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AN OVERVIEW OF THE ROLE OF CYTOCHROME P450 ENZYME SYSTEM IN FOOD-DRUG INTERACTIONS AND POSSIBLE APPLICATIONS IN VETERINARY MEDICINE

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An overview of the role of Cytochrome P450 enzyme system
in food-drug interactions and possible applications in Veterinary Medicine

Abstract

Cytochrome P450 enzymes (CYP) are hemoproteins belonging to the group of monooxygenases and one of the main enzymatic systems responsible for drug metabolism.
In the present study, *in vitro* approach was applied to evaluate the relation of CYP-catalyzed activities between human, rabbit, minipig and mouse, using single substrate assays (MultiCYP 7-ethoxycoumarin 0-deethylase (ECOD), CYP1A1/2 7-ethoxyresorufin 0-deethylase (EROD), CYP2A6 coumarin 7-hydroxylase (COH), CYP3A4 midazolam 1-hydroxylase (OH-MDZ), and CYP2E1 chlorzoxazone 6-hydroxylase (OH-CLZ)). It was also studied plant extracts (*Pinus sylvestris*, *Angelica archangelica*, *Mentha sp.*, *Citrus grandis*) and phytochemicals (8-Hydroxybergapten, 5,6-dihydroxyangelicin, α, β-Thujone, α-Thujone, angelicin, bergamottin, bergapten, bergaptol, cnidilin, imperatorin, isobergapten, isopimpinellin, lanatin, phellopterin, psoralen, sphondin, xanthotoxin) as potential inhibitors in CYP-related activities of hepatic human microsomes (CYP1A1/2 (EROD), CYP2A6 (COH), CYP3A4 (OH-MDZ)).
This study showed that the lowest ECOD activity was detected in humans and there was no similarity between other species. CYP1A1/2 showed equivalent activities. The highest CYP activities in humans were found for CYP2A6 and CYP3A4. In CYP2E1 activity, two similar groups were recognized: human and mouse versus rabbit and minipig.
EROD reaction was the most inhibited CYP-mediated reaction. COH reaction was inhibited by few compounds. The highest inhibition was detected among angular furocoumarins. Linear furocoumarins group had the lower inhibitory concentration of CYP3A4. Thujone showed weak inhibition of CYP activities.

Keywords: drug metabolism, food-drug interactions, microsomal enzymatic activities, CYP inhibitors, plant extracts and phytochemicals.

Abordagem geral sobre o papel do sistema enzimático Citocromo P450 em interacções fármaco-nutriente e possível aplicabilidade em Medicina Veterinária

Resumo

As enzimas do sistema citocromo P450 (CYP) são hemoproteinas pertencentes ao grupo das monoxigenases e um dos principais sistemas enzimáticos responsáveis pela metabolização de fármacos.

Neste estudo foi avaliada a relação da actividade catalítica de diferentes CYPs entre humanos, coelhos, *minipig* e murganhos, recorrendo a substratos como sondas individuais *in vitro* para mensurar reacções especificas (MultiCYP 7-etoxicumarina 0-deetilase (ECOD), CYP1A1/2 7-etoxiresorufina 0-deetilase (EROD), CYP2A6 cumarina 7-hidroxilase (COH), CYP3A4 midazolam 1-hidroxilase (OH-MDZ), e CYP2E1 clorozoxazona 6-hidroxilase (OH-CLZ)). Também foram estudados extractos de plantas (*Pinus sylvestris*, *Angelica archangelica*, *Mentha sp.*, *Citrus grandis*) e fitoquímicos (8-hidroxibergaptem, 5,6-dihidroxiangelicina, α, β-tujona, α-tujona, angelicina, *bergamottin*, bergapteno, bergaptol, cnidilina, imperatorina, isobergapteno, isopimpinelina, *lanatin*, felopterina, psoraleno, *sphondin*, xantotoxina) como potenciais inibidores da actividade catalítica dos CYPs microssomais hepáticos humanos (CYP1A1/2 (EROD), CYP2A6 (COH), CYP3A4 (OH-MDZ)).

Neste estudo não foram detectadas actividades similires entre espécies na reacção ECOD e a actividade mais baixa foi detectada nos humanos. A reacção EROD dos CYP1A1/2 demonstrou actividades similares entre as diferentes espécies. As maiores actividades cataliticas verificadas nos humanos correspondem aos CYP2A6 e CYP3A4. No estudo da reacção do CYP2E1 foram determinados dois grupos distintos com actividades cataliticas similares: 1) humanos e murganhos, 2) coelhos e *minipigs*.

No estudo de potenciais inibidores dos CYPs, a reacção EROD foi a mais inibida. Pelo contrário, a reacção COH foi inibida por poucos compostos. A maioria das inibições ocorreu por exposição a furocumarinas angulares. O grupo de furocumarinas lineares teve a menor concentração inibitória da reacção OH-MDZ do CYP3A4. Os fitoquímicos α, β-tujona e α-tujona demonstraram ter um fraco poder inibitório na actividade dos CYPs analisados.

Palavras-chave: Metabolismo farmacológico, interacções farmaco-alimento, actividades enzimáticas microsomais, inibidores dos CYPs, extractos vegetais e fitoquimicos.

Contents

Figures

Tables

Equations

Abbreviations

ADME	Absorption, distribution, metabolism and excretion
ADME-Tox	Absorption, distribution, metabolism, excretion and toxicity
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ADR	Adverse drug reaction
AhR	Aryl hydrocarbon receptor
AUC	Area under the plasma concentration
C/EBP	CCAAT/enhancer-binding protein
CAR	Constitutive androstane receptor
COH	Coumarin 7-hydroxylase
CYP	Cytochrome P450
DDI	Drug-drug interaction
DME	Drug metabolizing enzyme
DMSO	Dimethyl sulfoxide
ECOD	7-ethoxycoumarin 0-deethylase
Em	Emission
EROD	7-ethoxyresorufin 0-deethylase
Ex	Excitation
F	Bioavailability
FAD	Flavin adenine dinucleotide
FDI	Food-drug interaction
FMN	Flavin mononucleotide
FR	Ferredoxin reductase
FXR	Farnesoid X receptor
GR	Glucocorticoid receptor
HNF	Hepatic nuclear factor
HPLC	High-performance liquid chromatography
IC50	Half maximal inhibitory concentration
ICH	Intracerebral haemorrhage;
K_m	Michaelis constant
LADME	Liberation, absorption, distribution, metabolism and excretion
LXR	Liver X receptor
MFO	Microsomal mixed-function oxidase

MRP	Multidrug resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate
OATP2	Organic anion transporting polypeptide 2
OH-CLZ	Chlorzoxazone 6-hydroxylase
OH-MDZ	Midazolam 1-hydroxylase
PAH	Polycyclic aromatic hydrocarbon
PAPS	3’phosphoadenosine-5’phosphosulfate
P-gp	P-glycoprotein
pKa	Negative logarithm of the acid dissociation constant
PPAR	Peroxisome proliferator-activated receptor
PUVA	Psoralen + UVA treatment
PXR	Pregnane X receptor
RXR	Retinoid X receptor alpha
SAH	Subarachnoidal haemorrhage
SD	Standard deviation
SDH	Subdural haematoma
TCA	Trichloroacetic acid
UDP	Uridine diphosphate
UDPGA	5'-diphospho-glucuronic acid
UGT	UDP-glucuronosyltransferase

Preface

This work was carried out as part of the project "Food-drug interactions: exploring and pre-empting intricate health risks", at the Department of Pharmacology and Toxicology, Institute of Biomedicine, University of Oulu, lead by Professor Olavi Pelkonen, head of the Department.

Olavi Pelkonen's research group develops generic *in vitro, in vivo* and *in silico* approaches to investigate metabolic fate and interactions of any chemicals, be they pharmaceuticals, nutritional components, carcinogens or others. Predictions to the *in vivo* situation are performed with the aid of modeling and simulation tools and targeted in-depth studies are undertaken for the confirmation of predictions.

The major goal of this project is to characterize the risks and reduce future cases of harmful food-drug interactions, particularly among the more sensitive sections of the population like the elderly and patients undergoing extensive drug treatments due to severe diseases. The specific objectives of the proposed research are:

1. Survey and selection of individual compounds and/or compound groups in selected local food sources (e.g. fruits, vegetables, berries, and beverages, as well as commonly used spices);
2. Studying the effects of the different compounds from the food extracts and similar sources on the activity of human cytochrome P450 monooxygenases (CYPs) and human UDP-glucuronosyltransferases (UGTs);
3. Assessing the effect of the different compounds on the expression level of major human CYPs and UGTs;
4. Analyzing the metabolism of phenolics compounds, such as flavonoids and coumarins, from fractionated food extracts by the different human CYPs and UGTs.

This thesis is based in two different studies. The first study was an independent project meant to compare CYP-catalyzed activities between different species, which led to the presentation of a communication to the 11th International Congress of the European Association of Veterinary Pharmacology and Toxicology (see annex I). The second study was part of the main project already described, which consisted in the analysis of plant extracts and phytochemicals as possible CYP inhibitors, using single substrate assays *in vitro*.

Introduction

In the modern world, man and other animals have been progressively more exposed to a huge diversity of chemical compounds, foreign to their normal composition and potentially toxic, known as xenobiotics. These compounds cover a wide range of drugs, pesticides, food additives and pollutants that can be available in the environment, food or part of medical treatments.

Animals possess several defense mechanisms able to act as barriers against the entry of chemical compounds or to eliminate them after being exposed. These mechanisms can be classified as mechanical, cellular or enzymatic systems.

After coming in contact with organisms, most of the foreign compounds undergo a number of chemical reactions performed by hepatic and extra-hepatic enzyme systems, known as metabolism. In case of drugs, the metabolism rate affects directly its bioavailability, the pharmacological effect, therapeutic response and most of the pharmacokinetic properties.

Most of the tissues and organs are well equipped with diverse and various drug metabolizing enzymes (DMEs), including phase I, phase II metabolizing enzymes, as well as phase III transporters. The later is characterized by transmembrane transporters that facilitate movement of drugs/metabolites across cellular membranes before and after phase I and/or phase II. So, the enzymatic pathways involved in metabolism can be classified in two main enzymatic systems: 1) Phase I enzymes catalyze functionalization reactions and are mainly constituted by Cytochrome P450 enzyme system (CYP); 2) Phase II enzymes involve conjugative reactions, mostly by UDP-glucuronosyltransferases and glutathione S-transferases, responsible for increase the hydrophilicity of the parent compound after phase I.

Usually, metabolism yield more polar derivatives (metabolites) which may leave the body via the urinary and biliary routes or be excreted by perspiration, tears, saliva, gastric juice, semen, milk or eggs. However in some cases, metabolism can be harmful to the body by leading to metabolic activation or by interference of drug interactions. The former involves the formation of reactive forms from inactive parent compounds that may lead to bioavailability problems, interindividual variation, metabolic interactions and idiosyncrasies. Drug interaction is a situation in which the activity of a drug is affected by another drug or a food component, in most of the cases by changing drug metabolism. One notable system involved in metabolic drug interactions is Cytochrome P450 enzyme system. This system may be affected by either enzymatic induction or inhibition.

So, the absence of detailed information regarding drug metabolism and drug interactions can result in adverse effects, therapeutic failure or toxicity from unanticipated overdose or metabolic reactions.

During the past decades, in human pharmacology and toxicology great emphasis have been placed upon a detailed knowledge of DMEs in laboratory animals and man. In contrast, knowledge of biotransformation enzymes and their variability in veterinary species, which include companion and farm animals, is still rather insufficient.

Nowadays, the importance of research on drug metabolism in veterinary species is growing, especially regarding the importance of companion animals in western countries. Because several therapeutic areas require long-term pharmaceutical intervention on the time scale of months or years (e.g. infectious disease including parasitism, pain and inflammation, cancer, behavior, cardiovascular, and endocrinology disorders), prolonged exposure and accumulation of drugs may increase the risk of drug-drug interactions. Also, the growing market for pet medicines devoted to improving quality of life rather than treating a specific disease is expected to increase the number of pets receiving pharmaceutical agents or a regular basis.

The importance of drug metabolism is also growing in farm animals, not only because of permanent exposure to industrial or agricultural contaminants, but also because of the frequent use of pharmacologically active substances. So, the relevance of metabolism in these species also concerns public health. For better understanding, in next chapters will be discussed the importance of pharmacokinetic, liver and DMEs in drug metabolism, and some data concerning CYPs and drug interactions.

The present thesis was aimed to:

1. Explore the available data on CYP regulation, expression, substrate specificity and potential interactions in animal species and in human medicine, illustrating the main role performed by CYP system in drug development, clinical treatments, food management and animal production.
2. Find similarities between CYP-related activities in humans and three different laboratory animal species (rabbit, minipig and mouse). According to the final results, it may help to identify possible extrapolations between species and to find a suitable animal model for human CYP-mediated metabolism.
3. Identify potential human CYP inhibitors among several plant extracts, including purified herbal compounds. This subject concerns public health and food safety, which can lead to harmful food-drug interactions in humans and, it may also affect, animal species.

1. Review of the literature

1.1. Pharmacology as a science

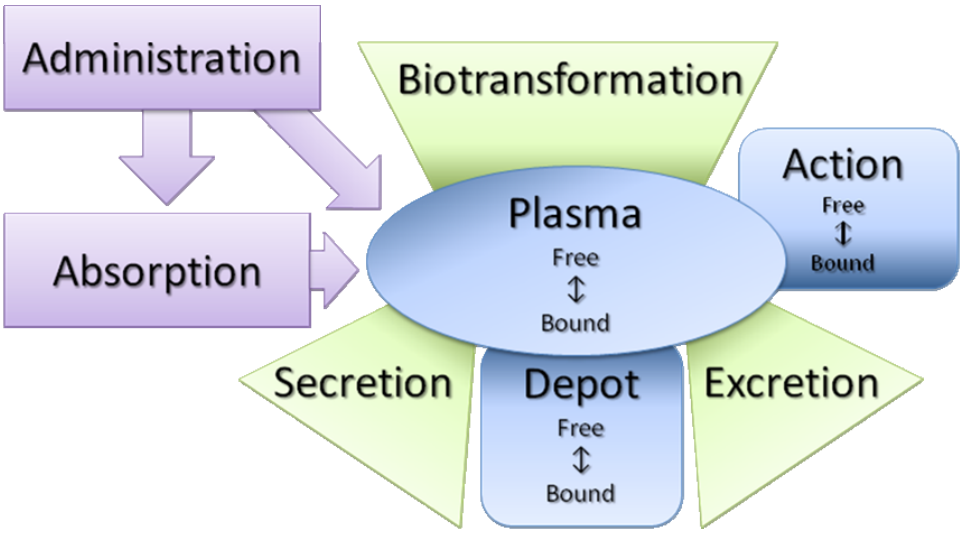
Pharmacology is the science field responsible for the study of drugs. The pharmacology field encompasses the drug's history, origin, composition and physicochemical properties, interactions, toxicology, therapeutic applications and mechanisms involved in their actions, such as absorption, distribution, biotransformation and excretion. All of these fields can be comprised in several subdisciplines or branches (Dimasi, Caglarcan, & Wood-Armany, 2001; Guimarães, Moura, & Silva, 2006; Roots *et al.*, 2004; Scatena, Bottoni, Botta, Martorana, & Giardina, 2007; Spinosa, Gorniak, & Bernardi, 2006):

- Pharmacodynamics (study of the manner in which drugs act on the body and their mechanisms and effects);
- Pharmacokinetics (study how the body affects drugs after administration and interactions of medications within the body);
- Pharmacognosy (study of the characteristics and uses of drugs);
- Pharmacy (science of preparing and dispensing medications);
- Pharmacotoxicology (study the toxic effect of xenobiotics, which also includes drugs, in living organisms);
- Pharmacogenetics (study or clinical testing of genetic variation that gives rise to differing response to drugs);
- Pharmacogenomics (application of genomic technologies to new drug discovery and further characterization of older drugs);
- Pharmaeconomics (compares the value of one pharmaceutical drug or drug therapy to another, evaluating the cost *versus* effects).

1.2. Pharmacokinetics and Toxicokinetics

During the biological disposition of a drug or other xenobiotic, in the pharmacokinetic or toxicokinetic phase respectively, there are four distinct processes which include the extent and rate of absorption, distribution, metabolism and excretion. This is commonly known by the acronym ADME (see figure 1). Nevertheless, in toxicokinetics is possible to use the acronym ADME-Tox or ADMET when the potential or real toxicity of the compound is taken into account (Ionescu & Caira, 2005; Lin *et al.*, 2003).

Figure 1. Schematic representation of the interrelationship of the four main processes - ADME (modified from Ionescu & Caira, 2005)



On the other hand, it should be noted that depending on their respective pharmacokinetics, a given dose of drug may be undergoing more than one of these processes simultaneously, e.g. metabolism of absorbed drug may commence while part of the administered dose is still being absorbed; or it can over lap metabolism being directly excreted (Guimarães, Moura, & Silva, 2006; Ionescu & Caira, 2005)

Despite the fact that ADME scheme is the most consensual, some authors include also the term liberation. This process is characterized by the dissolution rate of the drug from the solid particles, where the surface phenomena at the solid/ liquid interface play an important part in drug delivery. Therefore, pharmacokinetics can be also referred to as the LADME concept (Dredán, Antal, Rácz, & Marton, 2004; Ionescu & Caira, 2005; Spinosa, Gorniak, & Bernardi, 2006).

1.2.1. Absorption

Drugs and other xenobiotics can be introduced into the body by a wide variety of routes of administration. The most important administration routes are listed in table 1.

Table 1. Routes of administration of drugs (modified from Ahokas & Pelkonen, 2007)

Enteric routes	Parenteral routes	
	Percutaneous	Other mucous membranes
Mouth Sublingual Rectal Colonic	Subcutaneous Intramuscular Intravenous Intra-arterial Intrathecal	Nasal Conjunctival Urethral Vaginal Epidermis

After the drug being intake, in case of enteric routes and some parenteral routes (e.g. nasal), the pharmacologically active compound only produces effect if its site of action is reached. Therefore it needs to transpose biological membranes, undergoing in a process of movement from the site of application into the extracellular compartment of the body. Then it needs to be present in appropriate concentrations at its sites of action and remains there for an adequate period of time before being excreted. The main physiologic mechanisms of crossing membranes are filtration (through the small pores in the membranes), passive diffusion, facilitated diffusion, active transport and pinocytosis (Ahokas & Pelkonen, 2007; Guimarães *et al.*, 2006).

Thus, absorption can be defined as the sum of all processes that a compound may undergo after its administration before reaching the systemic circulation. Therefore, the blood concentration of active drug attained depends primarily upon the extent and rate of absorption. Although intravenous administration is an exception, since the drug is introduced directly in the bloodstream, there is no absorption (Ionescu & Caira, 2005; Spinosa *et al.*, 2006).

Besides the physicochemical properties of the drug molecules (e.g. lipophilicity and ionization) and membranes involved in absorption, there are other limiter factors such as the irrigation intensity and thickness of the membrane, the area, time and way of contact between the drug and membrane (Spinosa *et al.*, 2006).

1.2.2. Distribution

After the drug's administration, it distributes from the absorption place throughout the body via bloodstream. This process can be described as a combination of reversal transference of drug molecules from the systemic circulation to extravascular space, and interactions with macromolecules present in various body fluids and tissues.

