CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

PHASE II BIOTRANSFORMATION OF NSAID FLOBUFEN
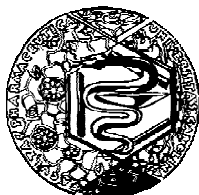
Dissertation Thesis

Mgr. Yogeeta Nautamlal Babú

Field: Pathobiochemistry and Xenobiochemistry

Supervisor: Doc. Ing. Vladimír Wsól, Ph.D.

Hradec Králové

2008

UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA BIOCHEMICKÝCH VĚD

DRUHÁ FÁZE BIOTRANSFORMACE FLOBUFENU

Disertační práce

Mgr. Yogeeta Nautamlal Babú

Studijní obor: Patobiochemie a Xenobiochemie

Školitel: Doc. Ing. Vladimír Wsól, Ph.D.

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This dissertation is an original work, written by myself and

dedicated to my loving parents

Bina and Nautamlal

The research described in this dissertation is part of a wide project and years of investigation that has been carried out at the Department of Biochemical Sciences of the Faculty of Pharmacy of Charles University in Hradec Králové, Czech Republic. This work would have not been possible without the support of many exceptional people.

- First, I would like to express my gratitude to Doc. Ing. Vladimír Wsól, Ph.D. for his supervision, advice and guidance from the very early stage of this project. I thank him for sharing with me his extraordinary knowledge and enriching my growth as a student and a scientist. Above all, I thank him and his family for their friendship.

- My deepest gratitude is due to Mrs. Alena Pakostová, not only for her excellent and invaluable technical assistance but most of all for her joyfulness, kindness and never ending friendship. These thanks are extended to her family, Alice and Pavel.

- My profound acknowledgment goes to PharmDr. Radím Král, Ph.D. for his exceptional scientist intuition and passion for science which triggered and nourished my intellectual maturity. I thank him for his supervision, guidance, his constructive critics and especially for his time, patience and endless friendship.

- My humble gratitude goes to Doc. Ing. Babora Szotaková, Ph.D. and Doc. RNDr. Lenka Skálová, Ph.D. for their guidance, for sharing their knowledge, for their precious advices and for being there whenever I needed.

- To Mrs. Božena Navratilová and Mrs. Ivana Široká, with whom I learned to speak Czech.

- To my dear friends and fellow Ph.D. students at "Dětsky Pokoj" – Iva Boušova, Michal Šavlik, Marek Link, Romana Novotná, Helena Kaiserová, Jakub Velík, Vendula Baliharová, Ladislava Schröterová, Bohumila Suchanová and Viktor Cvilink. With them I shared ideas, knowledge, scientific discussions as well as not so scientific

discussions, happiness, sense of humour, in another words, I thank them for sharing their lives with me.

1. ABSTRACT

Xenobiotika jsou chemické sloučeniny tělu cizí, které se obvykle tvoří syntetickými nebo abiotickými procesy. Syntetická xenobiotika mají často obrovský význam pro lidskou společnost a představují většinu chemických sloučenin z tak významných skupin, jako jsou petrochemikálie, pesticidy, plasty a léky, u kterých se obvykle ve vztahu ke xenobiotikům používá termín léčivo.

Biotransformace je hlavním mechanismem eliminace léčiv, protože ta se biotransformaci podrobí ihned po vstupu do organismu. Biotransformace, která téměř vždy poskytuje metabolity polárnější, než je výchozí látka, obvykle ukončuje farmakologický účinek parentního léčiva a prostřednictvím exkrece zvyšuje odstraňování léčiva z těla. Nicméně může mít i další důsledky včetně podobného nebo odlišného farmakologického účinku nebo toxikologického působení.

Je mnoho odlišných drah, kterými může být léčivo biotransformováno, a ty zahrnují mimo jiné oxidační, redukční, hydrolytické a konjugační reakce. Je důležité těmto drahám porozumět, protože cesta metabolismu léčiva může určovat jeho konečný farmakologický nebo toxikologický účinek.

Biotransformace léčiv se rozděluje do dvou fází: Fáze I neboli přípravné reakce a Fáze II čili konjugační reakce.

Kromě fyzikálně-chemických činitelů ovlivňujících metabolismus xenobiotik, hrají důležitou úlohu v biotransformaci léčiv také stereochemické faktory. Jejich účast se dá očekávat, protože enzymy metabolizující xenobiotika jsou ty samé enzymy, které metabolizují některé endogenní substráty, což jsou většinou chirální molekuly.

Biotransformace racemických léčiv může být stereoselektivní, protože se jednotlivé enantiomery mohou přeměňovat prostřednictvím odlišných metabolických drah. Tato stereoselektivita byla pozorována jak pro reakce I. fáze tak II. fáze.

Nesteroidní protizánětlivá léčiva/nesteroidní antiflogistika (NSAIDs) jsou skupinou analgeticky, protizánětlivě a antipyreticky působících léčiv, která jsou hojně používána v revmatologii. Ačkoliv nejsou všechna NSAIDs chirální, všechna léčiva patřící do největší chemické třídy derivátů 2-arylpropionové kyseliny mají chirální centrum. Chirální NSAIDs jsou pravděpodobně jednou z nejvíce studovaných skupin léčiv z hlediska enantioselektivity ve farmakokinetice.

Téma této dizertační práce je zaměřeno na II. fázi biotransformace potenciálního NSAID Flobufenu, který je odvozen od struktury 2-arylpropionové kyseliny. V této práci jsem zkoumala schopnost Flobufenu, jako chirálního léčiva, a/nebo jeho metabolitů podstoupit enantioselektivní konjugaci s využitím metod jako je LC-MS k separaci a identifikaci vznikajících metabolitů.

Xenobiotic chemicals are chemicals foreign to life that are usually derived synthetically or from an abiotic process. The synthetic xenobiotic chemicals are often of enormous value to human society and are usually the majority of the chemicals in such important groups of substances as petrochemicals, pesticides, plastics and pharmaceuticals, where the term drug is usually applied when referring to xenobiotics.

Biotransformation is a major mechanism for drug elimination, as they undergo biotransformation after they enter the body. Biotransformation, which almost always produces metabolites that are more polar than the parent compound, usually terminates the pharmacologic action of the parent drug and, via excretion, increases removal of the drug from the body. However, other consequences are possible, including similar or different pharmacologic activity, or toxicological activity.

The routes by which drugs may be biotransformed are many and varied and include oxidation, reduction, hydrolysis and conjugation reactions, among others. It is important that these pathways are understood, as the route of metabolism of a drug can determine its ultimate pharmacological or toxicological activity.

Drug biotransformation is divided into two phases: Phase I, or functionalisation reactions and Phase II, or conjugative reactions.

In addition to the physicochemical factors that affect xenobiotic metabolism, stereochemical factors play an important role in the biotransformation of drugs. This involvement is not unexpected because the xenobiotic-metabolizing enzymes are also the same enzymes that metabolize certain endogenous substrates, which for the most part are chiral molecules. Biotransformation of racemic drugs can be stereoselective, as the individual enantiomers undergo metabolism via different metabolic pathways. This stereoselectivity has been observed for both phase I and phase II reactions.

Non-steroidal anti-inflammatory drugs, NSAID, are a group of analgesic, anti-inflammatory and anti-pyretic drugs that are extensively used in rheumatology. Although not all NSAIDs are chiral, all of the drugs in its major chemical class, the 2-arypropionic acid, possess a chiral centre. As a collective group, the chiral NSAIDs are perhaps one of the most studied classes for enantioselectivity in pharmacokinetics.

This PhD project focused the study of Phase II Biotransformation of the potential NSAID Flobufen, structurally related to 2-arypropionic acids. As a chiral drug, this work investigated the ability of Flobufen and/or its metabolites to undergo enantioselective conjugation, employing techniques such as LC-MS to separate and identify the metabolites formed.

LIST OF ABBREVIATIONS

The following abbreviations were used throughout this dissertation:

2-APA	2-arylpropionic acid
Acetyl CoA	Acetyl-coenzyme A
ACN	Acetonitrile
AKR	Aldo-keto reductases
AR	Aldose reductase
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic acid
CID	Collision-induced dissociation
COMT	Catechol-*O*-methyltransferase
COX	Cyclooxygenase
CYP	Cytochrome P450
DHF	4-dihydroflobufen, 4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-4-hydroxybutanoic acid
DMSO	Dimethylsulphoxide
EA	Ethylacetate
ECD	Electrochemical Detector
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FAD	Flavin adenine dinucleotide
FLO	Flobufen, 4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid
FLO-GLUC	Flobufen glucuronide

FMO	Flavin monooxygenase
GSH	Glutathione
GST	Glutathione S-transferase
HPLC	High-performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
IR	Refractive index
LC-MS	Liquid chromatography-mass spectrometry
M/Z	Mass-to-charge ratio
M17203	2-(2′,4′-difluorobiphenyl-4-yl)-acetic acid
M17203-GLUC	M17203 glucuronide
M17203-TAU	Conjugate of M17203 with taurine
MFO	Microsomal mixed-function oxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADP$^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NSAID	Non-steroidal anti-inflammatory drugs
ODS	Octadecylsilane
ORF	Open reading frames
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PDA	Photo-diode array
POMT	Phenol-*O*-methyltransferase
ROS	Reactive oxygen species
RP	Reverse phase
SAM	S-adenosylmethionine
SDR	Short-chain dehydrogenase/reductase

SMZ	Sulfamethazine
SPE	Solid phase extraction
ST	Sulfotransferase
UDPGA	Uridine diphosphate glucuronic acid, UDP-glucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase, UDP-glucuronosyltransferase
UM	Unknown metabolite
UV	Ultra-violet

2. INTRODUCTION

2.1 Biotransformation

During their lives, humans take a wide, almost infinite variety of substances into their bodies, some of which are necessary for their well-being and provide the raw materials essential for the process of intermediary metabolism. However, other substances the body does not require are also ingested, inhaled, or absorbed. These substances have been described as <u>exogenous compounds</u>, <u>foreign compounds</u> or **<u>xenobiotics</u>** (from the Greek *xenos* = foreign), and include both manufactured and natural chemicals such as drugs, industrial chemicals, pesticides, pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites and toxins produced by moulds, plants and animals.

The physical property that enables many xenobiotics to be absorbed through the skin, lungs or gastrointestinal tract – the **lipophilicity** – is an obstacle to their elimination, because lipophilic compounds can be readily reabsorbed. Consequently, the elimination of xenobiotics often depends on their conversion to water-soluble chemicals by a process known as **biotransformation**, which is catalyzed by enzymes in the liver and other tissues.

An important consequence of biotransformation is that the physical properties of a xenobiotic are generally changed from those favouring absorption (lipophilicity) to those favouring excretion (hydrophilicity). Without biotransformation, lipophilic xenobiotics would be excreted from the body so slowly that they would eventually overwhelm and kill an organism.

A change in pharmacokinetic behaviour is not the only consequence of xenobiotic biotransformation nor, in some cases, is it the most important outcome. Xenobiotics exert a variety of effects on biological systems. These may be beneficial, in the case of drugs, or harmful, in the case of poisons. These effects are dependent on the

physicochemical properties of the xenobiotic. In many instances, chemical modification of a xenobiotic by biotransformation alters its biological effects. The importance of this principle to pharmacology is that some drugs must undergo biotransformation to exert their pharmacodynamic effect (i.e., it is the metabolite of the drug, and not the drug itself, that exerts the pharmacological effect). The importance of this principle to toxicology is that many xenobiotics must undergo biotransformation to exert their characteristic toxic or tumorigenic effect (i.e., many chemicals would be considerably less toxic or tumorigenic if they were not converted to reactive metabolites by xenobiotic-biotransforming enzymes). For this reason, the term *detoxification* which was used to refer biotransformation has been largely discarded. However, in most cases, biotransformation terminates the pharmacologic effects of a drug and lessens the toxicity of the xenobiotics (Testa 1995).

2.2 Xenobiotic biotransforming enzymes

Xenobiotic biotransformation is the principal mechanism for maintaining homeostasis during exposure of organisms to small foreign molecules, such as drugs. The chemical alterations of drugs, like the chemical changes taking place in normal metabolism, are not spontaneous reactions; they are **catalyzed reactions**. They take place in the presence of **enzymes**, protein catalysts which accelerate the reaction but remain essentially unchanged in the process.

The word *enzyme* occasionally denotes more than just a catalytic protein. Many enzymes require non-protein organic compounds called **cofactors**, which can be named as **prosthetic groups** or **coenzymes**, which play an intimate and frequently essential role in catalysis. Ordinarily, the term prosthetic group is reserved for groups which are bound firmly to the protein and cannot be readily removed without destroying the enzyme, whereas coenzymes refer to dissociable entities necessary for the reaction. Some enzymes also require small ions, such as Mg^{2+} for full catalytic activity.

In general, xenobiotic biotransformation is accomplished by a limited number of enzymes with broad substrate specificities. The synthesis of some of these enzymes is triggered by the xenobiotics (by the process of enzyme induction), but in most cases the enzymes are expressed constitutively (i.e., they are synthesized in the absence of a discernible external stimulus). The specificity of xenobiotic biotransforming enzymes is so broad that they metabolize a large variety of endogenous chemicals, such as steroid hormones, vitamins A and D, bilirubin, bile acids, fatty acids and eicosanoids. Indeed, xenobiotic biotransforming enzymes, or enzymes that are closely related, play an important role in the synthesis of many of these same molecules.

Xenobiotic biotransforming enzymes are widely distributed throughout the human body and are present in several subcellular compartments. In vertebrates, the

liver is the richest source of enzymes catalyzing biotransformation reactions, followed by lung, kidney, intestine, intestinal flora, skin and endocrine organs, such as gonads, placenta and adrenal (Krishna & Klotz 1994).

Within the liver, and most other organs, the enzymes catalyzing xenobiotics biotransformation reactions are located primarily in the endoplasmic reticulum (microsomes) or in the soluble fraction of the cytoplasm (cytosol) but also can be found in mitochondria, nuclei and lysosomes. Their presence in the endoplasmic reticulum can be rationalized on the basis that those xenobiotics requiring biotransformation for urinary or biliary excretion will likely be lipophilic, thus soluble in the lipid bilayer of the endoplasmic reticulum (Meeks & Harrison 1991).

By extracting and biotransforming xenobiotics absorbed by the gastrointestinal tract, the liver limits the systemic bioavailability of orally ingested xenobiotics, a process known as **first-pass elimination**. In some cases, xenobiotic biotransformation in the intestine contributes significantly to the first-pass elimination of foreign chemicals. Some extrahepatic sites contain high levels of xenobiotic biotransforming enzymes as, for instance, in the nasal epithelium. Although these enzymes are present in levels that rival those found in the liver, the role of the nasal epithelium is limited to the biotransformation of inhaled xenobiotic, whereas is quantitatively unimportant in the biotransformation of orally ingested xenobiotics (Brittebo 1993).

2.2.1 Enzyme kinetics

The term enzyme kinetics implies a study of the speed, rate or velocity of an enzyme catalyzed reaction, and of the various factors which may affect this.

In an enzymatic reaction, initially free enzyme **E** and free substrate **S** in their respective ground states combine reversibly to an enzyme-substrate complex – **ES**. The

ES complex passes through a transition state on its way to the enzyme-product complex – **EP** – and then on to the ground state of free enzyme **E** and free product **P** (Figure 1).

$$E + S \rightleftharpoons ES \longrightarrow EP \longrightarrow E + P$$
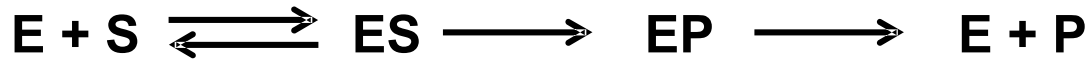
Figure 1 – Schematic representation of an enzymatic reaction.

From the formulation of the reaction sequence, a **rate law** can be derived. The first rate law was written in 1913 by Michaelis and Menten and therefore the corresponding kinetics is named the **Michaelis-Menten equation**.

