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Do Cytochrome P450 Enzymes Contribute to the Metabolism of Xenobiotics in Human?

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1. Introduction

The cytochromes P450 (CYP) comprise a large multigene family of hemethiolate proteins which are of considerable importance in the metabolism of xenobiotics and endobiotics. CYP enzymes in humans as well as in other species have been intensively studied during recent years (Pelkonen et al., 2008; Turpeinen et al., 2007). It is possible to characterize metabolic reactions and routes, metabolic interactions, and to assign which CYP is involved in the metabolism of a certain xenobiotic by different *in vitro* approaches (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Hodgson and Rose, 2007a). Risk assessment needs reliable scientific information and one source of information is the characterization of the metabolic fate and toxicokinetics of compounds. Toxicokinetics refers to the movement of a xenobiotic into, through, and out of the body and is divided into several processes including absorption, distribution, metabolism, and excretion (ADME). Metabolism is one of the most important factors that can affect the overall toxic profile of a pesticide. During metabolism, the chemical is first biotransformed by phase I enzymes, usually by the cytochrome P450 (CYP) enzyme system, and then conjugated to a more soluble and excretable form by phase II conjugating enzyme systems (Guengerich & Shimada, 1991). In general, these enzymatic reactions are beneficial in that they help eliminate foreign compounds. Sometimes, however, these enzymes transform an otherwise harmless substance into a reactive form – a phenomenon known as metabolic activation (Guengerich & Shimada, 1991).Exposure to pesticides is a global challenge to risk assessment (Alavanja et al., 2004; Maroni et al., 2006). On a world-wide basis, acute pesticide poisoning is an important cause of morbidity and mortality. In an extrapolation, WHO/UNEP estimated that more than 3 million people were hospitalized for pesticide poisoning every year and that 220 000 died; it particularly noted that two-thirds of hospitalizations and the majority of deaths were attributable to intentional self-poisoning rather than to occupational or accidental poisoning (Konradsen et al., 2005; WHO/UNEP, 1990). Humans are inevitably exposed to pesticides in a variety of ways: at different dose levels and for varying periods of time (Boobis et al., 2008; Ellenhorns et al., 1997).

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2. CYPs - the human xenobiotic-metabolizing enzymes

CYPs are found in high concentrations in the liver, but are present in a variety of other tissues, including lungs (Lawton et al., 1990), kidneys ((Hjelle et al., 1986; Tremaine et al., 1985), the gastrointestinal tract (Dutcher & Boyd, 1979; Peters and Kremers, 1989), nasal mucosa (Adams et al., 1991; Eriksson and Brittebo, 1991), skin (Khan et al., 1989) and brain tissue (Bergh & Strobel, 1992; Dhawan et al., 1990). CYPs are categorized into families and subfamilies by their sequence similarities. Humans have 18 families of cytochrome P450 genes and 44 subfamilies. The enzymes are thus identified by a number denoting the family, a letter denoting the subfamily and a number identifying the specific member of the subfamily. The example given below explains the system of nomenclature followed (Fig. 1).

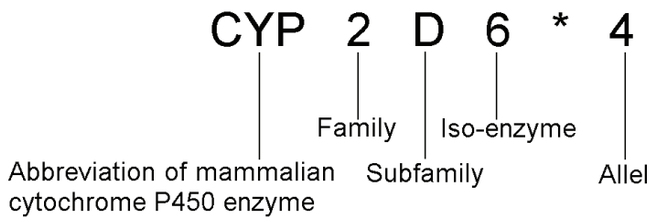


Fig. 1. An example of the nomenclature of the cytochrome P450 enzymes (modified from (Wijnen et al., 2007)).

The website http://drnelson.utmem.edu/CytochromeP450.html contains more detailed classification related to the cytochrome P450 metabolizing enzymes. The CYP enzymes in families 1-3 (Fig. 2) are active in the metabolism of a wide variety of xenobiotics including drugs (Pelkonen et al., 2008; Pelkonen et al., 2005; Rendic & Di Carlo, 1997).

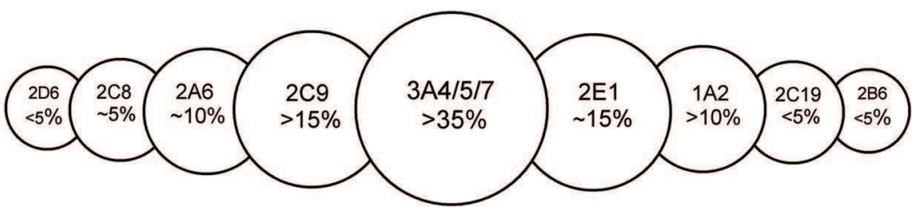


Fig. 2. Relative abundance of individual CYP forms in the liver (modified from (Pelkonen et al., 2008)).

2.1 CYP1A subfamily

CYP1A1 and CYP1A2 are members of the CYP1A subfamily. CYP1A1 is a major extrahepatic CYP enzyme and its level of expression in human liver is very low (Pelkonen et al., 2008; Guengerich & Shimada, 1991; Ding & Kaminsky, 2003; Edwards et al., 1998; McKinnon et al., 1991; Pasanen & Pelkonen, 1994; Raunio et al., 1995). In humans, CYP1A2 is expressed mainly in liver and in lower levels in lung along with CYP1A1 (Liu et al., 2003; Wei et al., 2001; Wei et al., 2002). CYP1A2 represents about 10% of total CYP enzymes in the human liver (Pelkonen & Breimer, 1994; Shimada et al., 1994). CYP1A2 enzyme levels in the human liver display some variability between individuals (Shimada et al., 1994).

2.2 CYP2A subfamily

The human CYP2A subfamily contains three genes i.e. CYP2A6, CYP2A7, and CYP2A13, and two pseudogenes (Hoffman et al., 1995; Honkakoski & Negishi, 1997; Pedro et al., 1995).