The rate and extent of the drug's distribution is directly influenced by interactions of the drug with body components, which depends on both the physicochemical properties of the drug in question (e.g. lipid solubility, ionization degree, pKa, molecular weight) and physiological parameters (e.g. pH, extent of plasma protein binding, permeability of membranes, blood flow, nature of the tissue) (Ahokas & Pelkonen, 2007; Gibson & Skett, 2001; Ionescu & Caira, 2005). Based on those determining factor, the main mechanisms involved in drug's distribution are summarized below (Ionescu & Caira, 2005; Uetrecht & Trager, 2007):

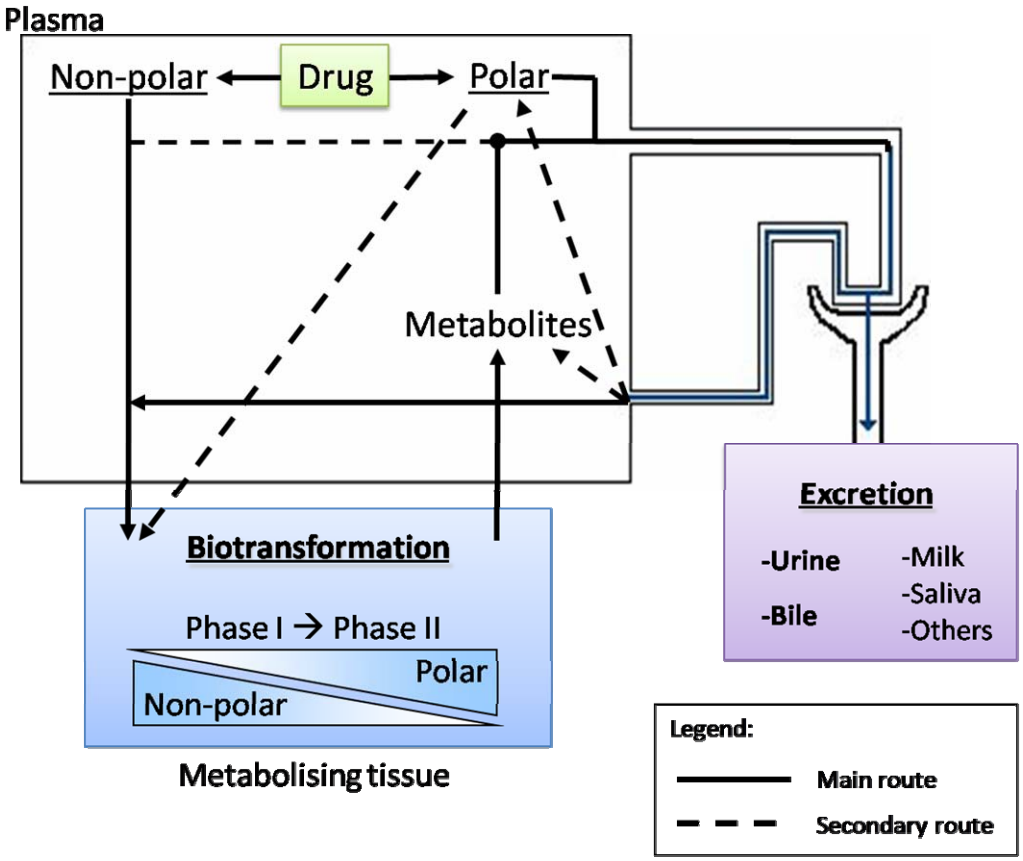
- Passive diffusion across lipid membranes is the main process by which most drugs across cell membranes. In this case, drug molecules move from a high to a low concentration area;
- Hydrostatic pressure represents a pressure gradient between the arterial end of the capillaries entering the tissue and the venous capillaries leaving the tissue;
- Presence of carrier-mediated active transport processes involving the xenobiotic. In this process, substances are moved across a cell membrane from a low to a high concentration region;
- Drug binding to plasma proteins (e.g. albumin, glycoproteins and hormonal transporters), blood cells (e.g. erythrocytes), lipids and various tissue proteins is very important in drug distribution. The fraction of the bound drug can be as high as > 90% or as low as < 20%. Drug-protein binding is influenced by several factors, such as drug concentrations and lipophilicity. Generally, the higher is the lipophilicity of a drug, the stronger is the binding to tissue protein and the greater its distribution (higher ability to cross the cell membrane). So, the bioavailability of the drug to pass through membranes is inversely related to their binding to plasma proteins.

1.2.3. Drug Elimination

As previously referred, animals have several defense mechanisms to eliminate foreign compounds, such as mechanical, cellular and enzymatic systems. Biotransformation is one of these defense mechanisms, where compounds (e.g. nutrients, amino acids, toxins, or drugs) undergo chemical changes for further elimination (Ionescu & Caira, 2005).

After being absorbed by the organism, drugs can be directly eliminated unmodified when soluble, via the urinary and biliary routes, or other excreta (generally in minor amounts). However, in most of the cases, it is necessary the conversion of drugs in more polar compounds (soluble), which undergo a number of chemical reactions known as drug metabolism (an example of biotransformation). In this process, parent drug is biotransformed into metabolites, being excreted then mainly by urine or/and bile (
figure **2**). Thus, the elimination (or clearance) of a drug refers to the sum of all clearance processes by all contributing organs, that remove or contributes to the removal of drugs or other foreign agents from the body. These include excretion and metabolism (Ahokas & Pelkonen, 2007; Guimarães *et al.*, 2006).

Figure 2. Elimination process (modified from Ahokas & Pelkonen, 2007)



1.2.3.1. Metabolism

The main goal of drug metabolism, as mentioned before, is to allow the excretion of non-polar or too lipophilic compounds from the body by changing their chemical structure (which includes active part of medications) making them more water-soluble and more readily excreted mainly by the kidney. The enzymatic systems involved in the drug's biotransformation are known as drug metabolizing enzymes (DMEs), being responsible for the drug's chemical conversion in metabolites. Paradoxically, DMEs can also metabolically activate biological inert compounds (bioactivation) to electrophilic derivates that can cause toxicity or even carcinogenesis (Gonzalez, 2005; Uetrecht & Trager, 2007).

Most of the tissues and organs, such as liver, lung, kidney, intestine and rumen (in case of ruminants) are well equipped with diverse and various DMEs (phase I, phase II metabolizing enzymes), as well as phase III transporters, which are present in abundance either at the basal non-stimulated level, and/or are inducible at elevated level after exposure to xenobiotics. However, these enzymes are mostly located in liver, which will be described in a later section (Fu, Xia, Lin, & Chou, 2004; Josephy, Guengerich, & Miners, 2005; Turpeinen, 2006).

DMEs are divided in two main enzymatic systems, according to their function and chemical reactions (Ionescu & Caira, 2005; Szakács, Váradi, Özvegy-Laczka, & Sarkadi, 2008; Xu, Li, & Kong, 2005):

- The phase I enzymes catalyze functionalization reactions (see table 2), which introduce a polar functional group to a parent molecule to form a metabolite.
- The phase II enzymes catalyze conjugation reactions, which conjugate a polar moiety to the parent compound or its phase I metabolite (see table 2). Therefore, the metabolites resulting from phase II are usually more polar than the parent molecule.

Table 2. Reactions of phase I and phase II (modified from Gibson & Skett, 2001)

Phase I	Phase II
Oxidation	Glucuronidation / glucosidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Isomerisation	Amino acid conjugation
Miscellaneous	Glutathione conjugation
	Fatty acid conjugation
	Condensation

Finally, phase III is constituted by transmembrane transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), organic anion transporting polypeptide 2 (OATP2), that facilitate movement of drugs/metabolites across cellular membranes after and before phase I and/or phase II. So, these transmembrane transporters are not only related with drug metabolism, but also provide a barrier against drug penetration, and play crucial roles in drug absorption, distribution and excretion (Xu *et al.*, 2005).

1.2.3.2. Excretion

Excretion is the final step in the xenobiotic elimination. Nonetheless, as already mentioned before according to the xenobiotic properties it may be excreted directly unchanged or it may previously require being biotransformed.

The most important organ of excretion is the kidney, although some substances can be excreted in the expired air (through the lungs), bile, perspiration, tears, saliva, gastric juice, semen, milk or eggs (Lin *et al.*, 2003; Spinosa *et al.*, 2006; Szakács *et al.*, 2008). Most of these drugs are excreted rather by these alternative routes owing to be more lipophilic.

Below is summarized the most important excretion routes:

- Renal excretion takes place mainly by glomerular filtration; however as the glomerular filtrate passes through the proximal tubule, some solute may be reabsorbed (tubular reabsorption) through the tubular epithelium and returned to the blood. Drugs also may be reabsorbed in the distal tubule, in which case the pH of the urine is extremely important in determining the reabsorption rate (in accord with the principle of non-ionic diffusion and pH partition). It should be take in consideration that the urinary pH, and hence drug excretion, may fluctuate widely depending on the diet, exercise level, drugs, day time and other factors (Guimarães *et al.*, 2006; Ionescu & Caira, 2005).
- Biliary excretion and fecal elimination include xenobiotics that are secreted into the bile usually pass into the intestine; from here, they may be reabsorbed and integrate the enterohepatic circulation (this system provides a reservoir for the drug). If a drug is not absorbed completely from the intestine, the unabsorbed fraction will be eliminated in faeces (Guimarães *et al.*, 2006; Ionescu & Caira, 2005).

- Alveolar excretion is another example of an alternative route of excretion, in which due to the large alveolar area and high blood flow at this level, it makes lungs ideal for the excretion of some compounds, e.g. gaseous and volatile anesthetics (Ionescu & Caira, 2005).

1.3. The role of liver in drug metabolism

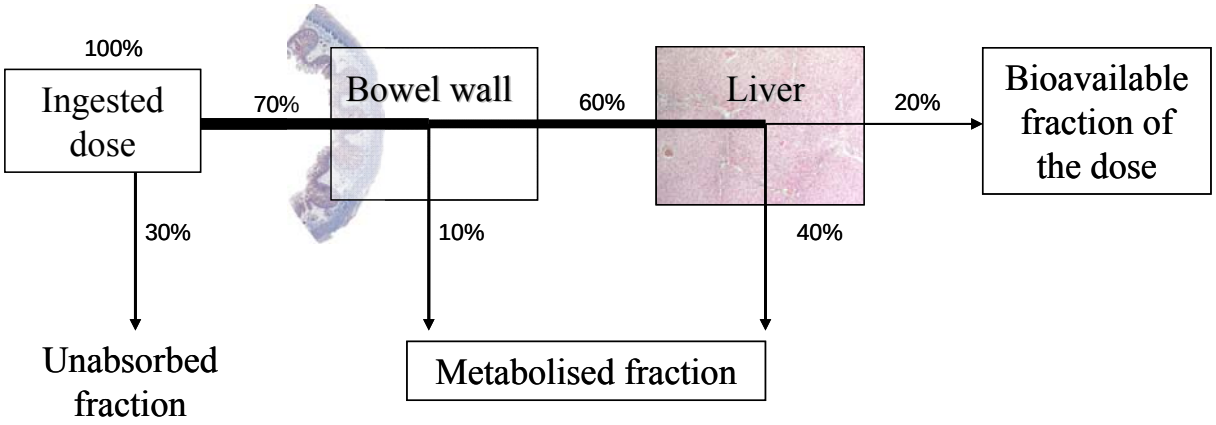
In drug metabolism, most of pharmacokinetic differences between and within species have it major determinant in chemical processes variability. The latter is mainly due to DMEs, which most of organs possess a wide range. Thus, DMEs plays a central role in pharmacokinetic, especially in the elimination phase (Hewitt et al., 2007).
In this way, liver represents one of the main organs in drug metabolism by his anatomic and histological singularity, cell specialization and by possessing the highest amount of DMEs. The previous feature is attributable to the combination of two characteristics - high concentration of DMEs in hepatocytes and the liver size (Spinosa *et al.*, 2006).

1.3.1. Hepatic clearance

One of the liver's functions is to use biotransforming enzymes to convert lipophilic agents in water-soluble enough to be cleared by the kidney. So, it must extract the drug from the circulation, biotransform (metabolize) it and then return the metabolite to the blood. The liver can also active clear or physically remove its metabolic products by excreting them in the bile, as already explained (Coleman, 2006).
The liver's capability to remove a specific compound from circulation (hepatic clearance) is mainly conditioned by his blood flow and the intrinsic hepatic clearance. The latter is determined by the excretory capacity, enzymatic activity and affinity. Nonetheless, these physiologic variables rely on interspecies differences and can be influenced by drug therapies, xenobiotics interactions, hepatic pathologies, as well in other organic systems (e.g. chronic kidney disease and congested heart failure) (Braz, 2005).
The hepatic biotransformation can intervene in two different stages of the drug's life, in a later stage during the distribution or immediately after the absorption owing to the anatomic position of the liver, which is interposed between the gastrointestinal tract and systemic circulation (Braz, 2005).

All drugs absorbed from the small intestine are transferred to the liver by portal circulation. To be absorbed from the intestine, before entering the hepatic portal circulation, the drug must pass through the intestine wall, where it can be pumped back out of the gut wall into the lumen by the efflux proteins and metabolized by various enzymes in the intestine wall cells. When the drug achieves the liver by the hepatic portal circulation, it may partially metabolized before entering the systemic circulation, whereby their concentration can be reduced before reach the site of action (see figure 3). This phenomenon is called first pass metabolism. Drugs having a high first pass rate (or poor absorption) are referred to have a low bioavailability (Braz, 2005; Coleman, 2006; Gibson & Skett, 2001).

Figure 3. The concept of first pass metabolism and bioavailability (Turpeinen, 2006)



In some drugs, most part of the administrated dose is lost before it reaches the systemic circulation. Thus, if the given dose and the amount that actually reaches the plasma are known, it is possible to know how much enters the system. This is known as the bioavailability (F) of the drug and it is calculated by the equation 1. For this reason, theoretically, drugs administered intravenously have a bioavailability of 100% (Ahokas & Pelkonen, 2007; Braz, 2005; Coleman, 2006).

Equation 1. Bioavailability equation (Ahokas & Pelkonen, 2007)

$$F = \frac{AUC_{0\rightarrow x(\text{oral})}}{AUC_{0\rightarrow x(\text{IV})}}$$

- $AUC_{0\rightarrow x}$: Area under the plasma concentration versus time curve from time 0 to time x after intravenous dosage (IV) / oral administration (oral).

1.3.2. Liver cells

The liver is composed of both parenchymal and nonparenchymal cells (Junqueira & Carneiro, 2005; Kuntz, 2006).

The nonparenchymal cells include:

- Endothelial cells: They delineate the sinusoidal spaces, allowing exchanges between blood and hepatocytes. These cells also produce prostaglandins, endothelins, nitric oxide and some extracellular components (Braz, 2005)
- Kupffer cells: These cells are stationary macrophages responsibles for immune surveillance and release inflammatory mediators (Kuntz, 2006).
- Ito cells: They are also known as lipocytes, fat storing cells or stellate cells, and serve to store vitamin A and lipids, but upon liver injury they can differentiate into fibroblasts, being able of producing collagen. These cells only represent 5-8% off all liver cells and they are located in space of Disse (or perisinusoidal space, which is located between a hepatocyte and a sinusoid) (Junqueira & Carneiro, 2005; Kuntz, 2006).

The parenchymal cells, commonly known as hepatocytes, constitute approximately 60% of all liver cells and 80% of the liver weight. They are also the main responsible for most of the liver's functions, being able of synthesizing blood components (e.g. serum albumin, fibrinogen, clotting factors), cholesterol, bile salts, phospholipids and their own structural proteins and intracellular enzymes. So, hepatocytes are provided with numerous mitochondria, and large amounts of rough and smooth endoplasmic reticulum, which are necessary to synthesize and secrete proteins. Regarding their unique metabolic characteristics and possessing all DMEs in liver, these cells are the major site of xenobiotics biotransformation (Junqueira & Carneiro, 2005; Kuntz, 2006).

1.3.3. Biotransforming enzymes of liver

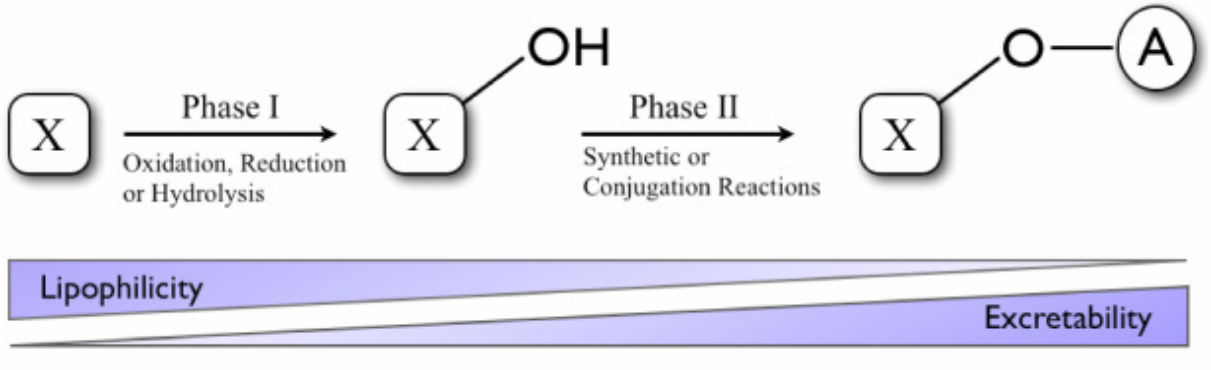
The DMEs constitutes the group of biotransforming enzymes, and as already referred, those are mostly located in the liver - more precisely in hepatocytes (Wexler & Anderson, 2005).

Phase I and II are the two main enzymatic systems that compose DMEs and the only enzymes truly responsible for biotransformation itself. So, both phases will be discussed in more detail in following topics (Ionescu & Caira, 2005).

1.3.3.1. Phase I Enzymes and Reactions (Functionalization Reactions)

The main function of phase I is to prepare the compound for phase II and not to prepare the drug for excretion. So, phase I enzymes catalyzes functionalization reactions, by adding or uncovering functional groups on xenobiotics with increasing polarity or hydrophilicity creating a suitable substrate for Phase II metabolism (see figure 4) (Gibson & Skett, 2001). Phase I metabolism includes oxidation, reduction, hydrolysis and hydration reactions, as well as other rarer miscellaneous reactions (see table 2) (Ahokas & Pelkonen, 2007; Josephy *et al.*, 2005).

Figure 4. Simple schematic depiction of the two main phases of drug metabolism (Ahokas & Pelkonen, 2007)



1.3.3.1.1. Oxidation

Oxidation involves the enzymatic addition of oxygen, removal of hydrogen or changing the oxidative state of the xenobiotic. These reactions are mainly catalyzed by CYPs, but other enzyme groups can be also involved (Olavi Pelkonen et al., 2008; Xu et al., 2005).

a. Oxidations involving CYPs (the microsomal mixed-function oxidase):

Oxidations performed by the microsomal mixed-function oxidase (MFO) system are considered separately, because of its major role and the diversity of reactions performed by this enzyme system (Gibson & Skett, 2001; Uetrecht & Trager, 2007).

The mixed-function oxidase system found in microsomes (small vesicle that is derived from fragmented smooth endoplasmic reticulum produced when tissues, such as liver, are homogenized) of many cells (mainly in liver, but also in kidney, lung and intestine) performs many different functionalization reactions (summarized in table 3) (Braz, 2005; Gibson & Skett, 2001).

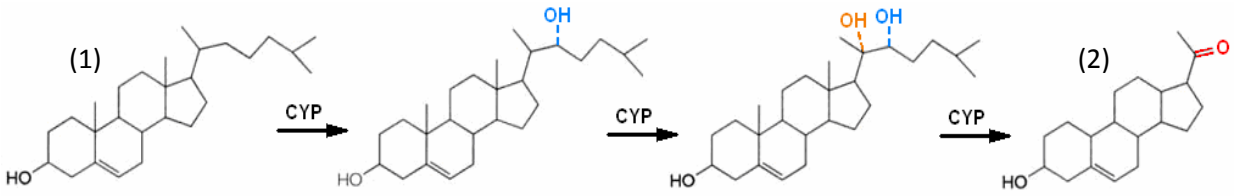
All of these reactions require the presence of molecular oxygen and NADPH as well as the complete mixed-function oxidase system (cytochrome P450, NADPH-cytochrome P450 reductase and lipid) (Gibson & Skett, 2001). All reactions involve the initial insertion of a single oxygen atom into the drug molecule. A subsequent rearrangement and/or decomposition of this product may occur, leading to the final product (Uetrecht & Trager, 2007).

