.5 hrs.

Work-up process was the same with TM1 as above. EM1 was obtained as a white solid (370 mg, yield 89%).

2.3.3 Preparation of standard and calibration curves

Standard solutions of *erythro*-flucetosulfuron, *threo*-flucetosulfuron, TM1 and EM1 were prepared as stock with 1 mM in acetonitrile. The stock was diluted at the concentration of 0.05, 0.1, 0.2, 0.5, 1.0, 10, and 100 μM

by serial dilution with acetonitrile. Concentrations of flucetosulfuron and metabolites were obtained by extrapolation of peak area from a calibration curve. The calibration curves were fitted with high linearity ($r^2 > 0.999$).

2.4 Optimization of microsomal reaction conditions

2.4.1 Optimization of incubation time

The incubation mixtures, containing 0.5 mg/ml of HLMs were pre-incubated in 50mM phosphate buffer (pH 7.4) without an NADPH-generating system for 30 min at 37℃ in a shaking water bath. After pre-incubation, the reaction mixtures were incubated for 10, 20, 30, 60, 120, 180 and 240 minutes with 10 μM of *erythro*-flucetosulfuron and *threo*-flucetosulfuron in total volume of 250 μL, respectively. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.4.2 Optimization of protein concentration

To determine the optimal protein (HLMs) concentration for the formation of the metabolite by HLMs, the reaction mixtures were incubated for 30 minutes with various concentrations of HLMs (0.16, 0.24, 0.32, 0.48, 0.64, 0.96 and 1.28 mg/ml) and 10 μM of *erythro*-flucetosulfuron and *threo*-flucetosulfuron in total reaction volume of 250 μL, respectively. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.4.3 Optimization of flucetosulfuron concentration

To determine the respective optimal flucetosulfuron concentration for the metabolism of *erythro*-flucetosulfuron and *threo*-flucetosulfuron by HLMs, the reaction mixtures with 0.16 mg/ml HLMs and various concentrations of each flucetosulfuron isomer were made up to the final volume of 250 μL and incubated for 30 minutes at 37℃. The concentrations of each flucetosulfuron isomer were 10, 20, 100, 200, 1000, 5000, and 10000 μM, respectively. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.5 Enzyme kinetics by HLMs

Various concentration of each flucetosulfuron isomer (10, 20, 100, 200, 1000, 5000 and 10000μM) was incubated with 0.16 mg/ml HLMs for 30 min at 37°C. Additionally, the percentages of formation of each metabolite from 100 μM of each flucetosulfuron isomer under optimized incubation conditions were tested, respectively. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.6 Identification of esterases by esterase selective inhibitors

Incubation mixtures containing 0.16 mg/mL HLMs and one of the following esterase-selective inhibitors [bis-nitrophenylphosphate (BNPP) (10, 100 μM), calcium chloride (1000 μM), cobalt chloride (1000 μM), dibucaine (100 μM), EDTA (3000 μM), eserine (10, 100 μM), magnesium chloride (1000 μM), mercuric chloride (100, 200 μM), and quinidine (100 μM)] were pre-incubated for 5 min at 37°C. Esterase-selective inhibitors were listed in Table 13.

Table 13. Esterase selective inhibitors

Inhibitors	Sensitive esterases	Concentration in reaction mixture	Reference
Ca^{2+} (Activator)	A-esterase	100 μM	Iatsimirskaia *et al.* 1997
CO^{2+}	A-esterase	1000 μM	Erdös *et al.* 1959
Mg^{2+}	A-esterase	1000 μM	Erdös *et al.* 1959, Lorentz *et al.* 1979
EDTA	A-esterase	3000 μM	Gonzalvo *et al.* 1997, Tang and Chambers 1999
Hg^{2+}	General esterase (-SH group)	100, 200 μM	Okada and Wakabayashi 1988, Dean *et al.* 1991
BNPP	Carboxylesterase	10, 100 μM	Okada and Wakabayashi 1988, McCracken *et al.* 1993b
Eserine	Cholinesterase	10, 100 μM	Ecobichon and Kalow 1963, McCracken *et al.* 1993a
Dibucaine	Cholinesterase	100 μM	Kalow and Genest 1957, Nigg *et al.* 1996
Quinidine	Cholinesterase	100 μM	Thakar *et al.* 1985

The reactions were initiated by adding each flucetosulfuron isomer (100 μM) to make total volume 250 μL and incubated for a further 30 min at 37°C in a shaking water bath. The reactions were terminated by adding 250 μL of acetonitrile. The samples were centrifuged at 10,770 x g for 5min at 4°C, and 10 μL of supernatant was analyzed by HPLC to quantify the form TM1 or EM1.

The percentage inhibition was calculated using the ratio of the amount of metabolite (TM1 or EM1) formed in the presence of the specific inhibitor to the amount formed under control conditions in the absence of inhibitor.

2.7 Enzyme kinetics of flucetosulfuron isomers by human carboxylesterases

The 0.16 mg/ml of each human carboxylesterase, CES1b, CES1c and CES2 was incubated with each flucetosulfuron isomer (100 μM) for 30 min at 37℃ (final volume of 250 μL). The test concentration of the human carboxylesterase was the same as that of HLMs which were recommended by supplier, BD Gentest Co. (Woburn, MA). For the enzyme kinetic studies, an increasing concentration of each flucetosulfuron isomer (10, 20, 100, 200, 1000, 5000, 10000 μM) was incubated with each human carboxylesterase, CES1b, CES1c and CES2 for 30 min at 37 ℃. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.8 Enzyme kinetics of flucetosulfuron isomers by Human Acetylcholinesterase

The 8.0 ug/mL of human acetylcholinesterase was incubated with each flucetosulfuron isomer (100 μM) for 30 min at 37℃ (final volume of 250 μL). The test concentration of the human acetylcholinesterase was recommended by supplier, Sigma-aldrich. For the enzyme kinetic studies, various concentration of each flucetosulfuron isomer (10, 20, 100, 200, 1000, 5000, 10000 μM) was incubated for 30 min at 37°C. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.9 Enzyme kinetics of flucetosulfuron isomers by Human Butyrylcholinesterase

The 23.4 ug/ml of human acetylcholinesterase was incubated with each flucetosulfuron isomer (100 μM) for 30 min at 37℃ (final volume of 250 μL). The test concentration of the human acetylcholinesterase was recommended by supplier, Sigma-aldrich.

For the enzyme kinetic studies, various concentration of each flucetosulfuron isomer (10, 20, 100, 200, 1000, 5000, 10000 μM) was incubated for 30 min at 37°C. The quantity of the metabolite formed in the reaction mixture was determined by HPLC analysis by injecting 10 μL.

2.10 Data analysis

The kinetic parameters of each flucetosulfuron isomer (V_{max} and K_m) were obtained by fitting a one-enzyme Michaelis-Menten equation, using Minitab 16 software. The CL_{int} was calculated as the V_{max}/K_m ratio. The calculations were performed using Minitab 16. The percentages of inhibition were calculated by the ratio of the amounts of metabolites formed with the specific inhibitor. For the kinetic study of HLMs and carboxylesterase, acetylcholinesterase, and butyrylcholinesterase, a simple Michaelis-Menten model was fitted to the formation rate of TM1 and EM1 to estimate the enzyme kinetic parameters.

Ⅲ. Results and Discussion

1. Preparation of flucetosulfuron isomers

To elucidate metabolism of flucetosulfuron by *in vitro* HLMs, the analytical method for flucetosulfuron was developed. During the method development of flucetosulfuron, there were two peaks of flucetosulfuron in the chromatogram of flucetoslufron technical. The facts that flucetosulfuron technical consists of *threo*- and *erythro*-flucetosulfuron and they could be separated on HPLC have been already known in the analytical method of flucetosulfuron (KOPAC 2009). To study *in vitro* metabolism of flucetosulfuron and compare the kinetic patterns of both flucetosulfuron isomers, they were necessarily required to be separated from flucetosulfuron technical.

First of all, the synthetic process through the patent (Kim et al. 1994) was tried to produce each enantiopure isomer. But mixture of flucetosulfuron isomers was synthesized and the purification process was additionally needed to get each the enantiopure isomer of flucetosulfuron.

Through the above analytical method of flucetosulfuron (KOPAC 2009), *threo*- and *erythro*-isomers were separated by the isocratic mobile phase with the retention time of 19 min and 23 min, respectively (Figure 10).

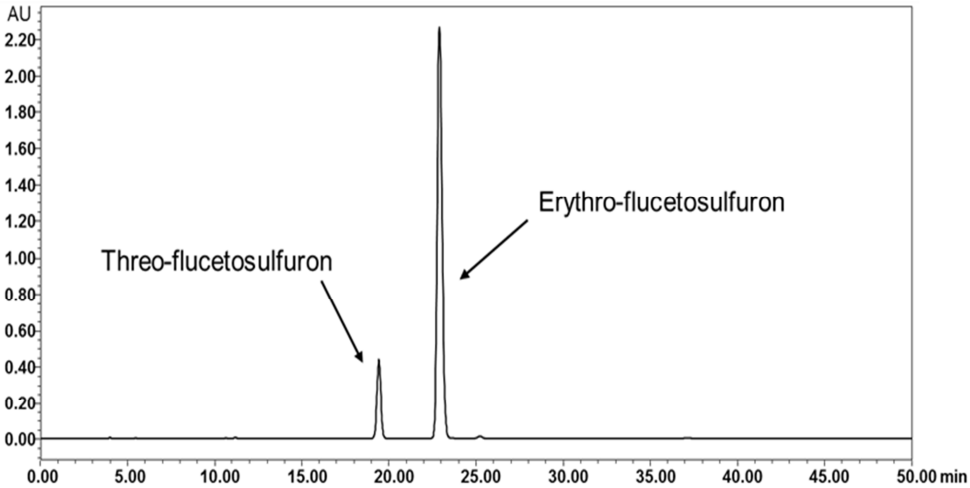


Figure 10. Chromatogram of flucetosulfuron by HPLC

1.1 Purification of *threo*-flucetosulfuron

To separate and collect each isomer of flucetosulfuron form flucetosulfuron technical, Waters Delta Prep-4000 Preparative Chromatography system coupled with UV 4890 detector was applied. After several trial and errors for developing the separation method to acquire both enantiopure isomers, *erythro*-flucetosulfuron and *threo*-flucetosulfuron were clearly separated with the retention time of 84 min and 100 min in the chromatogram of Preparative HPLC (Prep-HPLC), respectively (Figure 11). Each fraction was concentrated and gathered to produce the each enantiopure isomer.

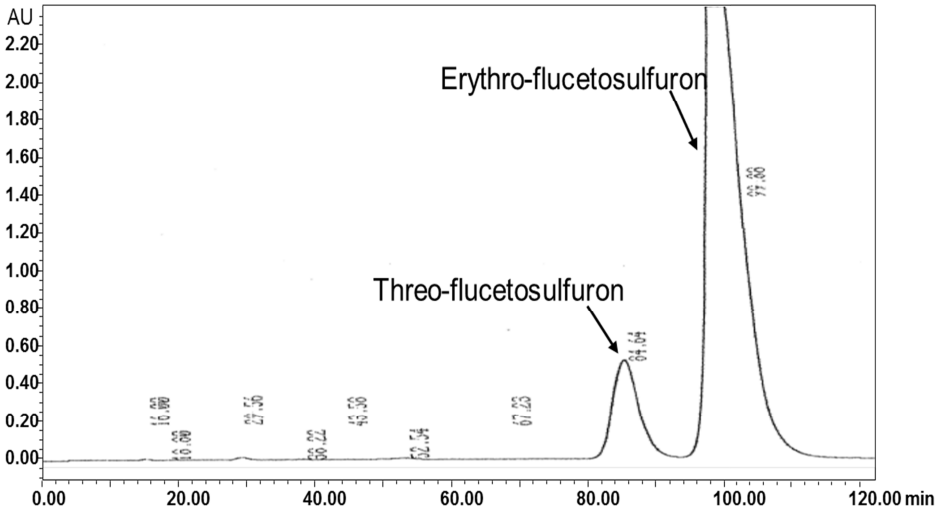


Figure 11. Chromatogram of flucetosulfuron by Prep-HPLC

After one cycle of the fractionation/purification, 2.6 ~ 4.0 mg of *threo*-flucetosulfuron was obtained. About 0.3g of *threo*-isomer was gathered from several repeat of the separation processes. To confirm the identity of *threo*-flucetosulfuron, LC-MS/MS analysis and ^{1}H and ^{13}C NMR were carried out. The purity of *threo*-isomer was also measured by quantitative analysis with working standard of flucetosulfuron. The MS/MS spectra of *threo*-flucetosulfuron ($[M+H]^{+}$ = m/z 488) showed fragmentation ions at m/z 156, 182, and 333 in Figure 12. From the MS/MS analysis, fragmentation ion at m/z 156 was assigned to positive ion of 4,6-dimethoxypyrimidin-2-amine, which could be formed by cleavage of sulfonylurea bridge. Fragmentation ions at m/z 182 and 333 were estimated to be cation forms of pyrimidine

moiety and sulfonylamide moiety, having carbonyl group by cleavage of urea brigde, respectively.

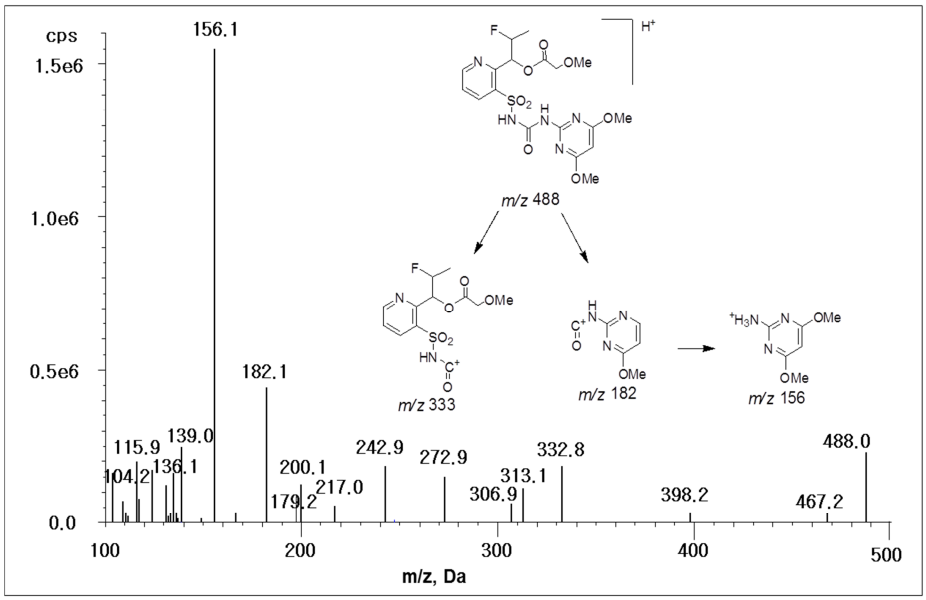


Figure 12. MS/MS Spectra and fragmentation of threo-flucetosulfuron isomer ([M+H]+ = m/z 488)

^{1}H NMR data and chemical structures of *threo*-flucetosulfuron are shown in Figure 13. In ^{1}H NMR of *threo*-fulcetosulfuron, Three proton signals at δ 8.83 (1H), 7.51 (1H), and 8.68 (1H) (having same coupling constant to each other) and one proton signal at 5.80(1H) were assigned to the pyridine ring and pyrimidine ring, respectively. Two proton signals at δ 7.31(NH) and 13.14 (NH) were assigned to urea bridge, adjacent to pyrimidine ring and sulfonyl moiety, respectively. One proton signals at δ 6.67 (1H), one proton at 5.26 (1H), and one proton at 1.28 (1H), indicating coupling with fluorine,

were assigned to proton on methine carbon, proton on carbon having fluorine, and methyl group, respectively. Two protons signal at δ 4.05 (2H) was assigned to ester bridge of methoxyacetyl moiety. Six protons at δ 3.96 (6H) and three protons at 3.98 (3H) were assigned to methoxy group of pyrimidine ring and methoxy acetyl moiety, respectively.

^{13}C NMR data and chemical structures of *threo*-flucetosulfuron are shown in Figure 14. The signals in ^{13}C NMR for *threo*-flucetosulfuron were assigned as follows: δ 140.9 (C1), 153.2 (C2), 148.7 (C4), 134.6 (C5), 123.4 (C6), 85.3 (C7), 170.5 (C8, C12), 153.5 (C10), 155.2 (C14), 73.7 (C18), 90.4 (C19), 16.7 (C21), 169.4 (C23), 69.2 (C24), 54.7 (C26, 27), and 59.0 (C28).