2.2.1.1 Michaelis-Menten equation

The Michaelis-Menten equation is a quantitative description of the relationship among the rate of an enzyme-catalyzed reaction v, the concentration of substrate $[S]$ and two constants, V_{max} and K_m (which are set by the particular equation – Figure 2). The symbols used in the Michaelis-Menten equation refer to the reaction rate v, maximum reaction rate (V_{max}), substrate concentration $[S]$ and the Michaelis-Menten constant (K_m).

$$v = \frac{V\max[S]}{Km + [S]}$$

Figure 2 – Michaelis-Menten equation.

The Michaelis-Menten equation can be used to demonstrate that at substrate concentration that produces exactly half the maximum reaction rate, i.e., $1/2$ V_{max}, the substrate concentration is numerically equal to K_m. In this derivation, the units of K_m are those used to specify the concentration of S, usually Molarity. This fact provides a

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simple, yet powerful bioanalytical tool that has been used to characterize both normal and altered enzymes, such as those that produce the symptoms of genetic diseases.

The Michaelis-Menten equation has the same form as the equation for a rectangular hyperbola; graphical analysis of reaction rate (v) versus substrate concentration [S] produces a hyperbolic rate plot (Figure 3).
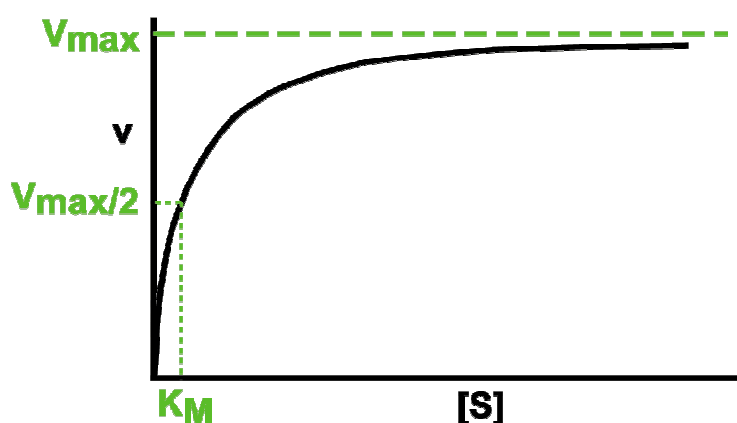
Figure 3 – Graphical scheme of Michaelis-Menten equation.

2.2.1.2 Deviations from Michaelis-Menten relationship

One of the assumptions of the Michaelis-Menten model implicit in applying the above scaling strategy is the premise that substrate enzyme interactions occur at only one site per enzyme and that each site operates independently from the others. There are evidences that for a number of xenobiotic catalyzing enzymes this is not the case (Izumi *at al.* 1997, Jong-Shik & Byung-Gee 2001, Uchapichat *et al.* 2004).

Two characteristic types of curves have been reported in the majority of the cases:

➢ **Sigmoidal**, when the enzyme has cooperative subunits: at very low substrate concentrations very few enzyme active sites will have substrate bound to them

and they will have a poor affinity for the enzyme. Therefore, the addition of more substrate causes only a small increase in reaction rate as the substrates bind very poorly. However, as more and more substrate molecules manage to bind, the positive cooperativity effect **increases** the ability of the enzyme to bind substrate and the graph starts to show this by sweeping upwards more sharply. Ultimately, just like a hyperbolic enzyme the enzyme will get close to saturation and the line will flatten out as it gets closer to the maximal velocity (Figure 4).
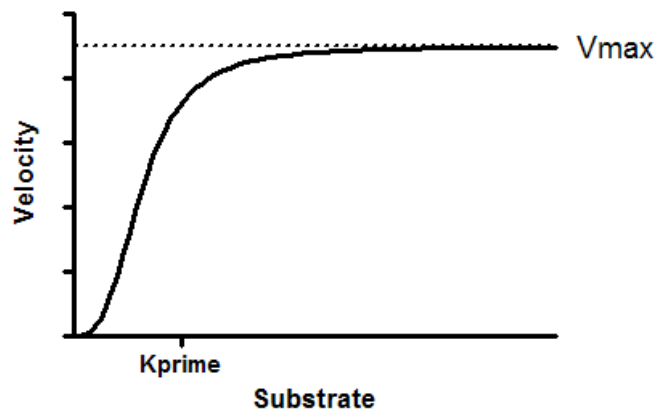
Figure 4 – Graphical scheme of sigmoidal kinetics.

> **Convex**, resulting from substrate inhibition where a hyperbolic-type curve is apparent at low concentrations, but there is no clearly defined plateau at high substrate concentrations and rates decrease as substrate concentrations are further increased. Substrate inhibition happens when *two* substrate molecules bind to the active site at the same time. They can only do this by approaching the active site in a fashion which prevents either of them from positioning itself in such a way that the enzyme can attack it. As long as both substrate molecules are attached to the active site the enzyme is effectively inactive, and therefore inhibited. For this process to occur, the second substrate must approach the active site very rapidly after the first, otherwise the first substrate would quickly

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attain the correct catalytic placement. As collisions between enzyme and substrate are completely random, this is only likely to occur at high substrate concentrations when the frequency of random collisions is greatly increased, so inhibition is only seen in the presence of excess substrate (Figure 5).
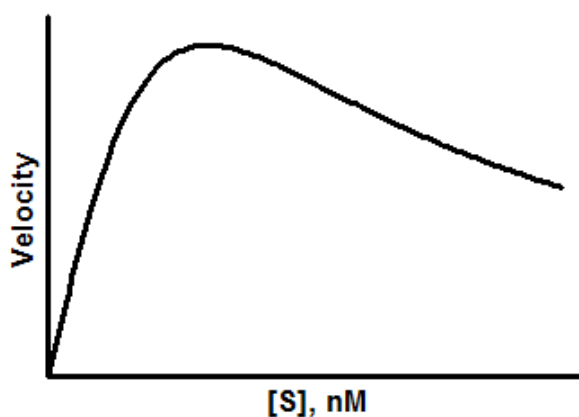
Figure 5 – Graphical scheme of substrate inhibition kinetics.

It is important not to underestimate these deviations as they may be possible constrains on the extrapolation of *in vitro* data on drug biotransformation to predict *in vivo* pharmacokinetic characteristics, such as metabolic stability and inhibitory drug interaction potential.

2.3 Pathways of Biotransformation

Pathways of biotransformation have been classified under the headings of **Phase I** and **Phase II reactions**. Phase I reactions involve oxidation, reduction and hydrolysis, and result in the exposure or introduction of a functional group (-OH, -NH$_2$, -SH or -COOH) and usually result in only a small increase in the hydrophilicity. Many of the metabolites formed are then able to participate in a subsequent biotransformation process, Phase II biotransformation, in which a polar hydrophilic moiety, such as glucuronic acid, sulfonate or amino acid is covalently linked – or **conjugated** – to the functional group introduced in phase I reaction. The usual overall result of these processes is the production of metabolites with progressively greater water-solubility, which facilitates their removal from the body.

Many drugs undergo a series of biotransformation and the combination of consecutive phase I and phase II reactions can produce a complex array of metabolites. Drugs already containing appropriate functional groups can undergo phase II reactions without the need of phase I biotransformation. For example, morphine is converted to morphine-3-glucuronide by directly conjugating with glucuronic acid.

In some cases, phase I metabolites may not be detected, owing to their instability or high chemical reactivity. The latter type are often electrophilic substances, or *reactive intermediates*, which frequently react non-enzymatically as well as enzymatically with conjugating nucleophiles to produce a phase II metabolite. A common example of this type is the oxidative biotransformation of an aromatic ring and conjugation of the resulting arene oxide (epoxide) with the tripeptide glutathione. Detection of metabolites from this pathway often points to the formation of precursor reactive electrophilic phase I metabolites, whose existence is nonetheless only inferred.

2.3.1 Phase I biotransformation reactions

2.3.1.1 Oxidation

➢ **Cytochrome P450 – the microsomal mixed-function oxidase (MFO)**

Among phase I biotransforming enzymes, the cytochrome P450 ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates. The MFO system is found at the highest concentrations in the liver endoplasmic reticulum (microsomes) but cytochrome P450 enzymes are present in virtually all tissues, except striated muscle and erythrocytes.

Microsomal cytochrome P450 is a two-enzyme complex of NADPH-cytochrome P450 reductase and cytochrome P450. During the MFO reaction, reducing equivalents derived from NADPH H^+ are consumed and one atom of molecular oxygen is incorporated in the metabolite and the other atom of oxygen is reduced to the level of water (Figure 6 and 7).

$$NADPH\ H^+ + O_2 + RH \xrightarrow{\text{Cytochrome P450}} NADP^+ + H_2O + ROH$$
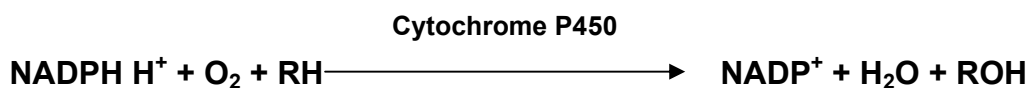
Figure 6 – Scheme of oxidation catalyzed by cytochrome P450. RH represents an oxidisable drug and ROH is the hydroxylated metabolite.
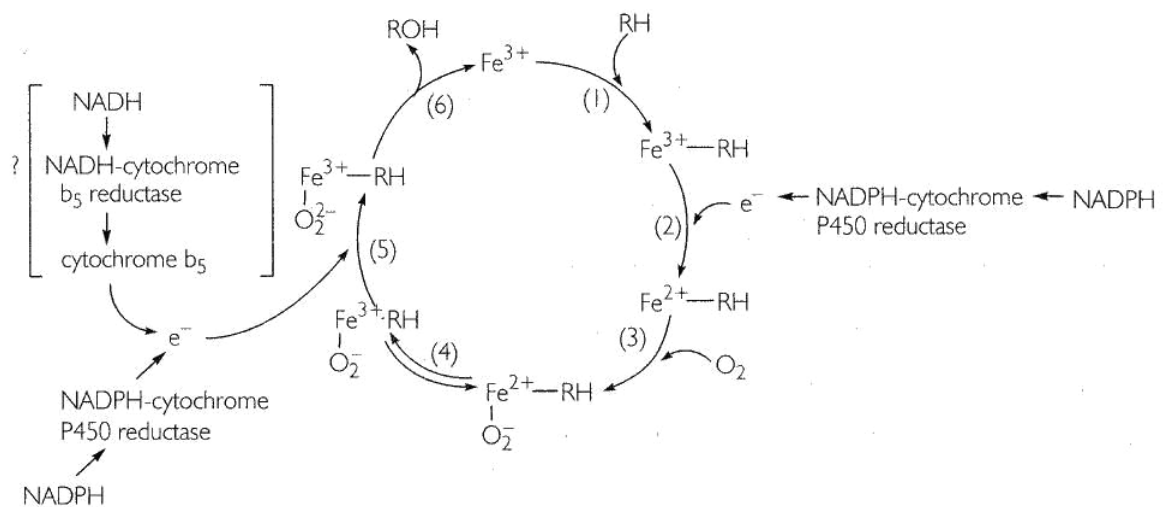
Figure 7 - Catalytic cycle of cytochrome P450. Taken from Gibson and Skett, 2001.

Cytochrome P450 is classified as a heme-containing enzyme (a hemoprotein) with iron protoporphyrin IX as the prosthetic group. This prosthetic group is common to other hemoproteins, but with substantially different biological functions, such as hemoglobin and myoglobin (oxygen transport proteins). Spectrally, it is a *b*-type cytochrome, but an unusual one in that it readily reacts with small molecular weight ligands and the spectral absorbance maximum of the ferrous-carbon monoxide adduct is 450 nm, as compared to around 420 nm for the majority of other hemoproteins.

The heme of the cytochrome is non-covalently bound to the apoprotein and the name **cytochrome P450** is derived from the fact that the cytochrome (or *pigment*) exhibits the above mentioned spectral absorbance maximum at 450 nm when reduced (Fe^{2+}-heme) and complexed with carbon monoxide. The hemoprotein serves at the locus for oxygen binding/activation – and the binding site for some, but not all, drugs – and in conjunction with its associated flavoprotein reductase – NADPH-cytochrome P450reductase – undergoes cyclic reduction/oxidation if the heme iron that is mandatory for its catalytic activity.

Cytochrome P450 is not a single enzyme, but rather consists of a family of closely related isoforms embedded in the membrane of the endoplasmic reticulum and exists as multiple forms of monomeric molecular weight of approximately 45-55 kDa. They are denoted as CYP (**cy**tochrome **P**450) and classified in the basis of their amino acid sequences and divided into gene families and gene sub-families. For example, *CYP3A4* uniquely describes only one particular gene encoding a specific protein, and it is the fourth gene to have been completely sequenced in the 3A sub-family (Gibson & Skett 2001).

Cytochromes P450 play a key role in the biotransformation of a great range of drugs and other xenobiotics and it has been demonstrated its participation in endogenous

metabolism, especially in the metabolism of lipophilic compounds such as the conversion of cholesterol to bile acids, fatty acids to eicosanoids and others (Lewis 2001).

➢ **Flavin Monooxygenases (FMO)**

Flavin-containing monooxygenases – FMO – constitute a multi-gene family of xenobiotic-biotransforming enzymes. The FMOs are polymeric proteins exhibiting a monomeric molecular weight of approximately 65 kDa and contain one mole of FAD per mole of protein monomer, hence the name **FAD-containing monooxygenase**. Like cytochrome P450, FMO are microsomal enzymes that require NADH or NADPH as source of reducing equivalents (being the latest the preferred cofactor) and molecular oxygen O_2, to catalyse the oxygenation of nucleophilic nitrogen, sulfur, phosphorous and selenium atoms in a range of structurally diverse compounds. FMO have been implicated in the biotransformation of a number of drugs, pesticides and toxicants (Lawton *et al.* 1994, Gibson & Skett 2001).

$$\text{NAD(P)H H}^+ + O_2 + \text{RN} \xrightarrow{\text{FMO}} \text{NAD(P)}^+ + H_2O + \text{RN-O}$$
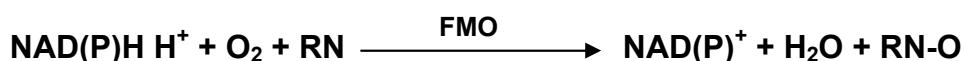
Figure 8 – Scheme of oxidation catalyzed by FMO. RN is an oxidisable, amine-containing substrate and RN-O is the *N*-oxidised metabolite.

Five mammalian forms of FMOs are now known and have been designated FMO1-FMO5, being FMO3 the major flavin monooxygenase in human liver microsomes (Cashman *et al.* 1999).

FMOs also play a major role in hepatic N- and S-oxidation of various endogenous compounds. FMOs are responsible in large part for the oxidation of the volatile odorous sulfur and nitrogen metabolites produced during the metabolism of

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dietary methionine and choline by intestinal microflora, to non-volatile hydrophilic metabolites, which are excreted predominantly in urine without any aroma. When the liver fails to oxidise the absorbed volatile substances and allows them to escape via breath, sweat and urine, foetor hepaticus, fish-odour syndrome or trimethylaminuria may occur (Morgan *et al.* 2001).
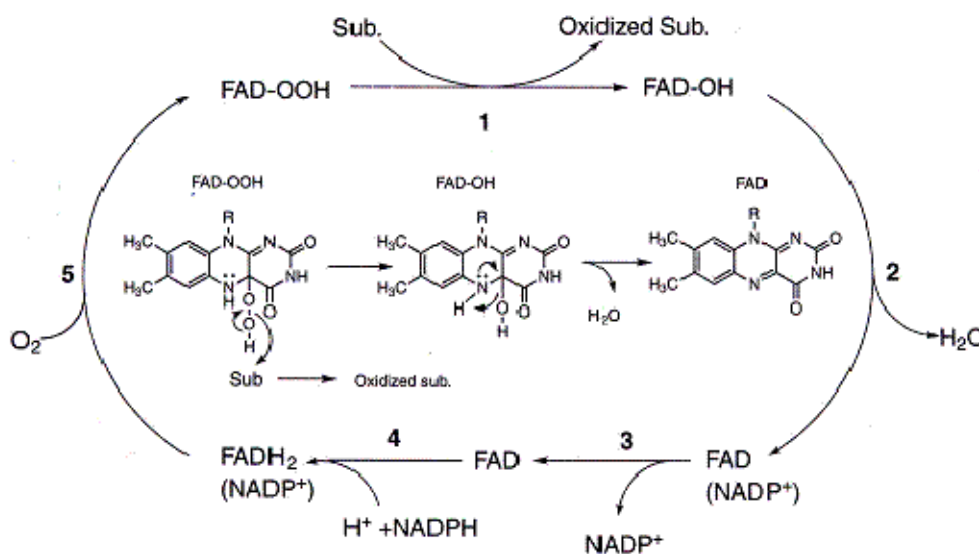
Figure 9 - Catalytic cycle of flavin monooxygenase. Taken from Williams and Lemke, 2002.