Table 3. Reactions performed by the microsomal mixed-function oxidase system (Ionescu & Caira, 2005)

Reaction	Substrate
Aromatic hydroxylation	Lidocaine
Aliphatic hydroxylation	Pentobarbital
Epoxidation	Benzo[a]pyrene
N-Dealkylation	Diazepam
O-Dealkylation	Codeine
S-Dealkylation	6-Methylthiopurine
Oxidative deamination	Amphetamine
N-Oxidation	3-Methylpyridine
S-Oxidation	Chlorpromazine
Phosphothionate oxidation	Parathion
Dehalogenation	Halothane
Alcohol oxidation	Ethanol

This enzymatic system is involved in both xenobiotics (drugs) and endogenous metabolism. The latter applies to the metabolism of steroid hormones, thyroid hormones, fatty acids, prostaglandins and derivates. Steroid biosynthesis is dependent on CYPs at many stages, where the rate-limiting step is the conversion of cholesterol to pregnenolone (see figure 5). Pregnenolone is the prohormone involved in the steroidogenesis of progesterone, mineralocorticoids, glucocorticoids, androgens and estrogens (Nebbia, 2001; Uetrecht & Trager, 2007).

Figure 5. The conversion of cholesterol [1] to pregnenolone [2] (modified from Gibson & Skett, 2001)



b. Oxidations not catalyzed by CYPs:

Some other enzymes in the body not related to CYPs can oxidize drugs. They are listed in table 4. Most of these enzymes are primarily involved in endogenous compound metabolism (Ionescu & Caira, 2005; Nebbia, 2001).

Table 4. Other oxidative enzymes

Flavin-containing monooxygenase system	Co-oxidation by peroxidases
Alcohol dehydrogenase	Amine oxidases
Aldehyde dehydrogenase	Aromatases
Xanthine oxidase	Alkylhydrazine oxidase

1.3.3.1.2. Reductive metabolism

Reductive reactions of certain functional groups are not as common as oxidative and some of them can be catalyzed by hepatic microsomal enzymes. These reactions usually require NADPH, however they are generally inhibited by oxygen, unlike the mixed-function oxidase reactions that use oxygen as a main reagent. A list of the types of compounds undergoing reduction is given in table 5 (Gibson & Skett, 2001; Nebbia, 2001).

Table 5. Compounds undergoing reduction by hepatic microsomes

Azo-compounds	N- and S-Oxides
Nitro-compounds	Disulfides
Epoxides	Alkenes
Heterocyclic ring compounds	Halogenated hydrocarbons

Azo- and nitro-reduction can be catalyzed by CYPs, but also by NADPH-CYP reductase, and can involve substrates such as chloramphenicol and prontosil red (forming sulfanilamide), which led to the discovery of the sulfonamides (Abass, Reponen, & Pelkonen, 2009; Nebbia, 2001).

1.3.3.1.3. Hydrolysis

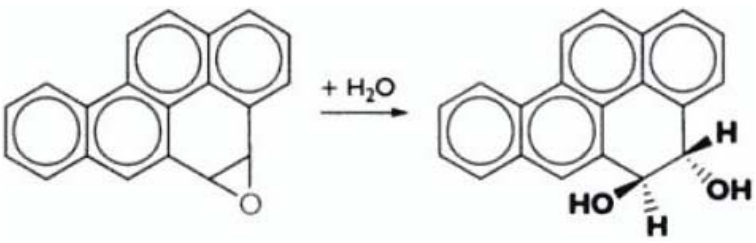
Hydrolysis reactions can be performed by several enzymes that readily catalyzed esters, amides, hydrazides and carbamates (Abass *et al.*, 2009; Ionescu & Caira, 2005).

- Ester hydrolysis is performed in plasma by non-specific acetylcholinesterases, pseudocholinesterases and other esterases or it can occur in liver by specific esterases for particular groups of compounds.
- Amides can be hydrolyzed by the plasma esterases (non-specific), but they are more likely to be hydrolyzed by the liver amidases.
- Hydrazides and carbamates are less common drugs functional groups that can be also hydrolyzed.

1.3.3.1.4. Hydration

This reaction can be considered as a specialized form of hydrolysis, where a molecule of water is added to the compound structure without leading to the compound dissociation. Hydration can be carried out by many enzymes, such as epoxide hydrolase hydrates (also known as Epoxide hydratase). This enzyme is present in large quantity in endoplasmic reticulum and converts epoxides from the degradation of aromatic compounds to trans-dihydrodiols which can be conjugated (see figure 6) and excreted from the body (Abass *et al.*, 2009; Gibson & Skett, 2001; Uetrecht & Trager, 2007).

Figure 6. Hydration of Benxo[a]pyrene-4,5-epoxide (Gibson & Skett, 2001).



1.3.3.1.5. Other reactions

There are many other reactions that have been proposed as possible routes of metabolism for specific drugs. Some of these reactions are listed in table 6 (Abass *et al.*, 2009; Uetrecht & Trager, 2007).

Table 6. Other reactions involved in drug metabolism (Abass *et al.*, 2009)

Reaction	Compound
Ring cyclization	Proguanil
N-Carboxylation	Tocainide
Dimerisation	N-OH-2-Acetylaminofluorene
Transamidation	Propiram
Isomerisation	α-Methylfluorene-2-acetic acid
Decarboxylation	L-Dopa
Dethioacetylation	Spironolactone

1.3.3.2. Phase II Enzymes and Reactions (Conjugation Reactions)

After phase I, products are not usually eliminated, but undergo in conjugation reactions to form a highly polar conjugates to turn them more easily excreted. The phase II reactions involve a diverse group of enzymes often requiring co-factors or substrate derivates (see table 7), which must be replenished through dietary sources (Josephy *et al.*, 2005; Xu *et al.*, 2005). Glucuronidation, sulfation and glutathione conjugation are the most prevalent reactions of phase II metabolism, which may occur directly on the parent compounds or, as normally happens, on the products of phase I (Gibson & Skett, 2001; Xu *et al.*, 2005).

Table 7. Conjugation reaction (Gibson & Skett, 2001)

Reaction	Enzyme	Functional group
Glucuronidation	UDP-Glucuronosyltransferase	-OH -COOH -SH_2 -SH
Glucosidation	UDP-Glucosyltransferase	-OH -COOH -SH
Sulfation	Sulfotransferase	-NH_2 -SO_2NH_2 -OH
Methylation	Methyltransferase	-OH -NH_2
Acetylation	Acetyltransferase	-NH_2 -SO_2NH_2 -OH
Amino acid conjugation		-COOH
Glutathione conjugation	Glutathione-S-transferase	Epoxide
Fatty acid conjugation		-OH
Condensation		Various

1.3.3.2.1. Glucuronidation

Glucuronidation (conjugation with α-D-glucuronic acid) represents the major route for sugar conjugation, although it is also possible to occur with other sugars (glucose, xylose and ribose, though less common). This reaction is the most frequent conjugation due to the relative abundance of the co-factor (UDP-glucuronic acid) and the ubiquitous nature of the enzyme (UDP-glucuronosyltransferase) (Abass *et al.*, 2009; Gibson & Skett, 2001).

The glucuronidation reaction consists in the transference of the glucuronosyl group from uridine 5'-diphospho-glucuronic acid (UDPGA) to the substrate (attached via a glycosidic bond), the resulting glucuronide product (also known as glucuronoside) has a much higher water solubility than the original substance (Gibson & Skett, 2001).

Glucuronide formation is quantitatively the most important form of drugs conjugation (alcohols, phenols, amines, thiols, hydrozylamines and carboxylic acids). It is also a common pathway of metabolism for many endogenous compounds (steroid hormones, catecholamines, bilirubin and thyroxin) (Ionescu & Caira, 2005).

1.3.3.2.2. Sulfation

Sulfation is a conjugation pathway for many drugs and endogenous compounds, which are constituted mainly by phenols, but also alcohols, amines or thiols. This conjugation is catalyzed by a sulfotransferase (present mainly in liver) in the presence of the drug or endogenous compound and 3'phosphoadenosine-5'phosphosulfate (PAPS) (Mizutani, 2003) . Most of the drugs and endogenous compounds metabolized by glucuronidation can also be sulfated, leading to the possibility of completion for the substrate between the two pathways. In general glucuronide conjugation occurs at higher rate than sulfation, due to the limited supply of PAPS in cells when compared with UDPGA levels (Gibson & Skett, 2001; Uetrecht & Trager, 2007).

1.3.3.2.3. Methylation

These reactions are mainly oriented to endogenous compound metabolism, but some drugs can be methylated by non-specific methyltransferases found in lung and by the physiological methyltransferases. However, unlike other conjugation reactions, methylation leads to less polar products, delaying the excretion of the drug (Abass *et al.*, 2009; Ionescu & Caira, 2005). Metals such as mercury can be converted in methyl mercury by this process. The latter is highly toxic, soluble in lipids and concentrates in neural tissues (Abass *et al.*, 2009).

1.3.3.2.4. Acetylation

Acetylation is performed by N-acetyltransferase, which is mainly located in Kupffer cells (not in hepatocytes, the most usual location), although it can also be found in reticuloendothelial cells of the spleen, lung and gut. The location of the enzyme may be related to its role in leukotriene biosynthesis (Coleman, 2006).

Acetylation reactions are involved in aromatic amines and sulfonamides metabolism, which require, as a co-factor, acetyl-CoA (Abass *et al.*, 2009).

1.3.3.2.5. Amino acid conjugation

This conjugation reaction is a special form of an N-acylation. This reaction involves metabolites containing a carboxylic acid and the amino group of amino acids. There are several enzymes implicated (cytosolic and mitochondrial) and cofactors (ATP and acetyl-CoA) that activate the carboxylic acid group and results in the formation of an amide bond (Gibson & Skett, 2001; Uetrecht & Trager, 2007).

1.3.3.2.6. Glutathione conjugation

Glutathione acts as a protective compound within the body, removing potentially toxic electrophilic compounds. Those can be drugs or phase I products. The glutathione conjugates may be excreted directly in urine or more usually in bile, but more often continue for other conjugation reactions (Gibson & Skett, 2001).
The enzymes catalyzing these reactions are glutathione-S-transferases, which are located in liver, kidney, intestines and other tissues .(Ionescu & Caira, 2005)

1.3.3.2.7. Fatty acid conjugation

The fatty acids involved - stearic and palmitic acids - are conjugated to drug by esterification reaction, being catalyzed by the microsomal fraction of the liver. Nevertheless, little is known about this reaction (Abass *et al.*, 2009; Gibson & Skett, 2001).

1.4. Cytochrome P450 (CYP) enzyme system

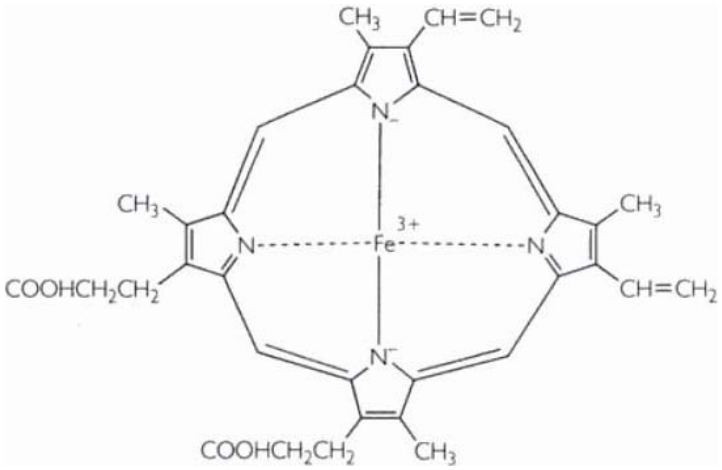
Cytochrome P450 (CYP) is not a single enzyme, but a numerous family of heme-containing (hemoprotein) enzymes with closely related isoforms, belonging to the group of monooxygenases. This enzymatic system is found in almost all organisms, being crucial for the oxidative, peroxidative, and reductive metabolism of exogenous and endogenous compounds (Gibson & Skett, 2001; Turpeinen, 2006).

In prokaryotes, CYPs usually perform a plastic function, whereas in eukaryotes their functions are very diverse. In mammals, are embedded in the membrane of the endoplasmic reticulum, working as a terminal oxidase component of an electron transfer system. CYPs are involved in biosynthesis and metabolism of many physiologically active substances (steroids, fatty acids, eicosanoids, vitamins and bile acids), and act as the major xenobiotic metabolizing enzymes (Arpiainen, 2007).

1.4.1. Chemical structure

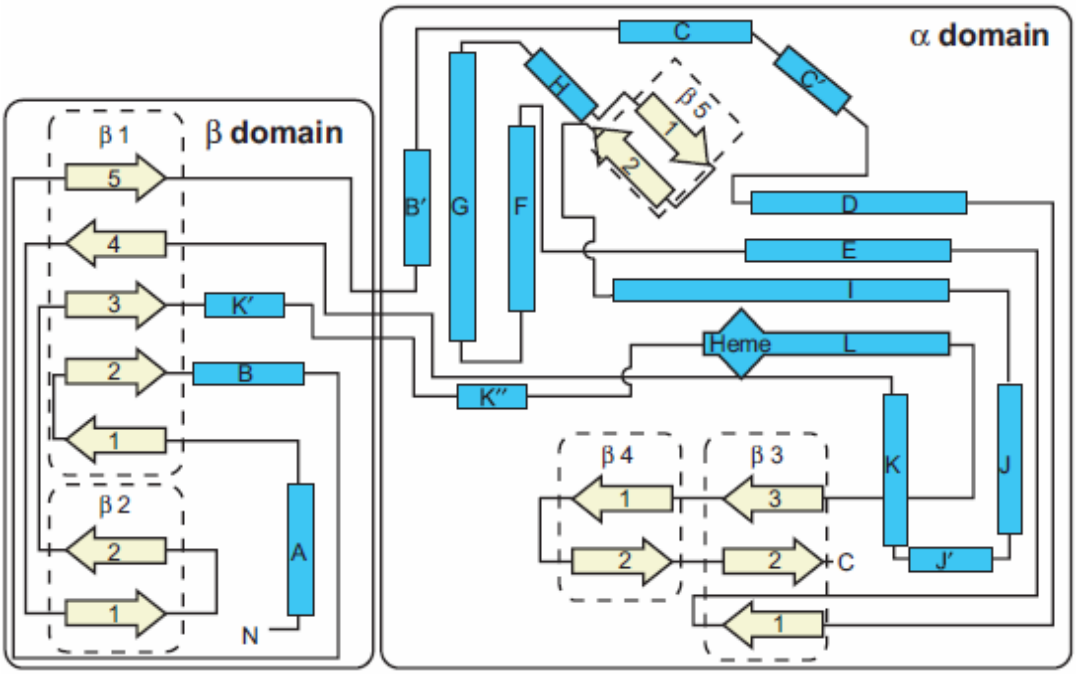
Cytochrome P450 is classified as a hemoprotein constituted approximately by 500 amino acids and by an iron protoporphyrin IX as the prosthetic group (non-protein component of a conjugated protein) (see figure 7). The monomeric molecular weight is approximately 45.000 to 55.000 daltons. This prosthetic group is common to other hemoproteins, but with different biological functions, such as hemoglobin and myoglobin (Gibson & Skett, 2001; Zuber, Anzenbacherová, & Anzenbacher, 2002).

Figure 7. Structure of iron protoporphyrin IX (Gibson & Skett, 2001).



Sequence identity among CYPs is often low (approximately 20%) and only have been identified three absolutely conserved amino acids. Highest structural conservation is found in the core of the protein around the heme and reflects a common mechanism of electron and proton transfer and oxygen activation. The most variable regions are associated with either amino-terminal anchoring or targeting of membrane-bound proteins, or substrate binding and recognition; the latter regions are located near the substrate-access channel and catalytic site and are often referred to as substrate-recognition sites. They are described as flexible, moving upon binding of substrate so as to favor the catalytic reaction. Other variations reflect differences in electron donors, reaction catalyzed or membrane localization (Gibson & Skett, 2001; Werck-Reichhart & Feyereisen, 2000).

Figure 8. Secondary and tertiary structure of CYP (adapted from Werck-Reichhart & Feyereisen, 2000).



The diagram in figure 8 shows the secondary structure and arrangement of a typical CYP. The α helices are represented by blue boxes. Groups of cream arrows outlined with dotted lines represent β sheets, lines, coils and loops. There are usually around four β sheets and 13 α helices defining one domain that is predominantly β sheets and one that is predominantly α helices. The first domain is often associated with substrate recognition and the access channel, the second with the catalytic center (Werck-Reichhart & Feyereisen, 2000).

1.4.2. Function

The most common reaction catalyzed by CYPs is a monooxygenase reaction of lipophilic compounds by inserting one atom of molecular oxygen into the parent compound (RH) while the other oxygen atom is reduced to water with the help of reducing equivalents from NADPH. The previous reaction can be described by the following scheme (Ionescu & Caira, 2005; Turpeinen, 2006):

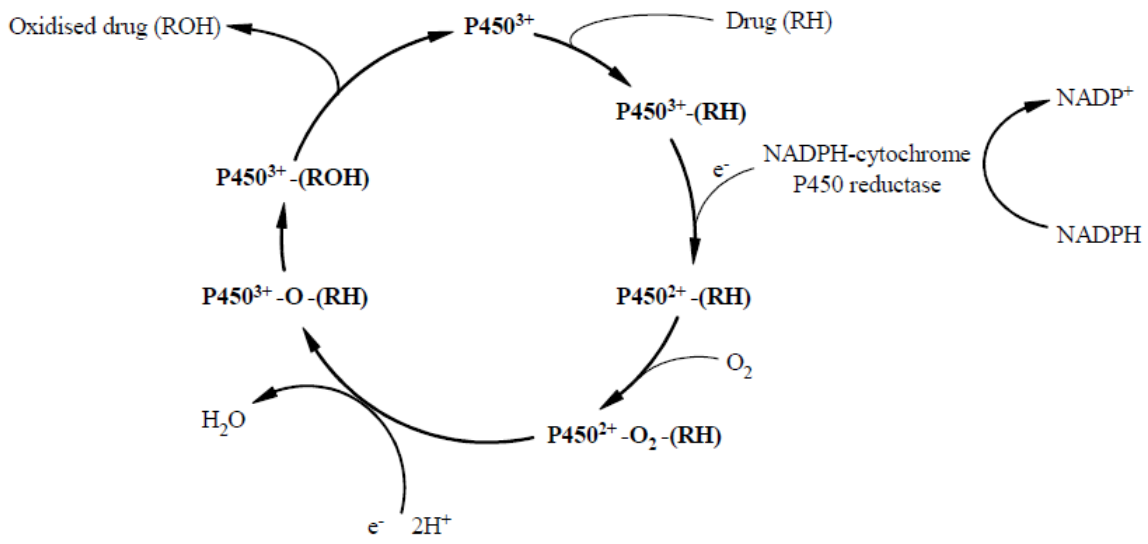
$$RH + NADPH + H^+ + \mathbf{O_2} \rightarrow R\mathbf{O}H + NADP^+ + H_2O$$

The attached hydroxyl group is used as a reactive group by other enzymatic systems for further modifications. Other reactions catalyzed by CYPs include epoxidations, peroxidations, deaminations, desulfurations, dehalogenations and reductive reactions (Turpeinen, 2006).
CYPs can be divided into four classes depending on how electrons from NAD(P)H are delivered to the catalytic site (Werck-Reichhart & Feyereisen, 2000):

- Class I: CYPs require both an FAD-containing reductase and an iron sulfur ferredoxin;
- Class II: CYPs require only an FAD/FMN-containing CYP reductase for transfer of electrons;
- Class III: Enzymes are self-sufficient and require no electron donor;
- Class IV: CYPs receive electrons directly from NAD(P)H.