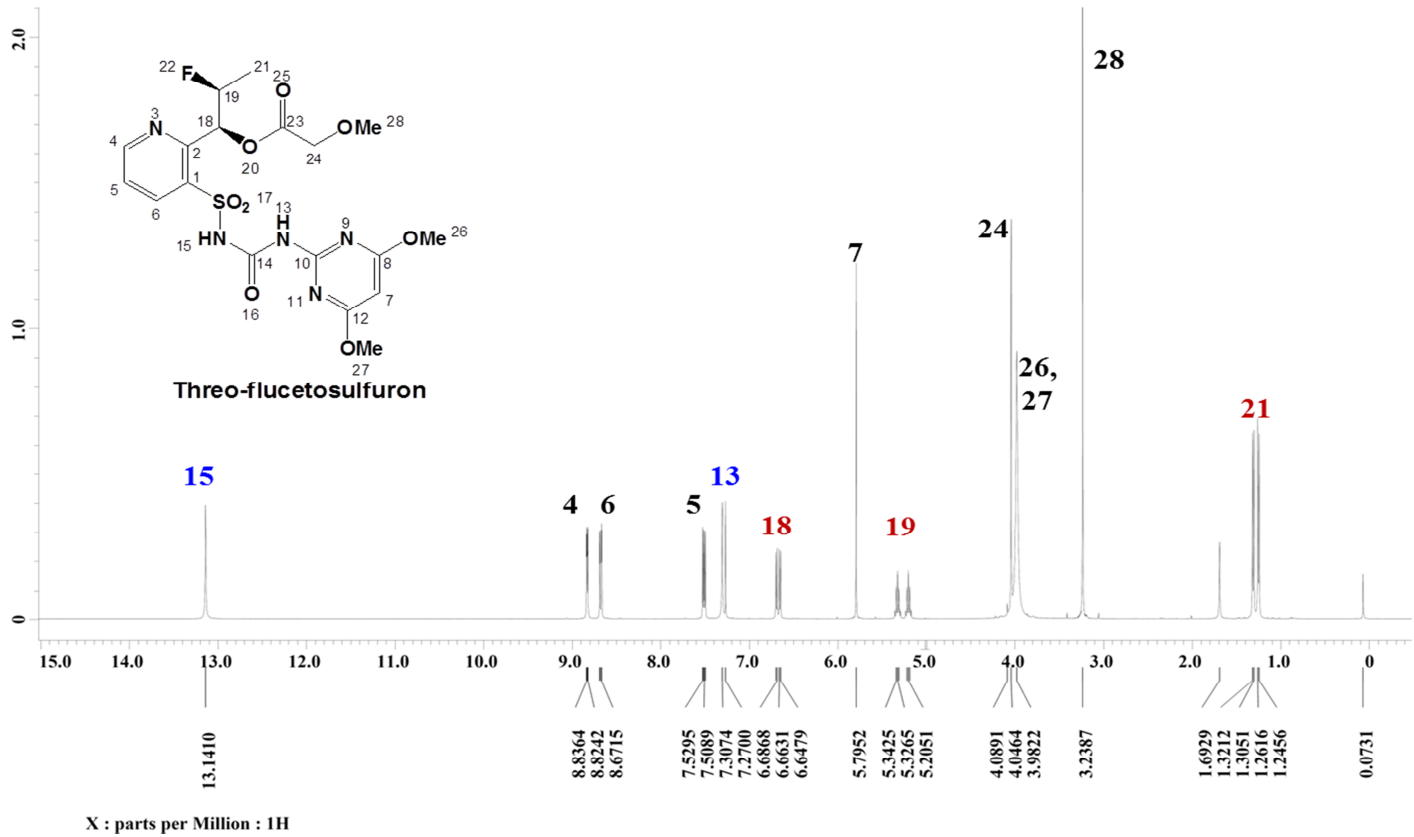


Figure 13. ^{1}H NMR of *threo*-flucetosulfuron isomer

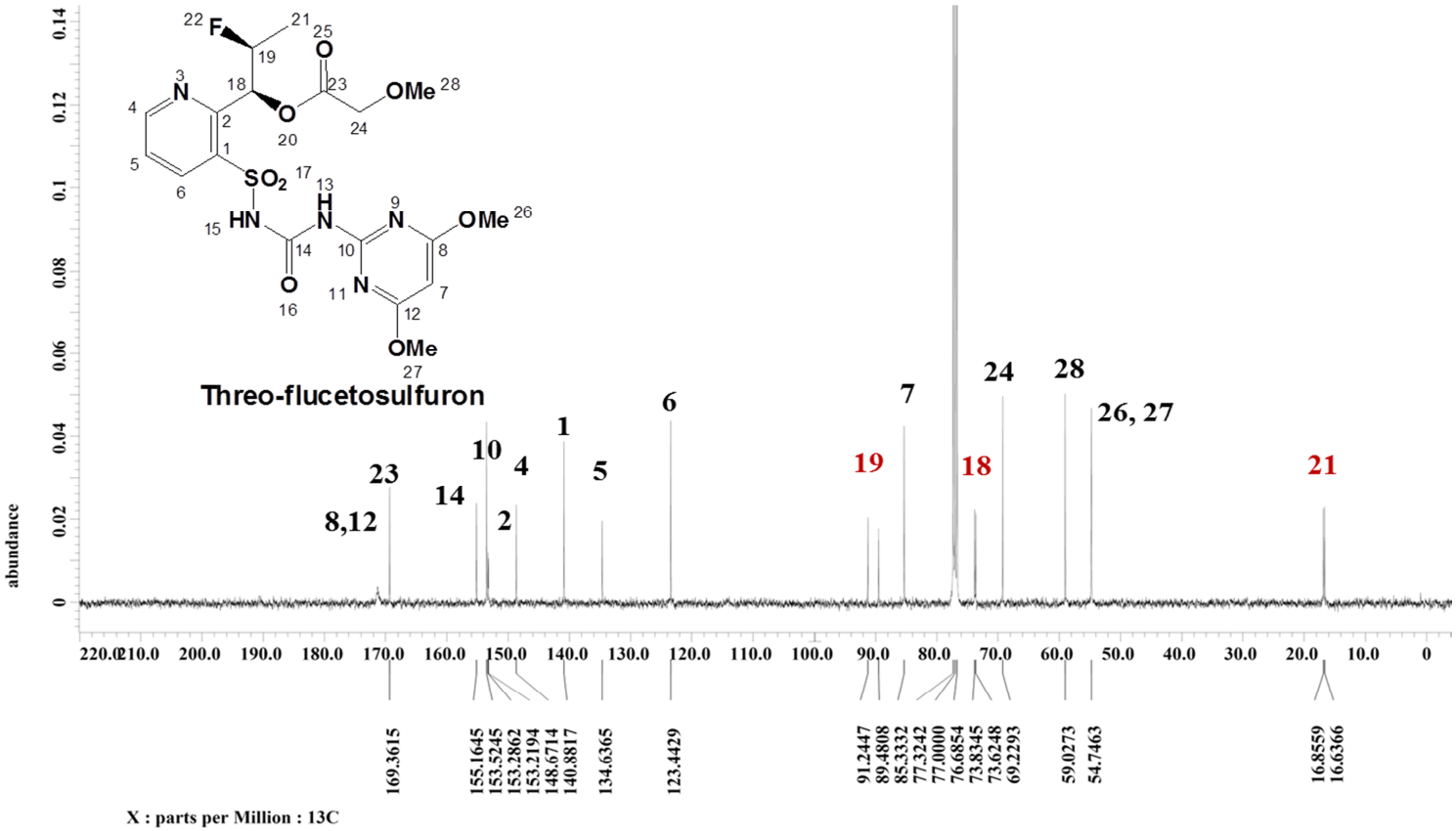


Figure 14. ^{13}C NMR of *threo*-flucetosulfuron isomer

1.2 Purification of *erythro*-flucetosulfuron

After the same procedure as for *threo*-flucetosulfuron, 23 ~ 26 mg of *erythro*-flucetosulfuron was obtained from one cycle of the fractionation/purification process. After several repeat of the same processes, about 2.0 g of *erythro*-isomer was obtained.

To confirm the identity of *erythro*-flucetosulfuron, LC-MS/MS analysis and ^{1}H and ^{13}C NMR were also carried out. The purity of *threo*-isomer was measured by quantitative analysis with working standard of flucetosulfuron, too. The MS/MS spectra of *erythro*-flucetosulfuron gave the same $[M+H]^{+}$ at m/z 488 and showed fragmentation ions at m/z 156, 182, and 333 as the same pattern of *threo*-isomer.

^{1}H NMR data and chemical structures of *erythro*-flucetosulfuron are shown in Figure 15. In ^{1}H NMR of *erythro*-fulcetosulfuron, three proton signals at δ 8.81 (1H), 7.50 (1H), and 8.64 (1H) (having same coupling constant to each other) and one proton signal at 5.78(1H) were assigned to the pyridine ring and pyrimidine ring, respectively. Two proton signals at δ 7.31(NH) and 13.29 (NH) were assigned to urea bridge, adjacent to pyrimidine ring and sulfonyl moiety, respectively. One proton signals at δ 6.67 (1H), one proton at 5.17 (1H), and one proton at 1.46 (1H), indicating coupling with fluorine, were assigned to proton on methine carbon, proton on carbon having fluorine, and methyl group, respectively. Two protons signal at δ 4.07 (2H) was assigned to ether bridge of methoxyacetyl moiety. Six protons at δ 3.98 (6H) and three protons at 3.29 (3H) were assigned to methoxy group of pyrimidine ring and methoxy acetyl moiety, respectively.

^{13}C NMR data and chemical structures of *erythro*-flucetosulfuron are shown in Figure 16. The signals in ^{13}C NMR for *erythro*-flucetosulfuron

were assigned as follows: δ 140.6 (C1), 153.1 (C2), 148.6 (C4), 134.6 (C5), 123.3 (C6), 85.3 (C7), 170.5 (C8, C12), 153.3 (C10), 155.3 (C14), 73.5 (C18), 89.7 (C19), 15.3 (C21), 169.4 (C23), 69.3 (C24), 54.7 (C26, 27), and 59.1 (C28).

The NMR data of flucetosulfuron isomers, *threo*-flucetosulfuron and *erythro*-flucetosulfuron are summarized in Table 14. On the basis of MS/MS spectra and NMR data, both *threo*- and *erythro*-flucetosulfuron were confirmed unambiguously as 1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl 2-methoxyacetate.

There were slight differences in the chemical shift values between two flucetosulfuron isomers in ^{1}H NMR and ^{13}C- NMR, whereas the exact confirmation of their chirality could not be revealed. Additionally to confirm a precise molecular conformation, i.e. the three-dimensional structure of flucetosulfuron isomers, NOE was carried out in two solvent systems, DMSO and $CDCl_3$, respectively. NOE was known to be useful in NMR spectroscopy for characterizing organic chemical structures and NOE, two-dimensional NMR spectroscopy could be used to determine the three-dimensional structures of biological macromolecules in solution (Anet and Bourn 1965). Theoretically it is also known that *"as the NOE differs from the application of spin-spin coupling in that the NOE occurs through space, not through chemical bonds, atoms that are in close proximity to each other can give a NOE, whereas spin coupling is observed only when the atoms are attached by more than two chemical bonds*" (Knowles 2002). But, NOE analysis could not give any supporting data to determine exact configurations of *threo*- or *erythro*- flucetosulfuron.

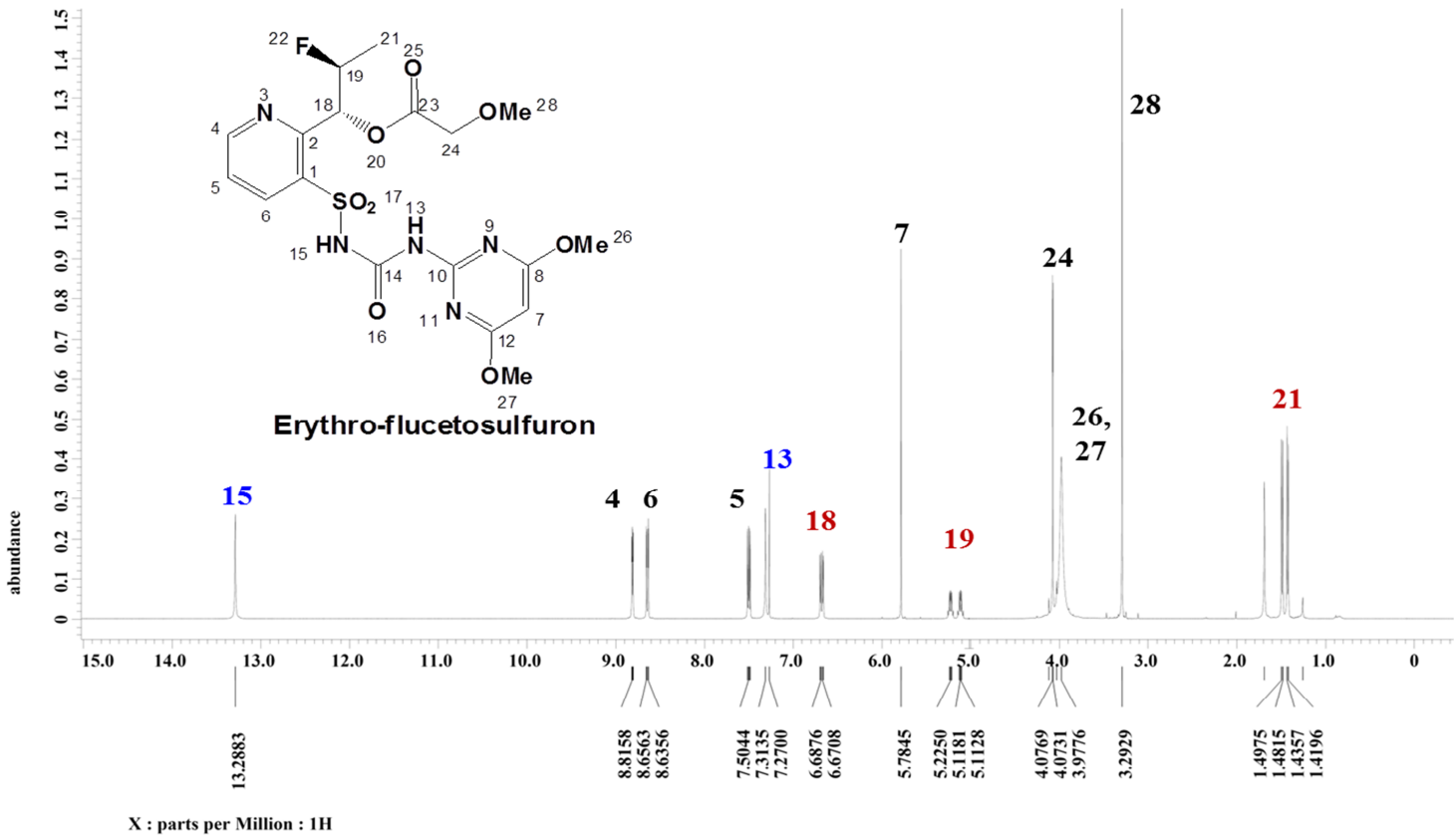


Figure 15. ^{1}H NMR of *erythro*-flucetosulfuron isomer

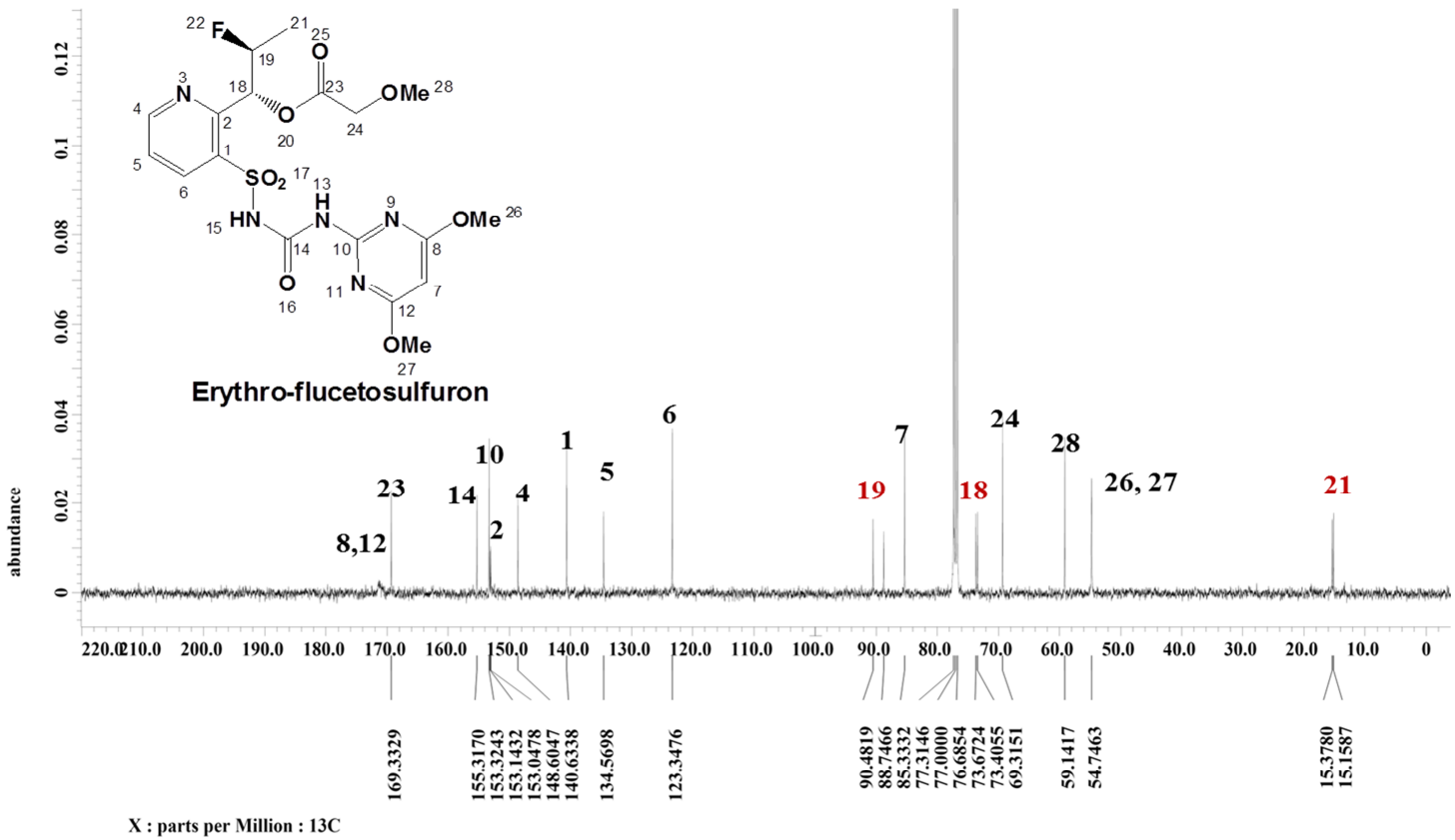


Figure 16. ^{13}C NMR of *erythro*-flucetosulfuron isomer

Table 14. ^{1}H and ^{13}C NMR data for *threo*- and *erythro*-flucetosulfuron

	^{1}H NMR Data			
	Threo-flucetosulfuron		*Erythro*-flucetosulfuron	
Proton	δ ($CDCl_3$)	*J* (Hz)	δ ($CDCl_3$)	*J* (Hz)
4	8.83 (dd)	1.5, 4.8	8.81 (dd)	1.5, 4.6
5	7.51 (dd)	4.7, 8.2	7.50 (dd)	4.6, 7.9
6	8.68 (dd)	1.5, 8.2	8.64 (dd)	1.5, 8.2
7	5.80 (s)		5.78 (s)	
13[a]	7.31 (s)		7.31 (s)	
15[a]	13.14 (s)		13.29 (s)	
18[b]	6.67 (dd)	6.1, 15.6	6.67 (1H, dd)	4.0, 10.7
19[b]	5.26 (m)		5.17 (m)	
21[b]	1.28 (3H, dd)	6.4, 23.8	1.46 (3H, dd)	6.1, 24.7
24	4.05 (2H, s)		4.07 (2H, s)	
26,27	3.98 (6H, br s)		3.98 (6H, br s)	
28	3.24 (3H, s)		3.29 (3H, s),	
	^{13}C NMR Data			
	Threo-flucetosulfuron		*Erythro*-flucetosulfuron	
Carbon	δ ($CDCl_3$)	*J* (Hz)	δ ($CDCl_3$)	*J* (Hz)
1	140.9		140.6	
2	153.2		153.1	
4	148.7		148.6	
5	134.6		134.6	
6	123.4		123.3	
7	85.3		85.3	
8, 12	170.5		170.5	
10	153.5		153.3	
14	155.2		155.3	
18[c]	73.7 (d)	21.0	73.5 (d)	26.7
19[c]	90.4 (d)	176.4	89.7 (d)	173.5
21[c]	16.7 (d)	21.9	15.3 (d)	21.9
23	169.4		169.4	
24	69.2		69.3	
26,27	54.7		54.7	
28	59.0		59.1	

[a]NH. [b]H-F coupling. [c]C-F coupling.

Each isomer was tried to form a crystal for X-ray crystallography to see the exact configuration depending on their chirality. But, it was also unfruitful works because they are unstable in volatile solvents.

The acquired enantiopure isomers, *threo*-flucetosulfuron and *erythro*-flucetosulfuron were measured by HPLC analysis, respectively (Figure 17).

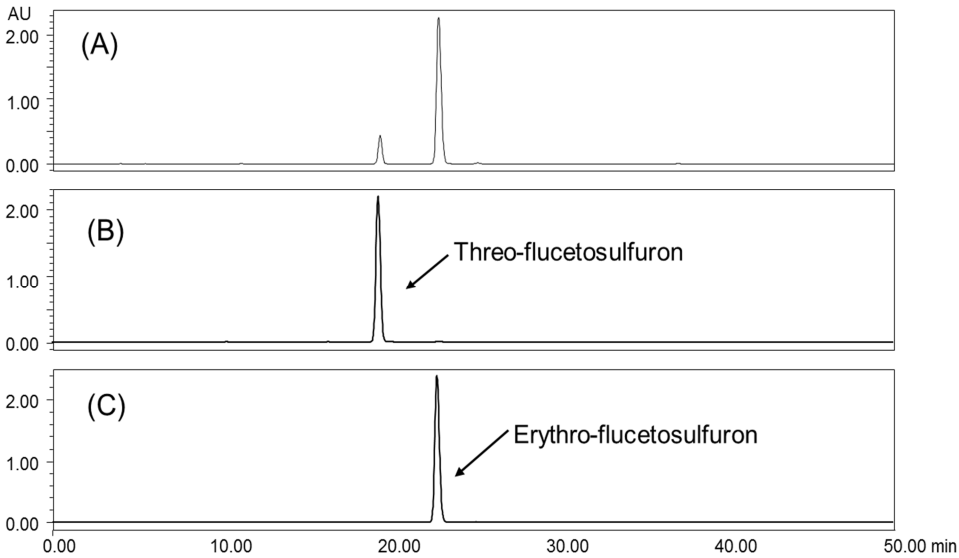


Figure 17. Chromatogram of flucetosulfuron technical (A), the obtained 99.8% *threo*-flucetosulfuron (B) and 99.7% *erythro*-flucetosulfuron (C)

The purity of the acquired *threo*-flucetosulfuron ((1S,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate) and *erythro*-flucetosulfuron ((1R,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetate) was measured as 99.8% and 99.7% by Prep-HPLC method, respectively.

2. *In vitro* metabolism of flucetosulfuron by HLMs

2.1 Analysis of metabolite of flucetosulfuron in reaction mixture

To see if there is metabolic reaction in the incubation mixture of flucetosulfuron by HLMs, *Threo*-flucetosulfuron and *erythro*-flucetosulfuron, which were isolated using preparative HPLC, were incubated with HLMs in the presence of NADPH generating system (Figure 18).

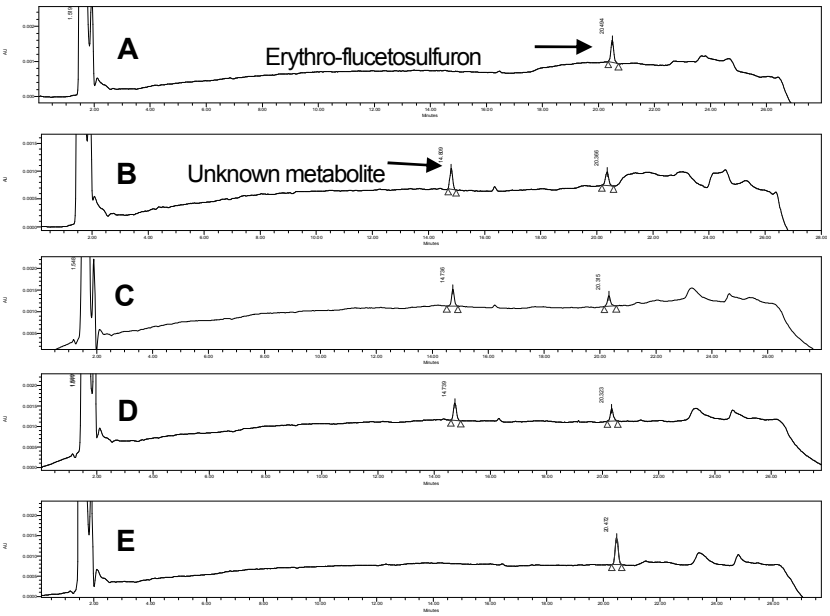


Figure 18. Chromatograms showing the formation of metabolite from *erythro*-flucetosulfuron after the reaction by HLMs

A: Prior to the microsomal reaction

B: After the microsomal reaction with NADPH generating systems

C: After the microsomal reaction without NADPH generating systems

D: After the microsomal reaction with the HLMs heated for 1min at 50°C and NADPH generating systems

E: After the microsomal reaction with the HLMs boiled for 5mins at 100°C and NADPH generating systems

The formation of *erythro*-flucetosulfuron metabolite in the presence of NADPH generating system by HLMs demonstrated that there is a metabolic reaction in incubations of *erythro*-flucetosulfuron by HLMs (Figure 18B).