➤ *Other oxidation enzymes*

A number of enzymes in the body not related to cytochrome P450 can oxidise drugs. Most of these enzymes are primarily involved in endogenous compounds metabolism although some of them are more intimately involved in drug biotransformation. These enzymes can be found in cytosol, mitochondria and microsomes and may require NADPH and/or NADH as source of reducing equivalents (Testa 1995).

- Alcohol and aldehyde dehydrogenase: oxidation of alcohols, aldehydes and ketones (Agarwal & Goedde 1992);

- Monoamine, diamine and polyamine oxidase (MAO, DAO, PAO): oxidative deamination of primary, secondary and tertiary amines (Benedetti & Dostert 1994);

- Cyclooxygenase and peroxidase: peroxidase-dependent cooxidation (Eling *at al.* 1990);

- Xanthine oxidase, aldehyde oxidase, superoxide dismutase and others.

2.3.1.2 Reduction

Reduction plays an important role in xenobiotic biotransformation and the enzymes catalyzing reduction are located mainly in the membrane of the smooth endoplasmic reticulum and cytosol. The intestinal microflora also possesses an important reduction potential.

These reactions require NADH or NADPH as cofactors, but are generally inhibited by oxygen, unlike the MFO system reactions, which require oxygen. Certain metals (e.g. pentavalent arsenic) and xenobiotics containing aldehyde, ketone, disulfide, sulfoxide, quinone, *N*-oxide, alkene, azo, or nitro group are often reduced *in vivo*.

The most important enzymatic systems responsible for reduction of the carbonyl group xenobiotics include SDR and AKR.

SDR or short-chain dehydrogenases/reductases are one-domain NADPH-dependent enzymes of typically 250 amino acid residues. SDRs are defined by distinct, common sequence motifs but constitute a functionally heterogeneous superfamily of enzymes with about 3000 known forms, including species variants. They display a wide substrate spectrum, ranging from steroids, alcohols, sugars and aromatic compounds to xenobiotics.

Using the defined sequence motifs as queries, 37 distinct human members of the SDR family can be retrieved. The functional assignments of these forms fall minimally into three main groups, enzymes involved in intermediary metabolism, enzymes participating in lipid hormone and mediator metabolism, and open reading frames (ORF) of yet undeciphered function (Oppermann 2001, Karlberg 2002).

AKR or aldo-keto reductases are a superfamily of cytosolic, monomeric proteins that catalyze mainly the NADPH dependent reduction. Within the range of substrates of AKRs are different steroids that are metabolized by hydroxysteroid dehydrogenases and some stereospecific double bond reductases.

Among the AKR superfamily, several groups of enzymes with related structure and function have been established. To date, more than 150 AKR proteins from bacteria, yeast, plants, invertebrates and vertebrates have been identified and form 15 families. Mammalian AKRs (13 enzymes) are distributed between three major families, AKR1, AKR6 and AKR7. Among the different AKR superfamily groups, the aldose reductase (AR), aldehyde reductase, and hydroxysteroid dehydrogenase (HSD) families have been profoundly characterized in mammals. The AR family has been suggested to be involved in the development of secondary diabetic complications because of its ability to reduce glucose to sorbitol, a hyperosmotic compound. Other roles in aldehyde detoxification, osmotic homeostasis, steroid conversion, and catecholamine metabolism have been also proposed for ARs (Crosas 2001, Gravidia 2002, Spite 2007).

2.3.1.3 Hydrolysis

Mammals contain a variety of hydrolytic enzymes that hydrolyze xenobiotics containing such functional groups as carboxylic acid ester, amide, thioester, phosphoric acid ester and acid anhydride.

Hydrolytic enzymes are present in blood plasma, erythrocytes and various tissues. As examples of hydrolytic enzymes, it is possible to name carboxyesterases, cholinesterases and organophosphatases (Casarett & Doull 2001).

2.3.2 Phase II biotransformation reactions

Phase II biotransformation reactions include glucuronidation, sulfonation (more commonly called sulfation), acetylation, methylation, conjugation with glutathione and conjugation with amino acids.

The cofactors for these reactions react with functional groups that are either present on the xenobiotic or are introduced/exposed during phase I biotransformation.

With the exception of methylation and acetylation, phase II biotransformation reactions result in a large increase in xenobiotic hydrophilicity, so they greatly promote the excretion of foreign chemicals.

Glucuronidation, sulfation, acetylation and methylation involve reactions with **activated or "high-energy" cofactors**, whereas conjugation with amino acids or glutathione involves reactions with **activated xenobiotics**.

Most of phase II biotransforming enzymes are located in the cytosol, except for the enzymes catalysing the conjugation with glucuronic acid, which are microsomal enzymes.

Phase II reactions generally proceed much faster than phase I reactions, such as those catalyzed by cytochrome P450. Therefore, the rate of elimination of xenobiotics whose excretion depends on biotransformation by cytochrome P450 followed by phase II conjugation is generally determined by the first reaction.

2.3.2.1 Glucuronidation

Glucuronidation is a major pathway for the inactivation and excretion of both endogenous compounds such as bilirubin and steroids as well as a multitude of xenobiotic compounds including drugs, carcinogens and others environmental pollutants.

The readily available supply of the required cofactor **uridine diphosphate glucuronic acid (UDPGA)** and the ubiquitous nature of the enzyme, **UDP-glucuronosyltransferase (UGT)** contribute to the importance of glucuronidation as a main biotransformation reaction.
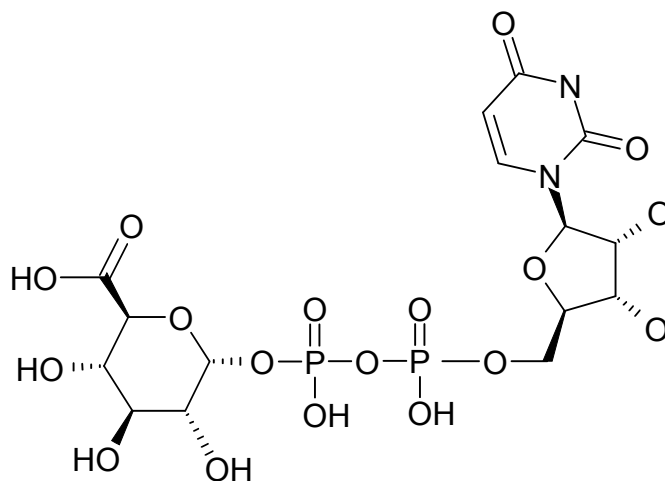
Figure 10 – Chemical structure of UDPGA.

UGTs are a family of membrane-bound enzymes of the endoplasmic reticulum. Many different genes and pseudogenes have been identified in the UGT superfamily and are subdivided into the UGT1A and UGT2B families based on sequence identity (Kuehl 2005).

Glucuronide formation is quantitatively the most important conjugation for drugs and endogenous compounds (e.g. bilirubin, some steroid hormones) containing an electron-rich nucleophilic heteroatom as O, N or S. Therefore, substrates for glucuronidation contain such functional groups as aliphatic, alcohols and phenols

(which form O-glucuronide ethers), carboxylic acids (which form O-glucuronide esters), primary and secondary aromatic and aliphatic amines (which form N-glucuronides) and free sulfhydryl groups (which form S-glucuronides).

Glucuronide conjugates of xenobiotics and endogenous compounds are polar, water-soluble conjugates that are eliminated from the body in urine or bile, depending on the size of the aglycone.

The cofactor for glucuronidation is synthesized from glucose-1-phosphate, and the linkage between glucuronic acid and UDP has an α-configuration. This configuration protects the cofactor from hydrolysis by β-glucuronidase. However, glucuronides of xenobiotics have a β-configuration. This inversion of configuration occurs because glucuronides are formed by nucleophilic attack by an electron-rich atom (O, N or S) on UDP-glucuronic acid, and this attack occurs on the opposite site of the linkage between glucuronic acid and UDP. For this reason, conjugates of xenobiotics with glucuronic acid are substrates for β-glucuronidase (mainly in the intestinal microflora), releasing the aglycone, which can be reabsorbed and enter enterohepatic circulation, which delays the elimination of the xenobiotic (Sahidan *et al.* 1994, Casarett & Doull 2001, Williams & Lemke 2002, Kuehl *et al.* 2005).

2.3.2.2 Sulfation

Conjugation of many xenobiotics, drugs and endogenous compounds with a sulfonate moiety is an important reaction in their biotransformation.

The major physiologic consequences of the conjugation of a drug or xenobiotic with a charged sulfonate moiety are increased aqueous solubility and excretion.

Although the major role of sulfation is in decreasing the biological activity of a compound, in some instances sulfate conjugation results in the bioactivation of a

compound to a reactive electrophilic species that is capable of covalently binding DNA and causing a mutagenic, teratogenic or carcinogenic response.

The reaction is catalyzed by **sulfotransferase – ST** - a large multigene family enzymes found primarily in the liver, kidney and intestinal tract. The cofactor for the reaction is **3'-phosphoadenosine-5'-phosphosulfate (PAPS)**, which provides the "active sulfate".
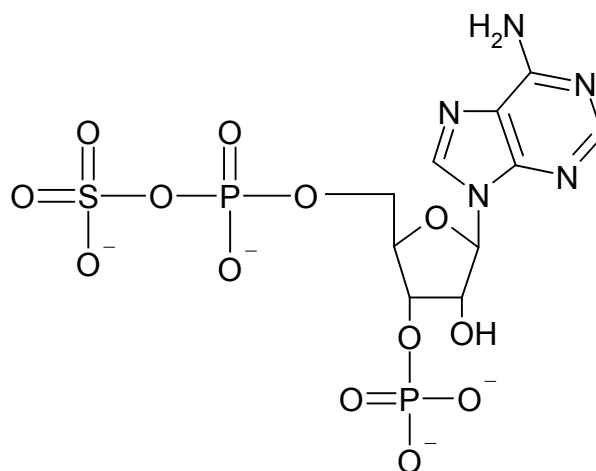
Figure 11 – Chemical structure of PAPS.

Sulfation involves the transfer of sulfonate, not sulfate, i.e., SO_3^- not SO_4^- , from PAPS to the xenobiotic. The conjugation reaction involves nucleophilic attack of oxygen or nitrogen on the electrophilic sulfur atom in PAPS with cleavage of the phosphosulfate bond.

Two distinct families of STs have been identified and characterized in human and animal tissues. These two families are the cytosolic STs, which are generally associated with drug and xenobiotic metabolism, and the membrane-bound STs localized in the Golgi apparatus of most cells. Several forms of membrane-bound STs are responsible for the sulfation of glycosaminoglycans, glycoproteins, and tyrosines in proteins and peptides in the Golgi apparatus of most cells.

Sulfation has long been recognized as an important reaction in the synthesis, transport, and metabolism of steroids in human tissues. Sulfate conjugation has an important role in decreasing the biological activity of steroids because steroid sulfates are not capable of binding and activating the appropriate steroid receptors.

Bile acid sulfation in the liver is an important mechanism for decreasing the toxicity and increasing the solubility of secondary bile acids such as lithocholic acid and chenodeoxycholic acid, especially during cholestasis when bile acid concentrations increase. Secondary bile acids are formed by bacteria in the intestine from primary bile acids secreted by the liver into the bile. Secondary bile acids are absorbed from the intestines into the portal circulation, and unless conjugated with amino acids, sulfated or glucuronidated, they have a detergent-like effect on hepatocytes (Falany 1997, Glatt 1997, Klassen & Boles 1997).

2.3.2.3 Glutathione Conjugation

Glutathione conjugation inactivates xenobiotic metabolites that are toxic to cells such as liver and kidney cells.

It is a non-specific reaction involving the combination of an electrophilic compound with the **tripeptide glutathione – GSH**, which is comprised of glycine, cysteine and glutamic acid - found in almost all tissues.
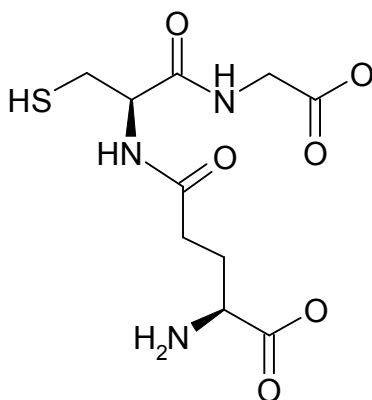
Figure 12 – Chemical structure of Glutathione.

Substrates for glutathione conjugation include an enormous array of electrophilic xenobiotics or xenobiotics that can be biotransformed to electrophiles.

Glutathione conjugates are thioethers, formed by nucleophilic attack of glutathione thiolate anion – GS⁻ - with an electrophilic carbon atom in the xenobiotic. Glutathione can also conjugate xenobiotics containing electrophilic heteroatoms – O, N, and S.

The conjugation of xenobiotics with glutathione is catalyzed by a family of **glutathione S-transferases – GST** – which represent a major group of detoxification enzymes. These enzymes are present in most tissues with high concentrations in the liver, intestine, kidney where they are located in the cytoplasm (> 95%) and endoplasmic reticulum (< 5%). All eukaryotic species possess multiple cytosolic and

membrane-bound GST isoenzymes, each of which displaying distinct catalytic as well as noncatalytic binding properties: the cytosolic enzymes are encoded by at least five distantly related gene families, whereas the membrane-bound enzymes, microsomal GST and leukotriene C4 synthetase, are encoded by single genes and both have arisen separately from the soluble GST. Evidence suggests that the level of expression of GST is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals and the individual isoenzymes contribute to resistance to carcinogens, antitumor drugs, environmental pollutants and products of oxidative stress. The most abundant mammalian GSTs are the class **alpha**, **mu**, and **pi** enzymes and their regulation has been studied in detail. The biological control of these families is complex as they exhibit sex-, age-, tissue-, species-, and tumour-specific patterns of expression.

In addition, GSTs are regulated by a structurally diverse range of xenobiotics and, to date, at least 100 chemicals that induce GSTs have been identified; a significant number of these chemical inducers occur naturally and, as they are found as non-nutrient components in vegetables and citrus fruits, it is apparent that humans are likely to be exposed regularly to such compounds. It also appears probable that GSTs are regulated *in vivo* by reactive oxygen species (ROS), because not only are some of the most potent inducers capable of generating free radicals by redox-cycling, but H_2O_2 has been shown to induce GST in plant and mammalian cells: induction of GST by ROS would appear to represent an adaptive response as these enzymes detoxify some of the toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress.

Glutathione conjugates formed in the liver can be excreted intact in bile or they can be converted to mercapturic acids in the kidney and excreted in urine. The conversion of glutathione conjugates to mercapturic acids involves the sequential

cleavage of glutamic acid and glycine from the glutathione moiety, followed by N-acetylation of the resulting cysteine conjugate (Hayes & Pulford 1995, Pandit 2007).

2.3.2.4 Methylation

Methyl conjugation of endogenous substrates such as histamine, amino acids, proteins, carbohydrates and polyamine is an important process in the regulation of normal cellular metabolism and accounts for the presence of this activity in mammalian cells.

However, it is generally a minor pathway of xenobiotic biotransformation. Only when a xenobiotic fits the requirements for the enzymes involved in these normal reactions does methylation become important in the biotransformation of foreign compounds.