In the endoplasmic reticulum, electrons are donated to CYPs by FAD/FMN-containing CYP reductase (also known as NADPH-cytochrome P450 reductase) (see figure 9). In mitochondria, ferredoxin acts as a single electron carrier from NADPH to FAD-containing ferredoxin reductase (FR) and CYP enzymes (Anzenbacher & Anzenbacherova, 2001; Gibson & Skett, 2001; Turpeinen, 2006).

Figure 9. Catalytic cycle of Cytochrome P450 (Turpeinen, 2006)



1.4.3. Nomenclature

The name cytochrome P450 (CYP) is derived from the unique optical absorption peak at wavelength 450 nm (around 420 nm for the majority of other haemoproteins) in the presence of a reducing agent and carbon monoxide (Turpeinen, 2006).

The first CYP named was CYP1A1 and since his discovery, it has been sequenced more than 7700 distinct CYP sequences of 700 families. The diversity of CYP enzymes has led to the need to classify single CYP forms into families and subfamilies. The nomenclature is based on amino-acid identity, gene organization and phylogenetic criteria (Nelson *et al.*, 1996): The amino acid sequences of enzymes in the same family are at least 40% identical, while members of subfamilies must share more than 55% amino acid identity. The abbreviation CYP refers to genes encoding CYP enzymes, and the enzymes themselves, followed by an Arabic numeral indicating the gene family, a capital letter indicating the subfamily, and another Arabic numeral representing the individual gene within the subfamily. Genes within a subfamily are numbered in order of discovery, regardless of species. The convention is to italicize the name when referring to the gene. However, some gene or enzyme names for CYPs may differ from this nomenclature, denoting the catalytic activity and the name of the compound used as substrate. In most of the species CYP gene names are written in capital letters, while mouse and Drosophila Cyp gene names are lower-case, except for the first letter. Pseudogenes (genes that have lost their protein-coding ability) are identified with 'P' or 'ps' in mouse and Drosophila, after the gene number (Turpeinen, 2006; Werck-Reichhart & Feyereisen, 2000).

There are blocks of family names reserved for different taxonomic groups: families in the range 1-49 and 301-499 are for animals, 51-69 and 501-699 are for lower eukaryotes, 71-99 and 701-999 are for plants, and 101-299 are for bacteria (Nelson *et al.*, 1996; D. Nelson, 2008; Werck-Reichhart & Feyereisen, 2000).

1.4.4. Classification and species differences

The basis of the CYPs unifying nomenclature system is founded on divergent evolution and sequence similarity, resulting in different families and sub-families. However, it does not encompass any classification or description of the substrate specificities or catalytic activities, although some broad generalizations can be done (see table 8) (Gibson & Skett, 2001; D. Nelson, 2008; Smith, Abel, Hyland, & Jones, 1998).

In mammals, CYP enzymes can be divided in two classes. One group of enzymes involved in biosynthesis of regulators of various biological functions (e.g. steroid hormones) and another group responsible for metabolism of xenobiotics (see table 8). Although CYP1, CYP2 and CYP3 families are considered to metabolize mainly xenobiotics, they also have endogenous substrates, such as steroids, fatty acids, bile acids, eicosanoids and retinoids (Turpeinen, 2006; Zuber *et al.*, 2002).

Despite the similarities within CYPs families, it is found a high polymorphism in CYP genes of the same species and also significant species differences in the expression level of individual enzymes and their substrate specificity. It has not been found inherited patterns of CYPs expression between groups of animals that share other physiological features (e.g. carnivorous species). For example, dogs possess several unique CYP450 isoenzymes such as a canine form of CYP1/2, CYP2B11, CYP2C21 and 2C41, 2D15, 3A12 and 3A26 (Fink-Gremmels, 2008).

Generally, studies comparing CYPs activity between species are conducted measuring the conversion of known CYP model substrates in liver microsomes. So, when the activity level of CYPs in the conversion of human model substrates is compared between species, it is obtained a wide range of different results. Therefore, the extrapolation of CYPs activity and specificity between species is limited and not well studied, especially in veterinary species. When the CYPs activity of different animal species is compared, it has been more consistent for CYP2E1>CYP1A2, CYP4A> CYP2D and CYP3A, however the extrapolation of substrate specificity has been inconsistent when applied to CYP2A, 2B and 2C families (Fink-Gremmels, 2008; Turpeinen *et al.*, 2007).

So, when a specific CYP reaction is compared between different species it may not correspond to the same CYP. For example, based on *in vitro* assays and antibody inhibition experiments the N-oxidation of irsogladine is catalyzed by CYP2C proteins in rat, dog and monkey microsomes but no metabolism was obtained by human CYP2C9 (Nakamura, Hirota, Morino, Shimada, & Uematsu, 1997).

Table 8. Generalized functions of CYPs (Gibson & Skett, 2001; Turpeinen, 2006)

CYP family	**Function**
CYP1-CYP3	Xenobiotic, drug and steroid metabolism
CYP4	Fatty acid, prostaglandin, leukotriene metabolism
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation and bile acid synthesis
CYP8	Prostacyclin and bile acid synthesis
CYP11	Cholesterol side chain cleavage + steroid 11β hydroxylase
CYP17	Steroid 17α-hydroxylation
CYP19	Aromatization of steroids
CYP21	Steroid 21-hydroxylase
CYP24	Vitamin D hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Vit.D3 hydroxylation, and cholesterol and bile acid synthesis,
CYP39	24-hydroxycholesterol 7-hydroxylase
CYP46	Cholesterol 24-hydroxylase
CYP51	Lanosterol 14-demethylase

1.4.4.1. CYP1 family

This family has been identified in 41 animal species and it is constituted by 3 subfamilies (CYP1A, 1B and 1C) (Cytochrome P450 Knowledgebase, 2006). In humans is formed only by 3 enzymes, which are codified by different alleles, but no pseudogenes have been found. The only enzyme of this family present in liver is CYP1A2, which is described in the next topic. The other two enzymes are CYP1A1 and CYP1B1, of which CYP1A1 is the major human extrahepatic CYP form; however it can be present at very low levels in human liver. CYP1B1 enzyme is undetectable in liver, but CYP1B1 mRNA is expressed, and it is present in almost every other tissue, such as kidney, prostate and breast (Ingelman-Sundberg, Daly, & Nebert, 2008; Phillips & Shephard, 2006; Turpeinen, 2006).

In other animals, CYP1A enzymes seem to be somewhat different from the human CYP. For instance, in dogs the antibodies against human CYP1A were shown to influence microsomal 6-hydroxylation of chlorzoxazone that is a typical activity of CYP2E1 in humans (Fink-Gremmels, 2008).

Enzymes in the CYP1 family are regulated by the AhR-receptor and are inducible after being exposed to polycyclic aromatic hydrocarbons (PAHs), found in cigarette smoke, and by 2,3,7,8-tetrachlorodibenzo-p-dioxin. The compound α-naphthoflavone acts as a potent inhibitor of this CYP family (Phillips & Shephard, 2006).

Besides detoxification, the CYP1 enzymes are often responsible for metabolic activation of PAHs and aromatic amines, which may be linked to chemical carcinogenesis (Sparfel, Van Grevenynghe, Le Vee, Aninat, & Fardel, 2006).

1.4.4.1.1. CYP1A2

The CYP1A2 has been identified at least in 15 animal species (e.g. mouse, guinea pig, rabbit, chicken, cat and dog) and they share highly homologous amino acid sequence (Cytochrome P450 Knowledgebase, 2006). The typical substrates are aromatic structures, preferably aromatic amines, but also polycyclic aromatic hydrocarbons and other planar structures (Lewis, 2003).

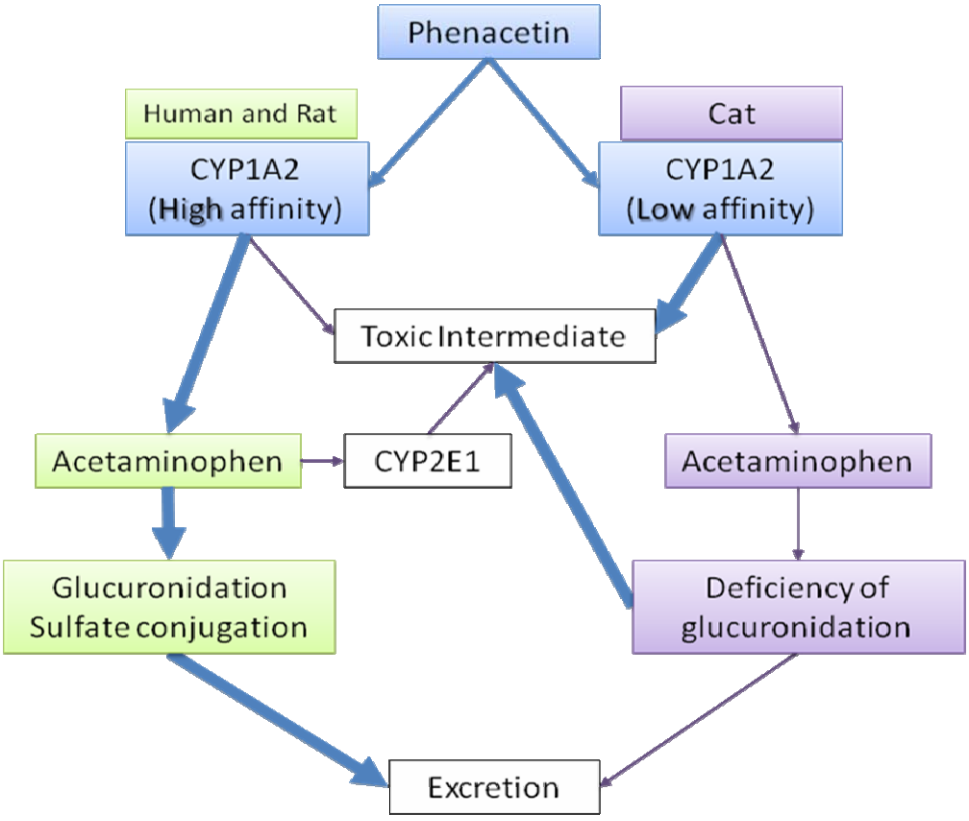
In humans is expressed mainly in liver and in a lower level in lungs along with CYP1A1. Despite the quite low quantitative proportion of CYP1A2 in liver, it has a major role in the metabolism of several compounds, such as ethoxyresorufin, caffeine (Rasmussen & Brosen,

1996), clozapine (Pirmohamed, Williams, Madden, Templeton, & Park, 1995), phenacetin (Tassaneeyakul *et al.*, 1993) and theophylline (Ha, Chen, Freiburghaus, & Follath, 1995).

This form is inducible by polycyclic aromatic hydrocarbons as 3-methylcholanthrene or by polychlorinated biphenyls, cigarette smoking, cruciferous vegetables and charcoal-grilled food (Lewis, 2003). Potent inhibitors of CYP1A2 include fluoroquinolones like ciprofloxacin (Batty, Davis, Ilett, Dusci, & Langton, 1995; Granfors, Backman, Neuvonen, & Neuvonen, 2004), fluvoxamine (Yao *et al.*, 2001), oral hormone replacement therapy and contraceptives (Lewis, 2003; Olavi Pelkonen et al., 2008).

In domestic animals, CYP1A2 is especial important in cats. This species is highly susceptible to toxic effects from many drugs, regarding his deficient ability to form glucuronide conjugates. On the other hand, in some cases it is due not only to deficiency in glucuronidation, but also to the kinetic properties of CYP1A2. For instance, phenacetin is metabolized slower by feline CYP1A2 when compared with other species. Thus, the toxic effects of phenacetin metabolites are prolonged and severe in domestic cats due to both glucuronidation deficiency and the catalytic properties of CYP1A2 (see figure 10) (Tanaka, Miyasho, Shinkyo, Sakaki, & Yokota, 2006).

Figure 10. Metabolic pathways of phenacetin (modified from Tanaka *et al.*, 2006).



1.4.4.2. CYP2 family

In CYP2 family has been identified at least 31 genes and 27 subfamilies among 10 animal species (*Cytochrome P450 Knowledgebase*, 2006). In humans 9 subfamilies are known (CYP2A, 2B, 2C, 2D, 2E, 2D, 2J, 2R, 2S and 2W), however they do not share any common regulation patterns and their substrate specificities and tissue expression vary substantially. At least five of those subfamilies (CYP2A-E) contribute significantly to drug metabolism. However the only functional enzymes in these subfamilies are CYP2B6, CYP2D6, and CYP2E1, whereas CYP2A contains two, and CYP2C four functional members. In other animal species, those human enzymes can not be identified but there are other active enzymes, such as in rat (CYP2B1/2), rabbit (CYP2B4/5) and dog (CYP2B11) (Ingelman-Sundberg, Sim, Gomez, & Rodriguez-Antona, 2007; Pasanen, 2004; Turpeinen, 2006).

1.4.4.2.1. CYP2A6

In humans, CYP2A6 is expressed in different levels among the population and quantitatively is a minor component among hepatic CYPs. It does not have a large substrate base; usually they are structurally small and planar molecules (e.g. coumarin and nicotin). Nevertheless, this enzyme has a main role in drug metabolism and is also involved in the bioactivation of some toxic compounds such as aflatoxin B1 (Kuilman, Maas, & Fink-Gremmels, 2000) and nitrosoamines (Bonate & Howard, 2004; Turpeinen, 2006).

Several inhibitors with variable selectivity are known (e.g. tranylcypromine and methoxysalen). However, the regulation mechanisms of this enzyme are not well known (Olavi Pelkonen et al., 2008).

This CYP is of special interest in human and pigs. In humans there are several variant CYP2A6 alleles with distinct frequencies between ethnic groups, which have been associated with altered nicotine pharmacokinetic and furthermore to differing smoking habits (Mwenifumbo & Tyndale, 2007; Olavi Pelkonen et al., 2008).

In pigs, CYP2A6 is linked to development of the phenomenon boar taint. This is characterized by high concentrations of androstenone and skatole (3-methylindole) stored in the fat tissues, which give undesirable taste and odor to the meat from sexually mature male swine. Sex and age differences in skatole level are related to an inhibitory effect of androstenone (and possibly some other sex steroids) on the expression of the skatole-metabolizing enzymes CYP2A6 and CYP2E1. The low CYP2A6 and CYP2E1 expression

lead to a reduced rate of the hepatic skatole clearance with the consequent accumulation of skatole in adipose tissue (Babol, Squires, & Lundstrom, 1998; Chen, Cue, Lundstrom, Wood, & Doran, 2008).

1.4.4.2.2. CYP2B6

CYP2B6 has a high polymorphic expression and it is affected by genotype, gender and ethnical group. This enzyme represents approximately 1-10 % of the total hepatic CYP and plays an important role in the hepatic metabolism of structurally diverse drugs, e.g. bupropion (Faucette et al., 2000), cyclophosphamide (Chang, Weber, Crespi, & Waxman, 1993) and ifosphamide (Granvil, Madan, Sharkawi, Parkinson, & Wainer, 1999). The usual substrates metabolized are non-planar, neutral, or weakly basic molecules with one or two hydrogen bond acceptors. However, in other species those substrates are metabolized by different CYPs. For instance, the canine orthologous of CYP2B6 (human) is CYP2B11 and the porcine homologue is CYP2B22 (72% of cDNA homology). Therefore, there is no clear ranking between species (Fink-Gremmels, 2008; Pasanen, 2004; Turpeinen, 2006).
This CYP in humans is inducible via CAR- and apparently PXR-associated mechanisms, by phenytoin (Ducharme, Bernstein, Granvil, Gehrcke, & Wainer, 1997) phenobarbital and rifampin (Chang, Yu, Maurel, & Waxman, 1997). There are also several drugs with ability to inhibit it, e.g. orphenadrine and antiretroviral drugs (ritonavir, efavirenz, and nelfinavir) (Olavi Pelkonen et al., 2008).

1.4.4.2.3. CYP2C subfamily

In humans, CYP2C family comprises about 20% of the total hepatic CYP and, as already referred, there are only four functional enzymes (2C8, 2C9, C18, 2C19), which have more than 80% identical amino acid sequences (D. Nelson, 2008; Phillips & Shephard, 2006).
In animals a huge species-dependent differences within this family can be observed. The level of activity of CYP2C family, between species, by comparing diclofenac-4'-hydroxylase activity, is higher in humans, followed by monkey and rat > rabbit > mouse > micropig > dog (Fink-Gremmels, 2008; Pasanen, 2004).

a. CYP2C8

CYP2C8 is present at relatively high levels in human liver and also plays a major role in drug metabolism, e.g. amodiaquine (X. Li, Bjorkman, Andersson, Ridderstrom, & Masimirembwa, 2002), paclitaxel (Václavíková, Horský, Šimek, & Gut, 2003), cerivastatin (Muck, 2000) and repaglinide (Bidstrup, Bjornsdottir, Sidelmann, Thomsen, & Hansen, 2003). The reaction quantified as a marker activity for polymorphically expressed CYP2C8 in humans is paclitaxel 6-hydroxylation (Phillips & Shephard, 2006). However using the same substrate specificity study no such counterpart has been identified in other species (Pasanen, 2004).
This CYP can be inhibit by several pharmaceutical compounds, e.g. quercetin (K.-A. Kim, Park, Kim, Ha, & Park, 2005), montelukast (K. Kim, Park, Kim, & Park, 2006), zafirlukast (Jaakkola, Backman, Neuvonen, Niemi, & Neuvonen, 2006) and trimethoprim (normal clinical dose can inhibit CYP2C8-mediated metabolic clearance by about 26% to 80%) (Mikko Niemi, 2004). CYP2C8 induction is mediated via multiple nuclear receptors (CAR, PXR, GR, and HNF4) (Ferguson, Chen, LeCluyse, Negishi, & Goldstein, 2005).

b. CYP2C9

CYP2C9 is the predominant CYP2C isoform expressed in human liver and highly polymorphic, which contributes to the wide interindividual variation in the pharmacokinetic of certain drugs. So, this enzyme is responsible for metabolize several clinically relevant drugs: celecoxib (Kirchheiner et al., 2003), cyclophosphamide (Timm *et al.*, 2005), ifosphamide (Chang, Yu, Goldstein, & Waxman, 1997), losartan (Stearns, Chakravarty, Chen, & Chiu, 1995), S-warfarin (Rai, Udar, Saad, & Fleisher, 2009) and several NSAID's (Phillips & Shephard, 2006). The importance of the CYP2C9 polymorphism has a major role in S-warfarin metabolism, which uses CYP2C9 as a major metabolic pathway and possesses a narrow therapeutic window with a fatal side-effect profile (Turpeinen, 2006).
The catalytic activity of this CYP can be quantified by measuring S-warfarin 7-hydroxylation, diclofenac 4'-hydroxylation or tolbutamide 4'-hydroxylation activity (Phillips & Shephard, 2006). However these reactions are performed by different CYP enzymes depending on the species, e.g. dog orthologous of CYP2C9 is CYP2C21. For instance, when tolbutamide 4'-hydroxylation activity levels among different species is compared higher activity has been found in horse than man and dog, and the lowest activity in cat (Fink-Gremmels, 2008).

Among the inhibitors known for CYP2C9 in humans are gemfibrozil (Tornio, Niemi, Neuvonen, & Backman, 2007), amiodarone, sulphaphenazole and certain other sulphonamides (Miners & Birkett, 1998).
The mechanism responsible for CYP2C9 induction is dependent on multiple regulatory elements (Olavi Pelkonen et al., 2008), e.g. rifampicin induces CYP2C9 via the PXR pathway (Youdim, Tyman, Jones, & Hyland, 2007).