To check whether CYP450s mediate the microsomal reaction of flucetosulfuron in HLMs, the microsomal reaction was tested without NADPH generating system, because it is known that metabolic reaction by CYP450 is NADPH-dependent (Guengerich 1993). However, HLMs without NADPH generating system also produced the *erythro*-flucetosulfuron metabolite, demonstrating that the metabolic reaction occur regardless of NADPH generating system (Figure 18C). CYP450 was determined not to be the mediating enzyme of the microsomal reaction of flucetosulfuron.

Additionally, to confirm whether FMOs mediate the microsomal reaction of flucetosulfuron in HLMs, heated HLMs (50 ℃ for 1min) was tested in the presence of NADPH generating system, because FMOs are heat-labile and can be inactivated in the absence of NADPH by warming microsomes to 50℃ for 1min (Rose 2002). The metabolites were also formed in reactions using heated HLMs (Figure 18D). However, Heated HLMs with NADPH generating system also produced the *erythro*-flucetosulfuron metabolite, demonstrating that the metabolic reaction occur regardless of FMOs (Figure 18D). FMOs were also determined not to be the mediating enzyme of the microsomal reaction of flucetosulfuron.

As expected, denatured HLMs (100 ℃ for 5 min) did not produced the metabolite, suggesting that enzymatic reaction were responsible for the formation of metabolites from flucetosulfuron isomers (Figure 18E).

Unknown metabolites, Uk1 and Uk2 were observed in the *threo*-flucetosulfuron reaction mixture and *erythro*-flucetosulfuron reaction mixture, respectively in Figure 19. Uk1 and Uk2 eluting at 10.2min and 10.6min respectively were clearly separated by the applied HPLC system. There were no other metabolite peaks or interfering peaks in the control metabolic incubations.

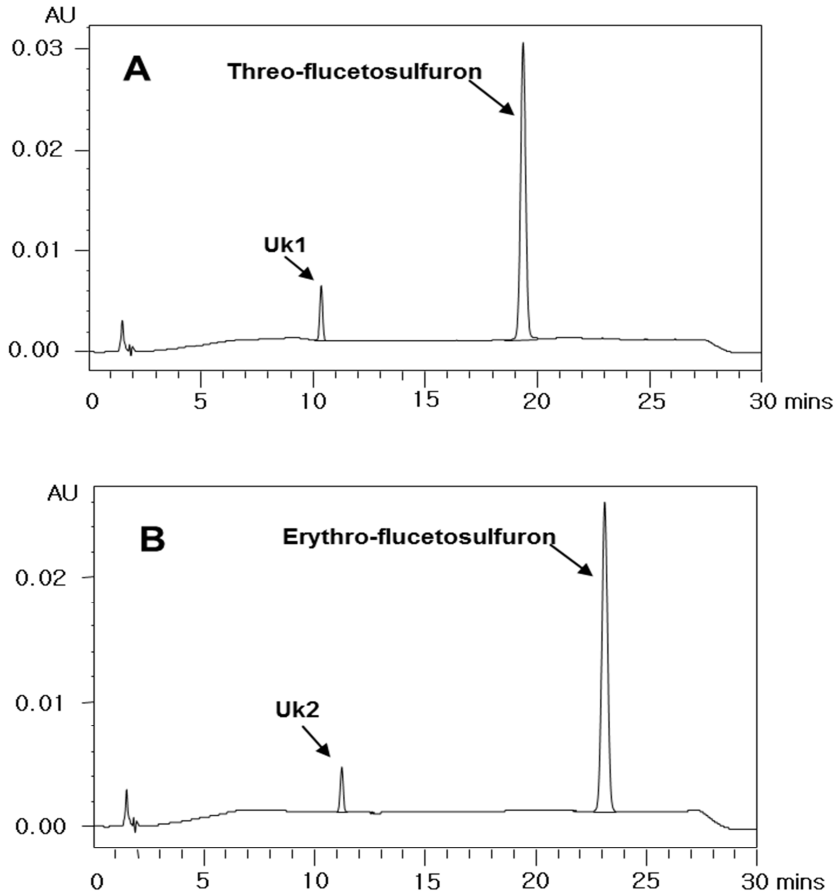


Figure 19. Formation of metabolite Uk1 from *threo*-flucetosulfuron (A) and Uk2 from *erythro*-flucetosulfuron (B) when flucetosulfuron isomers (100 µM) were incubated with 0.125 mg HLMs for 30 min at 37°C

2.2 Analysis of metabolite by LC-MS/MS

To identify the metabolites, the metabolic reaction mixtures were analyzed with LC-MS/MS. On LC-MS analysis, Uk1 and Uk2 gave the same $[M+H]^+$ at m/z 416, indicating a cleavage reaction, rather than insertion of oxygen, when compared with the parent flucetosulfuron isomers ($[M+H]^+$ = m/z 488). MS/MS analysis of flucetosulfuron ($[M+H]^+$ at *m/z* 488) in the microsomal reaction mixture showed fragment ions at *m/z* 156, 182, and 333 (Figure 20A), while the MS/MS spectrum of MI and M2 ($[M+H]^+$ = m/z 416) showed fragmentation ions at *m/z* 156, 182, and 261 (Figure 20B).

From these results, the metabolites were expected to be hydrolysis products, which could be formed by cleavage of the flucetosulfuron ester bond. The hydrolyzed metabolites tried to be synthesized.

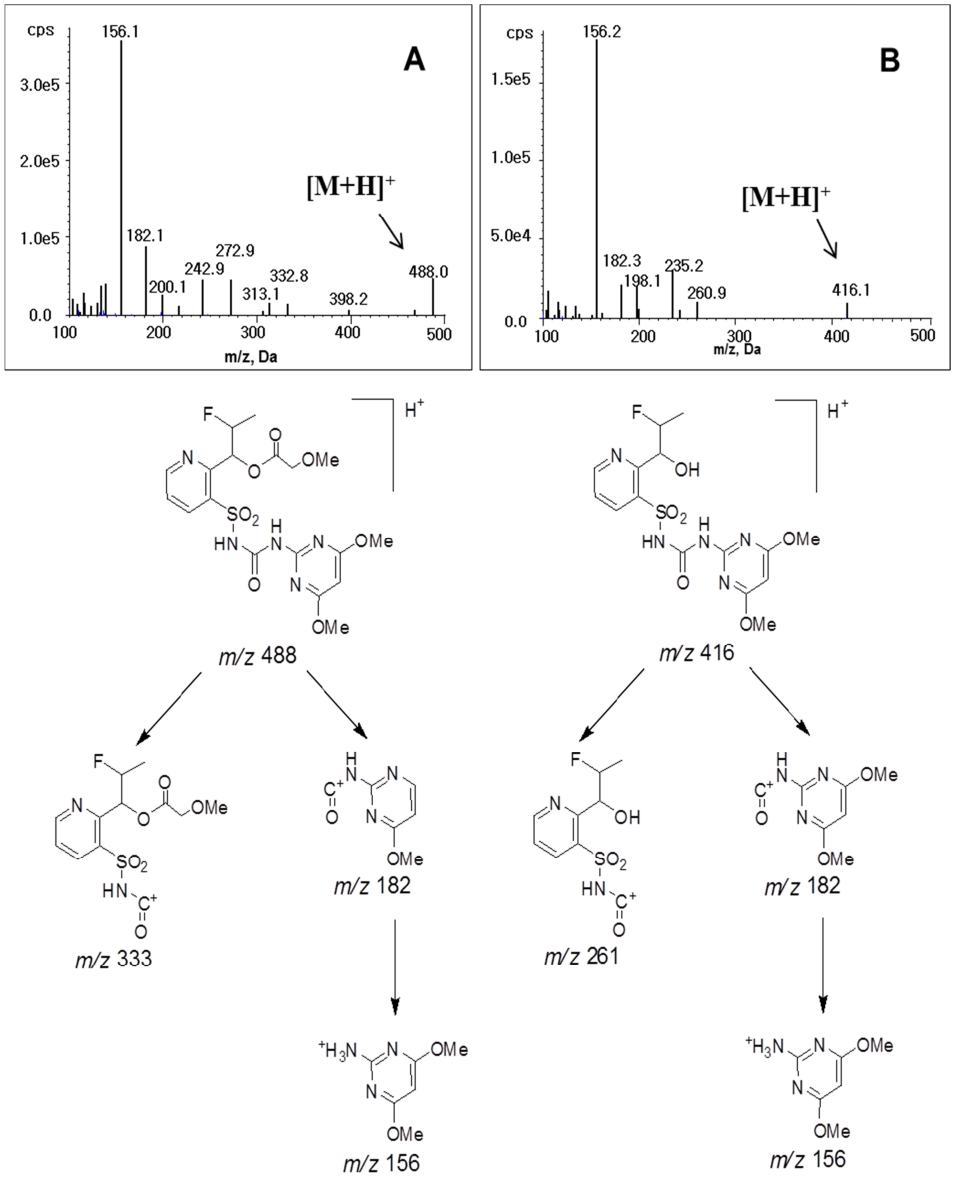


Figure 20. LC-MS/MS spectra and fragmentation of flucetosulfuron (A; $[M+H]^+$ = m/z 488) and its metabolite, Uk1 and Uk2 (B; $[MH]^+$ = m/z 416).

3. Synthesis of metabolites, TM1 and EM1

Since LC-MS/MS analysis indicated that Uk1 and Uk2 were expected to be hydrolysis products of the parent compounds, TM1 and EM1 were tried to be synthesized as the expected structure of Uk1 and Uk2, respectively.

They were synthesized by hydrolytic reaction from the purified *threo*-flucetosulfuron and *erythro*-flucetosulfuron. Both metabolites were also required not only to identify the Uk1 and Uk2 but also to measure the quantity of the formed metabolite in the metabolism of flucetosulfuron by HLMs.

3.1 Synthesis of TM1

TM1 was synthesized by hydrolysis of flucetosulfuron with base (NaOH). Acid is also a good hydrolyzing agent, but it can result in not only the hydrolysis of ester-bond but also the breakage of sulfonylurea bridge. The route for TM1 and EM1 from *threo*- and *erythro*-flucetosulfuron is the mechanism of base hydrolysis, in which ester bonds are hydrolyzed. Reaction time was traced to check the completeness of the reaction (Figure 21). The reaction for 30 min was insufficient to terminate the hydrolysis and 60 minutes was sufficient to terminate the reaction. But, to confirm the perfect hydrolysis of flucetosulfuron, 90min reaction time was finally performed. About 90mg of TM1 (MW 415.4: yield 70%) was obtained as a white powder. The MS/MS spectrum of TM1 ($[M+H]^+$ = m/z 416) showed fragmentation ions at m/z 156, 182, and 261 in Figure 22, indicating that the synthesized substance has the same molecular weight as TM1.

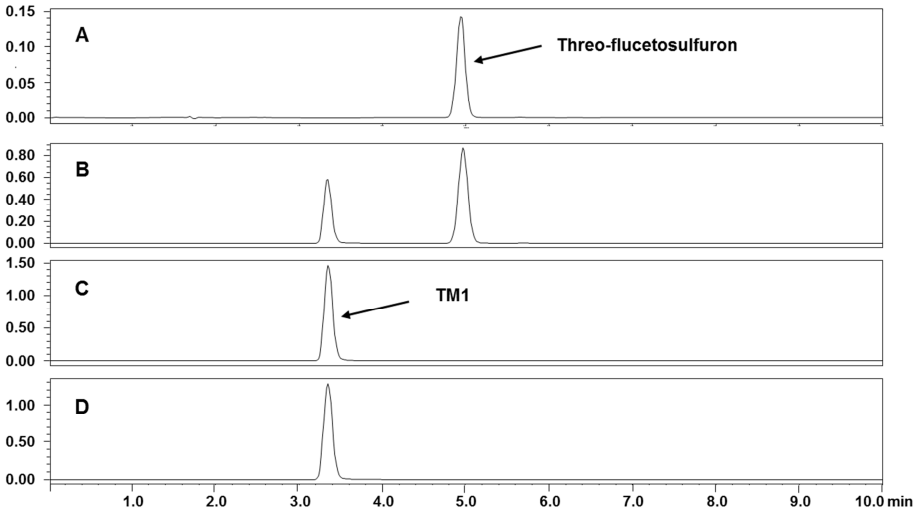


Figure 21. Chromatogram of metabolite-I from threo- flucetosulfuron depending on the reaction times; before reaction (A), 30 min later (B), 60 min later (C), and 90min later (D**)**

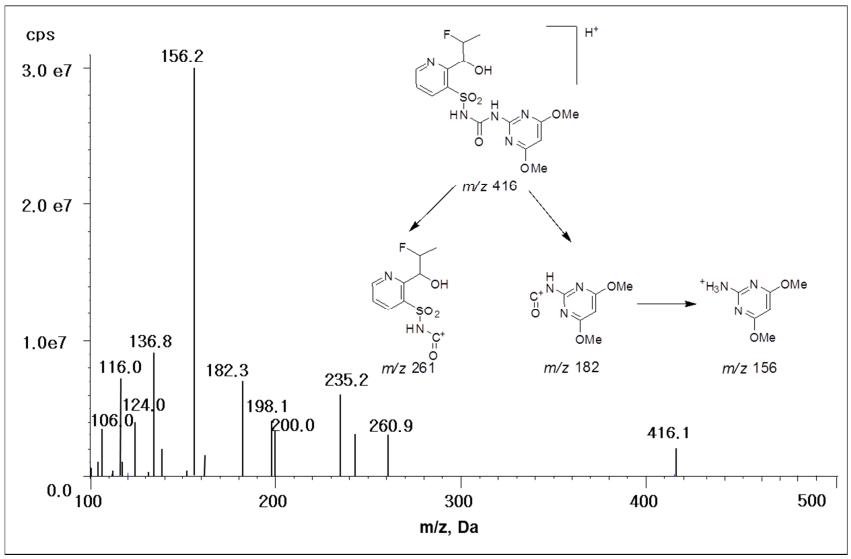


Figure 22. MS/MS Spectra and fragmentation of TM1and EM1 ($[M+H]^+$ = m/z 416)

^{1}H NMR data and chemical structures of TM1 are shown in Figure 23. In ^{1}H NMR, three proton signals at δ 8.83 (1H), 7.52 (1H), and 8.59 (1H), (having same coupling constant to each other) and one proton signal at 5.82(1H) were assigned to the pyridine ring and pyrimidine ring, respectively. Two proton signals at δ 7.27 (NH) and 12.99 (NH) were assigned to urea bridge, adjacent to pyrimidine ring and sulfonyl moiety, respectively. Two proton signals at δ 5.45 (1H) and 5.14 (1H) coupled with fluorine were assigned to proton on methine carbon and proton on carbon having fluorine, respectively. One proton signal at δ 4.39 (OH) and three protons at 1.47 (3H) were assigned to hydroxyl group and methyl group, respectively. Six protons at 3.96 (6H) were assigned to methoxy group of pyrimidine ring.

^{13}C NMR data and chemical structures of TM1 are shown in Figure 24. The signals in ^{13}C NMR for TM1 were assigned as follows: δ 140.6 (C1), 157.5 (C2), 148.7 (C4), 133.5 (C5), 123.3 (C6), 85.6 (C7), 172 (C8, C12), 152.7 (C10), 155.4 (C14), 72.7 (C18), 92.1 (C19), 17.7 (C21), and 55.0 (C25, C26).

On the basis of MS/MS spectra and NMR data, TM1 was confirmed unambiguously as N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)-2-(-2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide. Moreover, it was assigned N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)-2-((*1S,2S*)-2-fluoro-1-hydroxypropyl)-pyridine-3-sulfonamide, because this TM1 was synthesized by hydrolytic reaction from *threo*-flucetosulfuron having chiral center (*1S, 2S*).

The assay purity of TM1 was measured as 99.7 % by calculating the peak area in chromatogram of HPLC.

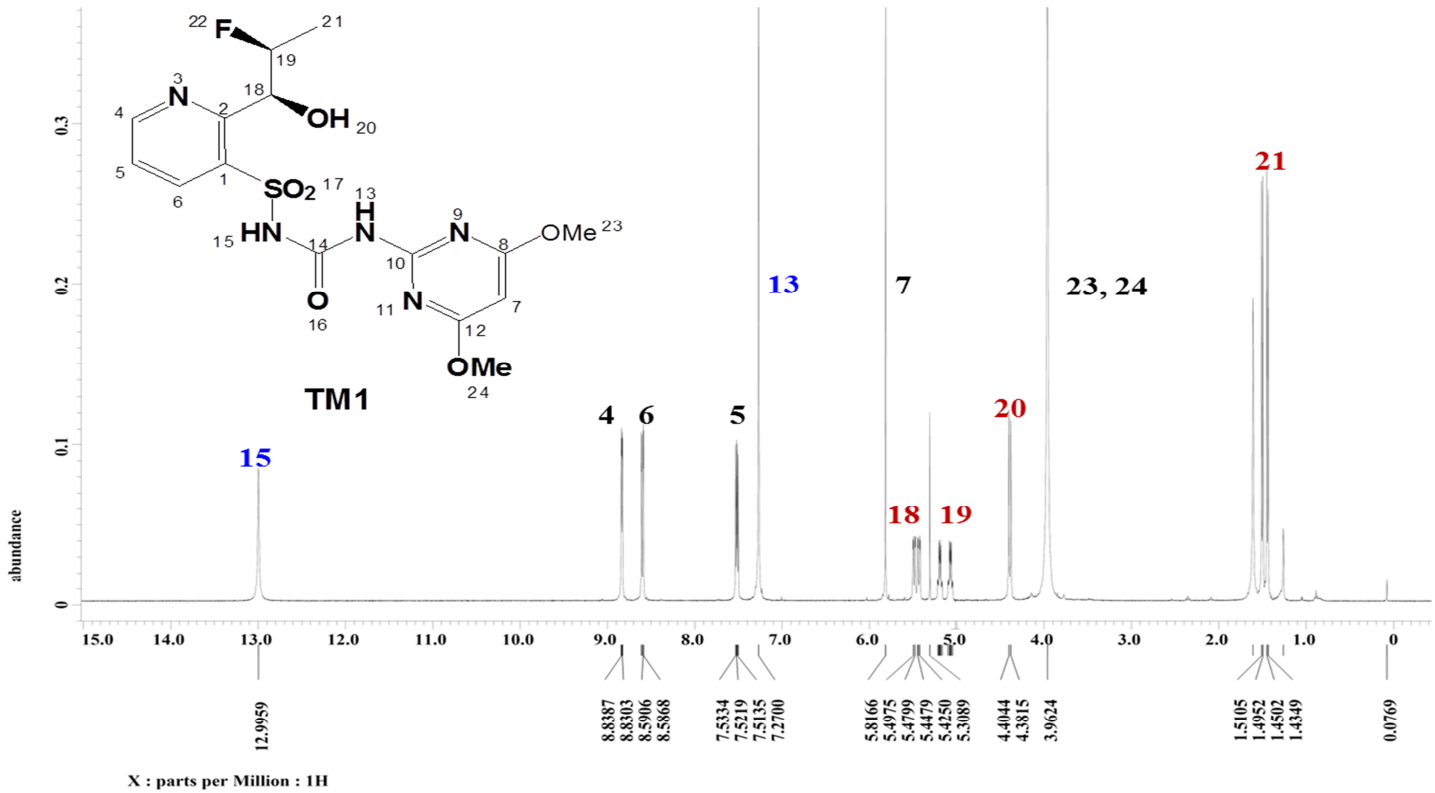


Figure 23. ^{1}H NMR of TM1

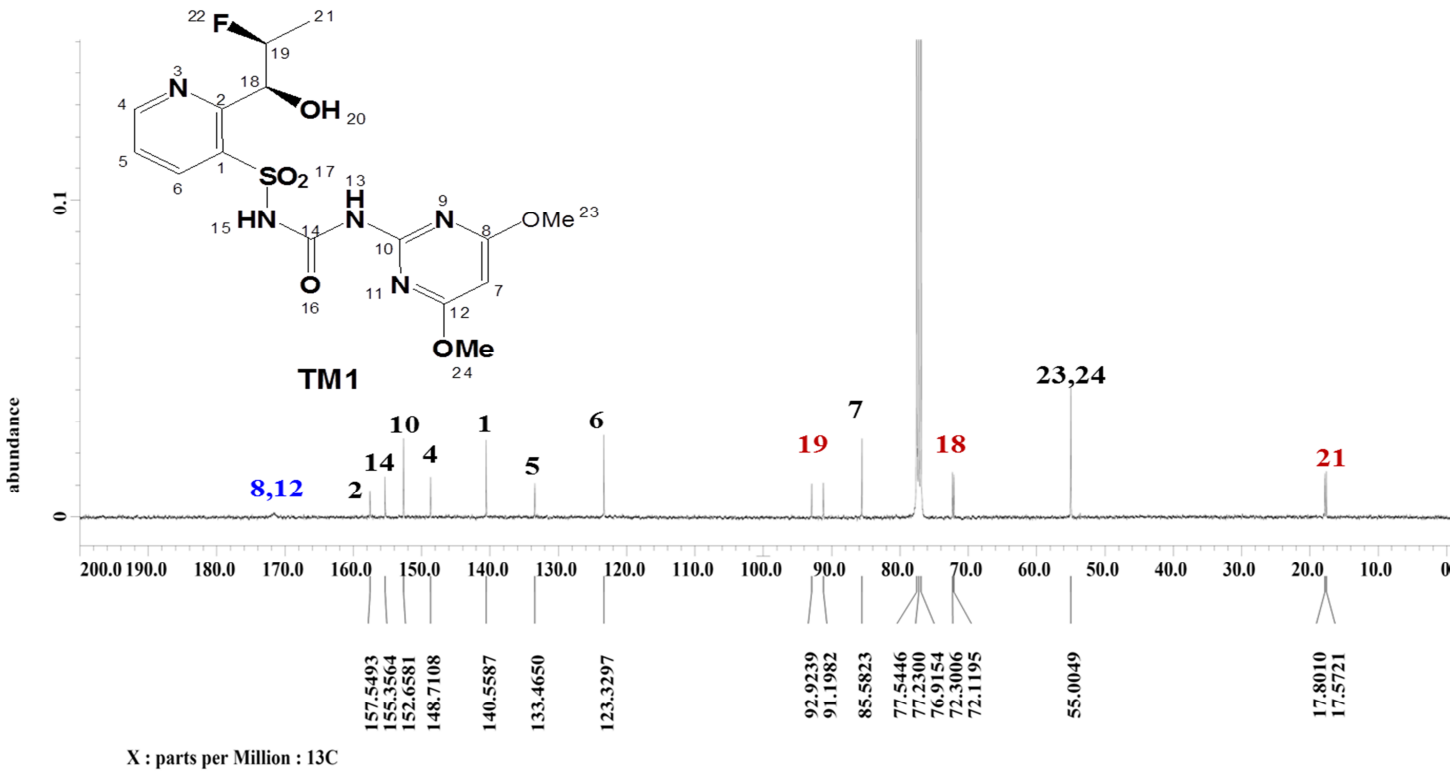


Figure 24. ^{13}C NMR of TM1

3.2 Synthesis of EM1

EM1 was also acquired by hydrolysis of flucetosulfuron with base. Reaction time was traced to check the completeness of the reaction, too (Figure 25). Reaction was terminated after 90min as done for the synthesis of TM1. About 370 mg of EM1 (MW 415.4: yield 89%) was obtained as a white powder.