The most important route by which xenobiotics can be methylated involves **methyltransferase**-catalyzed methylation that requires **S-adenosylmethionine – SAM** – as cofactor. Most biological methylations require SAM as the methyl donor. SAM is produced from *L*-methionine and ATP.
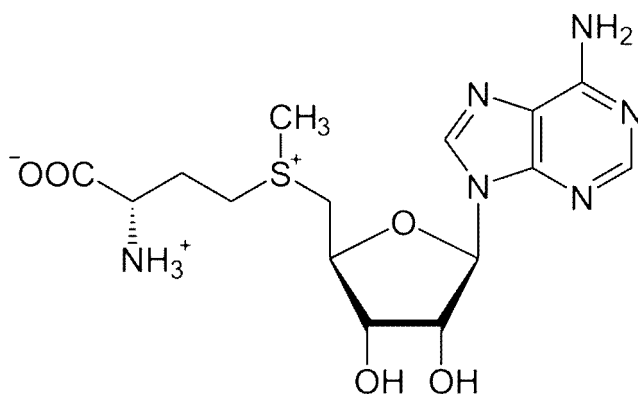
Figure 13 – Chemical structure of SAM.

The methyl group bound to the sulfonium ion in SAM has the characteristics of a carbonium ion and is transferred to xenobiotics and endogenous substrates by nucleophilic attack from an electron-rich heteroatom (O, N or S). During methylation reactions, SAM is converted to S-adenosylhomocysteine.

The non-specific **N-methyltransferase** is a soluble enzyme, playing an important role at lungs level and it is responsible for the methylation of xenobiotics as most of the methyltranseferases are specific for endogenous compounds.

The O-methylation of phenols is catalyzed by two different enzymes known as **phenol-O-methyltransferase – POMT –** and **catechol-O-methyltransferase – COMT**. POMT is a microsomal enzyme that methylates phenols but not catechols and COMT is both a cytosolic and microsomal enzyme with the converse substrate specificity. Substrates for COMT include several catecholamine neurotransmitters, such as epinephrine, noreepinephrine and dopamine and catechol drugs, such as L-dopa and methyldopa.

Methylation reactions can be stereoselective. For example, the (+)-R-enantiomer of nicotine is preferentially methylated over the (-)-S-enantiomer.
Methylation differs from conjugation processes as the products formed by methyl conjugation have the same or greater pharmacologic activity than the parent compound (Weinshilboum 1984, 1992).

2.3.2.5 Acetylation

Acetylation reactions are common for aromatic amines and sulfonamides. Like methylation, N-acetylation masks an amide with a nonionizable group, so that many N-acetylated metabolites are less water soluble than the parent compound. Nevertheless, N-acetylation of certain xenobiotics such as isoniazide, facilitates their urinary excretion.

The reaction involves the transfer of an acetyl group from the cofactor **acetyl-coenzyme A – acetyl CoA –** and it is catalyzed by *N*-**acetyltransferases**. It occurs in two sequential steps: in the first step, the acetyl group from acetyl-CoA is transferred to an active site cysteine residue within an *N*-acetyltransferase with release of coenzyme A; in the second step, the acetyl group is transferred from the acetylated enzyme to the amino group of the substrate with regeneration of the enzyme.
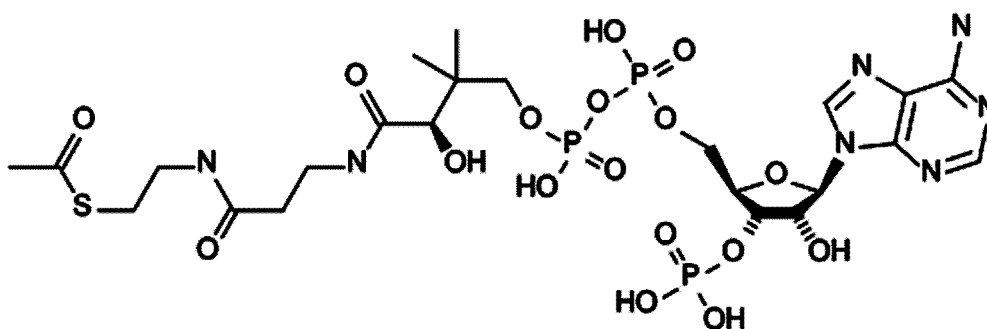
Figure 14 – Chemical structure of Acetyl CoA.

N-acetyltransferases are cytosolic enzymes found in the liver and many other tissues. In contrast to other xenobiotic-transforming enzymes, the number of *N*-acetyltransferases is limited. Some species, including humans, express only two *N*-acetyltransferases, known as NAT1 and NAT2.

Genetic polymorphisms for *N*-acetylation have been documented in several species. It has been established the existence of slow and fast acetylators.

The acetylation polymorphism is one of the most common inherited variations in the biotransformation of drugs and chemicals. Its association with drug toxicity and an increased risk to develop certain cancers has made it one of the oldest and best-studied examples of a pharmacogenetic condition. Forty to 70% of Caucasians in Europe and North America are of the "slow acetylator" phenotype and are less efficient than "rapid acetylators" in the metabolism of numerous drugs and chemicals containing primary aromatic amine or hydrazine groups. These include agents such as isoniazid, sulfamethazine (SMZ) and other sulfonamides, procainamide, hydralazine, dapsone, and caffeine, as well as several chemicals with carcinogenic potential such as benzidine, 2-aminofluorene, and f-naphthylamine, present in dyes, antioxidants, pesticides, and explosives. Highly mutagenic and carcinogenic arylamines also are generated during cooking of food. Slow acetylators are at higher risk to develop bladder cancer, whereas rapid acetylators are at higher risk for colorectal cancer (Blum *et al.* 1991, Casarett and Doull 2001).

2.3.2.6 Amino Acid Conjugation

Xenobiotics containing a carboxylic acid group can be conjugated with the amino group of amino acids such as **glycine, glutamine** and **taurine**, as well as ornithine, arginine, histidine, among others. The specific amino acid involved in the conjugation is both species and xenobiotic-dependent.
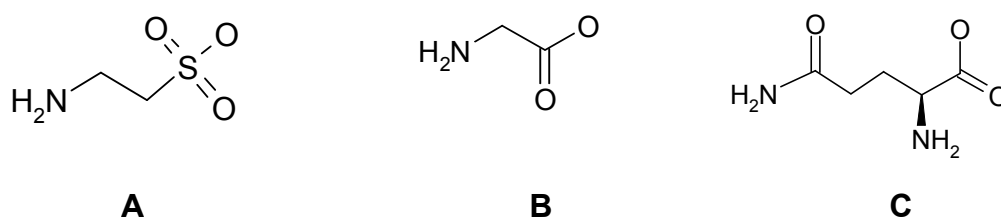
Figure 15 – Chemical structures of amino acids: A-taurine; B-glycine; C-glutamine.

The mechanism of amino acid conjugation involves three steps. The carboxylic acid is first activated by ATP to the AMP ester, which is converted to the corresponding coenzyme A thioester with CoASH; these first two steps are catalyzed by **acyl CoA synthetases (long-chain fatty acid-CoA ligases)**. The appropriate **amino acid:*N*-acyltransferase** then catalyzes the condensation of the amino acid and coenzyme A thioester to give the amino acid conjugate. The conjugation does not take place with the AMP ester directly because the AMP ester hydrolyzes readily.

The ability of xenobiotics to undergo amino acid conjugation depends on steric hindrance around the carboxylic acid group and by substituents on the aromatic ring or aliphatic side chain. In some species, such as rats, the major pathway of phenylacetic acid biotransformation is amino acid conjugation.

Bile acids are endogenous substrates for glycine and taurine conjugation. However, the activation of bile acids to an acyl-CoA thioester is catalyzed by a

microsomal enzyme, cholyl-CoA synthetase and conjugation with glycine or taurine is catalyzed by a single cytosolic enzyme, bile acid-CoA:amino acid *N*-acyltransferase. In contrast, the activation of xenobiotics occurs mainly in mitochondria, which appear to contain multiple acyl-CoA synthetases. The formation of the amino acid conjugate is catalyzed by cytosolic and/or mitochondrial forms of *N*-acyltransferase.

Another important difference between the amino acid conjugates of xenobiotics and bile acids is their route of elimination: bile acids are secreted into bile whereas amino acid conjugates of xenobiotics are eliminated primarily in urine (Casarett & Doull 2001).

2.4 Stereochemical aspects of Biotransformation

2.4.1 Chirality

The concept of **chirality** has been known in chemistry since the 1870's although it would be nearly a hundred years before chemists began using this term.

In extremely simple terms, chirality is **handedness** – that is, the existence of left/right opposition. For example, the left hand and the right hand are **mirror images** and therefore **chiral**. The term chiral is derived from the Greek *kheir* meaning *hand* and apparently was coined by Lord Kelvin in his Baltimore Lectures on Molecular Dynamics and the Wave Theory of Light in which he stated: …" *I call any geometrical figure, or group of points, chiral, and say it has chirality, if its image in a plane mirror, ideally realized, cannot be bought to coincide to itself.*"

Chiral molecules must not have an internal plane of symmetry and they must have a **stereocenter**. The two non-superimposable, mirror-images forms of chiral molecules are referred to as **enantiomers**. Chiral compounds exhibit optical activity, so enantiomers are also sometimes called **optical isomers**. The ability to rotate plane polarized light by equal amounts, but in opposite directions differentiates the pair of enantiomers, which have identical physical and physical-chemical properties - one isomer rotates this light "x" number of degrees to the right while the other isomer of the pair rotates this light to the left for the same number of degrees. A 50/50 mixture of the two enantiomers of a chiral compound is called **racemic mixture** and does not exhibit optical activity.

The majority of bioorganic molecules are chiral; living organisms, for example, are built of chiral compounds such as amino acids, sugars, proteins and nucleic acids. In nature, these biomolecules exist in only one of the two possible enantiomeric forms: sugars, like glucose, occur in the *D*-form, while amino acids are found in the *L*-form.

2.4.2 Chirality and Bioactivity

Many xenobiotics, especially drugs, are chiral compounds and the pure enantiomers often show different pharmacodynamic and pharmacokinetic behaviour.

Pharmacological activity and biotransformation usually require interaction of the drug with specific receptors or enzymes, which can be extremely chiral selective, or **stereoselective,** according to the steric configuration at the site of interaction. In this way, the enzymes will interact with each racemic drug differently and metabolize each enantiomer by a separate pathway to produce different pharmacological activity. Thus, and very often, one isomer may possess the desired therapeutic property whilst the other isomer may be inert, completely different in its pharmacological activity or even toxic (Mitchell 1998). As an example, report to the tragic case of the 1960's, when the sedative Thalidomide was marketed as a racemate. Its therapeutic activity resided exclusively in the (+)-(*R*)-enantiomer. Only after the birth of thousands of malformed babies, it was discovered that the (-)-(*S*)-enantiomer was teratogenic. In fact, neglect of stereochemistry in drug development was widespread until the middle of the 1980's, when E. J. Ariens wrote his review and stereochemistry achieved a prominent place in drug design.

2.5 Analytical assessment

2.5.1 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography – HPLC – is now firmly established as the premier technique for the analysis and purification of a wide range of molecules.

The enormous success of HPLC can be attributed to a number of inherent features associated with reproducibility, ease of selectivity manipulation and generally high recoveries. The most significant feature is the excellent resolution that can be achieved under a wide range of conditions for very closely related molecules, as well as structurally quite distinct molecules. This aspect arises from the fact that all interactive modes of chromatography are based on recognition forces that can be subtly manipulated through changes in the elution conditions that are specific for the particular mode of chromatography (Aguilar 2004).

In HPLC, the analyst has a wide choice of chromatographic separation methodologies, ranging from normal to reverse phase, affinity ion exchange and a whole range of mobile phases using isocratic or gradient elution techniques. Various detectors are also available for HPLC, including electrochemical (ECD), refractive index (IR), fluorescence, radiochemical and mass-sensitive detectors, although by far, the most popular remains the UV detector. This diversity of modes of separation and detection makes HPLC suitable for the analysis of a wide range of compounds in the pharmaceutical and biochemical industries.

Essentially, HPLC is a technique that enables the separation of the components of a mixture by virtue of their differential distribution between the mobile (liquid) phase and the stationary (solid) phase. Migration of a solute component can only occur while it is dissolved in the mobile phase. Thus, solutes that have a high distribution into the

stationary phase will elute more slowly that those that distribute more readily into the mobile phase and the two will therefore undergo chromatographic separation.

In HPLC, the sample is injected by means of an injection port into the mobile phase stream delivered by the high-pressure pump and transported through the column where the separation takes place. The separation is monitored with a flow through the detector (Niessen 2006).

Separation of the different components can be achieved by a variety of modes, namely normal phase chromatography, reverse phase chromatography and ion exchange chromatography, which will be briefly explained.

2.5.1.1 Normal Phase

It is carried out on polar stationary phases. The traditional stationary phases are **silica** and **alumina**, in which hydroxyl groups are involved in the interactions with solvent and solute molecules. Cyano, diol and amino bonded phases are preferable due to their greater stability. The mobile phase consists of a non-polar solvent, usually hexane or heptane and a polar modifier such as a short-chain alcohol or dichloromethane. There are two possible interactions by which the solute can be retained: displacement of solvent molecules or sorption of solute molecules, although in practice may occur the combination of both these processes. The retention of solutes on normal phase columns decreases with polarity.

2.5.1.2 Reverse Phase (RP)

In this type of chromatography, the conditions applied are, as the name implies, the reverse of that of a normal phase chromatography. The stationary phase is non-polar and is prepared by chemically bonding a relatively non-polar group onto the silica. The most frequent non-polar group bonded to the stationary support is **octadecylsilane – ODS or C18** – which gives a highly lipophilic stationary phase. In RP-chromatography the components are separated according to their relative partition coefficients between the mobile and the stationary phases. Highly lipophilic non-polar solutes will be better retained by the stationary phase and hence have longer retention times. In contrast, polar molecules will be less retained. The mobile phase is polar, generally consisting of water and a water miscible organic solvent, such as methanol or acetonitrile.

2.5.1.3 Ion exchange

The stationary phase for ion exchange chromatography is generally silica chemically bonded with anionic or cationic groups, usually aminopropyl, tetra-alkylammonium or sulfonic acid groups. The molecules are separated on the basis of their molecular charge on the principle of opposite charges attracting each other, i.e. cations would be retained on a negatively charged phase and vice versa. Resolution is influenced by pH and by the ionic strength of the buffer (Venn 2000).

Figure 16 - Example of a HPLC system.

2.5.2 LC-MS – Liquid Chromatography-Mass Spectrometry

One of the areas of interest in recent years has been in the use of coupled detectors, where the output of the column is connected directly to a detector, which can provide additional structure or identification information on the analyte. These detectors are usually well established and wisely used as stand-alone analytical instruments. In recent years, an increasing interest has been seen in these information-rich detection methods, especially LC-MS.

Mass spectrometry – MS – is a very sensitive detection method which can provide mass and structural information (Heftmann 2004).

2.5.2.1 Ionization

Perhaps the most widely accepted technique is **electrospray ionization** or **ESI**, where a solution of the sample is sprayed through a metal capillary held at high potential, assisted by a gas (typically nitrogen). This produces a spray of charged droplets, from which the solvent is evaporated, leaving gas-phase ions that will be subsequently analysed. The key advantages of ESI are that volatility of the sample is not

required, as it only needs to be soluble in a solvent to the extent of a few ppm, and it allows the investigation of positive and negative ions equally well (MacKay *et al.* 2002).

2.6 Solid Phase Extraction

Solid phase extraction – SPE - is a technique designed for rapid and selective sample preparation and purification prior to chromatographic analysis.

It is used to isolate and concentrate selected analytes from a liquid, fluid or gas by their interaction and transfer to a solid phase. After physical separation of the sorbent and sample medium, the analytes are recovered by liquid or fluid elution, or by thermal desorption. In addition, sorbent immobilization provides a mechanism for matrix simplification using selective desorption to remove co-extracted matrix components without displacing the analytes of interest. SPE provides the sample clean-up, recovery, and concentration necessary for accurate quantitative analysis.