CYP2A6 represents 10% of the total CYP content in liver (Pelkonen et al., 2008; Yun et al., 1991). CYP2A6 enzyme activity in the human liver displays a relatively large variability between individuals, and some Japanese are known to lack the functional protein completely (Pelkonen et al., 2008; Pelkonen et al., 2000; Shimada et al., 1996).

2.3 CYP2B subfamily

CYP2B6 represents approximately 1-10% of the total hepatic CYPs. A notable interindividual variability in the expression of CYP2B6 has been reported (Code et al., 1997; Faucette et al., 2000; Lang et al., 2001; Stresser & Kupfer, 1999; Yamano et al., 1989). CYP2B6 has a high polymorphic expression and it is affected by genotype and gender.

2.4 CYP2C subfamily

The CYP2C subfamily has four active members, namely 2C8, 2C9, 2C18 and 2C19. CYP2Cs are the second most abundant CYP proteins in human liver and the CYP2C subfamily consists of three members, comprising about 20 % of the total P450 enzymes. In humans, CYP2C9 is the main CYP2C, followed by CYP2C8 and CYP2C19, while CYP2C18 is not expressed in liver (Pelkonen et al., 2008; Edwards et al., 1998; Shimada et al., 1994; Gray et al., 1995; Richardson et al., 1997). CYP2C9 is a major CYP2C isoform in the human liver, and it is one of several CYP2C genes clustered in a 500kb region on the proximal 10q24 chromosomal region (Gray et al., 1995; Goldstein and de Morais, 1994). In Caucasian populations, the frequencies of the two variant alleles, CYP2C9*2 and CYP2C9*3, range from 7% to 19% (Furuya et al., 1995; Ingelman-Sundberg et al., 1999; Miners & Birkett, 1998; Stubbins et al., 1996; Sullivan-Klose et al., 1996; Yasar et al., 1999). CYP2C19, another member of the CYP2C enzyme family, represents approximately 5% of the total hepatic CYPs and metabolizes drugs that are amides or weak bases with two hydrogen bond acceptors (Pelkonen et al., 2008; Lewis, 2004; Musana & Wilke, 2005). Poor metabolizers with low CYP2C19 activity represent 3 to 5% of Caucasians and African-Americans, and 12 to 23% of most Asian populations (Goldstein, 2001).

2.5 CYP2D subfamily

CYP2D6 represents 1 to 5% of the total CYP, and approximately 3.5 and 5-10% of the Caucasian population are ultra-rapid and poor metabolize's for this enzyme, respectively. The CYP2D6 gene is clearly the most polymorphic of all known cytochrome P450s; more than 75 polymorphisms have been identified. Four alleles account for > 95% of the functional variation observed in the general population (Pelkonen et al., 2008; Shimada et al., 1994; Musana & Wilke, 2005; Al, Omari, A., & Murry, 2007; Ingelman-Sundberg, 2004; Zanger et al., 2004).

2.6 CYP2E subfamily

Only one gene belonging to this subfamily, namely CYP2E1, has been identified (Nelson et al., 1996; Nelson et al., 2004). CYP2E1 is one of the most abundant hepatic CYPs, represents 15% of the total P450 and it is also expressed in lung and brain (Pelkonen et al., 2008; Raunio et al., 1995).

2.7 CYP3A subfamily

In humans, the CYP3A subfamily contains three functional proteins, CYP3A4, CYP3A5, and CYP3A7, and one pseudoprotein, CYP3A34. The human CYP3 family constitutes approximately 30 % of total hepatic P450 and is estimated to mediate the metabolism of

around 50% of prescribed drugs as well as a variety of environmental chemicals and other xenobiotics. Because of the large number of drugs metabolized by CYP3A4, it frequently plays a role in a number of drug-drug interactions (Pelkonen et al., 2008; Shimada et al., 1994; Musana & Wilke, 2005; Bertz & Granneman, 1997; Domanski et al., 2001; Imaoka et al., 1996; Rostami-Hodjegan & Tucker, 2007).

CYP3A4 is the major form of P450 expressed in human liver. It is also the major P450 expressed in human gastrointestinal tract, and intestinal metabolism of CYP3A4 substrate can contribute significantly to first-pass elimination of orally ingested xenobiotics (Guengerich, 1995; Guengerich, 1999). X-ray crystallography studies demonstrated that CYP3A4 has a very large and flexible active site, allowing it to oxidize either large substrates such as erythromycin and cyclosporine or multiple smaller ligands (Scott & Halpert, 2005; Tang & Stearns, 2001).

CYP3A5 is a minor polymorphic CYP isoform in human liver in addition to the intestine (Lin et al., 2002; Paine et al., 1997) and kidney (Haehner et al., 1996). Functional CYP3A5 is expressed in approximately 20% of Caucasians and about 67% of African-Americans (Kuehl et al., 2001). CYP3A5 may have a significant role in drug metabolism particularly in populations expressing high levels of CYP3A5 and/or on co-medications known to inhibit CYP3A4 (Soars et al., 2006).

Expression of CYP3A7 protein is mainly confined to fetal and newborn livers, although in rare cases CYP3A7 mRNA has been detected in adults (Hakkola et al., 2001; Kitada & Kamataki, 1994; Schuetz et al., 1994).

3. Xenobiotic biotransformation

Xenobiotic biotransformation is the process by which lipophilic foreign compounds are metabolized through enzymatic catalysis to hydrophilic metabolites that are eliminated directly or after conjugation with endogenous cofactors via renal or biliary excretion. These metabolic enzymes are divided into two groups, Phase I and Phase II enzymes (Rendic & Di Carlo, 1997; Oesch et al., 2000).

Phase I products are not usually eliminated rapidly, but undergo a subsequent reaction in which an endogenous substrate such as glucuronic acid, sulfuric acid, acetic acid, or an amino acid combines with the newly established functional group to form a highly polar conjugate to make them more easily excreted. Sulfation, glucuronidation and glutathione conjugation are the most prevalent classes of phase II metabolism, which may occur directly on the parent compounds that contain appropriate structural motifs, or, as is usually the case, on functional groups added or exposed by Phase I oxidation (LeBlanc & Dauterman, 2001; Rose & Hodgson, 2004; Zamek-Gliszczynski et al., 2006).