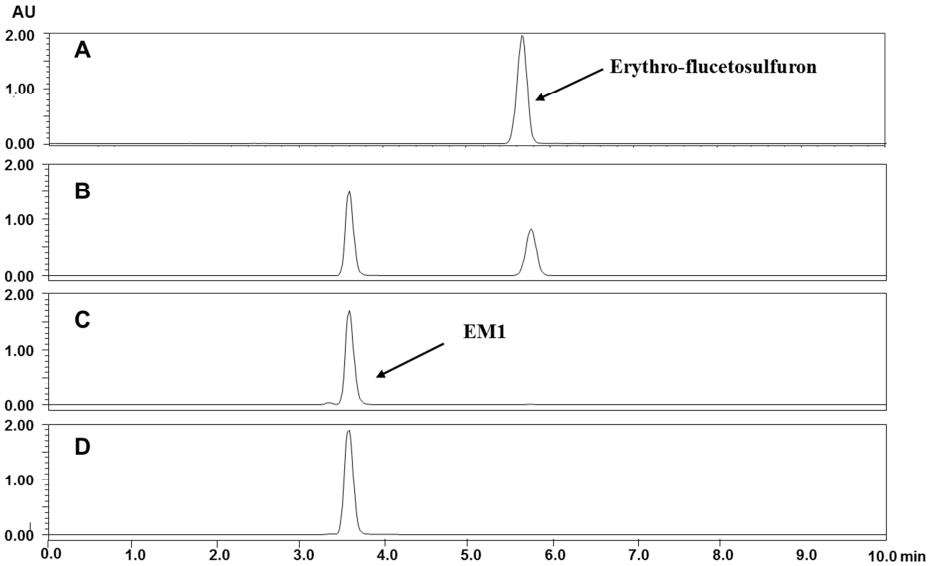


Figure 25. Chromatogram of metabolite-I from *erythro*- flucetosulfuron depending on the reaction times; before reaction (A), 30 min later (B), 60 min later (C), and 90min later (D)

The MS/MS spectrum of EM1 was the same as TM1 ($[M+H]^+$ = m/z 416) and the fragmentation ions appeared at m/z 156, 182, and 261, indicating that the synthesized substance is identical to EM1.

^{1}H NMR data and chemical structures of EM1 are shown in Figure 26. In ^{1}H NMR, three proton signals at δ 8.82 (1H), 7.52 (1H), and 8.67 (1H), (having same coupling constant to each other) and one proton signal at

5.80(1H) were assigned to the pyridine ring and pyrimidine ring, respectively. Two proton signals at δ 7.25 (NH) and 13.00 (NH) were assigned to urea bridge, adjacent to pyrimidine ring and sulfonyl moiety, respectively. Two proton signals at δ 5.65 (1H) and 4.83 (1H) were assigned to proton on methine carbon and proton on carbon having fluorine, respectively. One proton signal at δ 4.11 (OH) and three protons at 1.39 (3H) coupled with fluorine were assigned to hydroxyl group and methyl group, respectively. Six protons at 3.98 (6H) were assigned to methoxy group of pyrimidine ring.

^{13}C NMR data and chemical structures of EM1 are shown in Figure 27. The signals in ^{13}C NMR of EM1 were assigned as follows: δ 140.8 (C1), 157.5 (C2), 148.8 (C4), 134.5 (C5), 123.4 (C6), 85.6 (C7), 171 (C8, C12), 152.9 (C10), 155.4 (C14), 72.0 (C18), 92.6 (C19), 16.4 (C21), and 55.0 (C25, C26).

From these results, It was confirmed clearly as N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)-2-(-2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide, too. Finally, EM1 was assigned N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)-2-((*1R,2S*)-2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide, because this EM1 was synthesized by hydrolytic reaction from *erythro*-flucetosulfuron having chiral center (*1R, 2S*).

The assay purity of EM1 was measured as 96.5% by calculating the peak area in chromatogram of HPLC.

The NMR data of TM1 and EM1 are summarized in Table 15.

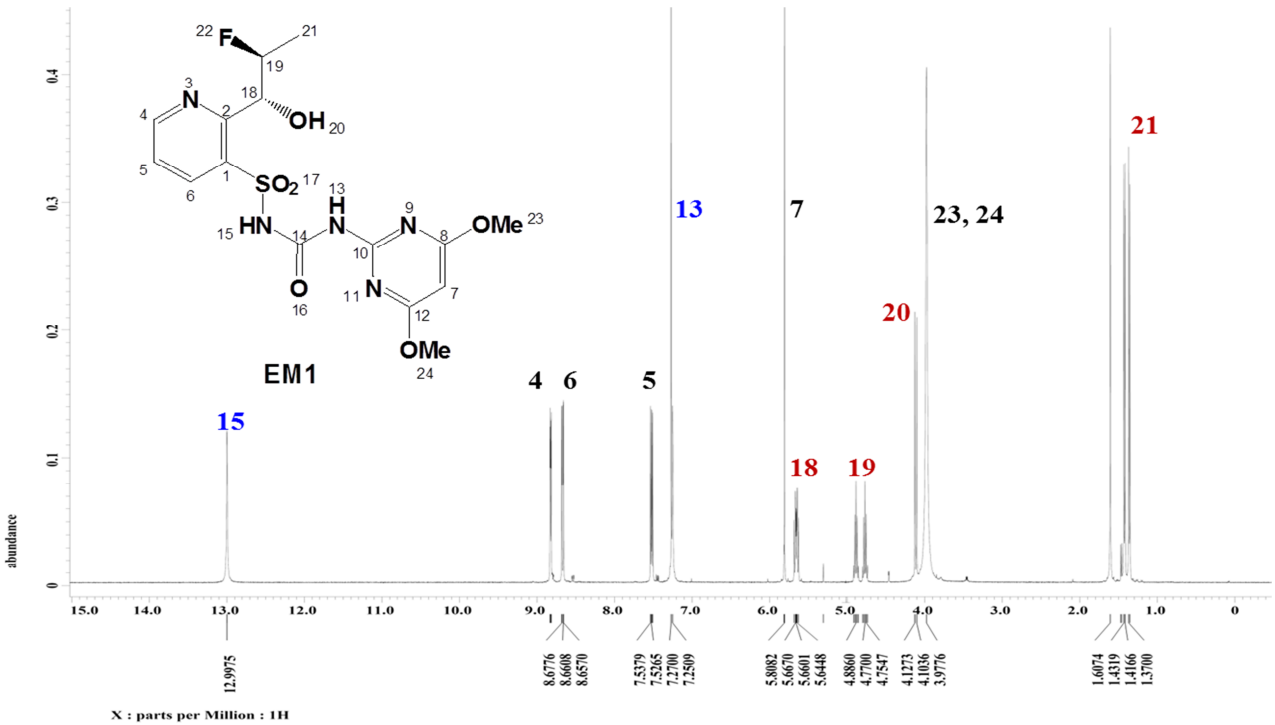


Figure 26. ^{1}H NMR of EM1

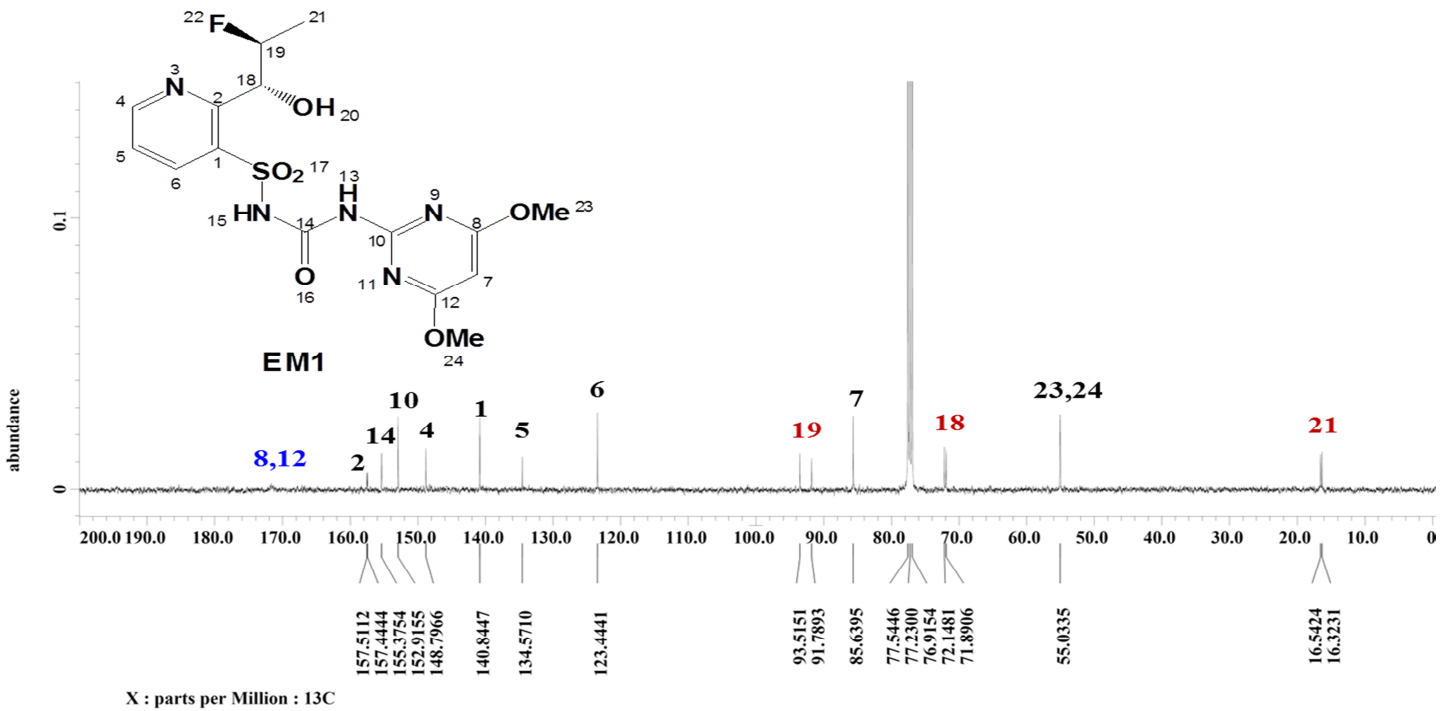


Figure 27. ^{13}C NMR of EM1

Table 15. ^{1}H and ^{13}C NMR data for TM1 and EM1

	^{1}**H NMR Data**			
	TM1		EM1	
Proton	δ ($CDCl_3$)	*J* (Hz)	δ ($CDCl_3$)	*J* (Hz)
4	8.83 (dd)	1.5, 4.8	8.82 (dd)	1.5, 4.8
5	7.52 (dd)	4.7, 8.0	7.52 (dd)	4.6, 8.2
6	8.59 (dd)	1.5, 8.0	8.67 (dd)	1.5, 8.2
7	5.82 (s)		5.80 (s)	
13[a]	7.27 (s)		7.25 (s)	
15[a]	12.99 (s)		13.00 (s)	
18[c]	5.45 (m)		5.65 (m)	
19[c]	5.14 (m)		4.83 (m)	
20[b,c]	4.39 (d)	9.1	4.11 (d)	9.5
21[c]	1.47 (3H, dd)	6.3, 24.1	1.39 (3H, dd)	6.1, 24.8
23,24	3.96 (6H, s)		3.98 (6H, s)	
	^{13}**C NMR Data**			
	TM1		EM1	
Carbon	δ ($CDCl_3$)	*J* (Hz)	δ ($CDCl_3$)	*J* (Hz)
1	140.6		140.8	
2	157.5		157.5	
4	148.7		148.8	
5	133.5		134.5	
6	123.3		123.4	
7	85.6		85.6	
8, 12	172.0		171.0	
10	152.7		152.9	
14	155.4		155.4	
18[d]	72.2 (d)	18.1	72.0 (d)	24.8
19[d]	92.1 (d)	72.6	92.6 (d)	182.6
21[d]	17.7 (d)	22.9	16.4 (d)	21.9
23, 24	55.0		55.0	

[a]NH. [b]OH. [c]H-F coupling. [d]C-F coupling.

3.3 Identification of Unknown metabolites, Uk1 and Uk2

Both metabolites, TM1 and EM1 were synthesized with good yields by chemical hydrolysis of each flucetosulfuron isomer. Uk1 and Uk2 showed the same MS/MS fragmentation ($[M+H]^+$ = m/z 416) pattern as TM1 and EM1. Finally, by comparing LC-MS and MS/MS fragmentation spectra and co-chromatography, Uk1 and Uk2 were identified as TM1 and EM1 (*N*-(4,6-dimethoxypyrimidin-2-ylcarbomoyl)-2-(2-fluoro-1-hydroxypropyl)pyrimidine-3-sulfonamid), respectively.

Chiral conversion was known to be occurred in aryloxyphenoxypropionates by microorganisms(Dicks *et al.* 1985, Wink and Luley 1988, Blaser and Spindler 1997, Harrison *et al.* 2003, Cai *et al.* 2008).The formation of only single metabolite, TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron by HLMs indicated that there was not any possibility of chiral inversion between flucetosulfuron isomers or TM1 and EM1 during the microsomal reaction.

Although studies on various pesticide metabolism by HLMs showed that NADPH-dependent oxidation by CYP450s was a major metabolic pathway (Lang *et al.* 1997, Coleman *et al.* 2000, Kim 2006, Lee *et al.* 2006, Mutch and Williams 2006, Abass *et al.* 2007), only a hydrolysis product was formed in the present study. Hydrolytic metabolism of sulfonylureas has only been reported in soil and water to date (Beyer *et al.* 1987, Brown 1990, Schneiders *et al.* 1993, Brown and Cotterman 1994, Strek 1998, Sarmah and Sabadie 2002).

Liver microsomes are a rich source of enzymes, such as CYP450s, FMOs, carboxylesterases, reductases (azo-, carbonyl, and quinone), and aldehyde dehydrogenases for phase I reactions (Parkinson 2001), and these results indicated the involvement of hydrolysis enzymes in HLM-mediated metabolism of flucetosulfuron.

4. Enzyme kinetics of flucetosulfuron by HLMs

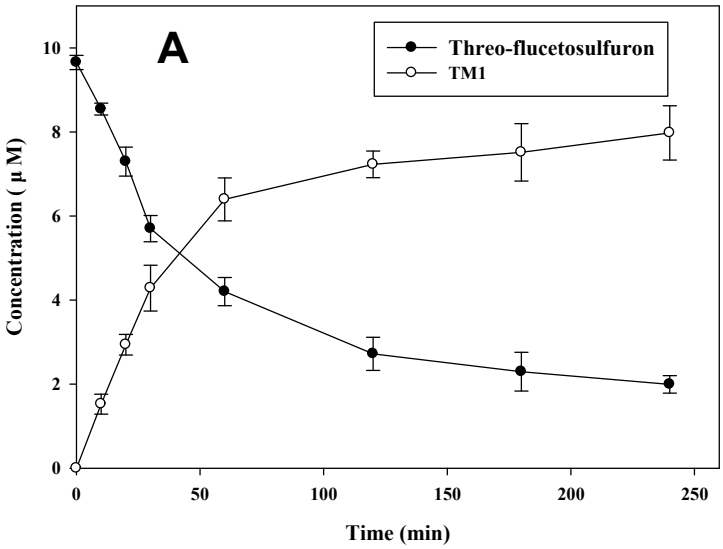
As enzyme kinetics is the key approach to study the mechanism of an enzyme-catalyzed reaction, most pesticide kinetics studies involve the use of hepatic microsomes, which contain a mixture of several xenobiotic-metabolizing enzymes with overlapping specificity, and the observed rates of reactions reflect the net effect of several protein-drug interactions.

To acquire the optimal metabolic conditions of flucetosulfuron by HLMs, optimization test was carried out in various combinations of factors, including incubation times, protein (HLMs) concentration and substrate (each flucetosulfuron isomer) concentrations. The optimal metabolic conditions of flucetosulfuron by HLMs were required to identify the mediating enzymes for flucetosulfuron metabolism. During the study, each flucetosulfuron isomer was dissolved and then serially diluted with acetonitrile to the required concentrations. Also, the solvent, acetonitrile did not exceed 0.5% in final reaction mixture.

4.1 Optimization of incubation time

To determine the optimal incubation time for the formation of metabolite by HLMs, The incubation mixtures, containing 0.5mg/ml of HLMs were activated in 50mM phosphate buffer (pH 7.4) without an NADPH-generating system for 30 min at 37℃ in a shaking water bath. The reaction mixtures were incubated for 10, 20, 30, 60, 120, 180 and 240 minutes with 10 μM of each flucetosulfuron isomer in total volume of 250 μL, respectively. The formation patterns of TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron on incubation times were similar to each other in that the formation rate of each metabolite was proportional to the incubation times up to 30 minutes (Figure 28).

Because the upper limit showing the linearity on the incubation times in this test for the optimization of incubation time was positioned between 30 and 60 min, 30 minutes was chosen to the optimal incubation time for flucetosulfuron metabolism by HLMs during the study.



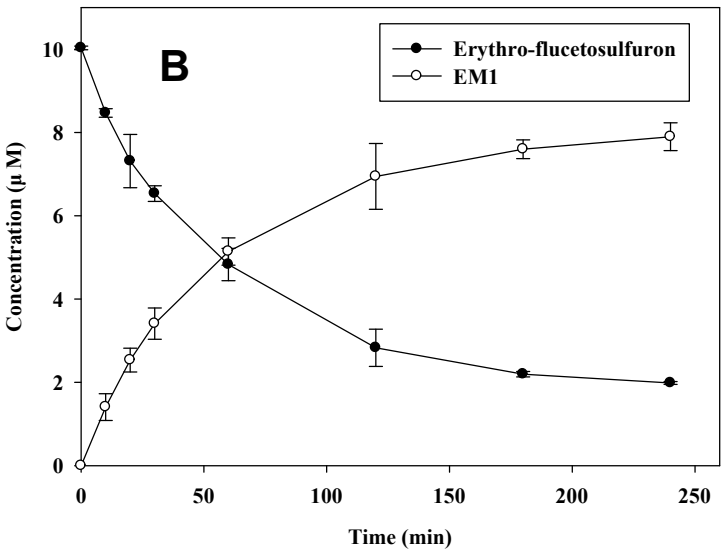


Figure 28. Formation of metabolites TM1 from *threo*-flucetosulfuron (A) and EM1 from *erythro*-flucetosulfuron depending on the incubation time by HLMs at 37°C

4.2 Optimization of protein concentration

To acquire the optimal protein (HLMs) concentration of each flucetosulfuron isomer, optimization test with various concentrations of HLMs was carried for the optimized incubation time, 30 min.