SPE uses the same type of stationary phases as are used in liquid chromatography columns which is contained in a glass or plastic column above a frit or glass wool. The column might have a frit on top of the stationary phase and might also have a stopcock to control the flow of solvent through the column.

Commercially available SPE cartridges have 1-10 ml capacities and are discarded after use.
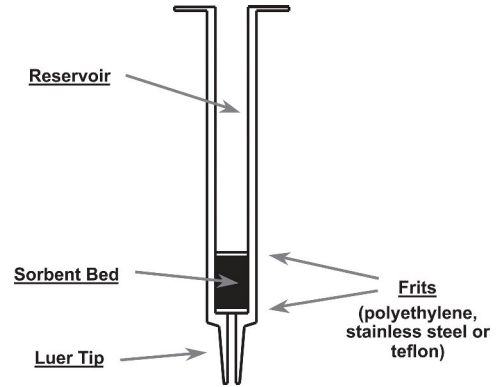
Figure 17 – Examples of SPE cartridges.

The picture below shows 1 ml cartridges on a vacuum manifold, which increases the solvent flow rate through the cartridge. Collection tubes are placed beneath the SPE cartridges (inside the vacuum manifold for the example in the picture) to collect the liquid that passes through the column.
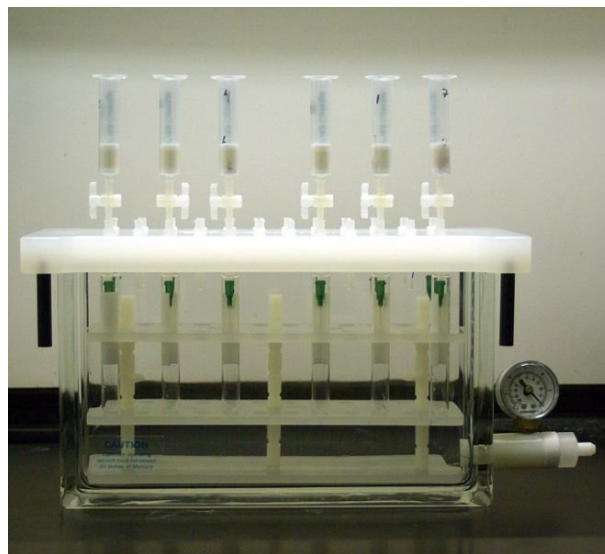
Figure 18 – Example of SPE manifold.

Similarly to liquid chromatography, we can distinguish three main types of SPE:

- **Reverse phase** – this type of separation involves a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase, such as alkyl- or aryl- bonded silicas. The analyte of interest is typically mid- to nonpolar;

- **Normal phase** – it typically involves a polar analyte, a mid- to nonpolar matrix and a polar stationary phase, which include polar functionalized bonded silicas;

- **Ion exchange** – it can be used for compounds that are charged when in a solution, usually aqueous, but sometimes organic. Anionic compounds can be isolated on aminopropyl bonded silicas or quaternary amine bonded silicas with Cl^- counterion. Cations can be isolated on sulfonic acid bonded silicas or carboxylic acid bonded silicas with Na^+ counterion.

Shortly, the general procedure consists of loading a solution onto the SPE cartridge, washing away the undesired and interfering components with an appropriate washing solution, and then elution of the desired analytes with a proper solvent into a collection tube. The washing solution and the elution solvent must be chosen in accordance to the nature of the analyte and the type of SPE applied.
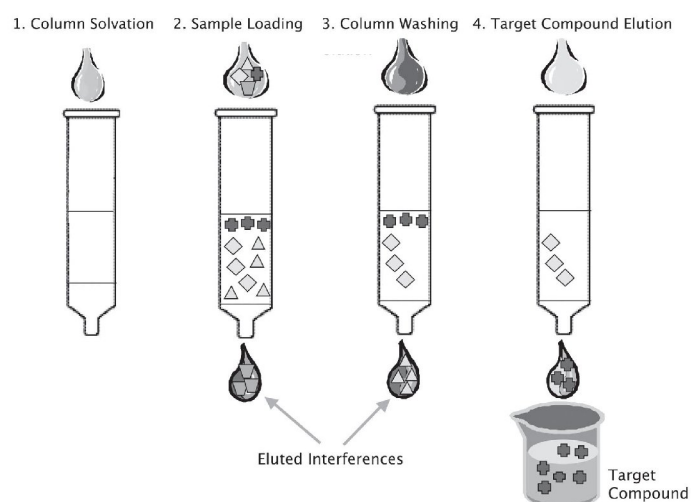
Figure 19 – SPE elution steps.

2.7 Non-Steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs – NSAID - are among the most widely used pharmaceutical drugs. Although being a heterogeneous group of compounds, all of them have in common, to a certain extent, the therapeutic properties as well as the side effects. Their mechanism of action consists predominantly of the inhibition of **cyclooxygenase – COX**.

COX is the enzyme that catalyzes the synthesis of prostaglandins, prime mediators of inflammation. COX is comprised of two isoenzymes:

- **COX-1, constitutive** – it plays an important role in the synthesis of prostaglandines, which have physiological activity, for instance, at the level of the gastric mucosa;

- **COX-2, induced form** – its synthesis is activated during inflammation processes.

The mechanism of action of NSAIDs consists predominantly of the inhibition of both forms of cyclooxygenase and therefore they inhibit the synthesis of prostaglandins and tromboxanes (Vane & Botting 1998).
Selective inhibitors of COX-2 decrease the inflammation reaction without influence on COX-1.

NSAIDs are comprised of molecules with different structures, as depicted in Figure 20:
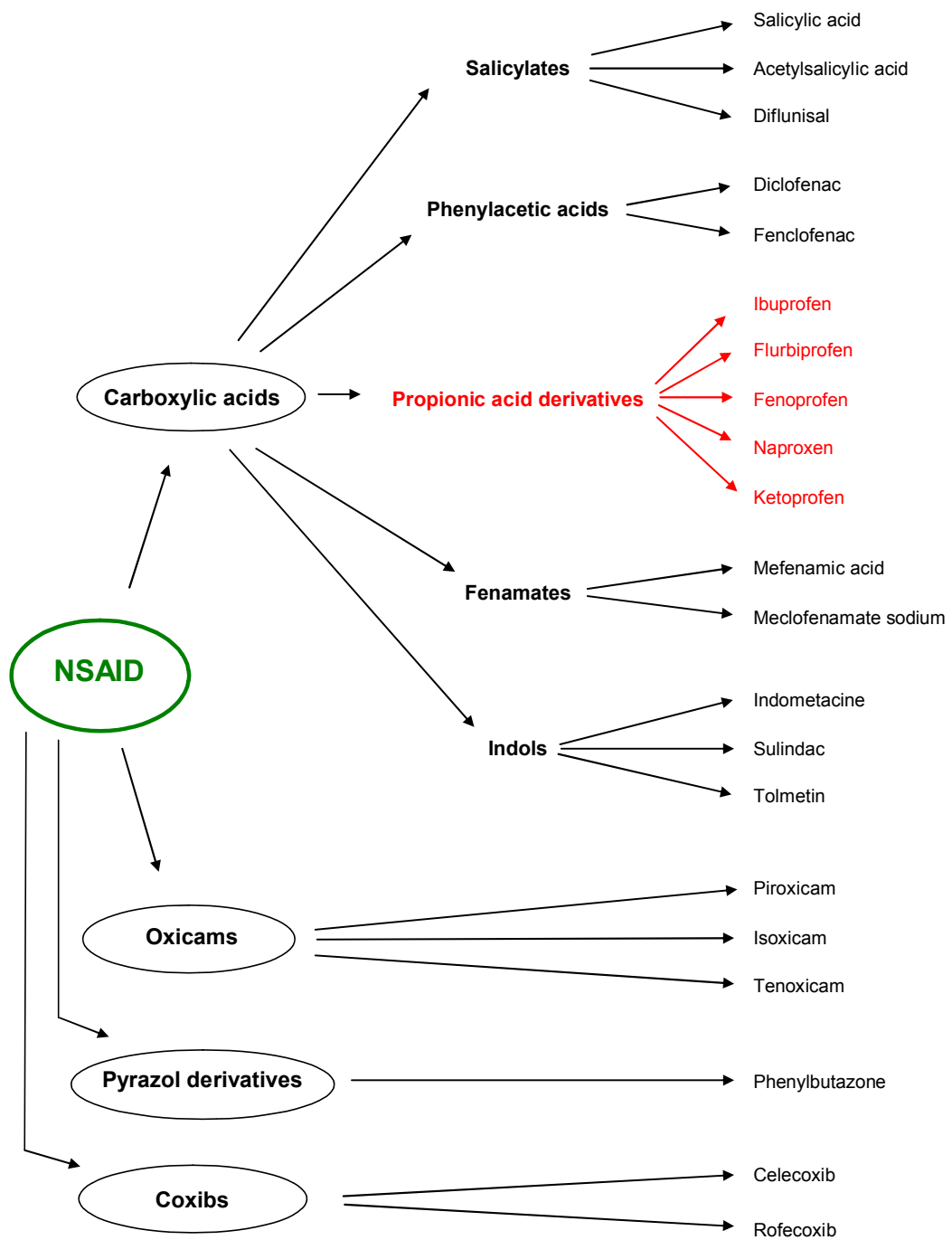
Figure 20 – The diverse chemical groups of NSAIDs.

The great majority of NSAIDs belong to the class of **2-arylpropionic acids (2-APA)** or **profens**. 2-APAs are chiral compounds and their racemic forms can be resolved into *R*- and *S*-enantiomers with different pharmacokinetic and pharmacological behaviour (Mueller *et al.* 1990). A particular characteristic of the metabolism of this class of compounds is the ability to undergo unidirectional inversion of the *R*- to the *S*-enantiomer, via the formation of an acyl-coenzyme A thioester, a reaction which is both species and drug dependent (Caldwell *et al.* 1998, Hall & Xiaotao 1994, Brugger *et al.* 2001). This can be associated with an important pharmacological implication: it is suggested that the *S*-enantiomer is responsible for the anti-inflammatory activity, whilst the *R*-enantiomer has a minor (if any) contribution (Rhys-Williams *et al.* 1998). Nevertheless, the clinically used 2-APAs are marketed as racemates, being naproxen the exception and, more recently, ibuprofen and ketoprofen, which have been marketed as *S*-enantiomer formulations (Glowka & Karazniewicz 2004).

Flobufen, the non-steroidal anti-inflammatory drug on which this project was focused, belongs to the group of aryloxobutyric acids, which are structurally related to the arylpropionic acids or profens.

2.8 Flobufen

2.8.1 Characteristics

Flobufen, 4-(2´,4´-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid, **FLO**, is a derivative from ω-bifenyl-ω-oxoalkanoic acid, together with fenbufen (Sunshine 1975), metbufen and itanoxon (Chanal *et al.* 1988) and belongs to the group of non-steroidal anti-inflammatory drugs. FLO arose on the 1980's as a result of structure and effect optimization of arylacetic acid (Kuchař *et al.* 1980, 1988). The molecular formula is $C_{17}H_{16}F_2O_3$ of molecular weight $M_r=304$ and it is presented as a white crystal. Its structure is depicted in Figure 21.
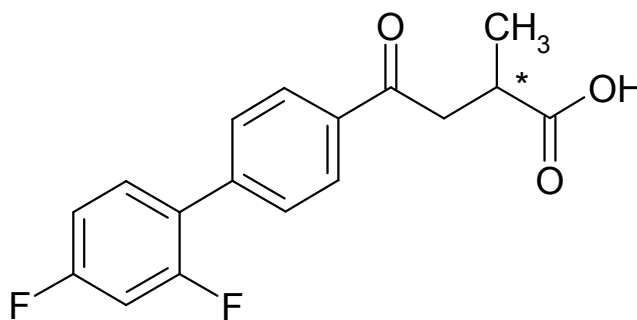
Figure 21 – Chemical structure of Flobufen. The * denotes the stereogenic centre.

FLO is a chiral molecule displaying one chiral centre and consequently exists in the form of two optical isomers: (+)-(*R*)-FLO and (-)-(*S*)-FLO. The fluorinated aromatic ring prevents undesired hydroxylation and, moreover, increases the lipophilicity of the drug (Kuchař *et al.* 1988).

FLO exhibits anti-inflammatory and anti-rheumatic activity, which results from the inhibition of enzymes necessary for the synthesis of the inflammation mediators, as are the prostaglandins, tromboxanes or leukotrienes. FLO can be characterized as a drug with combined inhibitory effect on 5-lipoxygenase and both forms of COX, although antagonist effects on LTB_4 receptors can also be observed (Kuchař *et al.* 1995).

FLO has demonstrated anti-inflammatory activity in standard models of inflammation in the rat. In the carrageenan paw edema assay, flobufen (10mg/kg *p.o.*) reduced the edema by 37% 1 h after carrageenan injection. Flobufen has a longer duration of action than fenbufen, reducing edema at 48 h after induction of inflammation (Fujitmoto 1999).

In addition to the anti-inflammatory activity, FLO also displays immunomodulatory properties, giving it a potential advantage over other NSAIDs. It has a profound anti-arthritic effect in the rat adjuvant arthritis, which seems the result of its anti-inflammatory and immunomodulatory characteristics: COX and 5-lipoxygenase inhibition, decrease of pro-inflammatory cytokine production and suppression of cellular immunity (Anonymous 2002).

Compared with other NSAIDs, FLO displays very good gastric tolerance. The low gastrotoxicity may be accounted to the combined inhibitory effect of prostaglandin and leukotriene production which retain the balance between protective prostaglandin and pro-ulcerous leukotriene effects (Anonymous 2002).

2.8.2 Biotransformation of Flobufen

Phase I biotransformation studies of FLO have been carried out. *In vitro* experiments concerning different species – mouse, rat, guinea pig, rabbit, mini-pig and dog – found 4-dihydroflobufen, **4-(2´,4´-difluorobiphenyl-4-yl)-2-methyl-4-hydroxybutanoic acid, DHF** to be its major metabolite (Kvasničková *et al.* 1999, Wsól *et al.* 2001). DHF is further metabolized to a higher or lesser extent in hepatocytes (Wsól *et al.* 2001, Král *et al.* 2003, Skálová *et al.* 2003) and *in vivo* (Wsól *et al.* 2001, Král *et al.* 2004) into the final metabolite **2-(2´,4´-difluorobiphenyl-4-yl)-acetic acid, M17203**, by a process similar to the β-oxidation of fatty acids (Král *et al.* 2003), being this metabolite much more potent than FLO itself (Kuchař *et al.* 1998).

Studies concerning the chiral aspects of phase I biotransformation of FLO have also been carried out, namely the stereochemical reduction of FLO into DHF in different species.

- In rat, the metabolism of (+)-(*R*)-FLO was found to be more extensive than the metabolism of (-)-(*S*)-FLO. The main metabolites present at microsomal level were determined to be (2*R*;4*S*)-DHF, arising exclusively from (+)-(*R*)-FLO and (2*S*;4*S*)-DHF, originating solely from (-)-(*S*)-FLO. In cytosol, the reduction of FLO into DHF was not detected. Assays on primary culture of hepatocytes showed that (2*R*;4*S*)-DHF was the main metabolite formed after incubation with *rac*-, (-)-(*S*)- and (+)-(*R*)-FLO, together with another metabolite, M17203 (Wsól *et al.* 2001).

- *In vitro* studies in guinea pig showed that, in opposite to rat, reduction of FLO into DHF occurred in microsomes as well as in cytosol, although to a more extent in the microsomes. The major metabolite in both sub-cellular fractions was found to be (2*R*;4*S*)-DHF and (2*S*;4*S*)-DHF, with the same substrate preference as observed in the rat. The main metabolite detected in primary culture of hepatocytes was, in similarity to rat, (2*R*;4*S*)-DHF. Moreover, it was also detected conversion of DHF stereoisomers (Král *et al.* 2004).