3.1 In vitro and human-derived techniques for testing xenobiotic metabolism

In order to study the metabolism and interactions of pesticides in humans we have to rely upon *in vitro* and human-derived techniques. *In vitro* systems have become an integral part of drug metabolism studies as well as throughout the drug discovery process and in academic research (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Lin & Lu, 1997). *In vitro* approaches to predict human clearance have become more frequent with the increase in the availability of human-derived materials (Skett et al., 1995). All models have certain advantages and disadvantages, but the common advantage to these approaches is the reduction of the complexity of the study system. However, the use of *in vitro* models is always a compromise between convince and relevance (Pelkonen et al., 2005; Brandon et al.,

2003; Pelkonen & Turpeinen, 2007; Pelkonen & Turpeinen, 2007). An overview of different *in vitro* models and their advantages and disadvantages are collected in Table 1.

Enzyme sources	Availability	Advantages	Disadvantages
Liver homogenates[a]	Relatively good. Commercially available. Human liver samples obtained under proper ethical permission.	Contains basically all hepatic enzymes.	Liver architecture lost. Cofactors are necessary.
Microsomes[a]	Relatively good, from transplantations or commercial sources.	Contains important rate-limiting enzymes. Inexpensive technique. Easy storage. Study of individual, gender-, and species-specific biotransformation.	Contains only CYP and UGTs. Requires strictly specific substrates and inhibitors or antibodies. Cofactor addition necessary.
cDNA-expressed individual CYPs[b]	Commercially available	The role of individual CYPs in the metabolism can be easily studied. Different genotypes. High enzyme activities.	The effect of only one enzyme at a time can be evaluated. Problems in extrapolation to HLM and *in vivo*.
Primary hepatocytes[c,d]	Difficult to obtain, relatively healthy tissue needed. Commercially available	Contains the whole complement of CYPs cellularly integrated. The induction effect can be studied. Well established and characterized. Transporters still present and operational.	Requires specific techniques and well established procedures. The levels of many CYPs decrease rapidly during cultivation. Cell damage during isolation.
Liver slices[e]	Difficult to obtain, fresh tissue needed.	Contains the whole complement of CYPs and cell-cell connections. The induction, morphology and interindividual variation can be studied.	Requires specific techniques and well established procedures
Immortalized cell lines[f]	Available upon request, not many characterized cell lines exist.	Non-limited source of enzymes. Easy to culture. Relatively stable enzyme expression level. The induction effect can be studied.	The expression of most CYPs is poor.

[a] (Kremers, 1999); [b] (Rodrigues, 1999); [c] (Guillouzo, 1995); [d] (Gomez-Lechon et al., 2004); [e] (Olinga et al., 1998); [f] (Allen et al., 2005).

Table 1. An overview of different in vitro models and their advantages and disadvantages (modified from (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Brandon et al., 2003; Pelkonen & Turpeinen, 2007)).

3.2 In vitro characterization of the metabolism and metabolic interactions of xenobiotics

The aim of *in vitro* characterization is to produce relevant and useful information on metabolism and interactions to anticipate, and even to predict, what happens in man. Each *in vitro* model has its own set of advantages and disadvantages as they range from simple to more complex systems: individual enzymes, subcellular fractions, cellular systems, liver slices and whole organ, respectively (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Brandon et al., 2003). To understand some of the factors related to xenobiotic metabolism that can influence the achievement of these aims, there are several important points to consider such as (Pelkonen et al., 2005; Pelkonen & Raunio, 2005; Pelkonen & Turpeinen, 2007; Hodgson & Rose, 2005):

- Determination of the metabolic stability of the compound
- Identification of reactive metabolites
- Evaluation of the variation between species
- Identification of human CYPs and their isoforms involved in the activation or detoxification
- Evaluation of the variation between individuals
- Identification of individuals and subpopulations at increased risk
- Overall improvement of the process of human risk assessment

An overview of different in vitro studies for the characterization of metabolism and metabolic interactions of xenobiotics are collected in Table 2.

In vitro test	*In vitro* model	Parameters	Extrapolations
Metabolic stability	Microsomes Homogenates Cells Slices	Disappearance of the parent molecule or appearance of (main) metabolites	Intrinsic clearance Interindividual variability Interspecies differences
Metabolite identification and quantitation	Microsomes Homogenates Cells Slices	Tentative identification by (e.g.) LC/TOF-MS	Metabolic routes Semi-quantitative Interspecies differences
Identification of metabolizing enzymes	Microsomes with inhibitors or inhibitory antibodies Recombinant CYPs Hepatocytes	Relative ability of enzymes to metabolize a compound	Prediction of effects of various genetic, environmental and pathological factors Interindividual variability
Enzyme inhibition	Microsomes Recombinant enzymes Hepatocytes	Inhibition of specific model substrate	Potential interactions
Enzyme induction	Cells Slices Permanent cell lines	Induction of CYP model activities or mRNA	Induction potential of a substance

Table 2. In vitro studies for the characterization of the metabolism and metabolic interactions of xenobiotics (modified from (Pelkonen et al., 2005; Pelkonen and Raunio, 2005)).