The formation patterns of TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron on HLMs concentrations were also alike and proportional to the HLMs concentration up to 0.48 mg/ml and 0.64 mg/ml, respectively (Figure 29). The lower protein concentration level than 0.48 mg/ml should be chosen, so the optimal protein concentration was selected 0.16 mg/ml, adjacent to the theoretical concentration of half the maximum velocity, for both flucetosulfuron isomers.

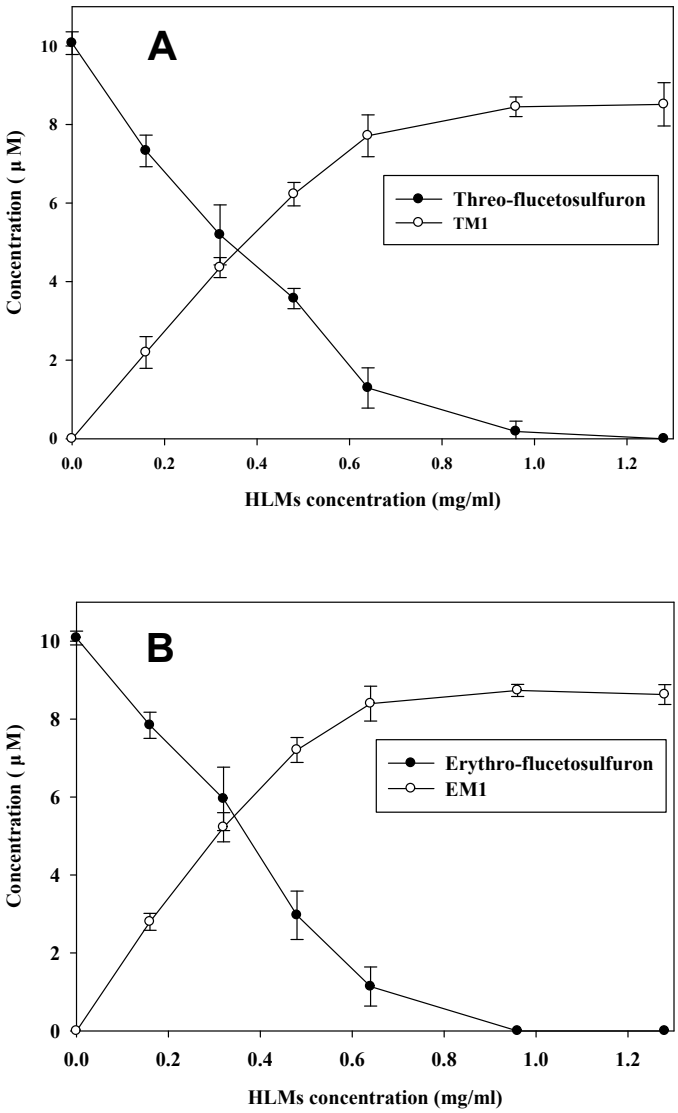


Figure 29. Formation of metabolites TM1 from *threo*-flucetosulfuron (A) and EM1 from *erythro*-flucetosulfuron (B) depending on the HLMs concentration for 30 min at 37°C.

4.3 Optimization of the concentration of flucetosulfuron isomers

To acquire the optimal concentration of each flucetosulfuron isomer, optimization test with various concentrations (10, 20, 100, 200, 1000, 5000 and 10000μM) of HLMs was carried with 0.16 mg/ml HLMs for 30 min at 37°C. The formation patterns of TM1 and TM1 from various concentration of each flucetosulfuron isomer on 0.16 mg/ml of HLMs for 30 min was proportional to the flucetosulfuron concentrations up to 10,000 μM (Figure 30).

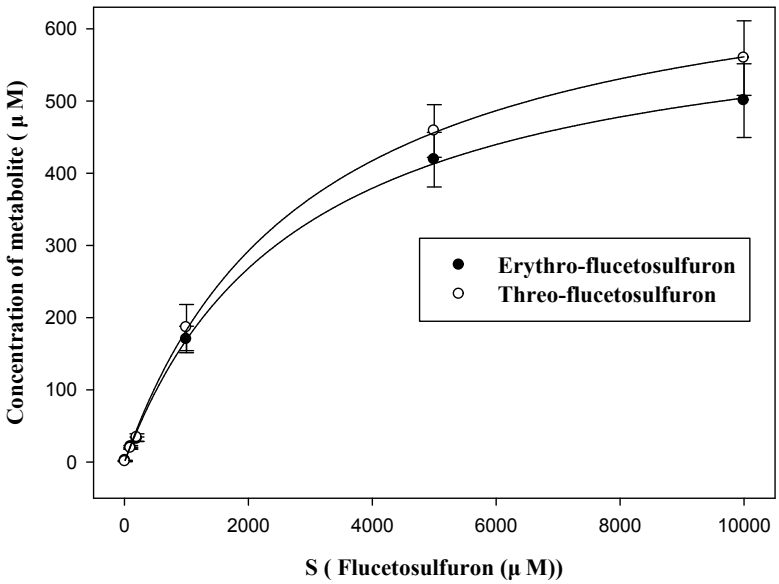


Figure 30. Formation of metabolites TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron depending on the concentration of flucetosulfuron with 0.16 mg HLMs for 30 min at 37°C.

10 μM of flucetosulfuron that has been treated microsomal reaction to determine the optimal incubation time was also in the linear range of the

formation of metabolite. But, 10 μM was too low concentration to quantify the exact concentration. So, 100 μM of each flucetosulfuron isomer was selected for the microsomal metabolism study.

On the basis of these results, 100 μM of each isomer of flucetosulfuron, 0.16 mg/ml of HLMs protein concentration and 30 minute of incubation time were obtained as the optimized incubation conditions.

The transformed percentages for TM1 and EM1 from 100 μM of each flucetosulfuron isomer under optimized reaction conditions were 25.8 % and 29.8%, respectively (Table 16).

Table 16. Formation of TM1 and TM1from 100 μM of each flucetosulfuron isomer under optimized reaction conditions

Substrate	HLMs (mg/ml)	Flucetosulfuron (μM)	TM1 or EM1 (μM)	Formation rate of metabolite (%)
Threo-flucetosulfuron	0.16	74.19 ± 2.79	19.16 ± 0.99	25.8
Erythro-flucetosulfuron	0.16	73.35 ± 1.20	21.91 ± 0.44	29.8

5. Kinetics of flucetosulfuron isomers by HLMs

Form the identification of metabolites, NADPH-independent hydrolytic pathway was elucidated to mediate the biotransformation of flucetosulfuron by HLMs. The metabolism of each flucetosulfuron isomer (10, 20, 100, 200,

1000, 5000 and 10000μM) was carried out under the optimized incubation conditions.

As enzyme kinetics is the key approach to study the mechanism of an enzyme-catalyzed reaction, most of pesticide kinetics studies involve the use of hepatic microsomes, which contain a mixture of several xenobiotic-metabolizing enzymes with overlapping specificity, and the observed rates of reactions reflect the net effect of several protein-drug interactions. The Michaelis-Menten equation can be used as the analysis method for many drugs metabolic kinetics (Miners *et al.* 1988, Chang *et al.* 1993, Stresser and Kupfer 1998, Casabar *et al.* 2006, Lee *et al.* 2006). Under these optimized metabolic conditions, the formation pattern of TM1 and EM1 from each flucetosulfuron isomer by HLMs was also best fitted to a Michaelis-Menten equation ($V = V_{max}*[S]/(K_m + [S])$), while the Hill model and two-enzyme model did not significantly improve the regression (Figure 31).

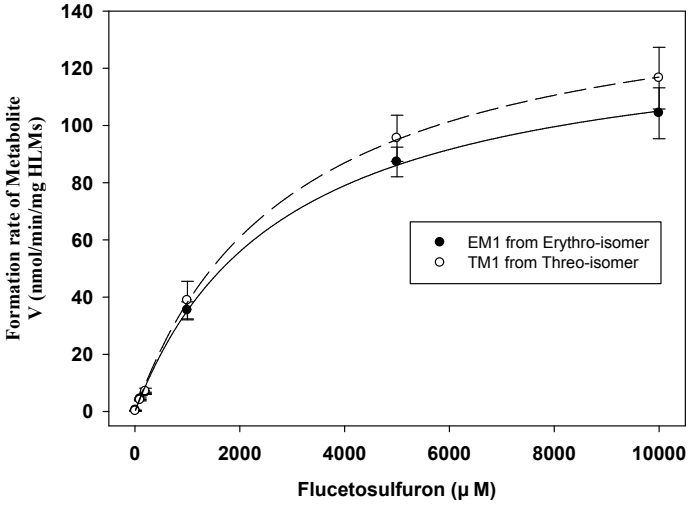


Figure 31. Formation pattern of metabolites, TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron, with 0.16 mg/ml HLMs for 30 min at 37°C ; Michaelis-Menten plot.

Under optimized metabolic conditions, the kinetic parameters for metabolite formation from *erythro*-flucetosulfuron and *threo*-flucetosulfuron were: V_{max}, 134.38 and 151.41 nmol/min/mg protein, respectively; K_m, 2798.53 and 2957.37 μM, respectively; and CL_{int}, 48.02 and 51.20 μL/min/mg microsomes, respectively (Table 17).

Table 17. Enzyme kinetic parameters for the production of the metabolites, TM1 from threo-flucetosulfuron and EM1 from erythro-flucetosulfuron under the optimized conditions

	Threo-flucetosulfuron	*Erythro*-flucetosulfuron
V_{max} (nmol/min/mg HLMs)*	151.41 ± 2.75	134.38 ± 2.48
K_m (μM)*	2957.37 ± 155.74	2798.53 ± 152.81
CL_{int} [V_{max} /K_m (μL/min/mg HLMs)]	51.20 ± 9.30	48.02 ± 8.86

* P value: 0.21and 0.24 for *threo*-flucetosulfuron and *erythro*-flucetosulfuron

CL_{int} value means the functional ability of HLMs to metabolize flucetosulfuron, was calculated from the maximum velocity, V_{max} and affinity constant, K_m. Hepatic pesticide clearance depends on the CL_{int} and hepatic blood flow. CL_{int} of the subsystem (whether enzyme, microsomes, or isolated cells) can be scaled to the corresponding *in vivo* parameter when the total content of enzyme (microsomal protein or hepatocellularity) for the liver is known (Hutson and Logan 1986, Sultatos 1991). So, CL_{int} can be re-estimated on a per kilogram body weight basis, for purposes of comparison, based on the assumptions that Human have 40g of liver per kg body weight (Davies and Morris 1993) and microsomal content of the livers is 52.5mg of microsomal protein per g of liver for humans (Iwatsubo *et al.* 1997). The re-estimated CL_{int} for *threo*-flucetosulfuron and *erythro*-flucetosulfuron were 107.6 and 100.8 mL/min/kg (body weight), respectively.

Godin et al. also calculated intrinsic clearance value for deltamethrin and esfenvalerate on the human body weight basis, indicating 162.1 and 108.2 ml/min/kg (body weight), respectively. As they determined that these values mean rapid elimination for deltamethrin and esfenvalerate by HLMs in human body, flucetosulfuron isomers having a similar intrinsic clearance values was also regarded to be rapidly eliminated by HLMs in human body.

Though the CL_{int} value of *erythro*-flucetosulfuron was slightly lower than that of *threo*-flucetosulfuron, no significant kinetic differences were observed between the formation of TM1 and EM1 from *threo*- and *erythro*-flucetosulfuron by t-test.

In this study, there was no difference in the kinetics of TM1 and EM1 formation, while previous metabolic studies of endosulfan (alpha-, beta-) showed stereoselectivity by CYP450s, and permethrin (cis- trans-) showed stereoselectivity of carboxylesterases (Lee *et al.* 2006, Ross *et al.* 2006).

6. Identification of enzymes metabolizing flucetosulfuron by selective esterase inhibitors

Since the metabolites were formed through ester bond cleavage and the major xenobiotic metabolizing esterases include A-esterases (paraoxonases/arylesterases), cholinesterases, and carboxyesterases (Kim 2006), identification of the esterase responsible for hydrolysis was performed through an inhibition study with class-specific esterase inhibitors (Ecobichon 1970). The concentration of each inhibitor was adopted from previous literatures of their inhibitory effects on their target esterases (Table 18).

Addition of 100 and 200 μM of $HgCl_2$ (Hg^{2+}) into the metabolic mixture significantly inhibited the production of TM1 to 80.6% and 91.5% and EM1 to 81.8% and 92.3%, respectively. These concentration related results suggested that general esterases containing an –SH group at their active center, such as arylesterases (Iatsimirskaia *et al.* 1997), carboxylesterases (Ali *et al.* 1985, Kim 2006), and cholinesterases (Frasco *et al.* 2007), were involved in the flucetosulfuron metabolism. Mercury induces the inactivation of cholinesterases with successive binding to the enzyme

surface and has several consequences, such as reversible inhibition, enzyme denaturation, and protein aggregation (Frasco *et al.* 2007).

However, treatment with an activator (Ca^{2+}) or inhibitor (Mg^{2+}, Co^{2+}, and EDTA) for A-esterases (Erdös *et al.* 1959) did not produce any significant effect on metabolite formation even at the relatively high concentrations, indicating that flucetosulfuron-metabolizing enzymes were not A-esterases (Gan *et al.* 1991, Gonzalvo *et al.* 1997).

The specific carboxylesterase inhibitor, BNPP (Simeon *et al.* 1988, Reiner *et al.* 1993, Kim 2006), suppressed more than 84% of metabolite formation at 100 μM and more than 60% even at 10 μM, indicating that the formation of TM1 and EM1 must be mediated by microsomal carboxylesterases. BNPP was known to be the strong inhibitor for many carboxylesterases at micromolar concentration, while had no inhibitory effect on cholinesterases at 100 μM.

Addition of eserine (Augustinsson 1958, Ecobichon 1970), dibucaine, and quinidine, which are specific cholinesterase inhibitors, also reduced metabolite formation. Eserine with HLMs inhibited strongly the formation of TM1 and EM1 to 78 ~ 83 % at 10 and to 88 ~ 89 % at 100 μM, respectively. Eserine was known to be a specific inhibitor for cholinesterases which was reported to be inhibited 100% at just 10 μM(Augustinsson 1958). These results indicated that that the formation of TM1 and EM1 is mediated by microsomal cholinesterases. Microsomal incubation with dibucaine and quinidine, known to be cholinesterases inhibitors, at 100 μM also inhibited

the formation of TM1 and EM1, supporting that metabolism of flucetosulfuron is mediated by microsomal cholinesterases, too.

These results indicated that flucetosulfuron metabolism must be mediated by microsomal carboxylesterases and cholinesterases.

Such hydrolytic reactions by carboxylesterases were reported with organophosphate pesticides (Devonshire and Moores 1982), pyrethroids (Ross *et al.* 2006, Crow *et al.* 2007), and pyribenzoxim (Kim 2006). Although cholinesterase-mediated pesticide hydrolysis has not been reported, cocaine and heroin were hydrolyzed by both carboxylesterase and cholinesterase (Stewart *et al.* 1977, Lockridge *et al.* 1980).

Table 18. Effects of esterase inhibitors on the production of metabolites, TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron, when flucetosulfuron isomers (100 μM) in the optimized condition of HLMs.

Inhibitor	Target esterase	Concentration (μM)	Specific activity (μmol/min/mg protein)*			
			TM1	Inhibition (%)	EM1	Inhibition (%)
Control	-	-	5.31 ± 0.08	-	5.28 ± 0.18	-
$HgCl_2$**	General esterases	100	1.03 ± 0.12	80.6	0.96 ± 0.35	81.8
		200	0.45 ± 0.09	91.5	0.41 ± 0.02	92.3
$CaCl_2$***	A-esterases	1000	5.15 ± 0.05	2.9	4.88 ± 0.20	6.4
$CoCl_2$	A-esterases	1000	5.05 ± 0.08	4.9	5.17 ± 0.05	7.6
$MgCl_2$	A-esterases	1000	5.09 ± 0.10	4.0	4.95 ± 0.40	6.2
EDTA	A-esterases	3000	5.46 ± 0.15	- 2.9	5.44 ± 0.19	- 3.1
BNPP**	Carboxylesterases	10	1.74± 0.04	67.2	1.79 ± 0.08	66.1
		100	0.84 ± 0.19	84.1	0.76 ± 0.02	85.7
Eserine**	Cholinesterases	10	1.15± 0.13	78.3	0.93± 0.41	82.4
		100	0.63 ± 0.40	88.2	0.57 ± 0.01	89.1
Dibucaine**	Cholinesterases	100	2.99 ± 0.15	43.6	3.18 ± 0.15	39.7
Quinidine**	Cholinesterases	100	2.89 ± 0.20	45.5	2.94 ± 0.20	43.3

* Each value represents the mean of two determinations

** Significant difference at $\alpha = 0.05$

*** A-esterase activator

7. Enzyme kinetics of flucetosulfuron by human esterases

As specific esterase inhibition of flucetosulfuron metabolism indicated that flucetosulfuron metabolism is mediated by microsomal carboxylesterases and cholinesterases, human recombinant metabolizing carboxylesterases such as human carboxylesterase 1 (CES1b and CES1c) and human carboxylesterase 2 (CES2) were applied to examine kinetics of the hydrolytic metabolism of flucetosulfuron. Additionally, purified esterases such as butyrylcholinesterase from human serum and acetylcholinesterase from human erythrocytes were also tested.

7.1 Kinetics of flucetosulfuron by human carboxylesterases

By inhibiting flucetosulfuron metabolism with selective esterase inhibitors, it was determined that carboxylesterases was the primary enzymes involved in the formation of metabolites, TM1 or EM1 from flucetosulfuron.

The kinetic parameters with human recombinant carboxylesterases, CES1b, CES1c and CES2 for each flucetosulfuron isomer are summarized in Table 19.

For *threo*-flucetosulfuron, the affinity (K_m value = 3176.40 μM) of CES1b of the formation of TM1 was slight lower than 2257.40 and 2608.20 for CES1c and CES2, respectively. But, the V_{max} value (nmol/min/mg protein), 44.33 of CES2 for *threo*-flucetosulfuron were evidently 3-fold and 4.3-fold higher than 12.66 of CESlb and 10.15 of CES1c. The intrinsic clearance value, CL_{int} (V_{max} /K_m (μL/min/mg protein)), of CES2 for the formation of TM1 from *threo*-flucetosulfuron was 16.98, which is higher than those of CES1b (3.99) and CESlc (4.50).

For *erythro*-flucetosulfuron, the K_m (μM) value of CES1b, CES1c and CES2 was 2695.38, 2185.55 and 2426.97, respectively. They were similar to that of HLMs (K_m =2812.78), but, their V_{max} values for *erythro*-flucetosulfuron were extremely lower (6.99, 6.70 and 42.29) than that of HLMs (134.38) and as a result, affected CL_{int} value to be relatively low. The CL_{int} values of CES1b, CES1c, and CES2 for the formation of EM1 from *erythro*-flucetosulfuron were 2.59, 3.07, and 17.43, respectively.

Table 19. Carboxylesterase kinetic parameters of the formation of metabolites, TM1 and EM1 from flucetosulfuron isomers

	Kinetic Parameter	*Threo*-flucetosulfuron	*Erythro*-flucetosulfuron
CES1b	V_{max} (nmol/min/mg CES1b)	12.66 ± 0.29	6.99 ± 0.12
	K_m (μM)	3176.40 ± 204.13	2695.38 ± 139.28
	CL_{int} [V_{max} / K_m (μL/min/mg CES1b)]	3.99 ± 0.98	2.59 ± 0.45
CES1c	V_{max} (nmol/min/mg CES1c)	10.15 ± 0.30	6.70 ± 0.25
	K_m (μM)	2257.40 ± 213.72	2185.55 ± 268.09
	CL_{int} [V_{max} /K_m (μL/min/mg CES1c)]	4.5 ± 1.21	3.07 ± 0.84
CES2	V_{max} (nmol/min/mg CES2)	44.33 ± 0.97	42.29 ± 1.62
	K_m (μM)	2608.20 ± 173.01	2426.97 ± 291.16
	CL_{int} [V_{max} /K_m (μL/min/mg CES2)]	16.98 ± 2.91	17.43 ± 3.34
HLMs*	V_{max} (nmol/min/mg HLMs)	151.41 ± 2.75	134.38 ± 2.48
	K_m (μM)	2957.37 ± 155.74	2798.53 ± 152.81
	CL_{int} [V_{max} /K_m (μL/min/mg HLMs)]	51.20 ± 9.30	48.02 ± 8.86

* Reference from HLMs kinetic parameters of flucetosulfuorn isomers

By comparing *threo*- and *erythro*-flucetosulfuron, kinetic parameters for CES2 showed that V_{max} and K_m of both flucetosulfuron isomers are quite similar to each other. CL_{int} values were 16.98 for *threo*-flucetosulfuron and 17.43 for *erythro*-flucetosulfuron. These results indicated that kinetics by CES2 did not produce significant difference between *threo*-flucetosulfuron and *erythro*-flucetosulfuron. On the other hand, CES1b and CES1c showed higher V_{max} values; 12.66 and 10.15 (μM) for *threo*-flucetosulfuron than 6.99 and 6.70 for *erythro*-flucetosulfuron. Considering the K_m value for each substrate and enzyme, CES1b and CES1c produced higher CL_{int} values for *threo*-flucetosulfuron (3.99 and 4.5 (μL/min/mg protein)) than for *erythro*-flucetosulfuron (2.59 and 3.07). Although these results showed that CES1b and CES1c produced slight difference in V_{max} and CL_{int} values between *threo*-flucetosulfuron and *erythro*-flucetosulfuron, it was difficult to clearly determine that there was some stereoselective activity of CES1b and CES1c on flucetosulfuron isomers.