- Primary culture of human hepatocytes were incubated with *rac*-, (-)-(*S*)- and (+)-(*R*)-FLO and the main metabolite was found to be (2*R*;4*S*)-DHF. Inversion of (+)-(*R*)-FLO into (-)-(*S*)-FLO was observed. Moreover, (2*S*;4*S*)-DHF and (2*S*;4*R*)-DHF reoxidated into (-)-(*S*)-FLO. Among the formed DHF stereoisomers, two characteristic inversions occurred: (2*R*;4*R*)-DHF into (2*S*;4*R*)-DHF and (2*S*;4*S*)-DHF into (2*R*;4*S*)-DHF (Skálová *et al.* 2003).

The metabolism of FLO in man is distinct from that observed in other animal species, as the final metabolite is DHF, which does not undergo the transformation into M17203 (Skálová *at al.* 2003). From this point of view, the complex metabolic study of FLO concerning human use differs from the studies in rat. While the use of FLO in human medicine is still open, its unique properties are being applied in veterinary medicine for several years, since the Virbac Company from Carros, France, obtained the license for commercialisation of FLO.

3. AIM OF THE PROJECT

The aim of this project was to elucidate the Phase II Biotransformation of the NSAID Flobufen and/or its phase I metabolites.

Rat was chosen as the target animal species because both phase I metabolites of Flobufen, DHF and M17203, arise in this species.

Hence, the main goals of this work were:

1. To determine which phase II biotransformation reactions could undergo the NSAID Flobufen, *in vitro* (incubations with microsomes, cytosol, mitochondria, isolated hepatocytes) and *in vivo* (urine and faeces);

2. To characterize the enantioselectivity of the conjugation reactions of Flobufen, *in vitro,* namely its glucuronidation;

3. To ascertain the phase II biotransformation of Flobufen metabolites, DHF and M17203, *in vitro* (microsomes, cytosol, mitochondria, isolated hepatocytes) and *in vivo* (urine and faeces);

4. To establish the full biotransformation pathway of Flobufen.

4. EXPERIMENTAL

In order to elucidate Phase II biotransformation of Flobufen and its metabolites in rat, several preliminary assays were performed, which led to a particular focus on two types of reactions:

- Glucuronidation of Flobufen and M17203 *in vitro*;

- Taurine Conjugation of M17203 *in vivo* and *in vitro*.

4.1 Materials and Methods

In this section, the different experimental methods applied in this project are detailed. Firstly, the materials and methods which were common for both sets of experiments will be described, followed by a more specific characterization of the techniques involved in each conjugation reaction.

> *Substrates*

The substrates standards, namely *rac*-FLO (*R/S* 50/50), (+)-(*R*)-FLO and (-)-(*S*)-FLO (optical purity > 99%), *rac*-DHF and M17203 were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic), gently provided by Dr. Miroslav Kuchař.

> *Chemicals*

Sigma-Aldrich (Prague, Czech Republic): UDPGA, acetyl-CoA, Ham F12 medium, William's E medium, foetal calf serum, collagen;

ICN (USA): Glycine, glutamine, taurine and ATP;

Riedel-deHaen (Germany): Dimethylsulfoxide (DMSO);

Merck (Prague, Czech Republic): Methanol and Acetonitrile (ACN) HPLC grade;

Lachema (Brno, Czech Republic): Ethylacetate (EA);

Sevapharma (Prague, Czech Republic): Collagenase

Millipore (Prague, Czech Republic): Water from the Milli-Q-RG Ultra-Pure Water System.

All the other chemicals were of the highest purity commercially available.

➤ *Animals*

This work focused on the metabolism of Flobufen in male Wistar rats (*Rattus norvegicus* var. Alba, 12-14 weeks, BioTest, Konárovice, Czech Republic). The animals were housed under a 12 h light/dark cycle, on a standard rat chow with free access to tap water.

All the experiments with the animals were carried out according to the Guide for the care and use of laboratory animals (Protection of Animals against Cruelty Act. No. 246/92 Coll., Czech Republic).

➤ *Solid phase extraction (SPE)*

The SPE cartridges (Discovery® DSC-18 LT 1.0 ml tube, 100 mg, Supelco, USA) were conditioned with 1.0 ml of methanol and 1.0 ml of monopotassium phosphate buffer (KH_2PO_4; pH 3.0; 25 mM). After loading the samples, the cartridges were rinsed with 1 ml of KH_2PO_4 (pH 3.0; 25 mM) and the retained compounds were eluted with 1 ml of methanol. The samples were then evaporated to dryness (Concentrator 5103 from Eppendorf, Medesa, Policka, Czech Republic) and reconstituted in 10% ACN and 90% water in a total volume of 150 μl.

➤ *Analytical Assessment*

The analytical assessments were conducted on a Surveyor LC system equipped with a quaternary gradient pump, an autosampler, a PDA detector and a LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA).

For the purpose of semi-quantification analysis, the PDA detector was used whereas the LCQ Advantage ion trap mass spectrometer was used for identification of the conjugates.

Mass spectrometry experiments were performed using ESI operated in negative ion mode as positive ionization showed weak or no corresponding ion. The electrospray voltage was set at -4.5 kV. The capillary voltage was set at -10 V and the temperature was maintained at 200 °C. The LC column effluent was nebulized using N_2 as sheath gas at the flow rate of 50 arbitrary units. The product ion spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with He with the relative collision energy set at 25%.

Reversed phase chromatography was run, where a Discovery® C18 150×2.1 mm I.D., 5 μm achiral column (Supelco, USA) coupled with a guard column Discovery® C18 20×2.1 mm I.D., 5 μm (Supelco, USA) was the stationary phase and the mobile phase consisted of 10 mM HCOOH (pH 5.0 ; NH_4OH) and ACN. Elution was accomplished with a gradient method: the mobile phase comprised of 30% ACN in the first 2 min followed by a linear increment to 90% until 4 min; the flow rate was changed from 300 μl/min to 200 μl/min at 4 min; an isocratic elution continued under these conditions (90% ACN and flow rate 200 μl/min) from the 4th to the 9th min. The temperature of the column compartment was set to 25 °C and the wavelength set at 275 nm for Flobufen and its possible conjugates or 240 nm for DHF/M17203 and its possible conjugates. Data acquisition and evaluation were carried out using the software Xcalibur, version 1.2. (Vienna, VA, USA). The LC conditions are summarized in Table 1.

Table 1 - LC conditions for the analysis of conjugates of flobufen and/or its metabolites.

Column	C18 150 × 2.1 mm 5 µm
Flow rate (µL/min)	
0 min	300
4 min	200
10 min	200
Wavelenght (nm)	275 for FLO and its conjugates
	240 for M17203 and its conjugates
Column temperature (ºC)	25
Injection volume (µL)	10
Gradient [a]	
0 min	70% A and 30% B
2 min	70% A and 30% B
4 min	10% A and 90% B
10 min	10% A and 90% B
Total run time (min)	10.0

[a] Mobile phase A: 10 mM formic acid (pH 5.0 ; NH$_4$OH); mobile phase B: ACN

Stereoselective HPLC analysis was employed to establish if there was no conversion of (-)-(*S*)-FLO to (+)-(*R*)-FLO, and vice versa, at all conditions tested.

The measurements were performed using a non-commercially available 150×4.6 mm I.D. 1-allyl-(5*R*,8*S*,10*R*)-terguride column (Institute of Microbiology, Academy of Sciences of Czech Republic, Prague, Czech Republic). A mixture of potassium acetate (10 mM) adjusted to pH 3.0 with acetic acid (1 M and 0.1 M, respectively) and ACN in a ratio 60:40 (*v/v*) was used as mobile phase. Retention times were 8.57 min for (-)-(*S*)-FLO, 9.71 min for (+)-(*R*)-FLO and 13.45 min for the internal standard which consisted of a methanolic solution of *S*-naproxen (c = 0.1 mg/mL). The calibration curve was linear up to 0.8 mg/mL for both (-)-(*S*)-FLO and (+)-(*R*)-FLO (Trejtnar *et al.* 1999).

4.2 Glucuronidation of Flobufen and M17203 *in vitro*

> *Isolation of microsomes*

Liver was homogenized with cold Na-phosphate buffer (pH 7.4 ; 0.1 M) in a 1:6 (*w/v*) ratio. The sub-cellular fractions were obtained by fractional ultra-centrifugation of the homogenates (Gillet 1971). A re-washing step (followed by a second ultra-centrifugation) was added at the end of the microsomes preparation procedure. Microsomes were finally resuspended in the homogenising buffer containing 20% glycerol (*v/v*) and were stored at $-80\ °C$.

Protein contents were determined by bicinchoninic acid (BCA) method (Smith *et al.* 1985).

> *Microsomal incubations*

The microsomes were pre-incubated with alamethicin, 50 µg/mg of microsomal protein, at 4 ºC for 20 min.

Standard incubation mixtures (total volume 300 µl) consisted of *rac*-FLO, (+)-(*R*)-FLO, (−)-(*S*)-FLO or M17203, 10 mM UDPGA, 1.0 mM $MgCl_2$ and Na-phosphate buffer (pH 7.4; 0.1 M). After pre-incubation of the medium at 37 °C for 2 min, the reaction started with the addition of the activated microsomal proteins, 1.0 mg/ml of reaction mixture. The assays were performed in triplicate. Total incubation time was 60 min and after this time the reaction was stopped with 0.1 ml H_3PO_4 (pH 2; 1 M) and cooling to 0 °C. All the samples were centrifuged for 10 min at 3500 g. The resulting supernatant was subjected to cleanup by solid phase extraction (SPE). Control reactions were also run and consisted of assays without UDPGA.

➢ *Kinetic Parameters Estimation*

The kinetic parameters were estimated using a non-linear least-square regression fit to the Michaelis-Menten equation or to the modified hill-substrate inhibition equation and the best model for each substrate was found on the basis of F test comparison ($P < 0.05$), by GraphPad Prism 4.00 for Windows, GraphPad Software (San Diego, California, USA, www.graphpad.com).

4.3 Taurine conjugation of M17203 *in vivo* and *in vitro*

➢ **In vivo** *studies*

The animals were kept in a glass chamber, which allowed the separation of faeces from urine. They were fasted for 12 h prior to the experiments, with free access to water.

A single oral dose of *rac*-FLO dissolved in 20% ethanol and 80% water was administered and the urine was collected after 24, 48 and 72 h and kept at –20°C.

Prior to LC-MS analysis, aliquots of urine were centrifuged (3500 g; 5 min) and the supernatant submitted to SPE procedure.

Faeces were treated as described by Král *et al.* (2004); briefly, the faeces were ground with water and acidified with 1 M H_3PO_4 to pH 1-2 and then extracted three times with EA. This extract was dried and dissolved in methanol, centrifuged (7000 g; 3 min) and the supernatant was separated. After its evaporation, it was prepared for LC-MS analysis. The aqueous phase of the extraction was centrifuged (7000 g; 3 min) and the supernatant subjected to SPE procedure.

➢ *Primary culture of hepatocytes*

The hepatocytes were obtained by a two-step collagenase method (Berry *et al.* 1991):

- in the first step, the whole liver was washed with 150-200 ml of a solution without calcium, with the purpose of removing the rest of blood and to allow the cell-cell junction to become weaker;

- in the second step, the hepatocytes were released by the action of collagenase (30 mg/100 ml) in the perfusion solution. The second perfusion lasted 4-6 min (re-circulation system). The isolated hepatocytes were rewashed three times and mixed together with the culture medium.

The culture medium consisted of a 1:1 mixture of Ham F12 and Williams' E, supplemented as described by Isom & Georgoff (1984). The viability of the cells,

measured by Trypan blue staining according to the Sigma protocol, was 75-90%. Three million viable cells in 3 ml of culture medium were placed into 60 mm plastic dishes pre-coated with collagen. The foetal calf serum was added to the culture medium (5%) to promote the cells attachment during the first four hours after plating. The medium was then exchanged with a fresh one without serum. The cultures were maintained at 37°C in a humid atmosphere of air and 5% CO_2.

➢ *MTT Test – Cytotoxicity Test*

MTT assay is a quantitative colorimetric assay for mammalian cell survival and cell proliferation. It has been widely used as a rapid and sensitive method for screening anticancer drugs as well as for the assessment of cytotoxicity of several substances.

MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, is a yellow salt which is metabolized by the mitochondria of living cells. Once metabolized, MTT is converted into blue formazan and the absorbance is measured; the amount of formazan produced is proportional to the number of living cells present in culture.

In order to evaluate the cytotoxicity of Flobufen and its metabolites, MTT test was performed on primary culture of hepatocytes.

➢ *Incubation of primary culture of hepatocytes*

The primary culture of hepatocytes was incubated with 50 and 100 μM *rac*-FLO, (+)-(*R*)-FLO and (-)-(*S*)-FLO. Aliquots of medium (0.5 ml) were collected at different times - 2, 4, 8 and 24 h - and submitted to SPE procedure prior to LC analysis.

> *Isolation of intact mitochondria*

The isolated liver was homogenized with 6 volumes of cold 0.25 M sucrose/10 mM Tris-HCl buffer pH 7.4. The homogenates were centrifuged at 700 g for 15 min and the supernatant subsequently centrifuged at 9700 g for 20 min.

The obtained mitochondrial pellet was resuspended in 0.2 M Tris-HCl buffer pH 8.5 and immediately used.

Protein contents were determined by the bicinchoninic acid (BCA) method (Smith *et al.* 1985).

> *Incubation of intact mitochondria*

The incubation mixture (total volume 350 μl) consisted of substrate in a concentration range 0.02-0.45 mM, 6 mM ATP, 1 mM acetyl-CoA, 20 mM amino acid (taurine, glycine or glutamine) and of 1 mM $MgCl_2$. After pre-incubation of the medium at 37 °C for 2 min, the reaction was started with the addition of the mitochondria, 1.2-1.4 mg protein/assay. Total incubation time was 45 min and after this time the reaction was stopped with 1 M H_3PO_4 (0.1 ml) and cooling to 0 °C. The precipitated proteins were removed by centrifugation for 5 min at 3500 g. The resulting supernatant was subjected to cleanup by SPE prior to LC analysis. Control reactions were also run and consisted of assays without substrate or assays without biological matrix.

5. RESULTS

5.1 Glucuronidation of Flobufen and M17203 *in vitro*

5.1.1 Optimization of incubation conditions - UGT activity

Incubation conditions for glucuronidation by microsomes usually include a detergent to disrupt the membrane barrier, and a divalent metal ion.

In order to determine the activity of UGT, preliminary spectrophotometric measurements of glucuronidation of p-nitrophenol was carried out. Substrate was incubated with native microsomes, SLOVASOL activated microsomes and alamethicin activated microsomes. Results show that UGT activity was 14 times higher with microsomes activated by alamethicin and 9 times higher with SLOVASOL activated microsomes, when compared with native microsomes.

UGT activity is also known to increase when Mg^{2+} is present in the incubation medium (Fisher *et al.* 2000) and it has been reported that the concentration of this ion inside the endoplasmic reticulum (ER) is approximately 1mM (Berg *et al.* 1995).

Based on these results, pre-treatment of microsomes with alamethicin, 50 μg per mg of protein – was chosen as standard incubation procedure. In addition, 1.0 mM Mg^{2+} was also included in all incubation mixtures.

5.1.2 HPLC Analysis

It is known that for the great majority of 2-APAs unidirectional chiral inversion of the (+)-(*R*)-enantiomer to the (-)-(*S*)-enantiomer occurs. This reaction involves the participation of CoA, where it plays a major role and it is thought to be catalyzed by long chains acyl-CoA synthetases (Caldwell *et al.* 1988, Hall & Xiaotao 1994, Brugger *et al.* 2001).

In preliminary studies using stereoselective conditions of HPLC analysis of Flobufen glucuronidation, it was shown that no chiral inversion occurs between its enantiomers

under the incubation conditions above mentioned. For this reason, it was chosen to perform all measurements under the achiral conditions referred in Materials and Methods section.