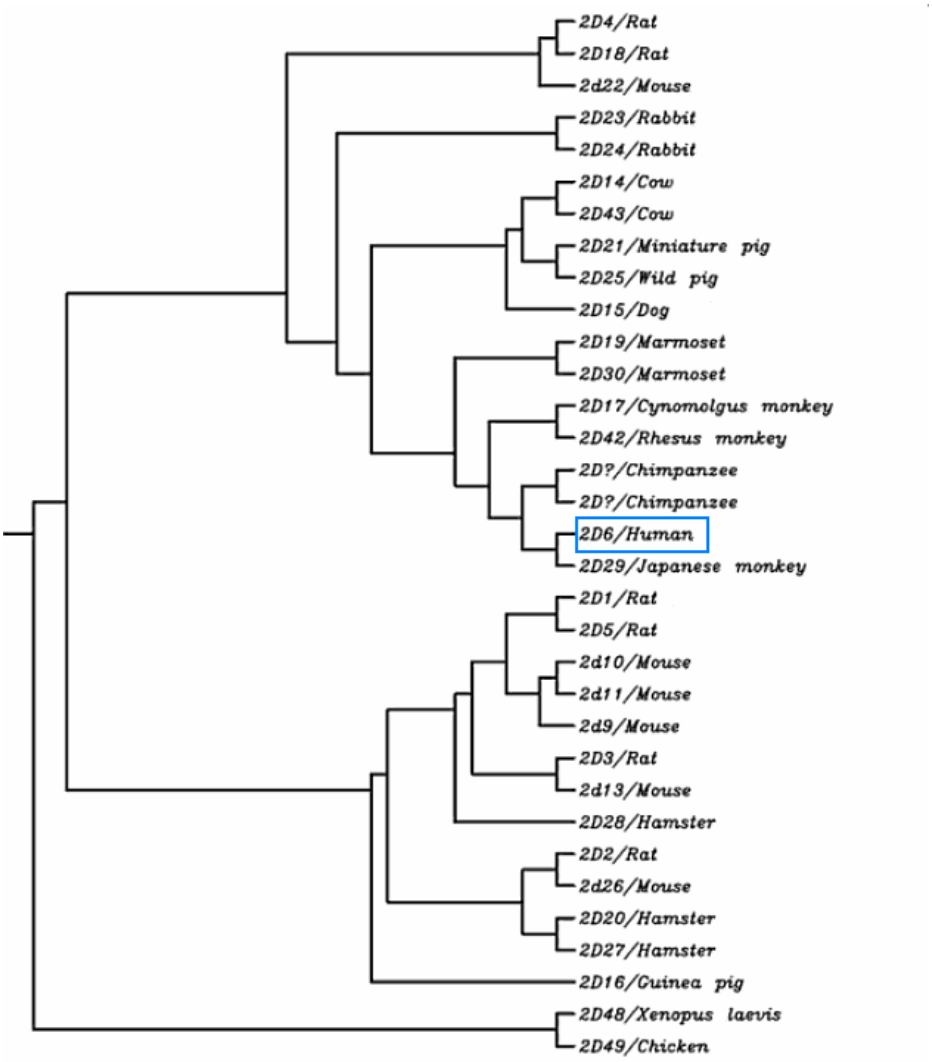
c. CYP2C19

CYP2C19 is only present in humans and handles a smaller proportion of all available drugs (~5%) (Musana & Wilke, 2005). So, CYP2C19 substrates include antiepileptics (phenytoin, mephenytoin), benzodiazepines (diazepam, flunitrazepam), tricyclic antidepressants (imipramine, clomipramine), selective serotonin receptors inhibitors (citalopram, sertraline) and proton pump inhibitors (lansoprazole, omeprazole, pantoprazole, rabeprazole) (FDA, 2006; Musana & Wilke, 2005; Olavi Pelkonen et al., 2008).
The reactions used as marker activities for CYP2C19 are S-mephenytoin 4-hydroxylation and tolbutamide methyl hydroxylation. No selective drug inhibitors for CYP2C19 have been found yet, only fluvoxamine and fluoxetine have been reported as moderate inhibitors (Jeppesen *et al.*, 1996). CYP2C19 is known to be inducible by chemicals, which act as ligands/activators for PXR and CAR, e.g. rifampin (Turpeinen, 2006).

1.4.4.2.4. CYP2D subfamily

Mammalian CYP2D genes are currently available for humans, apes, mouse, rat, rabbit, dog, pigs, cattle and others (see figure 11). Those vary tremendously among different species, with respect to the number of functional genes and pseudogenes, and the function and expression of their enzymes. However, in some species it can be found orthologous forms to human CYP2D enzymes with some similar functions (Cytochrome P450 Knowledgebase, 2006; Pasanen, 2004).

Figure 11. Phylogenetic tree of CYP2D subfamily genes. (Adapted from C. Ioannides, 2008).



One unusual feature in CYP2D subfamily is the extensive rodent subfamily, especially in mice, however the reason is not completely understood. The last data suggests that it may be due to selective pressure from the diet or/and an influence of endogenous pathways (C. Ioannides, 2008). Those could lead the evolution of CYP2D substrate specificities to an adaptive evolution, with the goal to fix mutations that enhanced protein-protein interactions as well as to fix mutations that allow the expansion of substrate specificity and increase the number of binding modes (Zawaira, Matimba, & Masimirembwa, 2008).

Another distinctive characteristic in this subfamily is the porcine CYP2D25, which shares 77% identity with human CYP2D6 (Hosseinpour & Wikvall, 2000). CYP2D25 has been recognized as a vitamin D3 25-hydorxylase, which is unique among CYP2D subfamily (Hosseinpour & Wikvall, 2000; C. Ioannides, 2008).

a. CYP2D6

CYP2D6 gene is the most polymorphic of all known human CYPs, with more than 75 polymorphisms identified (Musana & Wilke, 2005). It appears that 4 alleles account for >95% of the functional variation, being responsible for an important variability in CYP2D6 enzyme activity, ranging from complete deficiency (high risk for ADRs) to extensively increased activity (non-responsiveness to treatments) (Musana & Wilke, 2005; Turpeinen, 2006). Therefore, it is the most studied CYP with respect to pharmacogenetics (Turpeinen, 2006).

CYP2D6 metabolizes approximately 20% of all commonly prescribed drugs. Examples include beta-blockers (carvedilol, S-metoprolol, timolol), antidepressants (amitriptyline, clomipramine, desipraminea) and a variety of antipsychotics (amitriptyline, haloperidol, codeine, dextromethorphan, lidocain, metoclopramide) (FDA, 2006; Flockhart, 2008; Musana & Wilke, 2005). So, typical reactions performed by CYP2D6 include the codeine O-demethylation, haloperidol N-dealkylation and propranolol 4-hydroxylation (Uetrecht & Trager, 2007).

CYP2D6 is inhibited by a wide range of drugs, of which a large proportion is also a substrate. The stronger inhibitors (more than 80% decrease in clearance) are bupropion, fluoxetine, paroxetine and quinidine (Flockhart, 2008).

In contrast with most of other CYPs, no environmental agent or drug inducer are known – although physiological conditions such as pregnancy have been shown to increase the rate of CYP2D6-mediated metabolic reactions (Turpeinen, 2006).

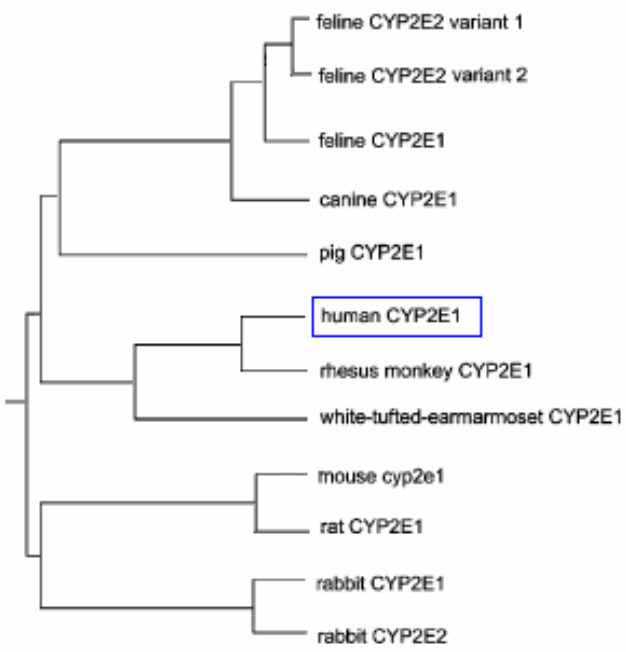
The species and CYP that share more similarities with human CYP2D6 is the Japanese monkey CYP2D29, however it shows higher liver microsomal expression than humans. In rats, CYP2D1 is the orthologous form to human CYP2D6, sharing 82% amino acid sequence. In mice, CYP2D22 was suggested to be the orthologue to human CYP2D6, but its catalytic properties towards some typical substrates and inhibitors were unique, which makes it more closely resemble to CYP3A4. The major CYP2D in dog is CYP2D15, which have some similar enzymatic activities with human CYP2D6 but showing some specific differences (e.g. very low debrisoquine hydroxylase activity) (Fink-Gremmels, 2008; Costas Ioannides, 2006; Pasanen, 2004).

So, regarding all species differences in CYP2D composition and substrate specificity, it has not been found a valid CYP2D6 polymorphism model (Costas Ioannides, 2006).

1.4.4.2.5. CYP2E1

CYP2E1 is a highly conserved gene and the only of its subfamily among most mammals, with the exception of rabbits and felines (see figure 12) (C. Ioannides, 2008; Tanaka et al., 2005). The enzyme is constitutively expressed in hepatic tissue and in a lower concentration by extrahepatic tissues (lung, kidney, brain, nasal mucosa, bone narrow and peripheral blood lymphocytes).

Figure 12. Phylogenetic tree of CYP2E subfamily of various species (Adapted from Tanaka *et al.*, 2005)



CYP2E1 exhibits very broad substrate specificity, but metabolizes only few drugs - contributing mainly to the metabolism of those that are metabolized primarily by other CYPs, for instance it can act as an alternative demethylation pathway for a variety of substrates metabolized by CYP3A4 (Tanaka *et al.*, 2005; Turpeinen, 2006). However, this enzyme is also responsible for the metabolic activation of a large number of compounds into toxic metabolites. The substrates of CYP2E1 usually consist of hydrophobic and low molecular weight compounds, such as chlorzoxazone, benzene, ethanol, carbon tetrachloride, chloroform and anesthetics (enflurane, halothane, methoxyflurane) (Gonzalez, 2005; C. Ioannides, 2008). For modeling purposes of CYP2E1, chlorzoxazone 6-hydroxylation (Khemawoot, Nishino, Ishizaki, Yokogawa, & Miyamoto, 2007), N-nitrosodimethylamine N-demethylation and p-nitrophenol hydroxylation (Phillips & Shephard, 2006) can be used to measure its catalytic activity.

This CYP plays a major role in xenobiotic metabolism and toxicity, owing to its substrate specificity. The main examples are: 1) major task in ethanol metabolism (C. Ioannides, 2008); 2) responsibility for initiating the cascade of toxic metabolites from acetaminophen; 3) requirement for the hepatotoxicity of chloroform and carbon tetrachloride; 4) ability to produce oxidative stress directly through NADPH oxidase activity and through metabolism of xenobiotics (Gonzalez, 2005).

CYP2E1 expression is altered in response to a variety of xenobiotics, as well many different conditions, including gender, circadian rhythm (Khemawoot *et al.*, 2007), nutrition (e.g. starvation and obesity), metabolic and endocrine disorders (e.g. diabetes), inflammation (in response to cytokines), viral infections (e.g. hepatitis C) and heppatocellular carcinoma (C. Ioannides, 2008).

Mechanisms responsible for CYP2E1 regulation are complex, and involve intracellular signaling, transcriptional and post-transcriptional events and post-translational modifications. This CYP is inducible by several of it own substrates (e.g. ethanol, acetone, imidazol, benzene and isopropanol). Inhibitors of CYP2E1 include diethyl-dithiocarbamate (Guengerich, Kim, & Iwasaki, 1991), disulfiram (Kharasch, Thummel, Mhyre, & Lillibridge, 1993) and pyridine (Turpeinen, 2006).

1.4.4.3. CYP3 family

The CYP3 gene family includes the CYP3A and CYP3B subfamilies, however only the former is expressed by mammals (McArthur *et al.*, 2003).

Members of the CYP3A subfamily represent the dominant CYP forms expressed in the digestive and respiratory tracts, which are the first targets for ingested and inhaled foreign compounds, providing a broad biochemical defense and an important role in first-pass clearance (Vaz, Klabunde, & Published by 2008).

This subfamily is responsible for the metabolism of a wide variety of chemical compounds (over 50% of all drugs in use today are substrates and/or inhibitors of mammalian CYP3A enzymes) and also metabolism of endogenous hormones, bile acids, fungal and plant products, and environmental pollutants (McArthur et al., 2003; Vaz et al., 2008).

As happens in other species, this subfamily contains the most abundant CYP enzymes of the human liver, representing about 30% of hepatic content, and is the only CYP subfamily present in substantive amount in the enteric mucosa. The substrate specificity among this subfamily is broad, being considered the most important CYP subfamily in drug metabolism

(C. Ioannides, 2008). It contains three functional enzymes (CYP3A4, CYP3A5, and CYP3A7) and one pseudoprotein (CYP3A43) (Bonate & Howard, 2004; Ingelman-Sundberg et al., 2008; Olavi Pelkonen et al., 2008; Turpeinen, 2006).

CYP3A5 is a minor polymorphic CYP isoform in human liver, with 25-40% of individuals expressing appreciable levels of this enzyme, but it is also expressed by other extrahepatic tissues (kidney, lung, colon, and oesophagus). It is structurally similar to CYP3A4, sharing most of its substrates and inhibitors, as well as with CYP3A7 (Pacifici & Pelkonen, 2001).

CYP3A7 is the predominant CYP form in embryonic, fetal, and newborn livers, in contrast with its minor expression in adult liver. The main role during the fetal stage is the hydroxylation of several endogenous substances like retinoic acid and steroid hormones (Pacifici & Pelkonen, 2001; Yaffe & Estabrook, 2000).

1.4.4.3.1. CYP3A4

CYP3A4 has the sixth most transcribed CYPs mRNA in human hepatocytes, and the most abundant form found in human liver (between 30 and 60% of total CYP in liver, depending on many factors, as genetics and food). Though it is also expressed in other several tissues, with the expression in the liver and small intestine of primary interest (Vaz et al., 2008; Zuber et al., 2002).

This CYP has an active site of sufficient size and topography to accommodate either large ligands or multiple smaller ligands (Scott & Halpert, 2005; Williams *et al.*, 2004). Thus, CYP3A4 substrates vary widely in size and structure from small molecules such as acetaminophen to extremely large compounds such as cyclosporine A. In addition, among the other substrates are several therapeutic classes of drugs, such as macrolide antibiotics (e.g. clarithromycin, erythromycin), benzodiazepines (e.g. diazepam, midazolam), prokinetic (cisapride) and calcium channel blockers (e.g. amlodipine, verapamil); as well as several endogenous agents including testosterone, progesterone, androstenedione, and bile acid. Hence, it is possible that many of those substrates may compete and the risk of drug interactions is possible (FDA, 2006; Ingelman-Sundberg et al., 2008; Olavi Pelkonen et al., 2008).

CYP3A4 expression can be modulated by several factors, like diet components, hormones, drugs and genetic polymorphism. In addition, the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have been shown to be involved in the inductive mechanism (Goodwin, Hodgson, D'Costa, Robertson, & Liddle, 2002; Rodrigues,

2008). This enzyme is inducible by a large number of substances, e.g. phenobarbital, glucocorticoids (dexamethasone), rifampin and St. John's wort. It can also be inhibited by several compounds including certain azole antifungal agents (ketoconazole, itraconazole), antibacterials (clarithromycin, erythromycin), antihypertensives (verapamil, diltiazem) and food constituents (grapefruit juice, bergamottin) (Pacifici & Pelkonen, 2001; Olavi Pelkonen et al., 2008; Rodrigues, 2008).

The most suitable animal CYP to an experimental model for CYP3A4-mediated metabolism was reported to be CYP3A29 in pigs. This CYP possesses a typical nifedipine hydroxylation and testosterone-6β-hydroxlylation activities (characteristic for CYP3A4) and it is inducible by phenobarbital, rifampin and dexamethasone (Soucek, Zuber, Anzenbacherova, Anzenbacher, & Guengerich, 2001; Zuber *et al.*, 2002).

1.5. Drug interactions

Drug interaction occurs when the activity of a drug is affected by another drug or a food component. Thus, drug interactions can be classified in drug-drug interactions (DDI) or food-drug interactions (FDI). These interactions are the consequence of alterations in pharmacokinetic or pharmacodynamics properties of the drug (Manzi & Shannon, 2005; Turpeinen, 2006).

Pharmacodynamics interactions can be the result of the administration of receptor agonists (pure or partial) or antagonists (competitive or non-competitive), or it can be by interference in signal transduction (Ionescu & Caira, 2005).

Pharmacokinetic interactions can take place at any level of the absorption, distribution, metabolism, or excretion process. The most clinically relevant interactions occur mainly in either absorption or metabolism phase (Olavi Pelkonen et al., 2008; Turpeinen, 2006).

In metabolism phase, CYPs are the main system involved in metabolic drug interactions. This may be affected by either enzyme induction or enzyme inhibition. Consequences of CYP-associated drug interactions resulting from reduced (inhibition) or increased (induction) rate and extent of the biotransformation will be discussed in more detail in following topics (Manzi & Shannon, 2005; Olavi Pelkonen et al., 2008).

1.5.1. Inhibition of CYP enzymes

Inhibition of CYP enzymes is the most common cause of DDI. The main interferences involved in CYP inhibitions are either the blocking of enzymatic synthesis, the destruction of pre-formed enzymes, or inactivation of the enzyme by their complexation with drug metabolites. The direct consequence can be: delay in drug metabolism, increased bioavailability of the parent compound; or prolongation of their pharmacological action by decreased elimination of compounds dependent on metabolism for systemic clearance. The type of CYP inhibition can be either irreversible or reversible (Ionescu & Caira, 2005; Olavi Pelkonen et al., 2008; Turpeinen, 2006).

The half maximal inhibitory concentration (IC50) is used as a measure of how effective is the inhibition, which can be determined constructing a dose-response curve and examining the effect of different concentrations of inhibitor on CYP-related activity. In a broad sense, the obtained IC50 values are used for ranking purposes within one compound series and binned into classes exhibiting a high (IC50<1 μM), a moderate (1 μM <IC50<10 μM) and a low (IC50>10 μM) potential for CYP inhibition (Krippendorff, Lienau, Reichel, & Huisinga, 2007).

a. Reversible inhibition

The latter is the most common type of enzyme inhibition. It is characterized by action of inhibitors that compete with substrates for occupancy of the active site. The binding forces involved are usually weak, which are both formed and broken down easily. Consequently, reversible inhibitors act fast, but do not permanently destroy the enzyme. Four different types of reversible inhibition can be distinguish (Ionescu & Caira, 2005; Olavi Pelkonen et al., 2008):

- Competitive inhibition: Both the inhibitor and the substrate compete for the same site on the enzyme. It may be prevented if the active site is already occupied by the substrate;
- Non-competitive inhibition: the active binding site of the substrate and inhibitor is different from each other;
- Uncompetitive inhibition: the inhibitor only binds to the enzyme-substrate complex and not to the free enzyme;
- Mixed-type inhibition: when elements of both competitive and non-competitive inhibition mode is observed.