4. The contribution of CYPs to the metabolism of xenobiotics in human

4.1 CYP1A subfamily

The catalytic activities of CYP1A2 have been reviewed by Pelkonen *et al.* (Pelkonen et al., 2008). CYP1A2 has a major role in the metabolism of many important chemicals such as caffeine (Butler et al., 1989; Tassaneeyakul et al., 1992), phenacetin (Sesardic et al., 1990; Venkatakrishnan et al., 1998), theophylline (Sarkar & Jackson, 1994; Tjia et al., 1996), clozapine (Fang et al., 1998), melatonin (Facciolá et al., 2001; von Bahr et al., 2000), and tizanidine (Granfors et al., 2004a; Granfors et al., 2004b). CYP1A1 is a major enzyme in the metabolism of a number of insecticides and herbicides (Lang et al., 1997; Tang et al., 2002; Abass et al., 2010; Abass et al., 2007c). CYP1A2 mediates herbicides (Lang et al., 1997; Abass et al., 2007c; Nagahori et al., 2000), insecticides (Tang et al., 2002; Stresser & Kupfer, 1998; Foxenberg et al., 2007; Mutch & Williams, 2006), and pyrethroids metabolism (Scollon et al., 2009).

4.2 CYP2A subfamily

It has been shown that CYP2A6 has a major role in the metabolism of nicotine *in vitro* and *in vivo* (Kitagawa et al., 1999; Messina et al., 1997; Nakajima et al., 1996a; Nakajima et al., 1996b; Yamazaki et al., 1999) and in the activation of aflatoxin B1 (Yun et al., 1991; Salonpää et al., 1993). More substrates and inhibitors currently known to be metabolized by or to interact with CYP2A6 *in vitro* and *in vivo* have been summarized by Pelkonen and co-workers (Pelkonen et al., 2008; Pelkonen et al., 2000). CYP2A6 participates in the metabolism of quite a few pesticides such as carbaryl, imidacloprid, DEET, carbosulfan and diuron (Tang et al., 2002; Abass et al., 2010; Abass et al., 2007c; Schulz-Jander and Casida, 2002; Usmani et al., 2002).

4.3 CYP2B subfamily

CYP2B6 is known to metabolize a large number of substrates including drugs, pesticides and environmental chemicals, many of which have been described in detail in reviews (see e.g. (Ekins & Wrighton, 1999; Hodgson & Rose, 2007b; Turpeinen et al., 2006)). Several clinically used drugs such as cyclophosphamide, bupropion, S-mephenytoin, diazepam, ifosamide and efavirenz are metabolized in part by CYP2B6 (Granvil et al., 1999; Haas et al., 2004; Huang et al., 2000; Jinno et al., 2003; Roy et al., 1999b; Roy et al., 1999a). CYP2B6 appears to activate and detoxify a number of precarcinogens (Code et al., 1997; Smith et al., 2003). CYP2B6 plays a major role in pesticides metabolism. CYP2B6 mediates herbicides N-dealkoxylation (Coleman et al., 2000); organophosphate insecticides desulfuration (Foxenberg et al., 2007; Mutch & Williams, 2006; Buratti et al., 2005; Leoni et al., 2008; Sams et al., 2000; Tang et al., 2001); orghanochlorine and carbamate insecticides sulfoxidation (Abass et al., 2010; Casabar et al., 2006; Lee et al., 2006); fungicide metalaxyl *O*-demethylation and lactone formation (Abass et al., 2007b).

4.4 CYP2C subfamily

CYP2C8 meditates amodiaquine N-deethylation, which is the selective marker activity, paclitaxel 6α-hydroxylation and cerivastatin demethylation (Li et al., 2002; Rahman et al., 1994). A few insecticides are mainly metabolized by CYP2C8 such as parathion, deltamethrin, esfenvalerate, and β-cyfluthrin (Mutch & Williams, 2006; Scollon et al., 2009; Mutch et al., 2003; Godin et al., 2007).

CYP2C9 is responsible for the metabolism of the S-isomer of warfarin (Rettie et al., 1992). CYP2C9 also metabolizes tolbutamide, the selective marker, glipizide, fluvastatin,

phenytoin, several non-steroidal anti-inflammatory agents and many other drug groups (Miners & Birkett, 1998; Doecke et al., 1991; Kirchheiner & Brockmoller, 2005; Rettie and Jones, 2005). CYP2C9 is found to be involved in the metabolism of pesticides such as pyrethroid insecticides (Scollon et al., 2009; Godin et al., 2007), as well as organophosphorus insecticides (Leoni et al., 2008; Usmani et al., 2004).

CYP2C19 participates in the metabolism of many commonly used drugs including the antiepileptics phenytoin and mephenytoin (Bajpai et al., 1996; Komatsu et al., 2000; Tsao et al., 2001; Wrighton et al., 1993)(Bajpai *et al.* 1996, Komatsu *et al.* 2000, Tsao *et al.* 2001, Wrighton *et al.* 1993), selective serotonin receptor inhibitors citalopram and sertraline (Kobayashi et al., 1997; von Moltke et al., 2001), the psychoactive drugs amitriptyline (Venkatakrishnan et al., 1998; Jiang et al., 2002) and diazepam, among others (Jung et al., 1997). Among the substrates of CYP2C19 are several widely used pesticides such as the phosphorothioate insecticides (Foxenberg et al., 2007; Mutch & Williams, 2006; Leoni et al., 2008; Tang et al., 2001; Usmani et al., 2004; Buratti et al., 2002; Kappers et al., 2001), as well as the pyrethroid insecticides (Scollon et al., 2009; Godin et al., 2007).

4.5 CYP2D subfamily

CYP2D6 metabolizes approximately 20 % of all commonly prescribed drugs *in vivo* (Brockmöller *et al.* 2000). For example, CYP2D6 contributes to the metabolism of betablockers metoprolol and timolol (Johnson & Burlew 1996, Volotinen *et al.* 2007) and the psychotropic agents amitriptyline and haloperidol (Coutts *et al.* 1997, Fang *et al.* 1997, Fang *et al.* 2001, Halling *et al.* 2008, Someya *et al.* 2003). Dextromethrophan *O*-demethylation is the most used *in vitro* model reaction for CYP2D6 activity (Kronbach *et al.* 1987, Park *et al.* 1984). Known pesticide substrates for CYP2D6 include phosphorothioate insecticides (Mutch *et al.* 2003, Mutch & Williams 2006, Sams *et al.* 2000, Usmani *et al.* 2004b) as well as (Johnson and Burlew, 1996; Volotinen et al., 2007) carbamate insecticide (Tang et al., 2002). CYP2D6 is also involved in the N-dealkylation of the atrazine and diuron herbicides (Lang et al., 1997; Abass et al., 2007c).