5.1.3 Identification of Glucuronides by LC-MS/MS

The glucuronides were eluted within 5 to 6 min (Figures 24 and 25). Due to a lack of reference material, relative glucuronidation rates were determined as LC-UV peak areas. *Rac*-FLO, (+)-(*R*)-FLO, (−)-(*S*)-FLO and M17203 as substrates were used in the microsomal incubations in a concentration range of 0.01 − 0.50 mM. All the four substrates were found to be glucuronidated. The chemical structures of the glucuronides are depicted in Figures 22 and 23.
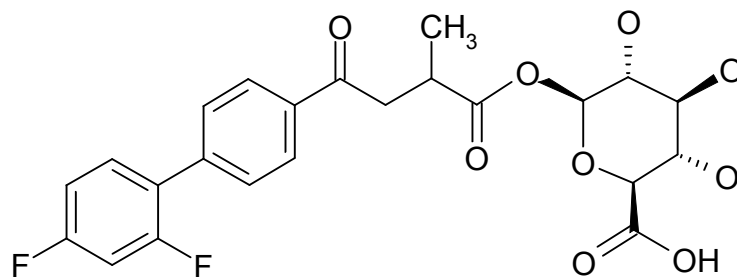

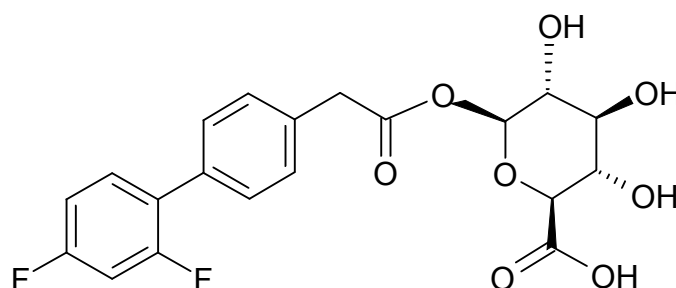
Figure 22 − Chemical structure of FLO-glucuronide.

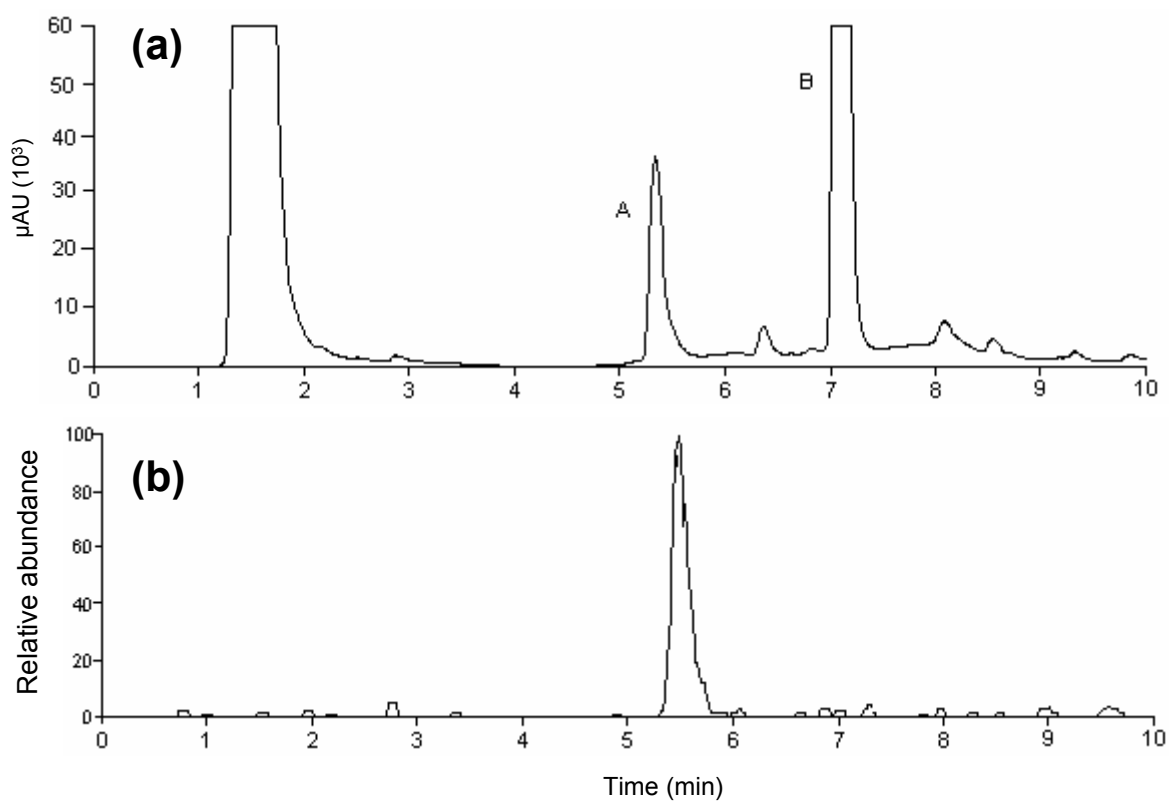Figure 23 − Chemical structure of M17203-glucuronide.

Figure 24 - a) Chromatographic record of microsomal incubation of FLO with UDPGA – peak A
(RT 5.34 min): FLO-glucuronide ; peak B (RT 7.08 min): FLO and b) MS record of extracted ion *m/z*
479, corresponding to FLO-glucuronide.
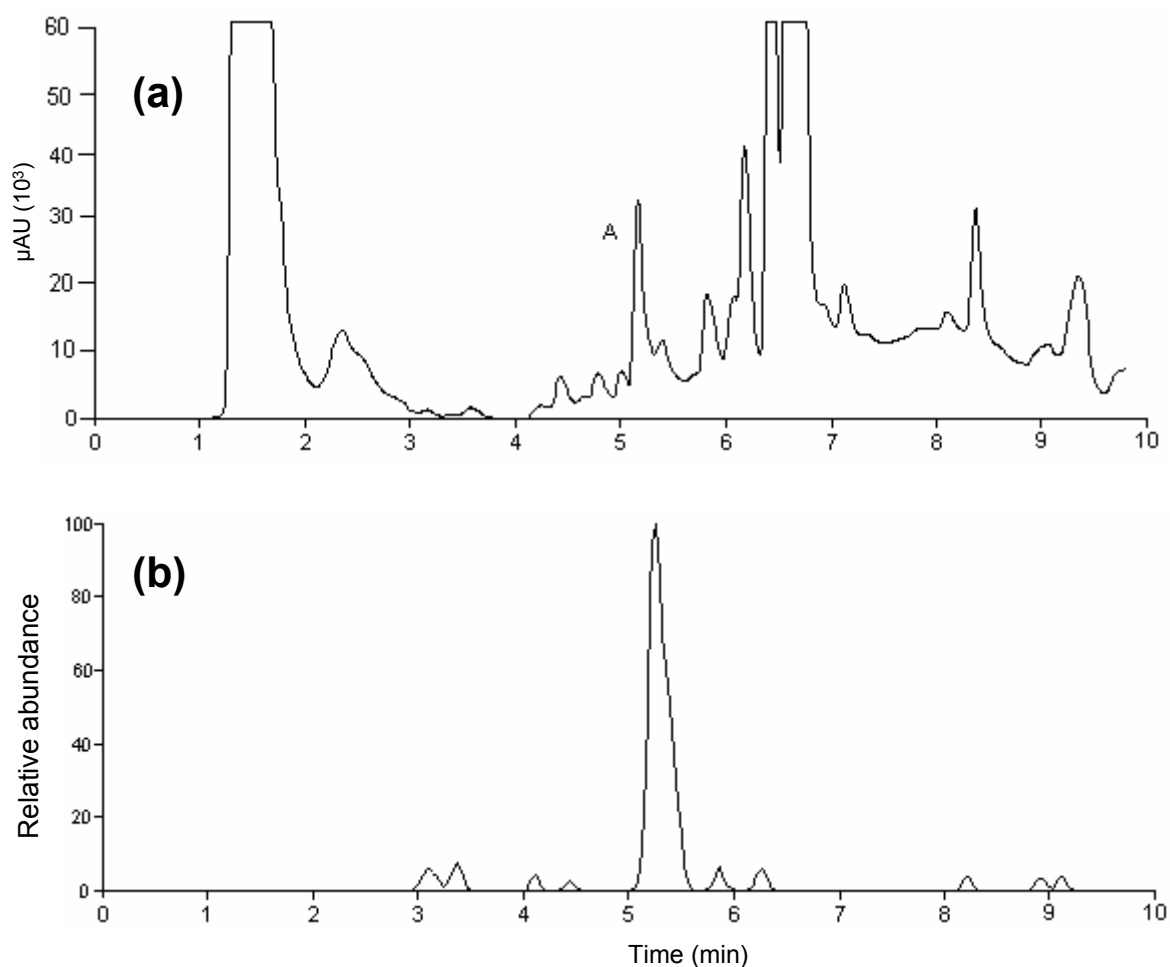
Figure 25 - a) Chromatographic record of microsomal incubation of M17203 with UDPGA - peak A (RT 5.28 min): M17203-glucuronide and b) MS record of extracted ion *m/z* 423, corresponding to M17203-glucuronide.

The ESI mass spectra of the peak typically formed after incubation of flobufen or M17203 with UDP-glucuronic acid in rat liver microsomes (Figures 26 and 27) showed a deprotonated molecule at *m/z* 479 and *m/z* 423 ([M-H]⁻), respectively, indicating the molecular weight of the metabolites as 480 and 424, respectively, corresponding to flobufen and M17203 glucuronides. The product ion spectrum of *m/z* 479 showed ions at *m/z* 303 and *m/z* 193 corresponding to deprotonated flobufen and deprotonated glucuronic acid, respectively. The single product ion at *m/z* 193

corresponding to deprotonated glucuronic acid was observed in the product ion spectrum of *m/z* 423.

Figure 26 - Product ion spectra and proposed fragmentation scheme of the [M-H]⁻ ion at *m/z* 479 of FLO-glucuronide; (a) ESI-MS spectrum and (b) ESI-MS/MS spectrum.

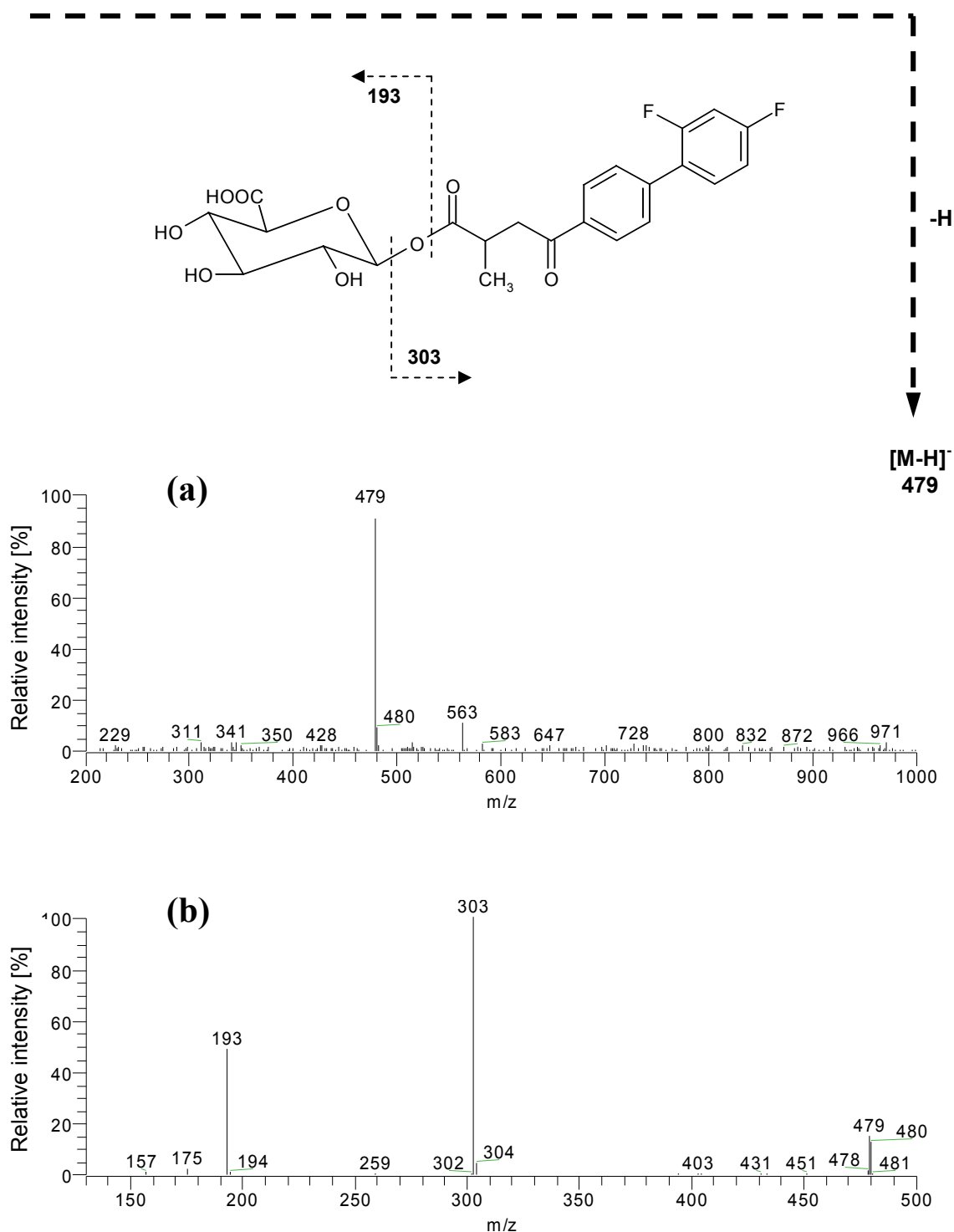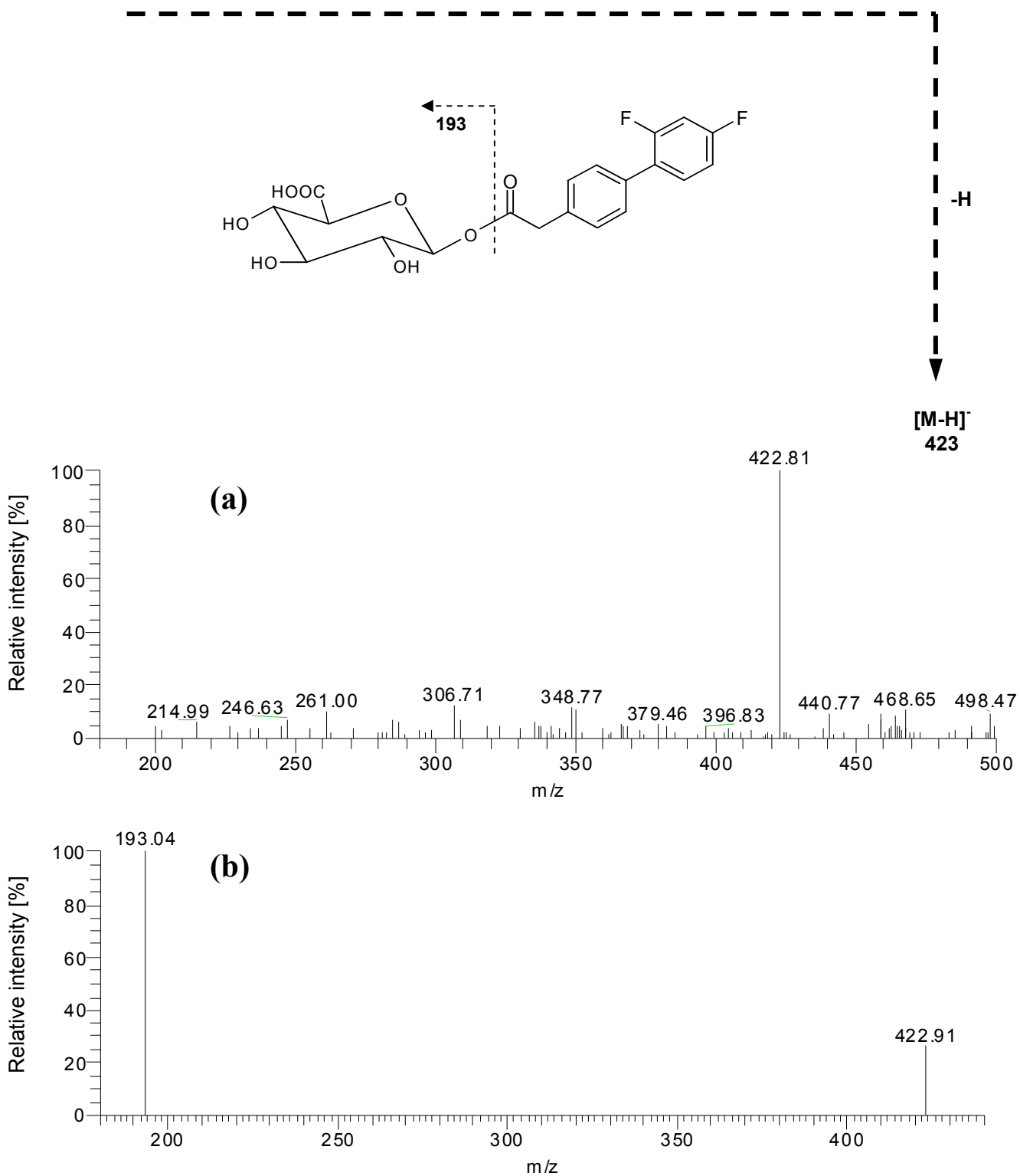Figure 27 - Product ion spectra and proposed fragmentation scheme of the [M-H]⁻ ion at *m/z* 423 of M17203-glucuronide; (a) ESI-MS spectrum and (b) ESI-MS/MS spectrum.