The kinetics pattern for flucetosulfuron isomers by human recombinant carboxylesterases (CES1b, CES1c and CES2) are shown in Figure 32.

Hosokawa et al. reported that CES1 family mainly hydrolyzes a substrate with a small alcohol group and large acyl group, whereas CES2 recognizes a substrate with a large alcohol group and small acyl group (Hosokawa 2008). As the structure of flucetosulfuron consists of large alcohol group and small acyl group, it was inferred that the intrinsic clearance value of CES2 for flucetosulfuron was much higher than those of CESl groups such as CES1b and CES1c.

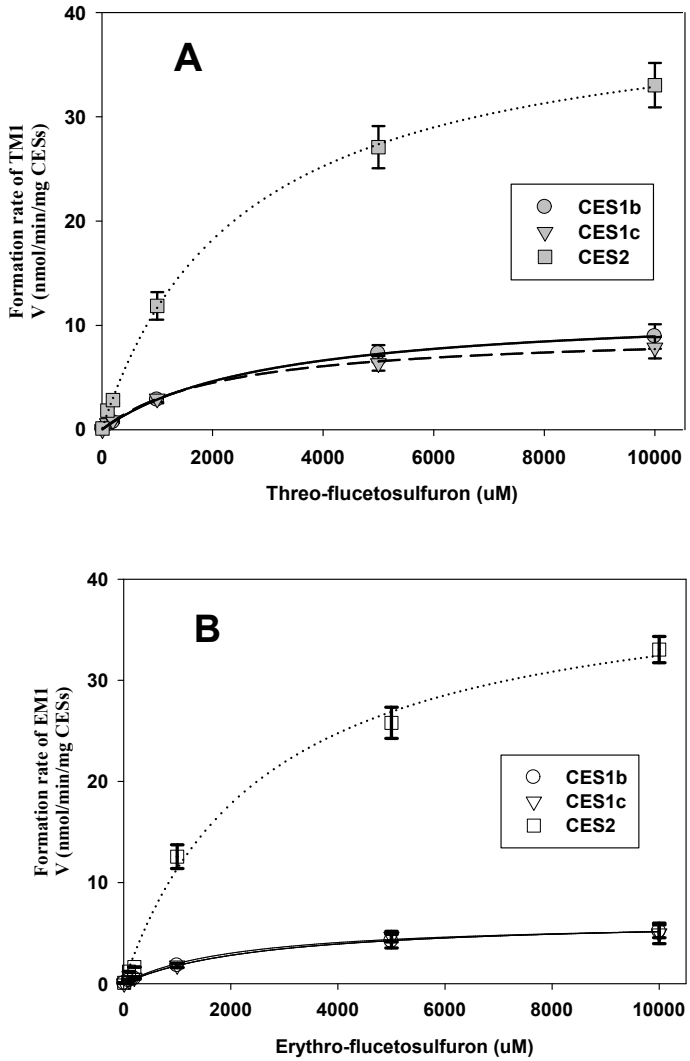


Figure 32. Kinetics for the formation rate of TM1 (A) and EM1 (B) from flucetosulfuron isomers with CES1b, CES1c, and CES2

Although Ross and Crow commented that CES1 enzymes such as CES1b or CES1c are found in higher amounts than CES2 (Ross and Crow 2007) in HLMs, the exact ratio of CES enzymes were not clearly known. Moreover, Maxwell et al. reported that carboxylesterases show different substrate specificities (Maxwell 1992), therefore, the percentages of TNR (%, total

normalized rates) of human carboxylesterases to investigate the contribution of each carboxylesterase isoforms in *in vitro* metabolism of flucetosulfuron by HLMs could not be calculated.

These studies on the kinetics of carboxylesterases in both flucetosulfuron isomers clearly demonstrated that CES1b, CES1c and CES2 are responsible for metabolizing flucetosulfuron.

7.2 Kinetics of flucetosulfuron by human cholinesterases

From the inhibition test of flucetosulfuron metabolism with selective esterase inhibitors, human acetylcholinesterase (AChE) and human butyrylcholinesterase (BChE) were also expected to be involved in the formation of TM1 or EM1 from flucetosulfuron. Because there was no available human recombinant acetylcholinesterase and human recombinant butyrylcholinesterase, AChE purified from human erythrocytes and BChE purified from human serum were purchased and tested.

However, concentrations from 10 μM to 10000 μM of both flucetosulfuron isomers were not sufficient to saturate AChE for the formation of metabolites, TM1 or EM1, showing continuous proportional increase of the formed metabolites. Consequently, Kinetics for the formation rate of metabolites, TM1 and EM1 by AChE could not give the exact K_m (μM) or V_{max} (nmol/min/mg protein) value (Figure 33). Nevertheless, kinetics pattern for both flucetosulfuron isomers were similar to each other.

On the other hand, the concentration ranges from 10 to 10000 μM of both flucetosulfuron isomers were sufficient to saturate BChE and to reach the maximum catalytic efficiency. Kinetic patterns of BChE for the formation

rate of each metabolite, TM1 or EM1, showed that the reactivity of BChE for *threo*-flucetosulfuron was remarkably better than that of *erythro*-flucetosulfuron (Figure 34).

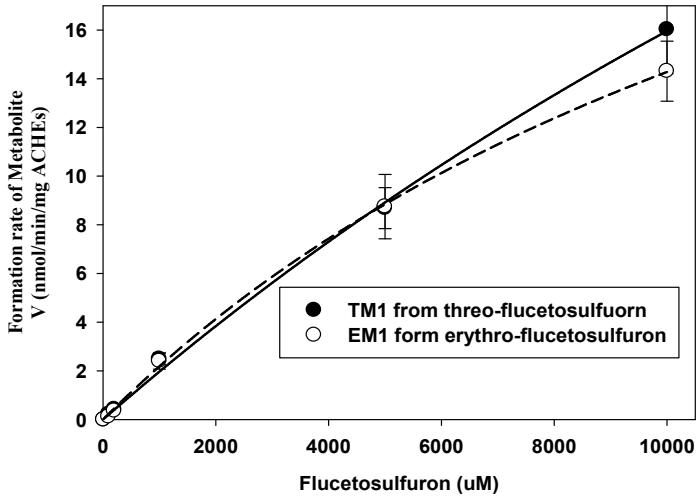


Figure 33. Kinetics for the formation rate of metabolite TM1 and EM1 from flucetosulfuron isomers by human acetylcholinesterase (AChE)

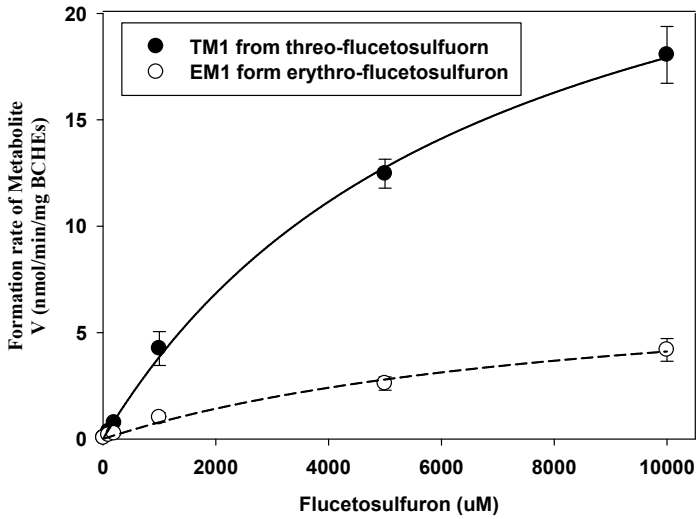


Figure 34. Kinetics for the formation rate of metabolite TM1 or EM1 from flucetosulfuron isomers by human butyrylcholinesterase (BChE)

The enzyme kinetic parameters with human acetylcholinesterase (AChE) and human butyrylcholinesterase (BChE) for each flucetosulfuron isomer were estimated (Table 20).

Table 20. Cholinesterases kinetic parameters of the formation of M1 (TM1 or EM1) from flucetosulfuron

	Kinetic Parameter	*Threo*-flucetosulfuron	*Erythro*-flucetosulfuron
AChE	V_{max} (nmol/min/mg AChE)	75.9 ± 20.6	37.00 ± 2.43
	K_m (μM)	37568.6 ± 12520.5	15939.6 ± 1573.20
	CL_{int} [V_{max} /K_m (μL/min/mg AChE)]	2.02 ± 0.57	2.32 ± 0.76
BChE	V_{max} (nmol/min/mg BChE)	30.11 ± 1.05	7.76 ± 0.94
	K_m (μM)	6800.20 ± 486.17	8875.09 ± 1974.27
	CL_{int} [V_{max} /K_m (μL/min/mg BChE)]	4.43 ± 0.77	0.87 ± 0.26
HLMs *	V_{max} (nmol/h/g HLMs)	151.41 ± 2.75	134.38 ± 2.48
	K_m (μM)	2957.37 ± 155.74	2798.53 ± 152.81
	CL_{int} [V_{max} /K_m]	51.20 ± 9.30	48.02 ± 8.86

*Reference from HLMs kinetic parameters of flucetosulfuorn isomers

For *threo*-flucetosulfuron, the K_m values of AChE and BChE were 37568.6 and 6800.2 and their V_{max} values were 75.9 and 30.11, respectively. The K_m and V_{max} values of AChE were also clearly higher than those of BChE. The CL_{int} value of BChE for the formation of TM1 from *threo*-flucetosulfuron was 4.43, which was also 2.2-fold higher than 2.02 of AChE.

For *erythro*-flucetosulfuron, the K_m and V_{max} value of AChE were 15939.6 and 37.0, which is higher than those of BChE at 8875.09 and 7.76. From these parameters, the CL_{int} value, 2.32 of AChE was 2.7-fold higher than

0.87 of BChE. V_{max} values for both AChE and BChE in the formation of TM1 from *threo*-flucetosulfuron were 2.0-fold and 3.8-fold higher than EM1 from *erythro*-flucetosulfuron, respectively.

CL_{int} value of AChE for *threo*-flucetosulfuron was 2.02, showing not much of a difference to 2.32 for *erythro*-flucetosulfuron. But, CL_{int} value of BChE for *threo*-flucetosulfuron was 4.43, showing 5-fold higher than 0.87 for *erythro*-flucetosulfuron.

Esterases kinetics with CES1b, CES1c, CES2, AChE, and BChE demonstrated that the metabolism of flucetosulfuron isomers is mediated by these esterases with the same results from the specific esterase inhibition. This study indicated that there is a possibility of stereoselective metabolism between flucetosulfuron isomers by BChE. Unfortunately, there was no other reference study about kinetics and stereoselective activity of pesticide by BChE. Although the stereoselective mechamism by BChE for *threo*- and *erythro*-isomers is not clear, the active site gorge of BChE is expected to contribute to the between the two isomers by restricting the structure of flucetosulfuron isomers for the acyl pocket of BChE (Shenouda *et al.* 2009). Namely, the larger active site gorge of BChE than that of AChE is ecpected to make possible to stoichiometrically bind and catalytically hydrolyze flucetosulfuron according to the conformational structure for flucetosulfuron isomers (Doctor and Saxena 2005).

On the basis of these results, the metabolic pathway of flucetosulfuron by HLMs is proposed as in Figure 35.

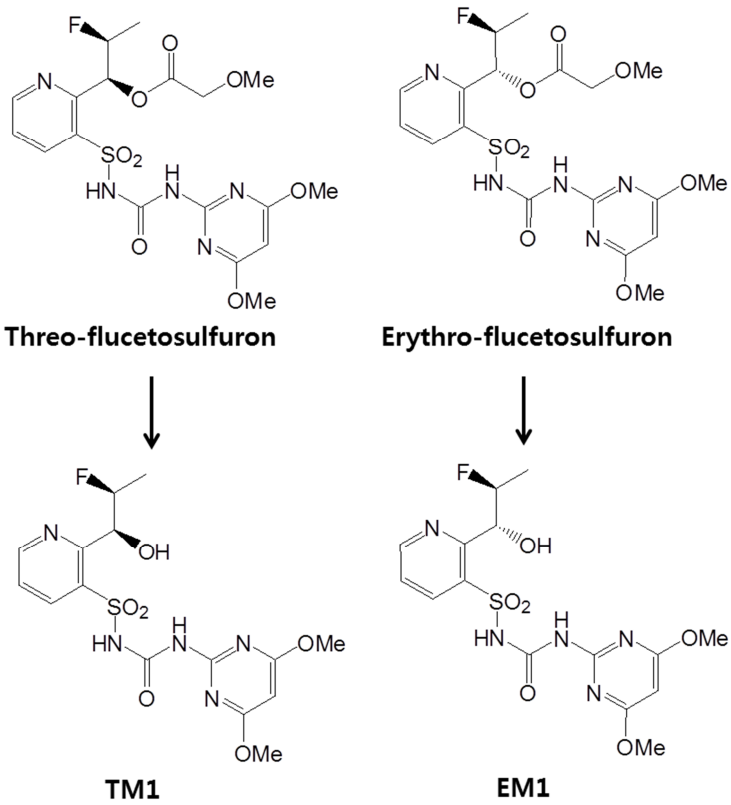


Figure 35. Proposed metabolic pathway of flucetosulfuron in *in vitro* metabolism by HLMs

Ⅳ. Conclusion

In vitro metabolism of flucetosulfuron was investigated with HLMs using *threo*- and *erythro*-isomers, respectively. *In vitro* metabolism of flucetosulfuron by HLMs produced M1, *threo*-metabolite (TM1) from *threo*-flucetosulfuron and *erythro*-metabolite (EM1) from *erythro*-flucetosulfuron. They were identified by LC-MS/MS and confirmed by cochromatographic method. Hydrolytic metabolism of flucetosulfuron was proved to be mediated by esterases rather than by P450s or FMOs, because TM1 and EM1 were produced by HLMs regardless of the NADPH generating system. After optimization of reaction time, HLMs concentration, and flucetosulfuron concentration, kinetics parameters of each flucetosulfuron isomers were obtained from Michaelis-Menten plot. The estimated V_{max} (nmol/min/mg HLMs) and K_m values were calculated 134.38 and 2798.53 for *threo*-flucetosulfuron and 151.41 and 3957.37 for *erythro*-flucetosulfuron. The intrinsic clearance values ($CL_{int} = V_{max} / K_m$ (μL/min/mg HLMs)) of the formation of TM1 from *threo*-flucetosulfuron and EM1 from *erythro*-flucetosulfuron were 51.20 and 48.02, respectively. Though the CL_{int} values from *erythro*-flucetosulfuron was slight lower than that of *threo*-flucetosulfuron, the difference by HLMs was not significant. Inhibition test with selective esterases inhibitors indicated thatwas the metabolizing esterases in hydrolysis of flucetosulfuron are carboxylesterases and cholinesterases. The kinetics with human recombinant carboxylesterases (CES1b, CES1c and CES2), human acetylcholinesterase (AChE), and human butyrylcholinesterase (BChE) demonstrated that the metabolism of

flucetosulfuron isomers is mediated by these esterases with the same results from the specific esterase inhibition. The CL_{int} values of CES2 for the formation of TM1 and EM1 showing the highest activity among the tested esterases were 16.98 and 17.43. The CL_{int} values of CES1b and CES1C were 3.99 and 4.5 for *threo*-flucetosulfuron and 2.59 and 3.07 for *erythro*-flucetosulfuron. CL_{int} value of AChE for *threo*-flucetosulfuron was 2.02, also showing not much of a difference to 2.32 for *erythro*-flucetosulfuron. However, CL_{int} value of BChE for *threo*-flucetosulfuron was 4.43, showing 5-fold higher than 0.87 for *erythro*-flucetosulfuron. The differences of the kinetic parameters between flucetosulfuron isomers indicated that there is a possibility of stereoselcetive metabolism between *threo*- and *erythro*-flucetosulfuron by esterase such as BChE.

On the basis of these results, the metabolic pathway of flucetosulfuron by HLMs was proposed.

PART II

In vitro Metabolism of Flucetosulfuron by Artificial Gastrointestinal Juices

Ⅰ. Introduction

In an effort to protect humans from the adverse effects of pesticides, tests of acute toxicity, carcinogenicity, and chronic toxicity in mammals are mandatory for the local registration of pesticides. These studies are evaluated by international scientific experts to gauge risk to humans and determine the acceptable daily intake level and maximum residue limit for the pesticides. Furthermore, to minimize the amount of pesticides in crops and to ensure that the residue level is below allowable levels, the pre-harvest interval for applying pesticides is recommended to farmers and pesticide applicators. According to a recent risk assessment of human safety in Korea, the current level of intake of pesticides through food is estimated to be very low (Lee *et al.* 2010, Yang *et al.* 2012). Nevertheless, pesticide poisoning cases occur frequently. The most frequent cause of pesticide poisoning is not through food or occupational intake, but by intentional oral ingestion of pesticides during suicide attempts (Jei 1992, Lee 2010, Chang *et al.* 2012, Roh 2012, Zhang and Li 2012). As a result, countries such as Finland, Sri Lanka, and Taiwan have banned the local use of highly toxic pesticides. This has resulted in reductions in the number of suicides because of pesticide ingestion (Chang *et al.* 2012). In order to adopt a similar plan in Korea, paraquat, a highly toxic pesticide frequently used in suicide attempts was banned by the Rural Development Administration (RDA) in 2013, despite its overwhelming market share in Korea. As a preventive measure, the legislation bans the sale of highly toxic pesticides; however, such measures have not been undertaken worldwide. Therefore, safety assessment of each pesticide is required.

Recent studies have focused on the *in vitro* metabolism of pesticides by HLMs. These studies reveal the metabolic patterns and metabolizing enzymes in human liver, allowing for the calculation of hepatic clearance and the detoxifying action of the liver. This enables a realistic risk assessment of pesticide toxicity (Lee *et al.* 2006). Human liver microsomal studies are limited to the metabolic processing of pesticides in the liver and do not provide any information on absorption from skin or gastrointestinal (GI) tract into the circulatory system. Thus, to thoroughly understand the metabolism after oral ingestion of pesticides, metabolism studies of pesticides by the GI tract in humans are also required.

As reported the unintentional ingestion quantity of soil by children in China can reach to 200 mg per day, thus, recent studies on mobilization of organochlorine pesticides (OCPs) and hydrophobic organochlorine pesticides in contaminated soils were carried out through an *in vitro* GI model (Tao *et al.* 2009, Tao *et al.* 2010). The study revealed that contaminated pesticides could be extracted by an *in vitro* GI model, providing a clue that the bound residue of OCPs could be mobilized.

In vitro metabolism of flucetosulfuron isomers by artificial GIs was carried out to understand stability and disintegration patterns of flucetosulfuron and to provide information for risk assessment of flucetosulfuron for human.

The main frame of the present study consists of as follows:

(1) investigation on the metabolism in artificial GIs

(2) identification and quantificaition of flucetosulfuron isomers and metabolites

(3) proposal of relevant metabolic pathway of flucetosulfuron, based on the metabolism by artificial GIs.

Ⅱ. Materials and Methods

1. Reagents and Materials

1.1 Flucetosulfuron and metabolites

Flucetosulfuron isomers, *threo*-flucetosulfuron and *erythro*-flucetosulfuron, were isolated and purified by preparative- high performance liquid chromatography (HPLC) method. *Threo*-metabolite-1 (*threo*-M1 or TM1) and *erythro*-metabolite-1 (*erythro*-M1 or EM1) were acquired by hydrolysis of each purified flucetosulfuron isomer. 2-(2-Fluoro-1-hydroxypropyl)pyridine-3-sulfonamide (M2) and 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl 2-methoxyacetate (M4), having both *threo*- and *erythro*-isomers, were provided by LG Life Sciences Ltd (Korea). 4,6-Dimethoxypyrimidin-2-amine (M3) was purchased from Sigma-Aldrich (USA) (Figure 36).

1.2 Chemicals and reagents

Ammonium acetate, acetic acid, and formic acid (above 99.9%) for HPLC and LC-MS/MS were purchased from Sigma-Aldrich. HPLC-grade acetonitrile and methanol were purchased from Burdick and Jackson (USA). α-Amylase, bile, mucin, pancreatin, pepsin, trypsin, urea, and uric acid for the preparation of artificial GI juices were products of Sigma-aldrich. Calcium chloride ($CaCl_2$), sodium chloride (NaCl), sodium isocyanide (NaSCN), sodium hydrogen carbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4), and potassium chloride (KCl) for the preparation of buffer

solution or artificial GI juices were the products of Daejung Chem (Korea). Potassium phosphate monobasic/dibasic and sodium hydroxide (NaOH) were purchased from Daejung Chem. The solvents and other chemicals used in the present study were of HPLC grade purchased from Burdick and Jackson (USA).