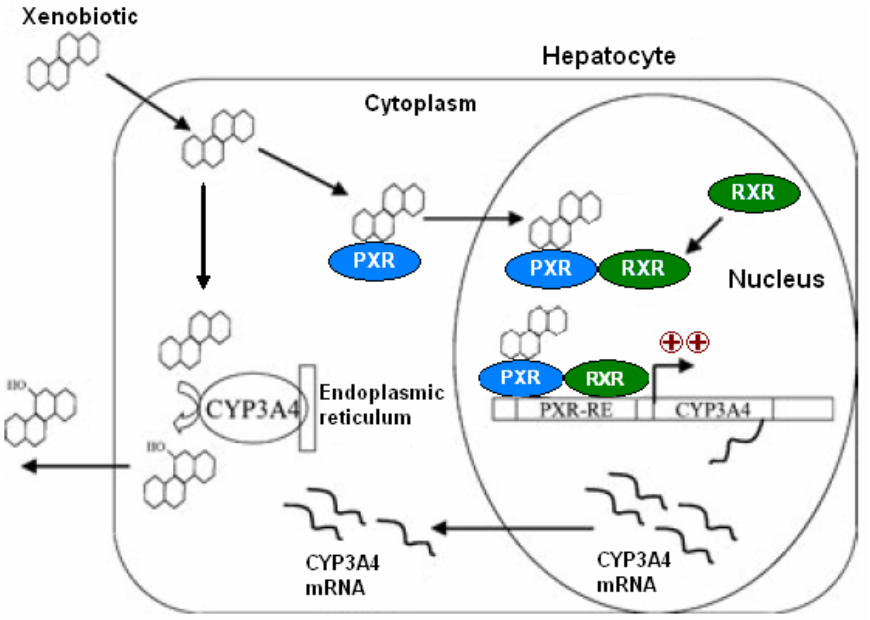
b. Irreversible inhibition

In contrast irreversible inhibition requires biotransformation of the inhibitor. So, the common step in this mechanism is the metabolic activation of a substrate into a metabolite. It can be trapped permanently within the active site of the CYP by: 1) forming a tightly bound complex, or; 2) via the strong covalent binding of reactive intermediates to the protein or heme of the CYP (Vaz et al., 2008). The only way to restore the enzymatic activity is though the new synthesis of the enzyme, that's why this mechanism is also called "mechanism-based inhibition", "metabolite base inhibition" or "suicide inhibition". Mechanism-based CYP inhibition is time dependent and it also depends on substrate concentration and many times it needs the presence of NADPH (Olavi Pelkonen et al., 2008).

1.5.2. Induction of CYP enzymes

The induction of CYPs is highly conserved and is found not only in humans but also among other animal species. Induction is defined as an increase in enzyme activity as a consequence of an increment in gene transcription and intracellular enzyme concentration. In contrast with CYP inhibition, induction is a slow regulatory process involving nuclear receptors - aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) (figure 13), constitutive androstane receptor (CAR) - but also other transcription factors including peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear factor (HNF) family members, glucocorticoid receptor (GR), and CCAAT/enhancer-binding proteins (C/EBPs) (Leslie M. Tompkins, 2007; Olavi Pelkonen et al., 2008).
The stimulation of enzyme activity represents a temporal process and a protective response increasing the detoxification activity. The direct consequence is an accelerated rate of biotransformation of both endogenous compounds and xenobiotics. Regarding drug metabolism, it usually leads to therapeutic failure (subtherapeutic levels) and contributes to inter- and intra-individual variation in drug efficacy and potential toxicity associated with drug-drug interactions.

Figure 13. Schematic model of a xenobiotic binding and activating PXR. The latter binds to DNA as a heterodimer with a retinoid X receptor alpha (RXR), leading to CYP3A4 induction (modified from Leslie M. Tompkins, 2007).



A typical characteristic of inducers is the ability to induce a wide spectrum of CYPs and in some cases it can lead to auto-induction, which can interfere with any other compounds metabolized by the same enzyme. Classical CYP-inducers in human include carbamazepine, phenytoin, rifampicin, and St. John's wort (Turpeinen, 2006). Also, in animals many CYP inducers have been characterized: polycyclic aromatic hydrocarbons (PAHs) (induces CYP1A), Phenobarbital (induces CYP2B and CYP3A), glucocorticoids and some of their antagonists (induces CYP3A), ethanol (induces CYP2E1) and peroxisome proliferators (induces CYP4A) (T. Li, 1997). However, most of those inducers may behave differently depending on the species. For instance, rifampin is a potent inducer in human and rabbit but is a very poor inducer in rat (Benedetti & Dostert, 1994). Another example is the benzimidazole derivative omeprazole, which is a potent inducer of CYP1A in human, but it doesn't act as inducer in rodents (Lu & Li, 2001). In contrast, pregnenolone 16α-carbonitrile is a potent inducer in rat CYP3A but not in either rabbit or human (LeCluyse, 2001; T. Li, 1997).

1.5.3. Food-drug interactions

The concept that food agents could enhance or reduce drug metabolism has gained importance in human medicine only gradually in the last two decades. However, the same didn't happen in veterinary medicine, which is still an unexplored field (Strandell, Neil, & Carlin, 2004).

The list of nutritious compounds involved in food-drug interactions embraces several food and herbal sources. For instance, regarding food interactions, the fish oil can be consumed as part of fish or as food supplement. It has been found in *in vitro* studies that commercial fish oil (constituted by eicosapentaenoic acid, docosahexaenoic acid, other omega-3 acids, and D-α-tocopherol) inhibits CYP2C19, 2D6 and 3A4. So, it may be possible to increase the bioavailability of some of their substrates (Strandell et al., 2004).

Concerning the major role and many well-known herbal extracts in drug interactions, they can be classified apart as herbal-drug interactions, but still belonging to the group of food-drug interactions (Cott, 2008; Nowack, 2008). So, in the following topics it will be presented: 1) examples of herbal-drug interactions in humans that can be used as basis for studies in veterinary sciences, owing to the lack of information in this field; 2) description of some plant extracts and phytochemicals as possible CYP inhibitors, which were used in the present study and could also be used in further studies involving veterinary species.

1.5.3.1. Herbal-drug interactions

The natural products grapefruit (*Citrus paradisi*) juice and St. John's wort (*Hypericum perforatum*) are usually the most cited examples responsible for drug interactions, involving either CYPs inhibition or induction. However, there are many other herbal components capable of affect CYPs activity, e.g. other close grapefruit relatives, wine, medicinal herbs and herbal teas (Nowack, 2008).

The first documented food-drug interaction, in 1989, when grapefruit juice was first found to increase the blood levels of felodipine, a calcium channel blocker. Since then, it has been described to interfere with many other drugs metabolism (Cott, 2008).

Grapefruit juice inhibits CYP1A2, CYP2C9 and most strongly intestinal CYP3A4 (Hidaka *et al.*, 2008). CYP3A4 inhibition is a mechanism-based inhibition. It is caused by furanocoumarins (bergamottin, bergaptol and geranycoumarin), which bind to the enzyme as suicide substrates, and to a minor extent by flavonoids (naringin/naringenin). So, this mechanism is responsible for increasing the bioavailability of many drugs metabolized by

CYP3A4, e.g. nifedipine, verapamil, simvastatin, lovastatin, cyclosporine, triazolam and midazolam (Dahan & Altman, 2004; Girennavar, Jayaprakasha, Jadegoud, Nagana Gowda, & Patil, 2007; Kupferschmidt, Ha, Ziegler, Meier, & Krähenbühl, 1995; Nowack, 2008).

The last studies suggest that the "grapefruit effect" extends also to pineapple juice and other close grapefruit relatives such as pomelos (*Citrus grandis*) and Seville oranges (*Citrus aurantia*), being also capable of CYP3A4 inhibition (Hidaka *et al.*, 2008).

Wine is a worldwide consumed drink with many phytochemically brands available. Red wine, but not white wine, is responsible for CYP3A4 inhibition. One of its constituents, revesterol acts as a mechanism-base inhibitor. It has been reported to decrease the bioavailability of cyclosporine in humans (Tsunoda, Harris, & Christians, 2001).

St. John's wort is widely used for the treatment of concussion depression (mild to moderate) (Stargrove, Treasure, & McKee, 2007). However, it has been reported to cause several herd-drug interactions. These interactions are caused by induction of the drug transporter P-gp and both intestinal and hepatic CYP3A4, through activation of the nuclear receptor PXR (Cott, 2008; Tannergren *et al.*, 2004). The CYP3A4 induction accelerates the clearance of its substrates, decreasing their bioavailability, e.g. cyclosporin, simvastatin, midazolam, omeprazole, theophylline and verapamil (Nowack, 2008; Tannergren et al., 2004).

In addition, St. John's wort can inhibit some CYPs (e.g. CYP1A2, 2C9, 2C19, 2D6 and 3A4). Therefore, first it may leads to an increased bioavailability of the CYP3A4 substrates (owing to the inhibition), but then is followed by a decrease in bioavailability as consequence of the PXR-mediated induction (slow regulatory process) (Nowack, 2008).

There are many other medicinal herbs or herbal teas which may induce or inhibit CYPs activity, such as: camomile (*Matricaria recutita*), peppermint (*Mentha piperata*), dandelion (*Taraxacum officinale*), ginkgo (*Ginkgo biloga*), siberian ginseng (*Eleutheroccus senticosus*), milkthistle (*Sylibum marianum*), saw-palmetto (*Serenoa repens*), echinacea (*Echinacea purpurea*), black cohosh (*Cimicifuga racemosa*), valerian (*Valeriana officinalis*), soya (*Glycine max*) and goldenseal (*Hydrastis canadensis*) (Nowack, 2008; Strandell et al., 2004). For instance, ginkgo has shown to inhibit a large number of CYPs (CYP1A2, 2C9, 2C19, 2D6 and 3A4) *in vitro* and is responsible for mechanism-based inhibition of CYP3A in rats (Zhou *et al.*, 2003). Goldenseal is a medicinal herb used in humans and animals regarding its immunomodulatory and anti-inflammatory properties. However, it is a strong inhibitor of several CYPs (CYP2C9, 2C19, 3A4/5, 2D6) (Wynn & Fougere, 2007).

1.5.3.2. Plant extracts with possible CYP inhibition properties

Regarding the importance of plants in human and animal diet, and the lack of knowledge in herbal-drug interactions, continued survey and research is essential. So, in the present thesis was analyzed five different plant extracts available in food supplies that can be ingested by both humans and animals. This study was conducted as a first surveillance test using human microsomes, owing to a bigger importance and development of this area in human medicine than in veterinary medicine. Nevertheless, the final results can also be used in further studies involving veterinary species.

The plant extracts analyzed are: 1) bark and phloem extracts from *Pinus sylvestris* (common name Scotch pine); 2) root extracts from *Angelica archangelica* (common name Angelica); 3) extracts from *Citrus grandis* (also known as *Citrus maxima* and by the common name Pomelo); 4) two different types of *Mentha sp.* (common name Mint) extracts by using water and ethanol as their solvents.

Most of these plants are used as food additives. For instance, *Pinus sylvestris* is used as a flavoring ingredient and adjuvant, permitted for direct addition to food for human consumption. In Scandinavian countries and Germany it is available as an aqueous extract from the pine needles. In many countries, *Angelica archangelica* is used as a flavoring ingredient and a food ingredient. *Mentha sp.* has many uses, such food ingredient, tea and oil extracts. Pomelo is an asian tree and only the fruits are consumed (Duke, 2008).

1.5.3.3. Phytochemicals with possible CYP inhibition properties

Nowadays, the use of purified phytochemicals as a food supplement is a common routine in the alimentary industry, or they can be used as part of medical treatments. In this study was analyzed compounds of two chemical groups (furocoumarins and monoterpenes), which will be described in the next topics.

1.5.3.3.1. Furocoumarins

Furocoumarins (or furanocoumarins) are a class of phytochemicals produced by a variety of plants as part of their defense mechanisms against predators, ranging from insects to mammals. Their chemical structure consists of a furan ring fused with coumarin. The former

may be fused in different ways producing several isomers. Some of the furocoumarins have been already identified as CYP inhibitors and responsibles for FDI (Zhou et al., 2003).

In this study, two structural classes of furocoumarins, angular and linear, were used. The angular furocoumarins used were: 5,6-dihydroxyangelicin, sphondin, lanatin, isobergapten and angelicin. The phytochemicals belonging to the linear class of furocoumarins used were: 8-hydroxybergapten, phellopterin, cnidilin, bergaptol, isopimpinellin, bergamottin, imperatorin, psoralen, bergapten and xanthotoxin.

5,6-Dihydroxyangelicin is a natural angular furocoumarins isolated from the root of *Angelica glabra makino* and from the fruits of *Ligusticum acutilobum* (Duke, 2008).

Sphondin is found in *Angelica archangelica* and also in the root of *Heracleum laciniatum* and *Pastinaca sativa*, being responsible for phototoxic reactions (Duke, 2008).

Lanatin is a natural furocoumarins extracted from *Heracleum thomsoni* (Banerjee, 1980).

Psoralen is a photosensitizing agent found in several plants and it has an important use in PUVA treatment (it is administered to sensitize the skin, after skin exposure to UVA light) for skin problems such as eczema, psoriasis, vitiligo, and mycosis fungoides. Some important psoralen derivatives are imperatorin, xanthotoxin and bergapten. Most furocoumarins can be regarded as derivatives of psoralen or angelicin.

Bergaptol, bergapten and bergamottin are present in grapefruit juice, but they inhibit principally CYP3A4 in the intestine (Duke, 2008).

Imperatorin and phellopterin can be found in several species of *Angelica sp*. and *Citrus sp*. (Duke, 2008). Specifically, imperatorin is present in the root of *Angelica dahurica*; in the fruits, leaves and roots of parsnip and *Ammi majus L.*; in lemon and lime oils; in the fruits of parsley, fennel and possibly coriander (Kleiner, Vulimiri, Starost, Reed, & DiGiovanni, 2002).

Isopimpinellin is found in healthy celery, in parsnip fruits, leaves, and roots; in the fruits of *Ammi majus L.* and in the rind and pulp of limes (Kleiner *et al.*, 2002).

Isobergapten and cnidillin are present in several plants, especially in *Angelica sp*. 8-hydroxybergapten is a characteristic compound found in *Angelica dahurica*.

1.5.3.3.2. Monoterpenes

As part of this study pure α-thujone and mixed α-, β-thujone were tested as possible CYP inhibitors. Thujone is a ketone and a monoterpene that exists in two stereoisomeric forms: α-thujone and β-thujone (more toxic). Thujone is a strong irritant and has cytotoxic properties.

Generally, it is considered to have antimicrobial, anthelmintic, uterine stimulant as well as psychedelic activity. Thujone is predominantly eliminated via kidneys and lungs. In nature it is present as a mixture of both and the proportion varies with the source. It occurs in the essential oils and parts of the plants of *Thuja occidentalis* (thuja), *Artemisia absinthium* (wormwood), *Salvia officinalis* (sage), *Salvia sclarea* (clary), *Tanacetum vulgaris* (tansy) and in *Juniperus sp.* and *Cedris sp*. Synthetic α-thujone is also available commercially in E.U.. However, this compound is most famous for being incorporated in the drink absinthe and some other alcoholic beverages. Owing to the possible neurotoxicity induced by thujone, in USA is not authorized for use as a flavoring substance and in E.U. it was set a maximum level (Annex II of Directive 88/388 EEC) (E.U., 2003).

The thujone metabolism occurs mainly via 7-hydroxylation with lesser amounts of other hydroxylated metabolites. Nevertheless, different metabolic pathways have been observed in different species, for instance there was found site specificity and species differences between in mouse, rat and human liver microsomes (E.U., 2003).

Thujone represents a major concern in veterinary medicine, owing to the ingestion of *Thuja occidentalis* and other plants containing thujone by wild and farm animals; and the use of ethanolic extraction of *Thuja occidentalis* as veterinary homeopathy treatment in food producing animals.

In the summary report of *Thuja occidentalis* evaluation by the Committee for Medicinal Products for Veterinary Use (CVMP one of the European Agency for the Evaluation of Medical Products - EMEA - Committees) it is stated that the calculated worst-case residues of thujone in meat or milk are considered negligible compared to maximum limits established for plant foodstuffs or following use of flavourings. Nevertheless, it is not known the possibility of CYP inhibition by thujone, leading to a herbal-drug interaction (EMEA/MRL, 1999).

2. Aims of the present study

This thesis was developed in collaboration with the project "Food-drug interactions: exploring and pre-empting intricate health risks", at the Institute of Biomedicine, University of Oulu. It consisted in the evaluation of plant extracts and phytochemicals as possible CYP inhibitors, using single substrate assays *in vitro* with human hepatic cromosomes. So, to establish a possible link or comparison between potencial inhibitions in humans and other animal species, two different studies were performed. The general aim was to explore the relation of CYPs activity between different species (human, rabbit, minipig and mouse) and to identify potential CYP inhibitors among several plant extracts, including purified herbal compounds. The specific goals included:

1. To compare hepatic drug metabolism characteristics of humans with three different laboratory animal species (rabbit, minipig and mouse), measuring *in vitro* CYP activities (MultiCYP 7-ethoxycoumarin 0-deethylase (ECOD), CYP1A1/2 7-ethoxyresorufin 0-deethylase (EROD), CYP2A6 coumarin 7-hydroxylase (COH), CYP3A4 midazolam 1-hydroxylase (OH-MDZ), and CYP2E1 chlorzoxazone 6-hydroxylase (OH-CLZ);
2. To investigate several herbal extracts and compounds as potential inhibitors of CYP1A1/2 (EROD), CYP2A6 (COH), CYP3A4 (OH-MDZ) activities *in vitro*.

3. Materials and methods

3.1. Materials

3.1.1. Chemicals

Midazolam was kind donation from F. Hoffman-La Roche (Basel, Switzerland). Plant extracts and phytochemicals (table 9) were acquired from Division of Pharmacy, Department of Biochemistry and Pharmacy, Abo Akademi University. 7-ethoxyresorufin, 7-ethoxycoumarin, bovine albumin, chlorzoxazone, coumarin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, NADPH, methanol, TCA and resorufin were obtained mainly from Sigma Aldrich (St. Louis, MO). The laboratory water was purified trough a Milli-Q system (Millipore S.A., Molsheim, France).

Table 9. List of phytochemicals and plant extracts used in the study

Phytochemicals		**Plant extracts**
8-Hydroxybergapten	Bergamottin	*Pinus sylvestris* (bark and phloem extract)
5,6-Dihydroxyangelicin	Imperatorin	
Phellopterin	Psoralen	*Angelica archangelica* (roots)
Sphondin	Angelicin	
Cnidilin	Bergapten	*Mentha sp.* [1]
Lanatin	Xanthotoxin	
Bergaptol	α, β - Thujone	*Citrus grandis*
Isobergapten	α - Thujone	
Isopimpinellin		

[1] Two types of extraction, one using water as a solvent and the other using ethanol

3.1.2. Liver samples

Human liver samples used in the study were obtained from the University Hospital of Oulu as surplus from cadaver kidney transplantation donors. The collection of surplus tissue was approved by the Ethics Committee of the Medical Faculty of the University of Oulu, Finland. All donors were Caucasians. Animal liver samples were left-over tissue from untreated male

animals used as controls in other studies. All liver samples of human and animal origin were transferred to ice immediately after the surgical excision, cut into pieces, snap-frozen in liquid nitrogen, and stored at –80°C until the microsomes were prepared by standard differential ultracentrifugation (O. Pelkonen, Kaltiala, Larmi, & Karki, 1974). The previous method is used to separate certain organelles from whole cells for further analysis of specific parts of cells, subjecting to repeated centrifugations, each time removing the pellet and increasing the centrifugal force. The final microsomal pellet (pooled sample from all livers) was suspended in 0.1 M phosphate buffer pH 7.4. The protein content was determined by the method of Bradford (1976), which is a spectrophotometric analytical procedure used to measure the concentration of protein in a solution. Detailed data for the animal and human liver specimens are summarized in tables 10 and 11.

Table 10. Characteristics of the animal liver samples used in the study.

Species	Number of liver samples	Strain	Age (weeks)
Mouse (*Mus musculus*)	10	DBA/2N	8
Minipig (*Sus scrofa domesticus*)	6	Goettingen	62–86
Rabbit (*Oryctolagus cuniculus*)	3	New Zealand white	52–96

Table 11. Characteristics of the human liver samples used in the study.