4.6 CYP2E subfamily

The metabolism of very few clinically important drugs such as paracetamol, caffeine, acetaminophen, enflurane and halothane seems to be mediated to some extent by CYP2E1 (Gu et al., 1992; Lee et al., 1996; Raucy et al., 1993; Thummel et al., 1993). Chlorzoxazone is probably the most used *in vitro* model substrate for CYP2E1 activity (Peter et al., 1990). Few pesticides have been reported to be metabolized at least in part by human CYP2E1 such as atrazine, carbaryl, parathion, imidacloprid and diuron (Lang et al., 1997; Tang et al., 2002; Abass et al., 2007c; Mutch & Williams, 2006; Schulz-Jander & Casida, 2002; Mutch et al., 2003).

4.7 CYP3A subfamily

CYP3A4 participates in the metabolism of several clinically important drugs such as triazolam, simvastatin, atorvastatin, and quinidine (Rendic & Di Carlo, 1997; Bertz & Granneman, 1997). Detailed characteristics of several CYP3A4 substrates and inhibitors were summarized recently by Liu *et al.* (Liu et al., 2007). The known pesticides mainly metabolized by CYP3A4 belong to several chemical groups such as, carbamate, phosphorothioate, chlorinated cyclodiene and neonicotinoid insecticides (Tang et al., 2002; Abass et al., 2010; Mutch & Williams, 2006; Schulz-Jander & Casida, 2002; Buratti et al., 2005;

Sams et al., 2000; Tang et al., 2001; Casabar et al., 2006; Lee et al., 2006; Mutch et al., 2003; Usmani et al., 2004; Buratti et al., 2002; Buratti et al., 2003; Buratti & Testai, 2007; Butler and Murray, 1997), herbicides (Abass et al., 2007c; Coleman et al., 2000), fungicides (Abass et al., 2007b; Abass et al., 2009; Mazur et al., 2007), and organotin biocide (Ohhira et al., 2006).
CYP3A5 mediates midazolam, alprazolam and mifepristone metabolism (Christopher Gorski et al., 1994; Galetin et al., 2004; Hirota et al., 2001; Huang et al., 2004; Khan et al., 2002; Williams et al., 2002). Alprazolam has been suggested as a selective probe for CYP3A5 (Galetin et al., 2004). The metabolism of a number of organophoshate and pyrethroid insecticides has been reported to be mediated by CYP3A5 (Mutch & Williams, 2006; Mutch et al., 2003; Godin et al., 2007).
CYP3A7 has similar catalytic properties compared with other CYP3A enzymes, including testosterone 6β-hydroxylation (Kitada et al., 1985; Kitada et al., 1987; Kitada et al., 1991).

5. The impact of modern analytical techniques in xenobiotics metabolism

5.1 Mass spectrometric methods in metabolism studies.

Traditionally metabolism studies were performed using gas chromatography-mass spectrometry (GC-MS). Because metabolites are usually polar molecules with high molecular masses, they have to be derivatised before measurement. After derivatisation the measured analyte is not anymore original metabolite but a less polar compound which is possible to vaporise and use in GC. Derivatisation can cause errors to the measurements and it is usually the most time and labour demanding phase which causes extra costs (Sheehan, 2002). In metabolism studies biggest problem of GC-MS is its lower sensitivity compared with modern mass spectrometric methods. Nevertheless GC-MS is still a useful method also in metabolism studies especially with thermally stable volatile compounds.
Nowadays the primary method used in metabolism studies is liquid chromatogram-mass spectrometry (LC-MS). Liquid chromatography is an old technique to separate polar compounds in liquid phase. However, it took quite a long time to develop a reliable technique to connect LC to the mass spectrometer, because the eluent solvent has to be vaporized before actual MS measurements which are performed in high vacuum. However after the introduction of electrospray ionisation (ESI) development has been very rapid during last 20 years and the performance of instruments has steadily improved. Usually ESI is the best choice for the ionization of polar metabolites but there are also other common ionisation methods like APCI (atmospheric pressure chemical ionisation) and APPI (atmospheric pressure photo-ionisation), which can be used to ionise less polar compounds. ESI can be run either in positive or negative modes and the best choice is dependent on the specific analyte. During ionisation hydrogen is either combined with the analyte to produce $[M+H]^+$ ion or broken away to produce $[M-H]^-$ ion, which can be accelerated within electric field. In the same time usually also other adducts, like sodium and potassium adducts, are formed. Sometimes other adducts can cause problems or decrease the sensitivity of the method. In addition other compounds that elute at same time from LC flow can reduce or block totally the ionisation of the analyte and cause errors to the measurements. This phenomenon is called ion suppression and it is quite common in ESI (Jessome & Volmer, 2006).
The most useful sample handling procedure to be used in metabolism studies with LC-MS is protein precipitation. It is performed easily by addition of organic solvent, either methanol or acetonitrile, to the samples. Samples are mixed and centrifuged to get clear supernatant. Usually after protein precipitation samples are clean enough to be analyzed directly, but

also other sample handling method may be needed with samples containing a lot of lipids or salts (Rossi, 2002). Different extraction methods, like SPE (solid phase extraction) or liquid-liquid extraction are then a better choice. However they are more expensive and time consuming methods.

Already HPLC (high performance liquid chromatography) is able to separate metabolites directly without any modifications. Compared to the GC, the resolution of HPLC is quite poor. Because mass spectrometric methods can measure compounds coming to the instrument at the same time, this has not been so big a problem. During the last five years liquid chromatography has improved considerably after introduction of ultra performance liquid chromatography (UPLC). UPLC instruments can work in higher operation pressures (up to 15.000 psi) which makes possible to use smaller particles and diameters in columns and to improve resolution, speed and sensitivity of the method. A typical run in UPLC can be just 5 minutes to analyse several different compounds.