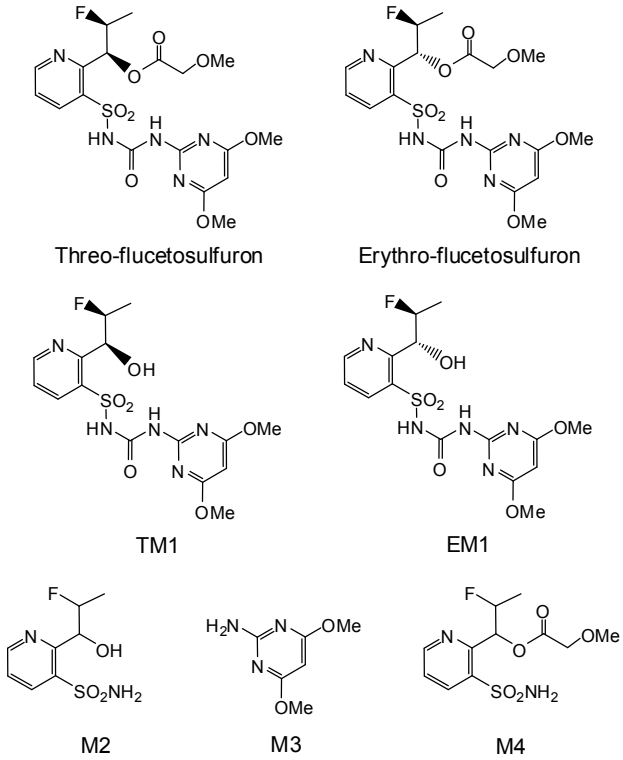


Figure 36. Structures of flucetosulfuron and its metabolites

1.3 Instruments

1.3.1 HPLC

HPLC, composed of Waters alliance 2690 HPLC and UV 4890 detector (Waters, USA) was used to analyze flucetosulfuron isomers and metabolites. The concentrations of flucetosulfuron isomers and its metabolites (TM1 and

EM1) were measured by Waters alliance 2690 HPLC equipped with a Capcell pak C18 UG120 column (4.6 mm i.d. x 150 mm, 3 μm; Shiseido, Japan) at 40℃. The mobile phases consisted of 20 mM ammonium acetate buffer containing 0.1 M acetic acid (A) and acetonitrile (B). The gradient condition was as follows: 20% B at 0 min, 27% B at 4 min, 30% B at 25 min, and 20% B at 28–33 min. The injection volume was 10 μL, and peaks were detected at the wavelength of 254 nm.

1.3.2 LC-MS/MS

LC-MS/MS, a tandem mass quadruple mass spectrometer (API2000 LC-MS/MS, Applied Biosystems, USA) coupled with an Agilent 1100 series HPLC system (Agilent, USA) was used with a Capcell pak C18 UG120 column (4.6 mm i.d. × 150 mm, 3 μm; Shiseido, Japan). The mobile phase consisted of 10 mM ammonium acetate containing 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The gradient condition was as follows: 20% B at 0 min, 27% B at 4 min, 30% B at 25 min, and 20% B at 28–33 min. The injection volume was 2 μL. Mass spectra were recorded by electrospray ionization with a positive mode. The turbo ion spray interface was operated at 4500 V and 550℃. The operating conditions were optimized by flow injection of analytes and were determined as follows: nebulizer gas flow, 50 psi; curtain gas flow, 10 psi; and collision energy, 30 eV. Quadrupoles Q1 and Q3 were set on unit resolution.

1.3.3 Centrifuge

Centrifuge, a Hanil Micro17TR model having temperature control system,

manufactured by Hanil Ltd (Korea), was used to acquire the supernatant of reaction samples.

2. Methods

2.1 Preparation of artificial GIs

Phosphate buffer at 50 mM (pH 7.4) was used as neutral control. As acidic control, pH 2 buffer solution was prepared by adjusting the pH of 50 mM phosphate buffer to 2.0 with 6 N HCl. Each composition of artificial GI solutions was weighed and solubilized to 1 L volume (Table 21).

Table 21. Composition of artificial GIs*

Artificial saliva	mg/L	Artificial gastric juice	mg/L	Artificial intestinal juice	mg/L
NaCl	500	NaCl	2900	KCl	300
NaSCN	150	KCl	7000	$CaCl_2$	500
Na_2SO_4	550	KH_2PO_4	2700	$MgCl_2$	200
$NaHCO_3$	150	Pepsin	1000	NaHCO3	1000
KCl	450	Mucin	3000	Trypsin	300
KH_2PO_4	600			Pancreatin	9000
$CaCl_2 \cdot 2H_2O$	150			Bile	9000
Mucin	750			Urea	300
α-amylase	250				
Urea	100				
Uric acid	10				

* Adenugba et al., 2008.

The artificial saliva and intestinal juices were adjusted to pH 7.4 with $NaHCO_3$, and artificial gastric juice was adjusted to 2.0 with 6 N HCl. The pH adjusted GI solutions were finally made up to total volume (100%). The mixture of saliva + gastric juice (1:4 *v/v*) was prepared by mixing artificial saliva and gastric juice with a ratio of 1:4. All solutions were stored at 4℃, and used within 24 h. Buffer solutions at pH 7.4 and 2 were tested as controls.

2.2 *In vitro* metabolism of flucetosulfuron by GIs

The concentration of flucetosulfuron in each GI solution (neutral control, acidic control, saliva, saliva + gastric juice (1:4 v/v), gastric juice, intestinal juice) was 100 μM and final volume of each GI juice was adjusted to 250 μL. The metabolic reaction was carried out in a shaking water bath with 80 cycle/min at 37℃. The times for each metabolic reaction were 5 min for saliva, 2 h for acidic control, saliva + gastric juice (1:4 v/v) and gastric juice, and 6 h for neutral control and intestinal juice. After metabolic reaction for each designed time, the reaction samples were terminated by addition of 250 μL acetonitrile. After immediately vortexing for 1 min, the samples were centrifuged at 10,770 x g for 5 min. Ten microliters of each supernatant of reaction mixtures were immediately analyzed by HPLC/UVD. The metabolic reaction was carried out in triplicate.

2.3 Identification of metabolites

Each 2 μL supernatant of saliva + gastric juice (1:4 v/v), gastric juice, intestinal juice were also analyzed by LC-MS/MS to identify the structure of

the unknown metabolites. LC-MS/MS fragmentation patterns based on the mass value $[M+H]^+$ of LC-MS/MS were analyzed, and the candidate structure of metabolites were drawn. In addition, tandem mass spectrometry (MS/MS) analyses of TM1, EM1, M2, M3, M4, and the parent flucetosulfuron isomers were conducted to compare their fragmentation patterns. Each solution of the metabolites and parent flucetosulfuron isomers were prepared in acetonitrile at 10 ppm level, and the corresponding metabolic reaction supernatant was analyzed using HPLC for confirmation.

2.4 Quantitation of flucetosulfuron and metabolites

After identification of metabolites, quantitative analysis was performed with an external standard calibration method. Standard solutions of *threo*-flucetosulfuron, *erythro*-flucetosulfuron, TM1, and EM1 were prepared as stock solutions with 1 mM in acetonitrile. The stock solution was diluted at the concentrations of 0.2, 0.5, 1.0, 10, and 100 μM with 1 mM in acetonitrile. Concentrations of flucetosulfuron and metabolites were obtained based on the peak area from each calibration standard. The calibration standards were fitted with high linearity (r^2 >0.999). Standard solutions of M2, M3, and M4 were also prepared as stock with 100 μM acetonitrile. The stock was diluted at the concentration of 0.5, 1.0, 10, and 100 μM. The stocks diluted at the concentration of calibration curves were fitted with high linearity (r^2 >0.997).

Ⅲ. Results and Discussion

1. Preparation of artificial GI juices

Prior to study on *in vitro* metabolism of flucetosulfuron by GI juices such as artificial saliva, gastric, and intestinal juices, attempt was made to prepare similar artificial GI juices to those in the human body. Because the human saliva, with pH ranging from 5.3 to 7.8, is 99.5% water and 0.5% other components such as proteins, enzymes, mucins, nitrogenous products, and electrolytes, including sodium, potassium, calcium, magnesium, bicarbonate, and phosphates, the artificial saliva that was prepared had pH 7.4 and contained all of the above components (Young and Schneyer 1981, Humphrey and Williamson 2001, Adenugba *et al.* 2008). To ensure optimum pH (1–3.5) for the denaturation of proteins from food intake and reaction of the enzyme pepsin, the gastric juice was prepared to pH 2 by using HCl, KCl, and NaCl with pepsin and mucin (Gray and Bucher 1941, Lian *et al.* 2003, Ganong and Barrett 2005, Adenugba *et al.* 2008). Intestinal juice was mixed with pancreatin, trypsin, bile, and electrolytes. Pancreatin and trypsin are secreted by the pancreas, whereas bile is released by the liver through the bile duct. Other components in intestinal juice are secretions by the intestinal glands in the small intestine (Borgström *et al.* 1957). Therefore, the intestinal juice was prepared by mixing pancreatin, trypsin, bile, urea, and electrolytes, and the pH was adjusted to 7.4 (Rao *et al.* 1989, Adenugba *et al.* 2008). Studies have shown that the compositions of each GI juice can be diversely modified according to the purpose of the study. For this study on the *in vitro* metabolism of flucetosulfuron, the composition of GI juices

was same as that of Adenugba et al. (2004), because the contents of several major enzymes and salts were close to the GI juice in the human body.

2. *In vitro* metabolic reaction of flucetosulfuron by artificial GI juices

To determine the flucetosulfuron metabolism in artificial saliva, saliva + gastric juice (1:4 v/v), gastric juice, and intestinal juice, the GI solutions with 100 μM of each flucetosulfuron isomer were incubated for a specific time. Metabolic reaction times to satisfy the proper digesting time in the body were determined to 5 min for saliva, 2 h for acidic control, saliva + gastric juice (1:4 v/v), and gastric juice, and 6 h for neutral control and intestinal juice, by considering the digesting time required by the GI organs such as the mouth, stomach, and small intestine. The metabolite peaks in the HPLC chromatogram of the incubated reaction with 100 μM *threo*-flucetosulfuron were identified for the artificial GI juices (Figure 37). *Threo*-flucetosulfuron did not show any metabolite in the control, which was a pH 7.4 buffer solution, whereas Uk2 and Uk3 were observed as unknown metabolites under acidic conditions, namely pH 2. In artificial saliva (5 min), *threo*-flucetosulfuron was stable. The reaction with artificial saliva + gastric juice (1:4 v/v) and artificial gastric juice for 2 h each produced some metabolites such as Uk1, Uk2, and Uk3, with no interfering peaks. The retention times of Uk1, Uk2, and Uk3 were 2.45, 4.05, and 5.03 min, respectively. TM1 was the only metabolite in the reaction mixture with intestinal juice for 6 h, indicating that flucetosulfuron can be catalyzed by trypsin, pancreatin, or bile, by the breakdown of its ester bond.

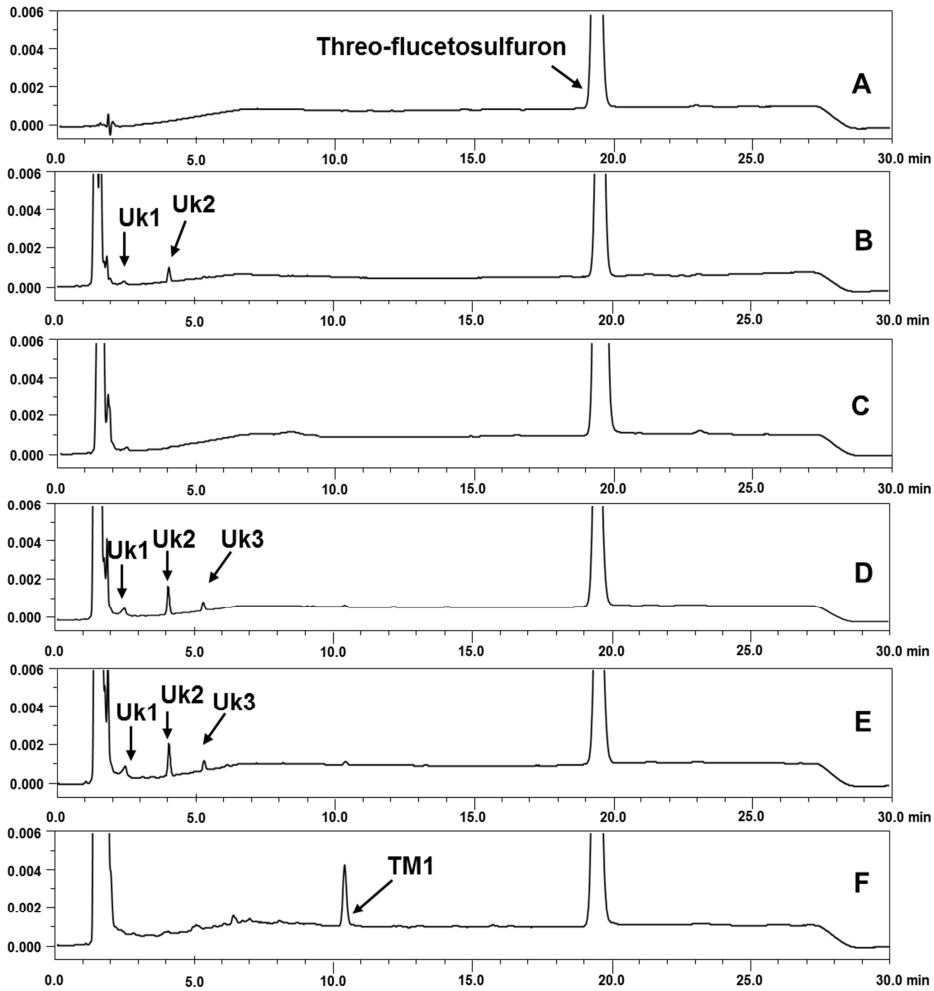


Figure 37. HPLC profiles of threo-flucetosulfuron with GI solutions, buffer solution pH7.4 (A), buffer solution pH2 (B), saliva (C), saliva+gastric juice (1:4 v/v) (D), gastric juice (E), and intestinal juice (F)

Erythro-flucetosulfuron also shows a similar metabolism pattern as that by the *threo*-isomer (Figure 38). Reaction mixtures with saliva + gastric juice (1:4 v/v) and artificial gastric juice for 2 h each produced metabolites

such as Uk4, Uk5, and Uk6, which eluted at 2.45, 4.05, and 4.95 min, respectively. No other metabolite peaks or interfering peaks were observed in the chromatograms.

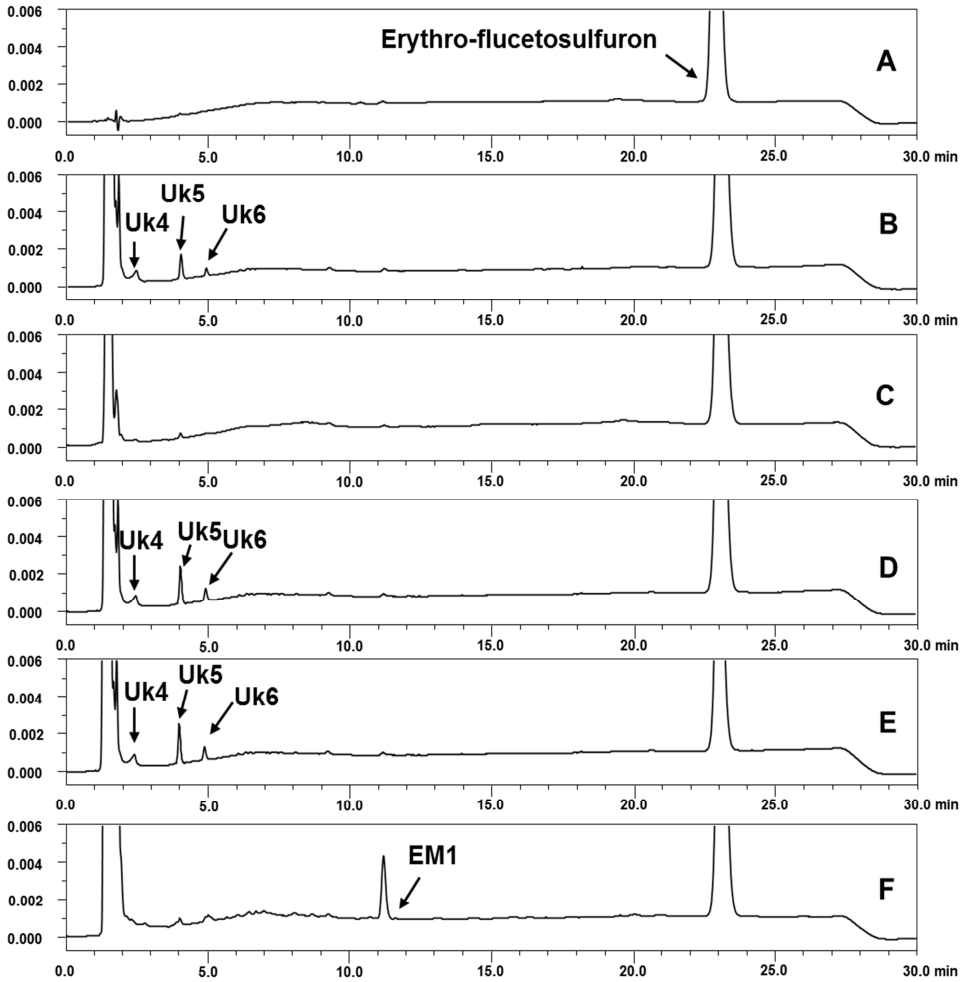


Figure 38. HPLC profiles of *erythro*-flucetosulfuron with GI solutions, buffer solution pH7.4 (A), buffer solution pH2 (B), saliva (C), saliva+gastric juice (1:4 v/v) (D), gastric juice (E), and intestinal juice (F)

3. Identification of unknown metabolites

To identify the structure of unknown metabolites, the metabolic reaction mixtures were analyzed using LC-MS/MS. From the LC-MS/MS fragmentation patterns, it was observed that the metabolites showed the same $[M+H]^+$ at m/z 416 as those after the *in vitro* metabolism of flucetosulfuron by HLMs, indicating that TM1 and EM1 can be formed in the intestinal juice (Figure 39A). TM1 and EM1 were already identified as the main metabolites of flucetosulfuron isomers in *in vitro* metabolism of flucetosulfuron by HLMs (Lee et al., 2014).

Furthermore, the LC-MS/MS analysis showed that Uk1 and Uk4 in the reaction mixture of gastric juice and saliva + gastric juice (1 + 4, v/v) had the same $[M+H]^+$ at m/z 235 as those of the fragment ions at *m/z* 106, 136, 200, and 217 (Figure 39B), indicating cleavage of the pyrimidine moiety from the parent flucetosulfuron isomers ($[M+H]^+$ = m/z 488). The results suggested that the metabolites, Uk1 and Uk4 are hydrolysis product, M2: *threo*-M2 (TM2) and *erythro*-M2 (EM2), respectively.

The LC-MS/MS spectra of Uk2 and Uk5 ($[M+H]^+$ = m/z 156) showed fragment ions at *m/z* 112 and 140 (Figure 39C). Uk2 and Uk5 were identified as M3, which may have been formed by the cleavage of the sulfonylurea bridge.

The LC-MS/MS spectra of Uk3 and Uk6 ($[M+H]^+$ = m/z 307) showed fragmentation ions at *m/z* 106, 136, 200, 217, and 247 (Figure 39D), indicating that Uk3 and Uk6 is M4, formed by the cleavage of the sulfonylurea bridge. Uk3 and Uk6 were in the threo (TM4) and erythro (EM4) forms, respectively.