Age (years)	Sex	Cause of death	Drug history	Liver pathology
n.a.	male	gun shot	no medication	none
n.a.	female	ICH[1]	no medication	none
54	male	ICH	diazepam[2]	none
44	male	ICH	phenytoin[2], alcohol abuse	cirrhotic
40	female	ICH	dexamethasone[2], nizatidine[2], phenytoin[2]	none
43	male	ICH	diazepam[2], smoker	none
47	male	ICH	no medication, smoker	none
33	male	astrocytoma	not known	none
70	female	melanoma	not known	steatosis
52	male	n.a.[3]	not known	steatosis
21	male	stroke	dexamethasone[2], smoker	none
39	female	ICH, SAH[4]	dexamethasone[2]	none
53	female	ICH, SAH	no medication	none
44	female	ICH, SAH	no medication	steatosis
62	male	ICH, SDH[5]	metformin, alcohol abuse, smoker	steatosis

[1] ICH, intracerebral haemorrhage; [2] Drugs were administered only during the last 24 h before death; [3] n.a., not available; [4] SAH, subarachnoidal haemorrhage; [5] SDH, subdural haematoma.

3.2. Methods

3.2.1. Evaluation of CYP-related activity

3.2.1.1. Incubation conditions

The following enzyme assays were employed: 7-ethoxyresorufin O-deethylation (ECOD), ethoxyresorufin-O-deethylation (EROD), coumarin 7-hydroxylation (COH), chlorzoxazone 6-hydroxylation (OH-CLZ) and midazolam 1-hydroxylation (OH-MDZ). The following procedures were described in detail by Taavitsainen *et al.* (2001).

All enzyme assays were carried out as individual assays and not as cocktail assays (with all substrate assays in the same incubation). Two parallel incubations and one control were performed for each liver sample. The reaction conditions were selected on the basis of experience with human livers. The substrate concentrations were chosen on the basis of known human K_m values and were within the linear part of time and protein concentration-dependence curve for the metabolite formations. The assay conditions and analytical methods are summarized in table 12.

Table 12. CYP-model activities assayed: incubation conditions and methods used

Activity	Protein in incubation ($mg.ml^{-1}$)	Substrate concentration (µM)	Incubation time (min)	Assay method
7-Ethoxycoumarin 0-deethylase	0,2	1	10	Fluorometric [1]Ex-365/Em-454 nm
Ethoxyresorufin 0-deethylase	0,2	1	5	Fluorometric [1]Ex-530/Em-585 nm
Coumarin 7-hydroxylase	0,2	10	10	Fluorometric [1]Ex-365/Em-454 nm
Chlorzoxazone 6-hydroxylase	0,5	100	20	UV-HPLC, 282 nm
Midazolam 1-hydroxylase	0,5	10	5	UV-HPLC, 245 nm

[1] Excitation (Ex) and emission (Em)

In 7-ethoxycoumarin O-deethylation (ECOD) and coumarin 7-hydroxylation (COH) reactions, each incubation comprised: hepatic microsomes (volume of protein equivalent to the amount showed in table 12), 100 µL of cofactor mixture (1 part of 2M KCl, 1 part of 0.1M $MgCl_2$, 2 parts of 0.03M glucose-6-phosphate, 1 part of 2.5mM NADP, 1 part of 0.114mg/mL glucose-6-phosphate dehydrogenase and 4 parts of 0.1M phosphate buffer pH=7.4) and

phosphate buffer pH=7.4 in a final volume of 0,5 mL. In the control samples was also added 500 µL of TCA 6%. It was used as standard a mixture of 125 µL of 7-OH-Coumarin and 375 µL of distilled water. All mixtures were pre-incubated for 2 min at 37°C in a shaking incubator block (Eppendorf Thermomixer 5436, Hamburg, Germany). The 7-ethoxycoumarin 0-deethylase and coumarin 7-hydroxylation reactions were started by the addition of 10 µL of substrate (0,5mM 7-ethoxycoumarin or 0,5mM coumarin) and terminated after 10 minutes adding 500 µL of TCA 6% (not necessary in the control tubes) and subsequently cooled in an ice bath to precipitate proteins. The mixtures were vortex mixed and spun at 10 000 G for 10 min. After the centrifugation 0.5 mL of supernatant was collected to be analyzed.

In ethoxyresorufin O-deethylation (EROD) reaction was used the same amount of hepatic microsomes, 100 µL of bovine albumin (12 mg/mL), 250 µL of cofactor mixture (already described) and 0.1M Tris-HCl pH=7.8 in a final volume of 550 µL. In the control samples was also added 2.5 mL of methanol. It was used as standard a mixture of 100 µL of 0.01mM of resorufin, 250 µL of cofactor mixture, 550 µL of 0,1M Tris-HCl pH=7.8 and 100 µL of bovine albumin (12 mg/mL). All mixtures were pre-incubated for 2 min at 37°C, also in a shaking incubator. The reaction was started by mixing 100 µL of the substrate (10 µM 7-ethoxyresorufin) and stoped after 5 minutes with 2.5 mL of cooled methanol. The mixtures were vortex mixed and spun at 10 000 G for 10 min.

Chlorzoxazone 6-hydroxylation (OH-CLZ) reaction was prepared using hepatic microsomes, 20 µL of 1mM chlorzoxazone and 0.1M phosphate buffer pH=7.4 in a final volume of 200 µL. In the control samples was also added 200 µL of methanol. All mixtures were pre-incubated for 2 min at 37°C, also in a shaking incubator. The reaction was started by mixing 40 µL of cofactor mixture (already described) and stoped after 20 minutes with 200 µL of cooled methanol. The mixtures were vortex mixed and spun at 10 000 G for 15 min. All supernatants were colleted after centrifugation and stored at -20ºC for 24H to precipitate proteins.

In midazolam 1-hydroxylation was used a similar procedure to chlorzoxazone 6-hydroxylation. However, it was used 20 µL of 0.1mM midazolam as substrate; the reaction was started by the addition of 20 µL of 10mM NADPH and was stoped after 5 minutes.

3.2.1.2. Assay methodology

3.2.1.2.1. Fluorimeter conditions

7-ethoxycoumarin O-deethylation (ECOD), ethoxyresorufin O-deethylation (EROD) and coumarin 7-hydroxylation (COH) metabolite formation were measured by using a standard 1 cm cuvette in a Kontron SFM5 fluorimeter with the excitation and emission values available in table 12. In ECOD and COH reactions, the analysis was performed by adding 2 mL of Glycine-NaOH and 0.5 mL of the supernatant already prepared. In EROD reaction, the analysis was performed directly in the supernatant isolated.
CYP activities were obtained according to the following equations:

Equation 2 and 3. Calculation of CYP-related activity

ECOD/COH activity:

$$pmol/\min/mg = \frac{1250 \times \mu}{Std \times 10 \times 0.1}$$

EROD activity:

$$pmol/\min/mg = \frac{1000 \times \mu}{Std \times 5 \times 0.2}$$

Legend:
μ - Average of fluorescence values of both parallel incubations, and minus the control incubation value
Std - Fluorescence value of the standard; In both equations, numbers 10 and 5 correspond to the incubation period (minutes); Numbers 0.1 and 0.2 correspond to the protein concentration ($mg.ml^{-1}$); The coeficient constants in each equation are respectively 1250 and 1000.

3.2.1.2.2. HPLC conditions

For midazolan 1-hydroxylase and chlorzoxazone 6-hydroxilation activity evaluation a Water Alliance chromatographic system with autosampler, vacuum degasser and column oven was used. The analytical column was a Waters XTerra MS C18, 2.1mm×50mm, 3.5 μm with Phenomenex Luna C18(2) precolumn, 4.0mm×2.0mm, 3.0 μm (Phenomenex, Torrance, California, USA). It was used an isocratic flow (the mobile phase composition remains constant), using a flow rate of 0.3 ml/min and the column oven temperature was 30ºC. The eluents used for chlorzoxazone 6-hydroxylation reaction were 70% 50 mM O-phosphoric acid-buffer and 30% acetonitrile. For midazolam 1-hydroxylase reaction the eluents were 60% water and 40% acetonitrile.
The quantification of metabolites, which represents the CYP-related activity, was obtained after being detected with HPLC. This quantification was directely provided by the

chromatographic system that performed a peak integration, using an internal calibration curve (it compares the unknown to a set of standard samples of known concentration).

3.2.1.3. Calculation of CYP-related activity

The catalytic activity for each CYP and species were determined by the average of duplicate incubations and compared with the respective control incubation (incubation with denatured microsomes) by statistical analysis using SPSS Statistics 17.0. Results are expressed as means ± standard deviation (SD) and coefficient of variation (CV%).

3.2.2. Evaluation of CYP inhibition

3.2.2.1. Incubation and assay conditions

Inhibition of human liver microsomes by various plant extracts and phytochemicals was studied in the incubation system described above: ethoxyresorufin-O-deethylation (EROD), coumarin 7-hydroxylation (COH) and midazolam 1-hydroxylation (OH-MDZ). However, it was added into the incubation system 2 μL (OH-MDZ) or 10 μL (EROD/COH) of phytochemicals in three different concentrations (1, 10 and 100 μM) and plant extracts (1, 10 and 50 mg/mL). The final results were compared with a normal incubation that ran in parallel.

3.2.2.2. Calculation of half maximal inhibitory concentration (IC50)

IC50 values were determined adding one phytochemical at final concentrations of 1, 10 and 100 μM or one plant extract at final concentrations of 1, 10 and 500 μg/mL to the incubation mixture. The resultant activities were compared with those from control incubations into which only solvent had been added. The solvent used was water (*Pinus sylvestris, Angelica archangelica, Mentha sp.*(water extraction) and *Citrus grandis*) or DMSO (*Mentha sp.* (ethanol extraction) and phytochemicals). The IC50 values (the concentration of inhibitor causing 50% inhibition of the original enzyme activity) were determined graphically by linear regression analysis of the plot of the logarithm of inhibitor concentration versus percentage of

activity remaining after inhibition using Origin, version 6.0 (Microcal Software Inc., Northampton, MA, USA).

4. Results and discussion

4.1. Interspecies comparison of in vitro activities of CYP-mediated metabolism

This study was focused on the determination of CYPs activities in humans, rabbits, minipigs and mouses, towards probe substrates.

It should be emphasized that all probe substrates used as enzymatic model reactions are mainly targeted to human CYPs. Nevertheless, similar probes have already been used to obtain animal CYP data in other individual studies (Fink-Gremmels, 2008; Pasanen, 2004; Turpeinen *et al.*, 2007).

Table 13. Estimated CYP-associated activities in hepatic microsomes of different species [1]

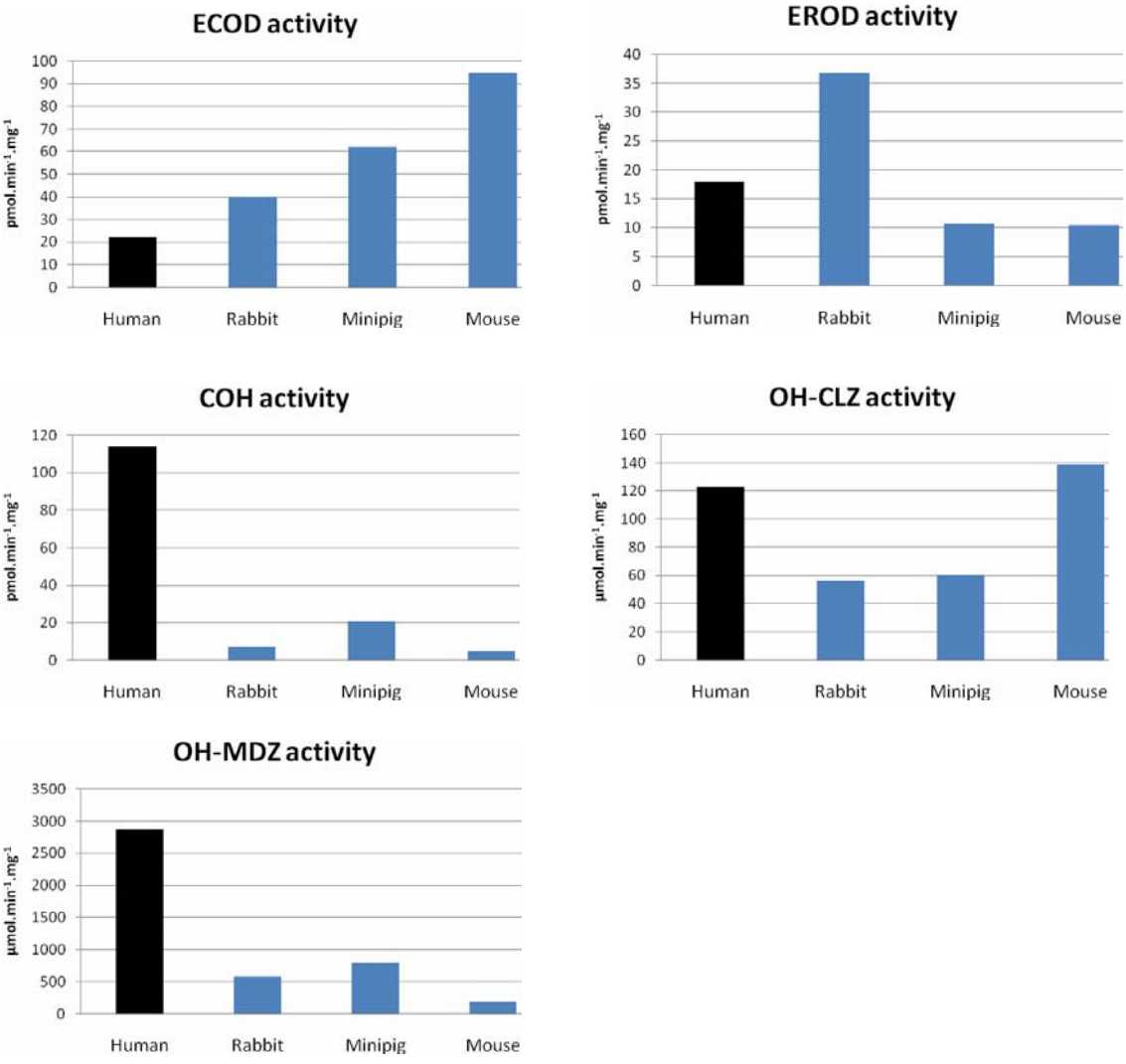
Activity and metabolite abbreviation	Human		Rabbit		Minipig		Mouse	
	$\mu \pm \sigma$ (1)	CV% (2)	$\mu \pm \sigma$ (1)	CV% (2)	$\mu \pm \sigma$ (1)	CV% (2)	$\mu \pm \sigma$ (1)	CV% (2)
7-Ethoxycoumarin O-deethylation (ECOD) [3]	22.09 ± 1.10	5,0	39.86 ± 0.19	0,5	62.18 ± 1.09	1,8	95.21 ± 0.95	1,0
Ethoxyresorufin O-deethylation (EROD) [3]	18.04 ± 0.54	3,0	36.71 ± 0.18	0,5	10.67 ± 0.01	0,1	10.32 ± 0.54	5,2
Coumarin 7-hydroxylation (COH) [3]	113.8 ± 2.05	1,8	7.295 ± 0.00	0	20.43 ± 1.23	6,0	4.748 ± 0.32	6,7
Chlorzoxazone 6-hydroxylation (OH-CLZ) [4]	122.9 ± 6.84	5,6	56.36 ± 0.00	0	60.20 ± 8.25	13,7	138.4 ± 23.6	17,1
Midazolam 1-hydroxylation (OH-MDZ) [4]	2877 ± 245.4	8,5	578.5 ± 31.2	5,4	786.9 ± 264	33,5	183.5 ± 0.00	0

[1] Each value is the mean ± standard deviation of two replicates; [2] Coefficient of variation; [3] Activity measured in $pmol.min^{-1}.mg^{-1}$; [4]Activity measured in $\mu mol.min^{-1}.mg^{-1}$

Table 13 illustrates the results of different CYPs activities - 7-ethoxycoumarin 0-deethylase, ethoxyresorufin O-deethylation, coumarin 7-hydroxylation, chlorzoxazone 6-hydroxylation, and midazolam 1-hydroxylase - among different species in this study. All CYP activities

show low coefficients of variation. OH-MDZ in minipig shows the highest variation, followed by OH-CLZ in mouse. The standard deviation is zero in three cases, because in those situations only one of two replicates was not valid.

Figure 14. Model CYP activities for each species studied. Values are the mean. Duplicate incubates for each of the different liver samples analyzed



Despite the fact that it wasn't performed any inferential statistics, it is still possible to find some equalities between different species, as illustrated in table 13 and figure 14.

7-Ethoxycoumarin-O-deethylation (ECOD) reaction is catalyzed in humans by several CYPs isoforms. Although it was found to have the lowest ECOD activity, which corresponds to 23% of the value obtained in mouse liver microsomes that displayed the highest activity.

Ethoxyresorufin-O-deethylation (EROD) reaction showed the highest similarity between mouse and minipig. Moreover, it only represents 30% of the activity in rabbit and 60% of the activity in humans.

Coumarin 7-hydroxylation (COH) reaction is catalyzed in humans by CYP2A6, in mice by CYP2A5 (Aoki *et al.,* 2006), by CYP2A in minipigs (Soucek *et al.*, 2001) and CYP2A10 in rabbits (Lee, Obach, & Fisher, 2003). Human CYP2A6 was found to have the highest COH activity and mouse CYP2A5 the lowest, which represents only 4% of COH activity in humans. Nevertheless, it represents 65% of rabbit CYP2A10 activity and 23% of minipig CYP2A activity.

CYP2E1, which is highly conserved in mammals, is responsible for chlorzoxazone 6-hydroxylation (OH-CLZ) reaction. In line with previous studies (Fink-Gremmels, 2008; Turpeinen *et al.*, 2007), this reaction showed similar activities among the tested species, being possible to organize in two groups: human and mouse (which human CYP2E1 has 90% of mouse activity) *versus* rabbits and minipigs (which rabbit CYP2E1 has 94% of minipig activity). On the contrary OH-CLZ reaction in humans is 218% higher than in rabbits, and in minipigs is 230% higher than in mice.

The midazolam 1-hydroxylase (OH-MDZ) reaction is performed by CYP3A4 in humans, which has demonstrated to have the highest catalytic activity. Mice have the lowest activity, only 6% of human OH-MDZ activity, followed by the rabbit and minipig that have 20% and 27% each of them, when compared also with humans.

The present results show extensive species differences in each catalytic activity studied, being possible to establish only sporadic catalytic similarities.

4.2. CYP-related activity in the presence of plant extracts and phytochemicals

The aim of this study was to identify plant compounds capable of inhibit CYP1A1/2 (ethoxyresorufin-O-deethylation), CYP2A6 (Coumarin 7-hydroxylase) and/or CYP3A4 (midazolam 1-hydroxylation), by measuring in vitro human CYPs activity in the presence of plant extracts.

As already explained, the potencial inhibition of CYPs resulting of interactions with these compounds, was measured using the half maximal inhibitory concentration (IC50) values and classifying them in classes, exhibiting a high (IC50<1 μM), a moderate (1 μM <IC50<10 μM) and a low (IC50>10 μM) potential for CYP inhibition (Krippendorff *et al.*, 2007).

4.2.1. Plant extracts

Five different plant extracts were used as possible CYP inhibitors. They were bark and phloem extracts from *Pinus sylvestris* (common name Scotch pine), root extracts from *Angelica archangelica* (common name Angelica), extracts from *Citrus grandis* (also known as *Citrus maxima* and by the common name Pomelo) and two different types of *Mentha sp.* (common name Mint) extracts by using water and ethanol as their solvents.

In the table below is presented CYP-related activity in the presence of plant extracts, wich data was used to determine the remain activity of the CYP and IC50 value, as presented in table 15 and figure 15.