Mass spectrometry is a superior method in the metabolism studies because of its high sensitivity. Although mass spectrometry is usually understood as one concept, it actually consist of several different types of instruments and techniques. Different types of instruments have specific advantages and consequently each individual type suits best for certain kind(s) of measurements. In the identification of metabolites time of flight mass spectrometry (TOF) is the best option. It can detect all ionized compounds simultaneously which improve the sensitivity compared to scanning instruments (Fountain, 2002). With help of the TOF instruments accurate mass of the analyte (±5 ppm) can be measured and elemental composition can be calculated. Modern instruments can easily reach 1 ppm mass accuracy and use isotope patterns of analytes to solve the right elemental composition with few potential possibilities. This kind of identification can be used to find different metabolites in samples, because masses of potential metabolites can usually be calculated before measurements. There are also softwares, such as Metabolynx (Waters Corp., Milford, MA, USA), which can search potential metabolites automatically from mass chromatograms and help a lot in data processing.

Additional structural information can be achieved with help of Q-TOF or triple quadrupole instruments. Measured analytes can be decomposed by collision with gas molecules (CID, collision induced dissociation) to produce fragment ions. In Q-TOF instruments accurate mass of fragment ions can be also measured to resolve molecular masses of fragments. In most cases fragmentation produces information about location of possible biotransformations. Because fragmentation is compound-specific, fragments can be used for identification purposes if they are known from previous measurements. However fragmentation is not as universal as in EI-ionisation (electron ionisation) of GC-MS instruments because it is partly instrument specific. With ion trap instruments even produced fragment ion can be selected and collided again to produce new smaller fragment ions. To resolve the structure of a metabolite completely other methods like nuclear magnetic resonance (NMR) or x-ray crystallography are usually required.

Knowledge about fragmentation of the analyte is useful also in quantitative measurements. Quantifications are usually performed in triple quadrupole instruments, where fragmentation can be used to increase selectivity of the measurements. The mode of the measurement is called multiple reaction monitoring (MRM), because several compounds can be measured simultaneously. In triple quadrupole instruments the first quadrupole selects the measured analyte, the second one decomposes it and the third passes the formed fragment to the detector. Because fragmentation is specific to every analyte, only right one is

measured even if compounds with the same molecular mass come to the instrument at the same time. This kind of high selectivity makes also possible to measure several compounds at the same time even when they are not separated in liquid chromatography. In triple quadrupole instruments dynamic range is usually at least 5 orders of magnitude what is enough for quantification purposes. Earlier TOF instruments were saturated at quite low concentrations and could not be used for quantification purposes. Modern TOF instruments however can be used for quantification at least to a certain extent.

Newest technological addition to mass spectrometry is ion mobility. Ion mobility is a small gas filled drift tube in instruments, which ions travel through within electric field. Drift tube will separate compounds based on their shape and size in addition to mass and charge as in conventional instruments. Ion mobility is quite an old technique but just recently it has been combined with commercial mass spectrometers like Synapt HDMS (Waters Corp., Milford, MA, USA) (Kanu et al., 2008). Ion mobility can be used to clean important analytes from sample matrix and to separate very similar compounds like isomers from each other. Because technique is so new, its real practical significance in metabolism studies is still unclear.

Figure 3 presents a practical example about mass spectrometric measurements of the pesticide profenofos and its metabolite hydroxypropylprofenofos (Abass et al., 2007a). Accurate mass measurements were performed by Micromass LCT-TOF (Micromass, Altrincham, UK) using leucine enkephalin ($[M+H]^+$ at m/z 556.2771) as a lock mass compound. Error in accurate mass measurements of hydroxypropylprofenofos was 4.3 mDa. Fragmentations of hydroxypropylprofenofos were determined by Micromass Quattro II triple quadrupole instruments. In the first fragmentation hydroxypropylprofenofos loses ethanol to produce fragment of m/z = 343 Da. In the second step propanol is released to produce fragment of 285 Da. Difference in molecular masses of these two fragments indicates that hydroxylation has to be located in S-propyl moiety of the metabolite. Finally quantifications were performed in multiple reaction monitoring mode (MRM) of triple quadrupole instruments. Quadrupole 1 passes only hydroxypropylprofenofos (molecular mass 389 Da) or compounds with the same molecular mass. After quadrupole 1 hydroxypropylprofenofos will fragment in collision cell with help of argon gas and collision energy (CE= 20 eV) to produce a specific fragment of m/z 343 Da. In the final step only fragment 343 will pass quadrupole 3 and its amount is determined in the detector of the instrument. After calibration of the instrument with reference standards, the real amount of hydroxypropylprofenofos can be determined.

5.2 Nuclear Magnetic Resonance spectrometry in the metabolism studies.

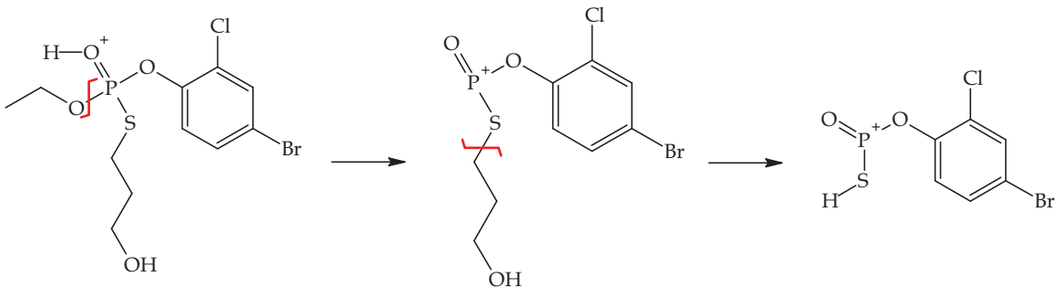
Nuclear Magnetic Resonance spectroscopy (NMR) is a powerful analytical tool in studies of solid, gaseous and liquid samples. The versatility of the technique and the long relaxation times of the nuclear spins allow for probing various different properties of the samples. An even normal, simple one-dimensional spectrum contains valuable information about the sample concentration, electron distributions of the molecule, spatial proximities of different chemical sites and electrostatic connectivities between different nuclei of the molecule. The full potential of NMR can be unleashed by going into higher dimensional NMR spectroscopy. In typical two-, or three-dimensional NMR spectra one can probe spatial proximities of various nuclei, do diffusion separated spectroscopy, probe for heteronuclear connectivities multiple bonds away, or characterize intermolecular dipolar interactions at the protein-ligand complex interface.