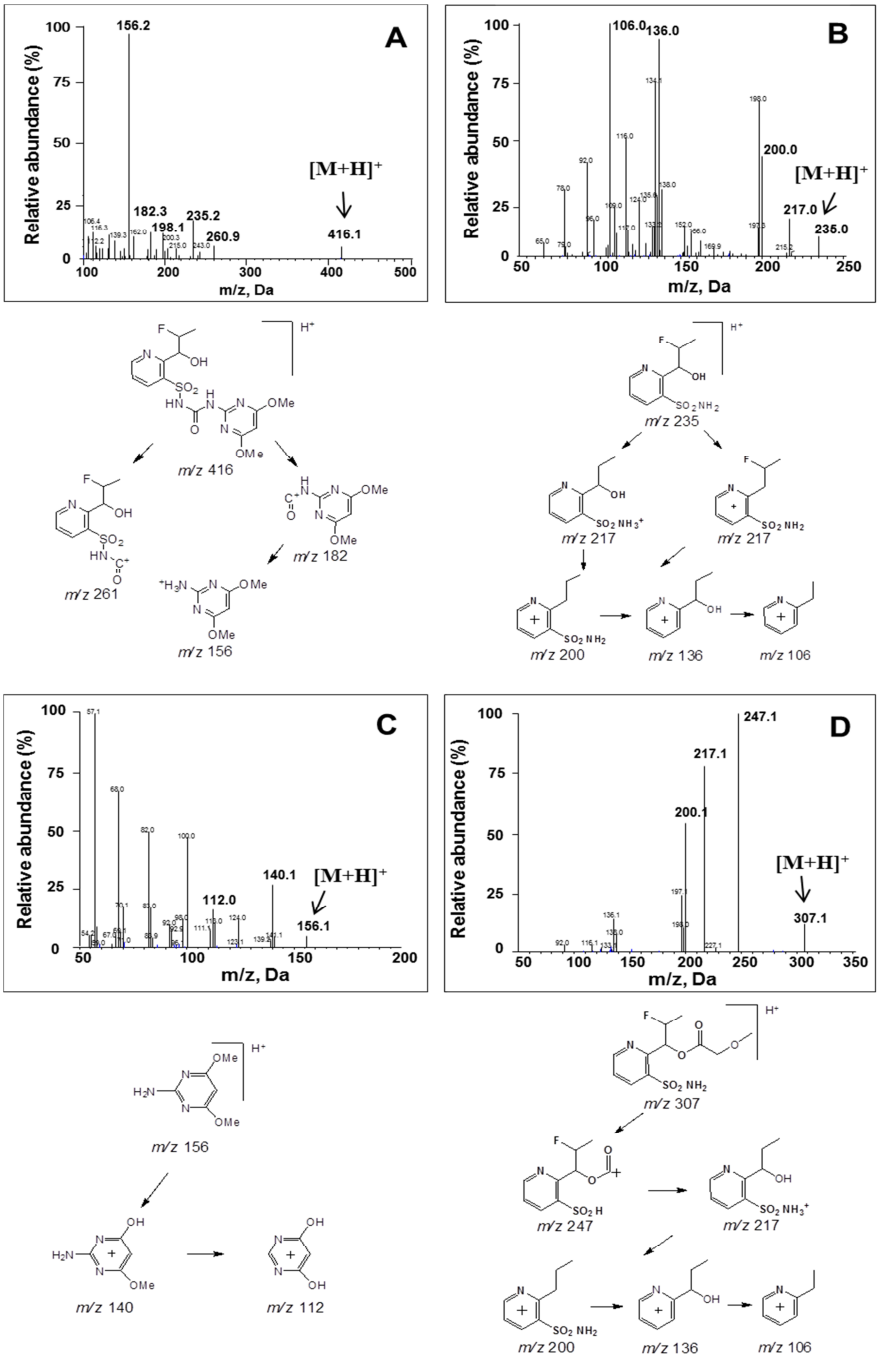


Figure 39. LC-MS/MS Spectra and fragmentation of metabolites, M1 (A; $[M+H]^+$ = m/z 416), M2 (B; $[M+H]^+$ = m/z 235), M3 (C; $[M+H]^+$ = m/z 156), and M4 (D; $[M+H]^+$ = m/z 307).

By comparing the LC-MS/MS fragmentation spectra and co-chromatography, the metabolites that formed in the GI juices were found to be identical. Thus, in the case of oral intake of flucetosulfuron, It was determined that the metabolites of flucetosulfuron in the GI juices were easily hydrolysable at the sulfonylurea bridge or ester bond, under normal digestion conditions.

The metabolite formation patterns, with only *threo*-forms—TM1, TM2, and TM4 from *threo*-flucetosulfuron and only *erythro*-forms—EM1, EM2, and EM4 from *erythro*-flucetosulfuron by artificial GI juices, indicated no possibility of chiral conversion between the flucetosulfuron isomers or their metabolites during metabolic reaction with the GI juices.

4. *In vitro* metabolism of flucetosulfuron by GIs

The degradation patterns of flucetosulfuron isomers in each artificial GI juice are shown in Figure 40. Flucetosulfuron isomers were relatively stable in the buffer solution of pH 7.4 and in artificial saliva, whereas they were easily degraded under acidic condition at pH 2 within 2 h.

In the incubation mixture of artificial Saliva + Gastric juice (1:4 v/v) for 2 h, flucetosulfuron isomers were not stable and their residues were 68.9~76.1%. In Gastric juice for 2h, their remaining residues were declined to 14.5% for *threo*-flucetosulfuron and 13.1% for *erythro*-flucetosulfuron, indicating that both isomers were extremely unstable in artificial Gastric juice.

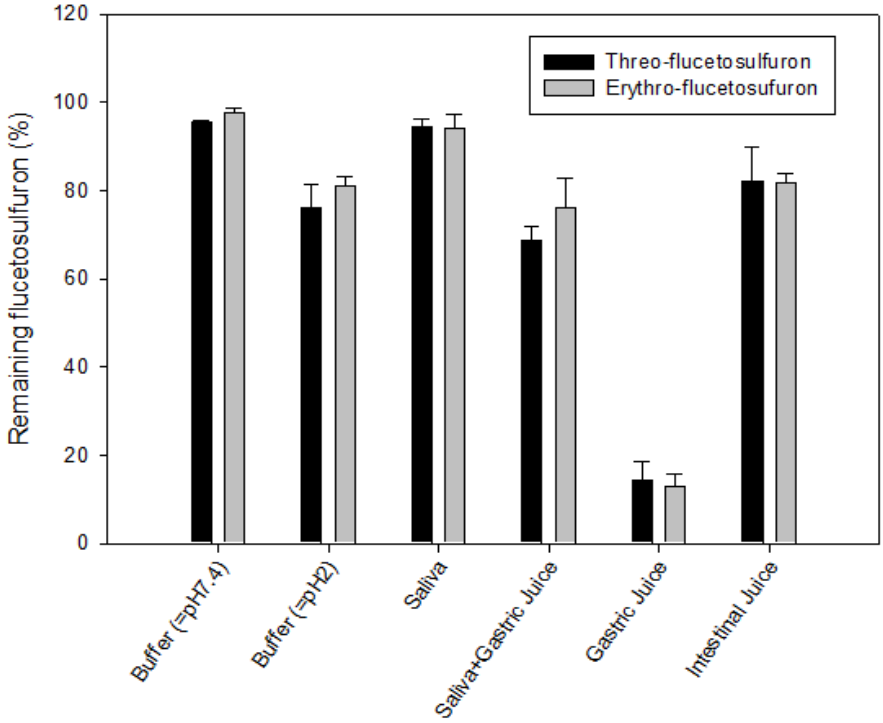


Figure 40. Remaining flucetosulfuron after metabolic reaction with artificial gastrointestinal juices.

The metabolites M2 (TM2 or EM2), M3, and M4 (EM4 or TM4) were produced in the reaction mixture of gastric juice and saliva + gastric juice (1:4 v/v), indicating that acidic pH and pepsin had catalytic effects on the formation of metabolites through breakdown of the sulfonylurea bridge and ester bond. M1 (TM1 or EM1) was also observed under acidic conditions such as saliva + gastric juice (1:4 v/v); however, the level was under the limit of quantification, indicating that the sulfonylurea bridge is the most prone to be hydrolyzed under acidic conditions (Table. 22).

Table 22. Metabolism of flucetosulfuron isomers (100 μM) in GIs for optimal times

	Gastrointestinal Juices (GIs)	Optimal time	Concentration (μM)				
			M1 (TM1 or EM1)	M2 (TM2 orEM2)	M3	M4 (TM4 or EM4)	Flucetosulfuron
***Threo*-flucetosulfuron**	Buffer (pH7.4)	6hrs	-	-	-	-	95.7 ± 2.9
	Buffer (pH2)	2hrs	-	0.75 ± 0.13	0.58 ± 0.16	-	76.1 ± 5.3
	Saliva	5mins	-	-	-	-	94.7 ± 1.7
	Saliva + Gastric Juice (1:4 v/v)	2hrs	<0.2	2.48 ± 0.12	1.91 ± 0.87	1.71 ± 0.10	68.9 ± 1.8
	Gastric Juice	2hrs	<0.2	2.12 ± 0.11	2.86 ± 0.02	1.42 ± 0.26	14.5 ± 2.0
	Intestinal Juice	6hrs	4.44 ± 0.59	-	-	-	82.1 ± 7.7
***Erythro*-flucetosulfuron**	Buffer (pH7.4)	6hrs	-	-	-	-	95.2 ± 3.3
	Buffer (pH2)	2hrs	-	0.70 ± 0.18	1.30 ± 0.14	-	81.3 ± 0.2
	Saliva	5mins	-	-	-	-	94.2 ± 3.3
	Saliva + Gastric Juice (1:4 v/v)	2hrs	<0.2	2.35 ± 0.71	2.70 ± 0.32	1.17 ± 0.17	76.1 ± 6.9
	Gastric Juice	2hrs	<0.2	2.99 ± 0.14	2.82 ± 0.05	1.26 ± 0.30	13.1 ± 2.6
	Intestinal Juice	6hrs	5.09 ± 0.13	-	-	-	81.8 ± 2.2

On the other hand, the residues of flucetosulfuron in the metabolic reaction mixture of artificial intestinal juice were 82.1% for *threo*-isomer and 81.8% for *erythro*-isomer, indicating that flucetosulfuron is slightly unstable in artificial intestinal juice. TM1 from *threo*-flucetosulfuron and EM1 form *erythro*-flucetosulfuron were the main metabolites in the intestinal juice, with no other metabolites observed.

Considering that the pH of the artificial intestinal juice was adjusted to 7.4, a stable condition for flucetosulfuron, it was estimated that trypsin, pancreatin, and bile in the intestinal juice exerted hydrolytic effects on flucetosulfuron. No significant differences were observed in the degradation patterns between flucetosulfuron isomers in each artificial GI juice.

Because it is impossible to perform *in vivo* metabolism of pesticides in humans, the current *in vitro* metabolism of flucetosulfuron was designed to provide an ideal estimation of the *in vivo* situation in humans. In this model, both flucetosulfuron isomers were rapidly degraded within 2 h in the gastric juices, and the degradation rate was higher than 85% of flucetosulfuron.

To date, some researchers have suggested pesticide biomonitoring approach by using animal and human salivas or artificial GI juices. But, there was limited information on metabolism of pesticides by GI juices (Nigg *et al.* 1993, Lu *et al.* 2003, Timchalk *et al.* 2007, Lee 2008, Esteban and Castaño 2009). In fact, chlorpyrifos metabolism in human intestinal tissue was investigated and the results suggested that following oral exposure, the intestinal bioactivation of chlorpyrifos, although much lower when compared to the total hepatic metabolism, could play a role in the pre-systemic chlorpyrifos clearance (Leoni *et al.* 2012).

This study showed that flucetosulfuron could be degraded in the GI organs, especially in the stomach (~85% degradation) and intestines (~18% degradation), before reaching other metabolic organs such as the liver or cytosol through the blood stream.

This *in vitro* study was designed on the basis of the secreted gastric juice in stomach having pH 2, while the acidity may be weakened by dilution with food in real digestion condition in stomach (Melander 1978, Weitschies *et al.* 2005). Because flucetosulfuron can be more stable with intaking with food, there are still some possibility that the more flucetosulfuron can be transferred from GI tracts to the blood stream, resulting the higher bioavailability than this study.

On the basis of these results, the proposed metabolic pathway of flucetosulfuron by artificial gastric juices is shown in Figure 41. The present study showed that there is no significant difference in the degradation patterns between flucetosulfuron isomers in each artificial GI juice. Considering the rapid degradation of flucetosulfuron by artificial GI juices in the *in vitro* metabolism study, the availability of flucetosulfuron from the GI tract into the blood stream is considered limited in the case of accidental oral intake of flucetosulfuron, and hence, the possibility of inducing any serious human toxicity is expected to be negligible.

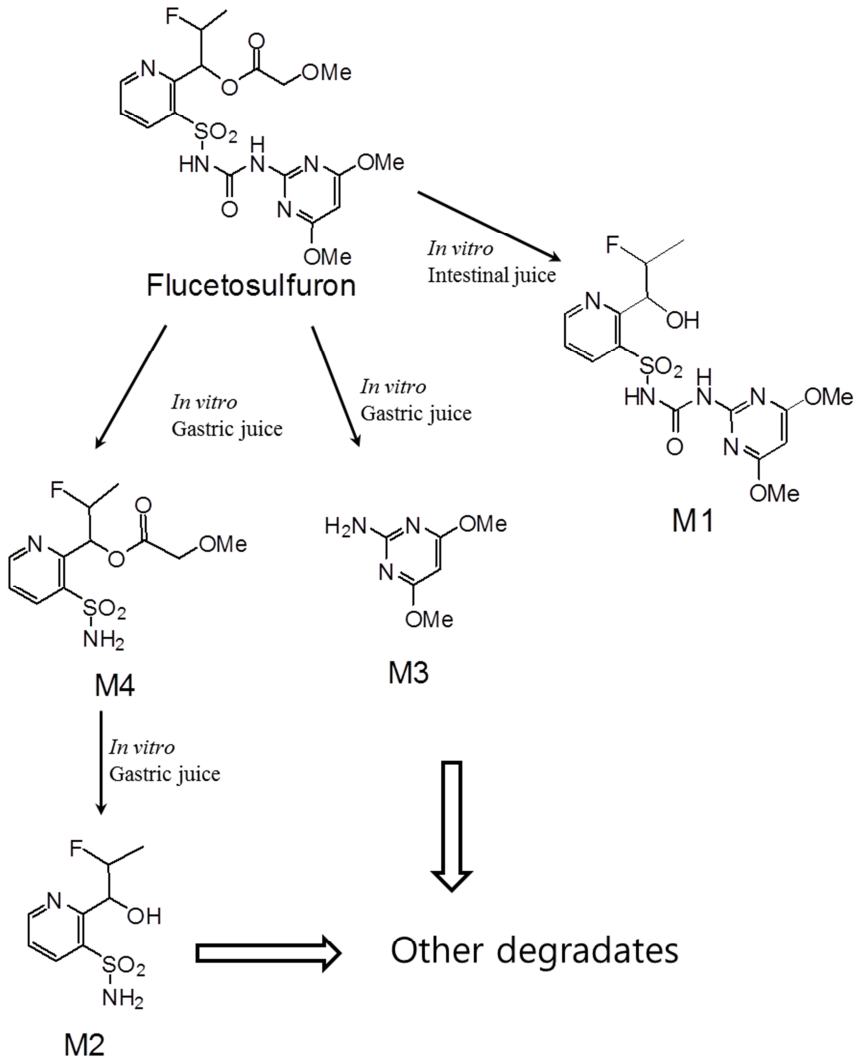


Figure 41. Proposed metabolic pathway of flucetosulfuron in *in vitro* metabolism by artificial GIs

Ⅳ. Conclusion

The *in vitro* metabolism of herbicide, flucetosulfuron was investigated by artificial GIs using *threo*- and *erythro*-isomers, respectively. The metabolites produced in each reaction mixture of artificial GI juices were unambiguously identified using liquid chromatography-tandem mass spectrometry. Flucetosulfuron was observed to be stable in saliva. However, in the intestinal juices, approximately 18% of flucetosulfuron was degraded, producing N-(4,6-dimethoxypyrimidin-2-ylcarbomoyl)-2-(2-fluoro-1-hydroxypropyl)pyrimidine-3-sulfonamid (M1). In artificial gastric juices, about 85% of flucetosulfuron was rapidly degraded, producing the metabolites 2-(2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide (M2), 4,6-dimethoxypyrimidin-2-amine (M3), and 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl 2-methoxyacetate (M4). These results indicate that the sulfonylurea bridge and ester bond of flucetosulfuron are hydrolyzed in artificial GI juices. *In vitro* metabolism of each flucetosulfuron isomer with artificial GIs (GIs) showed that there were not any significant differences in the degradation patterns between flucetosulfuron isomers in each artificial GIs. Considering the rapid degradation of flucetosulfuron *in vitro* by artificial GI juices, it is likely that there would be no significant absorption of flucetosulfuron from the GI tract into the blood stream after oral administration and hence the possibility of inducing ng serious human toxicity by flucetosulfuron is expected to be negligible. During the study, there was not any chiral conversion between flucetosulfuron isomers or metabolite isomers.

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국문 요약

본 연구에서는 국내에서 최초로 개발된 신규 sulfonylurea 제초제인 flucetosulfuron 의 인체간마이크로좀(Human liver microsomes; HLMs)과 인공소화액(Artificial gastrointestinal juices)에 의한 *in vitro* 생체대사연구를 수행하였다. Flucetosulfuron 은 *threo* 와 *erythro* 인 부분입체이성질체로 구성되어 있기 때문에, 각각의 분리과정을 거쳐서 99.7% 순도의 *threo*-flucetosulfuron 과 99.8% 순도의 *erythro*-flucetosulfuron 을 확보하여 대사연구에 적용하였다. HLMs 을 이용한 *in vitro* 대사시험에서 생성된 대사물은 유일하게 M1 이 생성되었고, M1 은 *threo*-M1(TM1; (1S,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl)pyridin-2-yl)-2-fluoropropyl-2-methoxyacetat)과 *erythro*-M1 (EM1; (1R,2S)-1-(3-(N-(4,6-dimethoxypyrimidin-2-ylcarbamoyl)sulfamoyl) pyridine-2-yl)-2-fluoropropyl-2-methoxyacetate)이었다. TM1 과 EM1 은 각각 *threo*-flucetosulfuron 과 *erythro*-flucetosulfuron 으로부터 생성되었으며, LC-MS/MS 와 NMR 분석 및 합성한 TM1 과 EM1 의 Cochromatography 방법을 통해 확인하였다. TM1 과 EM1 은 가수분해를 통해서 생성되며, 이를 담당하는 효소는 cytochrome P450 이나 flavin-containing monooxygenase 가 아닌 esterases 임을 확인하였다. 최적화된 조건에서 HLMs 의 동력학시험을 실시하여 flucetosulfuron 과 대사물들을 정량분석한 결과, *threo*-flucetosulfuron 의 V_{max} (nmol/min/mg HLMs)와 K_m (μM)과 CL_{int} (V_{max}/K_m (μL/min/mg HLMs)) 는 각각 134.38 과 1798.53, 51.2 였고, *erythro*-flucetosulfuron 은 각각 151.41 과 3957.37, 48.02 로 나타났으나, HLMs 의 대사연구에서 두 이성질체의 구조적 차이에 따른 유의성있는 차이를 볼 수 없었다. Esterases 의 선택적 저해시험을 통해 flucetosulfuron 의 가수분해에 직접적으로 관여하는 효소는 Carboxylesterases 와 Cholinesterases 임을 확인하고, 각각의 human acetylcholinesterase (AChE)와 human butyrylcholinesterase (BChE), 그리고 3 종의 재조합 human carboxylesterases (CES: CES1b 와 CES1c, CES2)의 효소들을 이용한 대사시험을 실시하여, HLMs 대사시험과 동일한 M1 (TM1 과 EM1)이 생성됨을 검증하였다. 따라서, 상기의 효소들에 의한 동력학시험을 실시하고, flucetosulfuron 과

대사물들의 정량분석을 하였다. CES2 의 CL_{int} (V_{max} /K_m (μL/min/mg HLMs)는 *threo*-와 *erythro*-flucetosulfuron 에서 각각 16.98 과 17.43 수준으로, 시험한 재조합 CES 효소들 중에 가장 flucetosulfuron 에 대한 높은 가수분해활성을 보여주었다. *Threo*-flucetosulfuron 에 대한 AChE 와 BChE 의 V_{max} 는 *erythro*-flucetosulfuron 보다 각각 2.0 배와 3.8 배로써, BChE 에서 더 큰 차이를 보였다. 또한, BChE 에서의 *threo*-flucetosulfuron 의 CL_{int} 는 4.3 으로, 0.87 을 보인 *erythro*-flucetosulfuron 보다 5 배수준으로 빠르게 대사됨을 보여주었다. CES1b 와 CES1c, CES2, AChE 와는 달리, BChE 에서는 flucetosulfuron 이성질체들의 선택적인 대사차이가 유의성 있게 나타남을 알 수 있었다. 인공소화액인 타액, 위액과 장액을 이용한 *in vitro* 대사시험에서는 HLMs 보다 다양한 대사물이 생성되었다. 인공타액에서는 분해가 없었으나, 인공위액에서는 설포닐유레아결합과 에스터결합의 분해산물들인 2-(2-fluoro-1-hydroxypropyl)pyridine-3-sulfonamide (M2)와 4,6-dimethoxypyrimidin-2-amine (M3), 2-fluoro-1-(3-sulfamoylpyridin-2-yl)propyl 2-methoxyacetate (M4)가 관찰되었고, 인공장액에서는 M1 이 주요대사물로 관찰되었다. Flucetosulfuron 과 대사물들의 정량분석결과, 인공위액에서 85%이상의 빠른 분해를 보였고, 장액에서는 18%의 분해를 보였으나, flucetosulfuron 의 이성질체간의 분해의 양상의 차이는 없었다. Flucetosulfuron 이 경구를 통한 체내 유입될 경우, 인공소화액에서 분해가 빠르게 진행되므로, 실제 체내 흡수량이 미미할 것으로 판단되었다. 본 연구결과, HLMs 과 인공소화액에 의한 플루세토설퓨론의 대사양상이 제안되었다.

주요어: flucetosulfuron, 인체간마이크로좀, esterase, 인공소화액, 대사시험

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