Table 14. CYP-related activity in the presence of plant extracts

Plant extracts	**µg/mL**	**CYP1A1/2 (EROD) [1]**	**CYP2A6 (COH) [1]**	**CYP3A4 (OH-MDZ) [2]**
Control		65,73	706,65	185,00
Pinus sylvestris	1	65,48	671,73	230,00
	10	36,18	556,73	105,00
	50	14,17	327,22	175,00
Angelica archangelica	1	9,12	608,35	159,50
	10	3,00	284,21	99,00
	50	0,88	383,83	26,00
Mentha sp. (Ethanol extration)	1	43,64	663,95	166,00
	10	11,39	574,59	136,50
	50	3,17	208,49	57,00
Mentha sp. (Water extration)	1	59,06	654,68	179,50
	10	27,57	612,91	198,00
	50	1,94	272,08	173,50
Citrus grandis	1	47,19	658,36	214,50
	10	17,06	675,93	272,00
	50	3,89	534,21	200,50

[1] $pmol.min^{-1}.mg^{-1}$; [2] $\mu mol.min^{-1}.mg^{-1}$

The ethoxyresorufin-O-deethylation (EROD) reaction performed by CYP1A1/2 is the most inhibited among the reactions analyzed. The *Angelica archangelica* extract has the highest

inhibitory effect in EROD reaction (lowest IC50). In general, the extract of this plant inhibits more effectively two out of three CYPs activities than the other plant extracts, as shown in table 15.

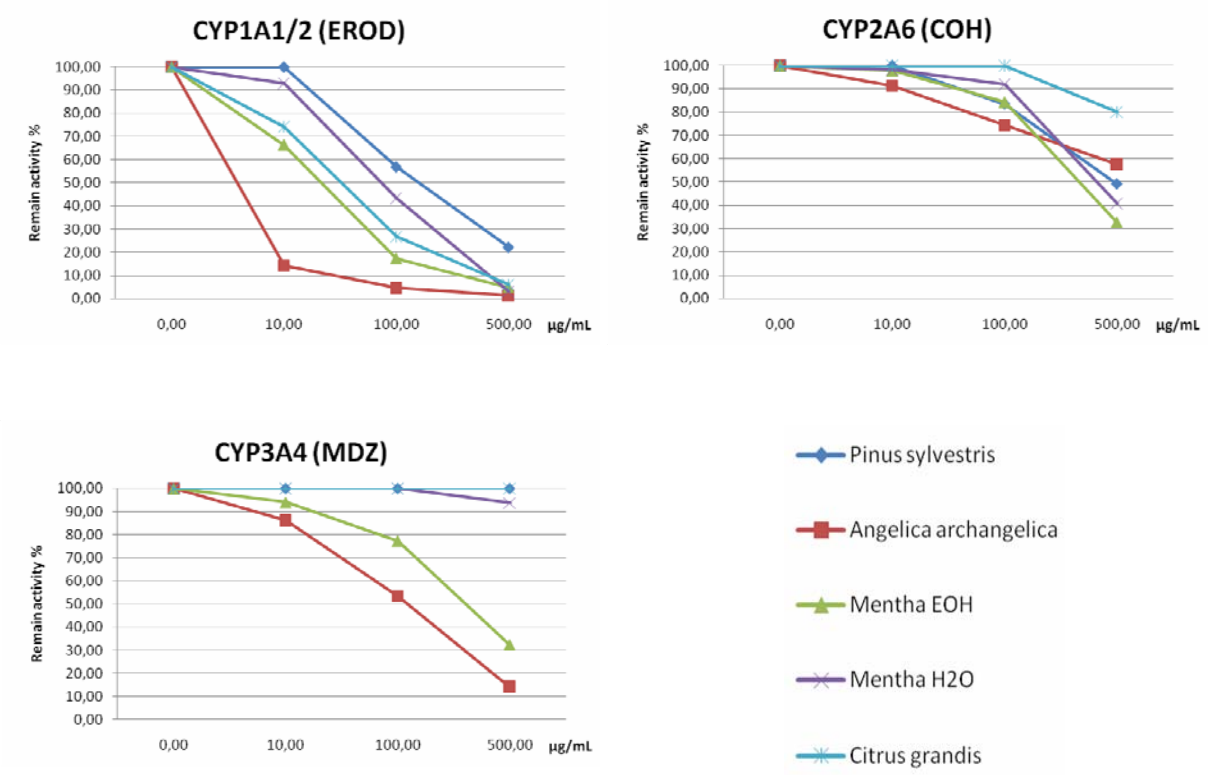
On the contraries with the latter statement, the *Pinus sylvestris* interacts in a less potent manner with the CYPs studied. Nevertheless, it has been reported that a phytochemical (quercetin) in this plant is capable of inhibiting CYP1A2 (Duke, 2008).

CYP3A4 (midazolam 1-hydroxylation), which is the most important human CYP in drug metabolism and susceptible of FDI, only suffers inhibition by high levels of the *Angelica archangelica* extract.

The ethanol extract of *Mentha sp.* caused high inhibition of all CYP activities studied, which may be due to the presence of more non-polar compounds in that extraction type. Especially OH-MDZ reaction (CYP3A4) was inhibited, as illustrated in figure 15.

Both water extract of *Mentha sp.* and *Citrus grandis* extract were found to have the lowest inhibitory effect on CYP3A4 (OH-MDZ) activity.

Figure 15. The effects of plant extracts on CYP-catalyzed activities in human liver microsomes



The IC50 values presented in table 15 were determined graphically based in figure 15, as already explained before.

Table 15. IC50 of plant extracts for CYP1A1/2, CYP2A6 and CYP3A4 activities in human microsomes. Values measured in μg.mL^{-1}

Plant extract	CYP1A1/2	CYP2A6	CYP3A4
Pinus sylvestris	179,65	489,92	>500
Angelica archangelica	0,59	>500	134,318
Mentha sp. (Ethanol extration)	34,14	365,48	341,802
Mentha sp. (Water extration)	86,73	429,01	>500
Citrus grandis	51,63	491,23	>500

4.2.2. Furocoumarins

In this study, two structural classes of furocoumarins, angular and linear, were used. The angular furocoumarins used were: 5,6-dihydroxyangelicin, sphondin, lanatin, isobergapten and angelicin. The phytochemicals belonging to the linear class of furocoumarins used were: 8-hydroxybergapten, phellopterin, cnidilin, bergaptol, isopimpinellin, bergamottin, imperatorin, psoralen, bergapten and xanthotoxin.

5,6-Dihydroxyangelicin on basis of its IC50 values (presented in table 16) provokes a low inhibition (IC50 >10 μM) in in CYP3A4 (OH-MDZ). Sphondin was found to be a high inhibitor (IC50 < 1 μM) of CYP1A1/2 and CYP2A6, but a weak inhibitor of CYP3A4. However, it proved to be the strongest inhibitor of the angular furocoumarins present in this study. Lanatin was found to be only a strong inhibitor of CYP1A1/2.

Table 16. IC50 of angular furocoumarins for CYP1A1/2, CYP2A6 and CYP3A4 activities in human microsomes. Values measured in μM.

Angular furocoumarins	CYP1A1/2	CYP2A6	CYP3A4
5,6-Dihydroxyangelicin	47,78	82,40	100,0
Angelicin	0,694	3,940	>100,0
Sphondin	0,824	0,850	64,76
Isobergapten	3,628	27,69	>100,0
Lanatin	0,720	>100,0	>100,0

The ECOD reaction (CYP1A1/2) is the most susceptible to inhibition when compared with other CYP-mediated reactions. It is inhibited by almost all angular and linear furocoumarins, expect for 5,6-dihydroxyangelicin and bergaptol (see table 16 and 17table 17).

Table 17. IC50 of linear furocoumarins for CYP1A1/2, CYP2A6 and CYP3A4 activities in human microsomes.. Values measured in μM.

Linear furocoumarins	**CYP1A1/2**	**CYP2A6**	**CYP3A4**
8-Hydroxybergapten	5,107	64,21	53,10
Bergaptol	75,29	>100,0	>100,0
Bergamottin	0,617	>100,0	52,97
Bergapten	0,537	>100,0	>100,0
Cnidillin	0,539	>100,0	>100,0
Isopimpinellin	0,513	>100,0	64,84
Imperatorin	0,539	77,55	2,537
Phellopterin	0,487	>100,0	12,71
Psoralen	0,591	>100,0	>100,0
Xanthotoxin	0,5646	0,69	>100,0

The most relevant inhibition by psoralen and angelicin were found toward CYP1A1/2 (EROD).

Bergaptol, bergapten and bergamottin inhibit mainly CYP3A4 in the intestine (Duke, 2008). In this study these compounds were found to be a weak inhibitor of hepatic CYP3A4 (OH-MDZ).

The stronger inhibitor among the linear furocoumarins of this study was imperatorin, followed by phellopterin with a moderate inhibition (IC50 between 1 and 10 μM).

Isopimpinellin was found to be a strong inhibitor of CYP1A1/2.

The only linear furocoumarins found in this study with a strong inhibition of CYP2A6 was xanthotoxin, which can be found in all parts of the plant *Angelica archangelica* (Duke, 2008).

Isobergapten and cnidillin are present in several plants, especially in *Angelica sp*. The former was found to be a moderate inhibitor of CYP1A1/2 and the latter to be a strong inhibitor.

8-hydroxybergapten is a characteristic compound found in *Angelica dahurica*. In this study it was found to be a moderate inhibitor of CYP1A1/2 and a weak inhibitor of CYP2A6 and CYP3A4.

4.2.3. Monoterpenes

As part of this study pure α-thujone and mixed α-, β-thujone were tested as possible CYP inhibitors.

In this study the two stereoisomeric forms of thujone showed weak inhibition of CYP activities studies (see table 18 and 19). Only, the IC50 value of coumarin 7-hydroxylase (CYP2A6) could be precisely determined. As illustrated in figure 16, both pure α-Thujone and α, β-Thujone mixture had the same inhibitory pattern. Nevertheless, α, β-Thujone mixture showed a lower IC50 of CYP2A6 (COH) than pure α-Thujone.

Table 18. CYP-related activity in the presence of plant extracts

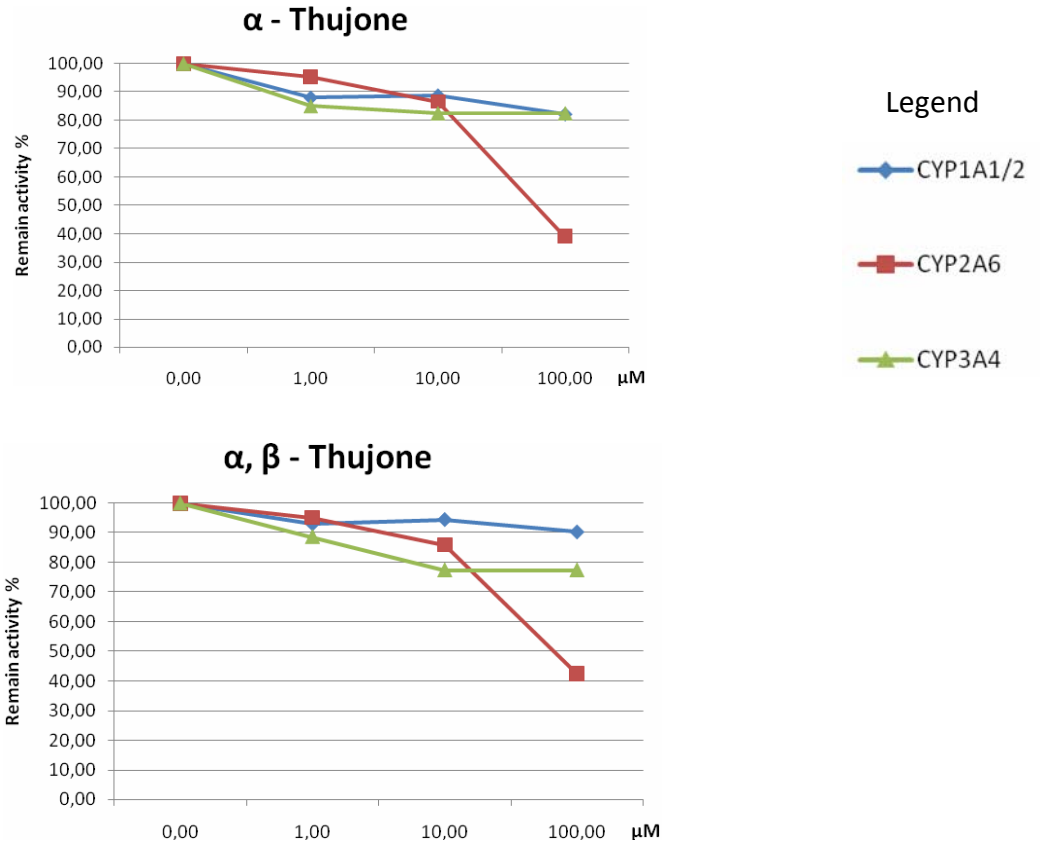
Plant extracts	µg/mL	CYP1A1/2 (EROD) [1]	CYP2A6 (COH) [1]	CYP3A4 (OH-MDZ) [2]
Control		65,73	706,65	176,50
α-β-Thujone	1	61,10	672,37	140,00
	10	62,06	607,75	136,50
	100	59,30	300,46	136,00
α-Thujone	1	57,87	673,90	150,00
	10	58,38	611,99	145,50
	100	54,03	275,20	145,00

[1] $pmol.min^{-1}.mg^{-1}$; [2] $\mu mol.min^{-1}.mg^{-1}$

Table 19. IC50 of thujone isomers for CYP1A1/2, CYP2A6 and CYP3A4 activities in human microsomes.. Values measured in µM.

Compound	CYP1A1/2	CYP2A6	CYP3A4
α, β-Thujone	>100	78,88	>100
α-Thujone	>100	84,51	>100

Figure 16. The remain CYP activity after incubation with α-, β- thujone



5. Conclusion

The presented results, related to the comparison of CYP activities between different species, suggest the existence of significant inter-species differences in substrate models for individual CYP-catalyzed activities. Even with similar patterns for some CYP-catalyzed activities between species no link could be established to all group of CYP belonging to different species. For example, in ECOD reactions no possible relation was establish between species.

It should be emphasized that all enzymatic model reactions studied are mainly targeted to human CYPs. However, similar probes but with slightly different experimental set-ups have been used to obtain animal CYP data in individual studies (Fink-Gremmels, 2008).

In the present study, all enzymatic analyses were carried out as individual determinations using only two replicate incubations and not including the true variation within species, e.g. different levels of CYP expression depending on gender and race.

Based on these data, to overtake some of these limitations, it should be used pools of hepatic microsomes divided at least in gender and age.

The impossibility to perform extrapolations with all CYP-related activities of one species with another is one of the main consequences. Concerning animal models for human CYP-mediated metabolism, it faces the same problem. In this case, it needs to be used different animal species depending on the human CYP selected.

Concerning the variability of CYP activities and specificity verified between species, it is necessary more detailed studies, e.g. CYP genes sequencing for identification of CYPs involved in enzymatic activity and development of enzymatic model reactions targed to animal CYPs, because different species normaly catalyze the same reactions but by different CYPs and magnitude.

Another aspect of this study was related with the identification of possible interferences of herbal compounds in CYP-related activities in human microsomes. The EROD reaction, performed by both CYP1A1 and CYP1A2, showed to be the most inhibited CYP-mediated reaction, especially by *Angelica archangelica* and most of the phytochemicals tested. Product of *Angelica archangelica* extraction was also the plant component with highest inhibition in CYP3A4 (OH-MDZ) activity.

The COH reaction, catalyzed by CYP2A6, was inhibited by few compounds with low IC50 values. The highest inhibition for this reaction was detected among the angular furocoumarins.

As already mentioned, CYP3A4 is one of the main responsible CYPs involved in drug metabolism. In this study, phytochemicals with lower inhibitory concentration were found in the linear furocoumarins group, especially by imperatorin.

Based on the first study presented, owing to the similar activities found in some CYPs (e.g.CYP1A1/2) between the species analyzed, it may be possible to find comparable interferences with the same plant extracts and phytochemicals. In addition, the same kind of interferences is also expected to occur in other veterinary species, which include companion and farm animals.

From all phytochemicals tested, thujone has been already identified as a major concern in veterinary medicine, owing to the ingestion of plants by wild or farm animals; and the use of ethanolic extraction of thuja as veterinary homeopathy treatment. In this study, the highest CYP inhibition of thujone in humans was found on CYP2A6, but the IC50 value represents a low inhibition (IC50 > 10 μM). Nevertheless, human CYP2A6 was found to have the highest COH activity (mouse CYP2A5 only possess 4%, minipig CYP2A has 23% and rabbit CYP2A10 has 65% of human CYP2A6 COH activity). The same applies to EROD reaction which has similar activities in the animals studied and for which most of furocoumarins and *Angelica archangelica* have a high inhibition. So, it may be possible to verify in these animals some inhibition by thujone and the other compounds at least in COH and EROD reactions, but more specific studies are needed.

Taking in consideration all potential CYP inhibitions by plant extracts and phytochemicals tested, the information collected is still preliminary. Nevertheless, more detailed studies will be needed to understand the comparison of CYP-catalyzed activities between different species, the importance of these compounds in animal diets, and to know if these *in vitro* interactions have any real effect *in vivo* and consequently an impact in public health or medical care. For instances: 1) food-drug interactions can decrease metabolism delaying drug elimination, which represents a risk for public health in farm animals; 2) food-drug interactions can increase metabolism changing the eficacy of the treatment, which can lead to therapeutic failure or toxicity even with death of the pacient.

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Annex I

Comparison of *in vitro* activities of biotransformation enzymes in human, rabbit, minipig and mouse

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Abstract

Cytochrome P450 enzymes (CYP) are primarily membrane-associated proteins that belong to the superfamily of heme-thiolate enzymes. The main role attended by the CYP system in human and veterinary medicine is based mostly in the CYP mediated drug metabolism, which can result in either detoxification or metabolic activation, i.e. the formation of reactive forms from inactive parent compounds and it can lead to bioavailability problems, interindividual variation, metabolic interactions and idiosyncrasies. So, the absence of detailed information regarding biotransformation processes can result in adverse effects, therapeutic failure and toxicity from unanticipated overdose or metabolic reactions, such as drug-drug interactions (DDIs) and food-drug interactions (Fink-Gremmels, 2008; Nebbia, 2001; Olavi Pelkonen, 2002).

In contrast to the extensive data available for human and rodents, the characterization of the CYP system in other animal species is still incomplete. This is of importance in veterinary and human medicine, as many drugs are used in more than one species and also animal models can be used as experimental models of drug metabolism. Consequently, major obstacles are the significant inter-species and intra-species differences in substrate models for individual CYP-mediated reactions (Fink-Gremmels, 2008; Turpeinen, 2006; Zuber *et al.*, 2002).

The present study compared hepatic drug metabolism characteristics of humans with three different laboratory animal species (rabbit, minipig and mouse), measuring CYP activities *in vitro* (MultiCYP 7-ethoxycoumarin 0-deethylase (ECOD), CYP1A1/2 7-ethoxyresorufin 0-deethylase (EROD), CYP2A6 coumarin 7-hydroxylase, CYP3A4 midazolam 1-hydroxylase, and CYP2E1 chlorzoxazone 6-hydroxylase).

The ECOD activity is catalyzed by several CYP450 forms in human liver microsomes, being used for general measure. The lowest activity is detected in humans and there is no similarity

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between other species. The major human CYP family involved in EROD is CYP1A1/2, which may be different in other animal species (CYP2C?) (Turpeinen *et al.*, 2007), shows equivalent activities. The highest CYP activities in humans, when compared with the other species, are found for CYP2A6 (e.g. 60x than mouse) and CYP3A4 (e.g. 15x than mouse). In CYP2E1 activity, two similar groups can be recognized: human and mouse versus rabbit and minipig.

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