Accurate mass measurement of hydroxypropylprofenofos by time-of-flight mass spectrometer:

LC/MS-ESI+, MH^+=373Da
Accurate mass: 372.9477, Calc. mass: 372.9430
Profenofos
O-(4-bromo-2-chlorophenyl)-O-ethyl-S-propyl phosphorothioate

LC/MS-ESI+, MH^+=389Da
Accurate mass: 388.9422, Calc. mass: 388.9379
Hydroxypropylprofenofos
O-(4-bromo-2-chlorophenyl)-O-ethyl-S-hydroxy -propyl phosphorothioate

The fragmentation of hydroxypropylprofenofos by triple quadrupole mass spectrometer:



m/z = 389 Da m/z = 343 Da m/z = 285 Da

The quantification of hydroxypropylprofenofos by triple quadrupole mass spectrometer:

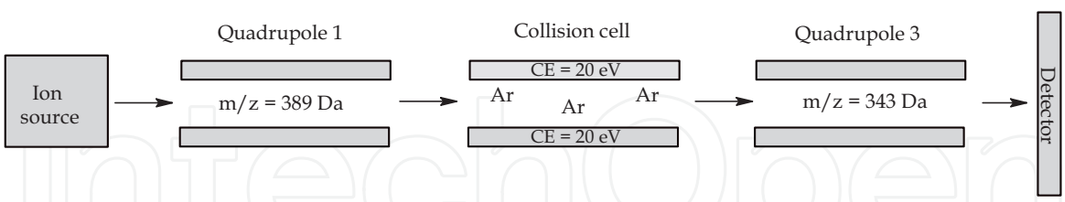


Fig. 3. Mass spectrometric measurements, accurate mass, fragmentations and quantifications, performed to study hydroxylation of profenofos in human hepatic subcellular fractions.

The biggest drawback of the NMR spectroscopy is its inherently low sensitivity, because the observed signal arises from the population difference of spin states. This population difference follows Boltzmann distribution and is quite low even at reasonably high magnetic fields used at modern NMR spectrometers. As the experiments are performed close to room or physiological temperature, there is only population difference of about 10 spins for every million spins in the sample.

Two recent sensitivity enhancing methods are cryogenic cooling of the probe-head electronics and miniaturization of the sample size. Equipment for both of these have been

commercially available now for several years and when combined the resulting cryo-microprobe would give up to 15 fold increase in sensitivity compared to regular room temperature probe head (Kovacs et al., 2005). Several articles have recently been published where full NMR analyses of complex natural products have been made using only nanomoles of material (would be equal to 1 μg if molecular mass is 1000) (Dalisay & Molinski, 2009; Choi et al., 2010a; Choi et al., 2010b; Djukovic et al., 2008).

If one wishes the acquisition parameters of NMR experiment can be set to provide quantitative spectrum. Typically the delay between individual transitions needs to be lengthened to allow full relaxation of spins before next transition. In simplest form the integral values of the individual resonances in spectrum give information of how many equivalent spins are present. Whenever there are modifications in chemical structure the change in integral provides valuables information on the chemical site of the modification (Holzgrabe, 2010).

The Chemical shift is a sensitive measure of the electronic surrounding of individual nuclei of a molecule. Even smallest changes in the chemical structure can cause peaks to resonate at slightly different frequency at the chemical shift range. Addition of the electronegative substituents to the molecule changes the chemical shifts of the resonances of the nearby nuclei. In favorable case the change in chemical shift can be observed for several nucleus many bonds away from the origin of modification site.

The signal fine structure, the splittings caused by spin-spin couplings, provides additional sensitive measure of the topology of the nucleus in the molecule. Change in number of nearby nucleus or even just a conformational change can be detected as a change observed coupling pattern caused by the spin-spin coupling.

In metabolic studies NMR spectroscopy is best utilized when used as a complementary technique to the mass spectrometric techniques. For instance the position isomerism studies are often quite tricky or even impossible to solve by mass spectroscopy e.g. what is the substitution pattern of the aromatic ring or which carbon of the aliphatic chain was hydroxylated. These questions can occasionally be answered in minutes by single ^{1}H NMR spectrum. Of course more challenging structural questions take longer and might require acquisition of several multi-dimensional data sets.

For the illustration of the position isomerism detection powers of NMR spectroscopy several simulated NMR spectra of the profenofos and the hydroxypropylprofenofos are displayed in figure 4. On the left are the aromatic signals of the profenofos and the corresponding spectrum if the bromine was in ortho position to the chlorine. The difference in signal positions and splitting patterns is clear. On the right are spectra of the propyl moiety of the profenofos and the hydroxypropyl moiety of the hydroxypropylprofenofos where the hydroxylation has occurred on terminal carbon 3 or in carbon 2.

6. Conclusion

The cytochrome P450 (CYP) superfamily comprises a broad class of phase I oxidative enzymes that catalyze many hepatic metabolic processes. Human CYPs have broad substrate specificity and enzymes in families 1-3 function mostly in the metabolism of a wide variety of xenobiotics. In human liver, CYP3A4 is found in the highest abundance and it metabolizes the greatest number of drugs and a very large number of other xenobiotics. CYP enzymes in humans as well as in other species have been intensively studied during recent years. It is now possible to characterize metabolism, metabolic interactions and to

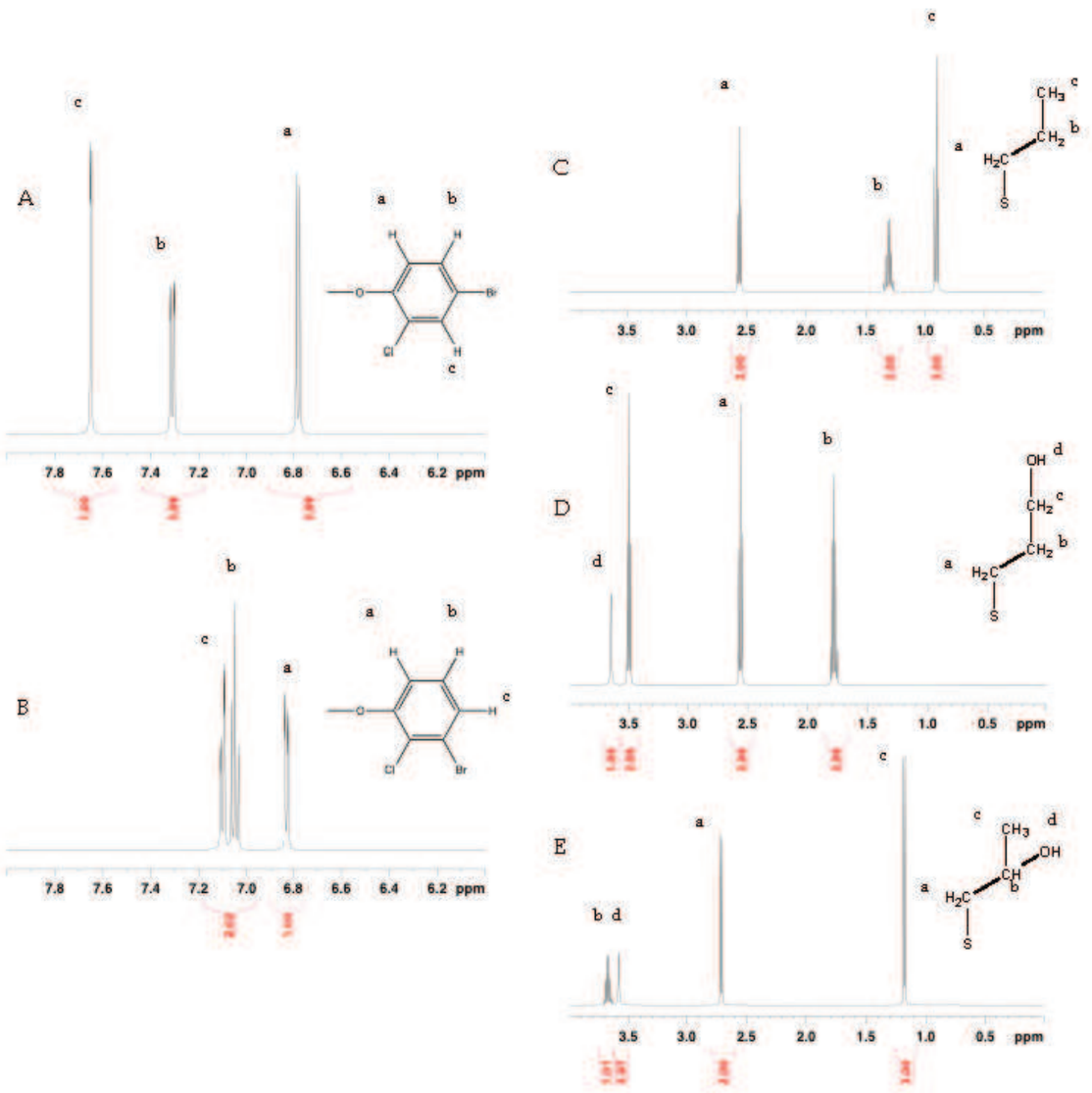


Fig. 4. An example of the effect of small changes in molecular structure to the outlook of ^{1}H NMR spectrum illustrated by 5 simulated NMR spectra. The values used for chemical shifts and coupling constants are only approximate and are presented for illustration purposes only. The values of signal integration are displayed below the frequency scale.

determine which P450 is involved in the metabolism of a certain xenobiotic by different in vitro approaches. The toxicity of many types of pesticides is mediated by enzymatic biotransformation reactions in the body. Recently, a number of papers have been published on the activity of human P450s involved in the metabolism of pesticides and these activities may result in activation and/or detoxification reactions.

The aim of in vitro characterization is to produce relevant and useful information on metabolism and interactions to predict what happens in vivo in human. To understand some of the factors related to xenobiotics, including pesticides, metabolism that can influence the achievement of these aims, there are several important points to consider such as metabolic stability, metabolic routes and fractional proportions, metabolizing enzymes

and potential interactions. In this review we described the human xenobiotic- metabolizing enzymes CYPs system; briefly illustrate in vitro human-derived techniques for studying xenobiotic metabolism and in vitro characterization of metabolic characteristics; review the role of CYPs in the metabolism of xenobiotics, including drugs and pesticides, in human in vitro; and finally describe the impact of modern analytical techniques in xenobiotics metabolism.

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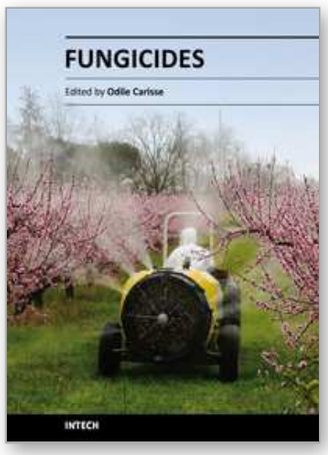

Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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