Glucuronidation of all four diastereoisomers of DHF was also studied at different incubation conditions, but no glucuronide formation for any diastereoisomer was detected under the analytical conditions used.

5.1.4 Glucuronidation of FLO Enantiomers: Empirical Kinetic Model

Glucuronidation of (−)-(S)-FLO and *rac*-FLO followed a Michaelis-Menten model (Figure 28) and the kinetic parameters were calculated using equation 1

$$v = \frac{V\max[S]}{Km + [S]} \tag{1}$$

where v is the rate of the reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant (substrate concentration at $V_{max}/2$) and $[S]$ is the substrate concentration.

On the other hand, glucuronidation of (+)-(R)-FLO exhibited atypical kinetics (Figure 28), namely substrate inhibition kinetics. In this type of inhibition, high concentrations of substrate lead to a decrease in the rate of product formation. The model of substrate inhibition kinetics is described by equation 2 (Gangloff *et al.* 2001)

$$v = \frac{V\max[S]}{Km + [S]\cdot\left(1 + \frac{[S]}{Ks}\right)} \tag{2}$$

where K_s is the constant describing the substrate inhibition interaction. At low concentrations of substrate, i.e. when $[S] << K_s$, the $[S]/K_s$ term becomes negligible and this equation changes into equation (1), and therefore Michaelis-Menten kinetics is observed for the first part of the plot.

However, using this model, it was not possible to obtain a good fit of the data (based on 95% confidence intervals, r^2), yielding physically unrealistic values for all kinetic parameters.

For this reason, it was necessary to explore other models and find a suitable equation which could give a good fit to the experimental data for this type of substrate inhibition. The Hill equation (Cornish-Bowden 1995)

$$v = \frac{V\max[S]^{nH}}{S_{50}^{nH} + [S]^{nH}}$$

(3)

where S_{50} is the substrate concentration resulting in 50% of V_{max} (analogous to K_m in the previous equations) and nH is the Hill coefficient, describes sigmoidal kinetics, typical for allosteric enzymes.

Evaluating the experimental data and truncating the data to the lower substrate concentrations, it was possible to verify that the Hill equation displayed the best fit, with a value of nH=3.2.

Based on this assumption and relating to the inter-conversion of equation 1 into equation 2, a combined Hill-substrate inhibition equation was generated, which ultimately obtained the best fit for the experimental data:

$$v = \frac{V\max[S]^{nH}}{Km^{nH} + [S]^{nH}\left(1 + \frac{[S]^{nH}}{Ks^{nH}}\right)}$$

(4)

The fitted parameters for this model closely match the values that would be derived by visual inspection. $K_m = 16.5 \pm 0.5$ μM and $K_s = 192.0 \pm 30.3$ μM are comparable to the [S]/2 values for the ascending and descending arms of the curve, respectively. The fitted

Hill coefficient (*nH*=4.0) also agrees with the one obtained from the Hill plot. All calculated kinetic parameters are resumed in Table 2.
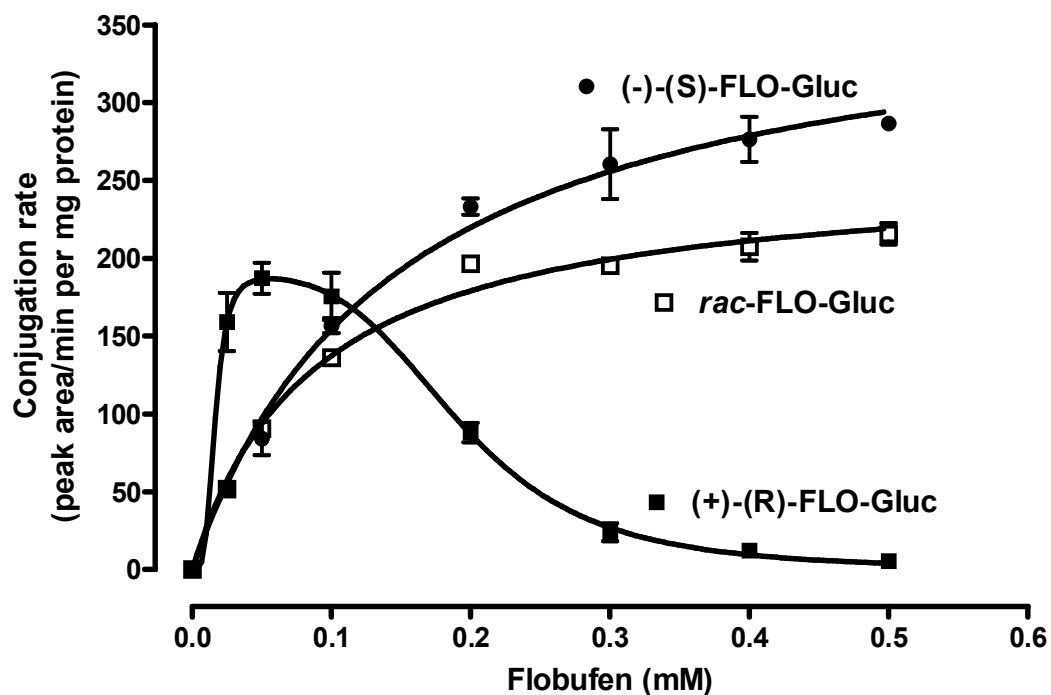
Figure 28 - Kinetic profiles of FLO-glucuronide formation from *rac*-FLO, (+)-(*R*)-FLO and (–)-(*S*)-FLO catalyzed by rat liver microsomes. Values are given as mean ± S.D. of triplicates.

Table 2 - Apparent kinetic parameters for O-glucuronidation of rac-FLO, (+)-(R)-FLO, (–)-(S)-FLO and M17203 in rat liver microsomes. Values of V_{max} are given in peak area/min per mg of protein; K_m, K_s and S_{50} in μM. (a) Fits to the Hill equation for (+)-(R)-FLO using truncated data.

	(-)-(S)-FLO-Gluc	rac-FLO-Gluc	(+)-(R)-FLO-Gluc	M17203-Gluc
Michaelis-Menten				
V_{max}	380.5 ± 15.8	257.3 ± 9.7	–	–
K_m	146.0 ± 16.7	87.2 ± 11.2	–	–
r^2	0.995	0.992	–	–
Hill equation (a)				
V_{max}	–	–	194.4 ± 8.2	38.0 ± 4.3
nH	–	–	3.2 ± 0.4	1.6 ± 0.4
S_{50}	–	–	16.3 ± 0.7	378.0 ± 44.3
r^2	–	–	0.997	0.991
Empirical model				
V_{max}	–	–	189.6 ± 2.5	–
nH	–	–	4.0 ± 0.2	–
K_m	–	–	16.5 ± 0.5	–
K_s	–	–	192.0 ± 30.4	–
r^2	–	–	0.999	–

5.1.5 Glucuronidation of M17203

Glucuronidation of M17203 also displayed atypical kinetics (Figure 29) and best fitted to the Hill model – equation 3 - with a value of nH=1.6. The calculated kinetic parameters are summarized in Table 2.
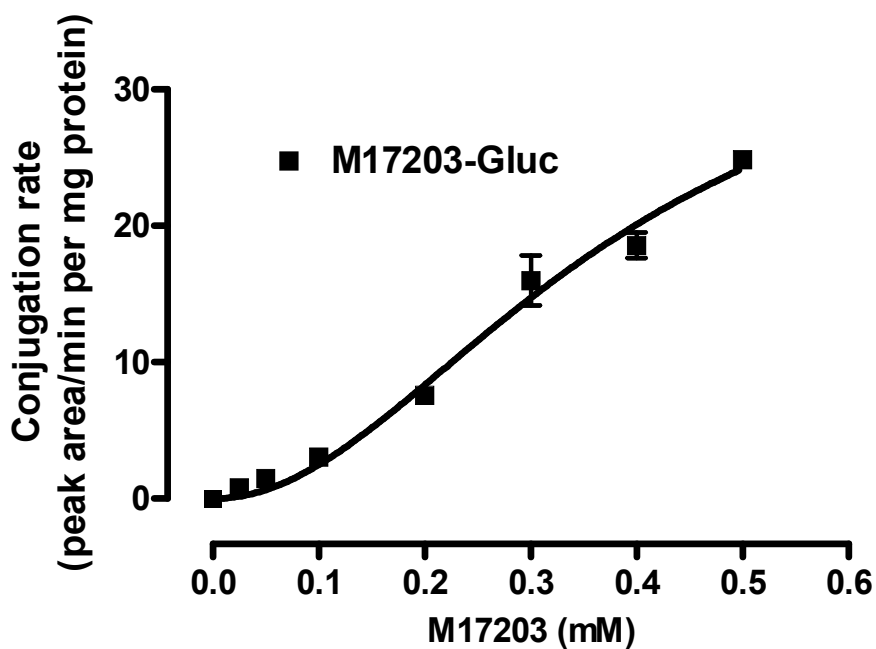
Figure 29 - Kinetic profile of M17203 glucuronide formation catalyzed by rat liver microsomes. Values are given as mean ± S.D. of triplicates.

5.2 Taurine conjugation of M17203 *in vivo* and *in vitro*

5.2.1 *In vivo* experiments

After administration of a single oral dose of 50 mg/kg of *rac*-FLO to rats, urine was collected at 24, 48, 72 h and only one conjugate was found to be excreted in rat urine (Figures 30 and 31). Regarding to faeces, no phase II metabolite of FLO was detected.
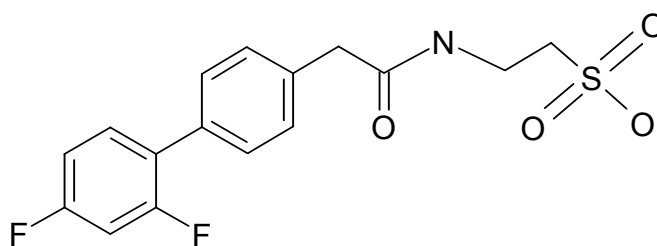
Figure 30 – Chemical structure of M17203-TAU.
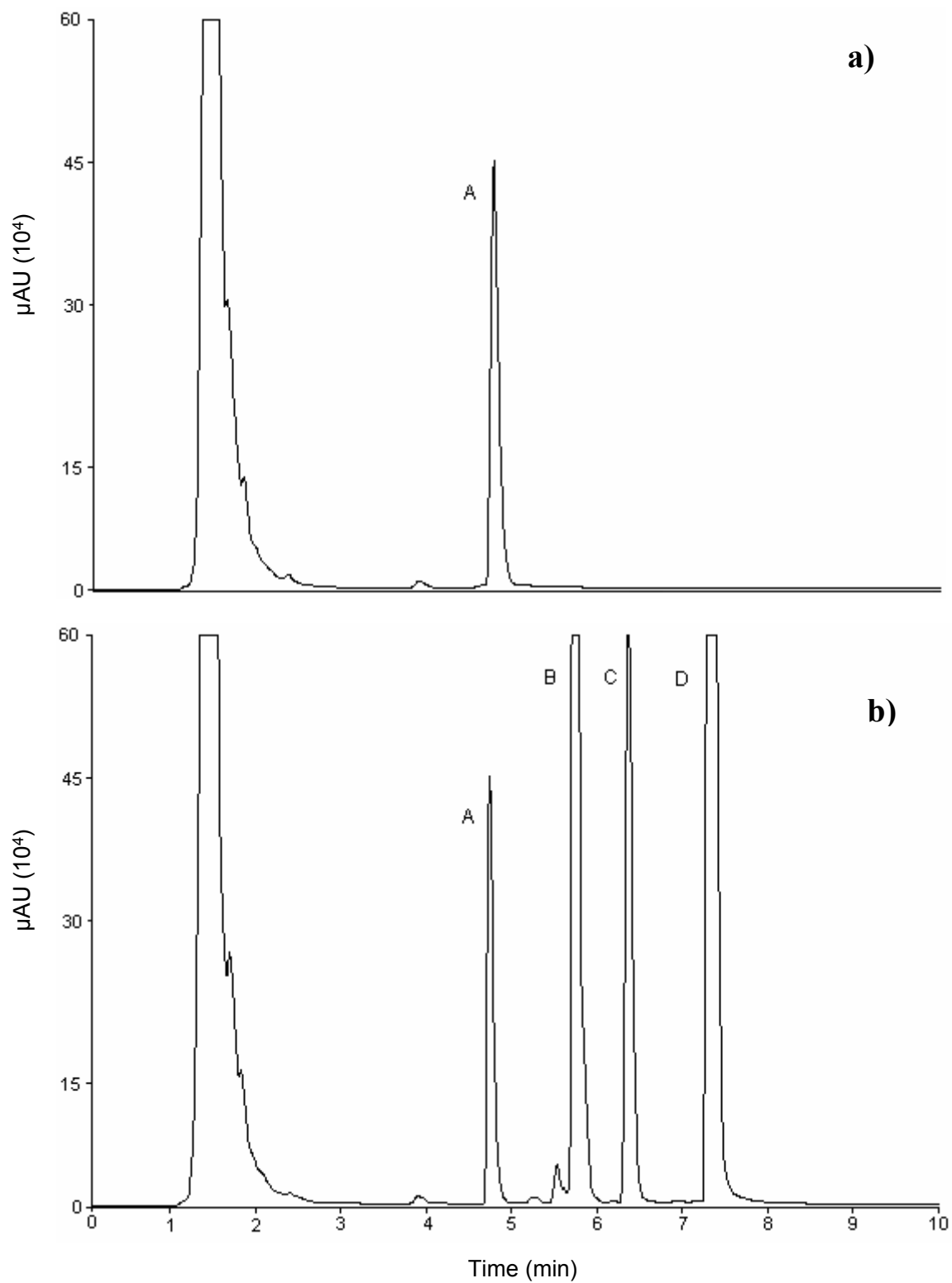
Figure 31 - Chromatographic record of sample of rat urine 24h-collection. a) non-spiked sample; b) sample spiked with standards (M17203, *rac*-FLO, *rac*-DHF). A: M17203-TAU; B: M17203; C: *rac*-FLO; D: *rac*-DHF.

5.2.2 Incubation with primary culture of rat hepatocytes

In incubations with primary culture of hepatocytes, *rac*-FLO, (+)-(*R*)-FLO and (-)-(*S*)-FLO were chosen as substrates and two concentrations of each substrate were used: 50 and 100 μM. A typical chromatogram of medium sample after 24h-incubation is demonstrated in Figure 32.

FLO was almost completely metabolized to its phase I metabolites, especially into M17203 and, to a less extent, into DHF. Furthermore, a new metabolite was identified, which corresponded to a conjugate of M17203 with taurine.

No significant difference was found in the formation of the conjugate after incubation with *rac*-FLO, (+)-(*R*)-FLO, (-)-(*S*)-FLO (Figure 33).
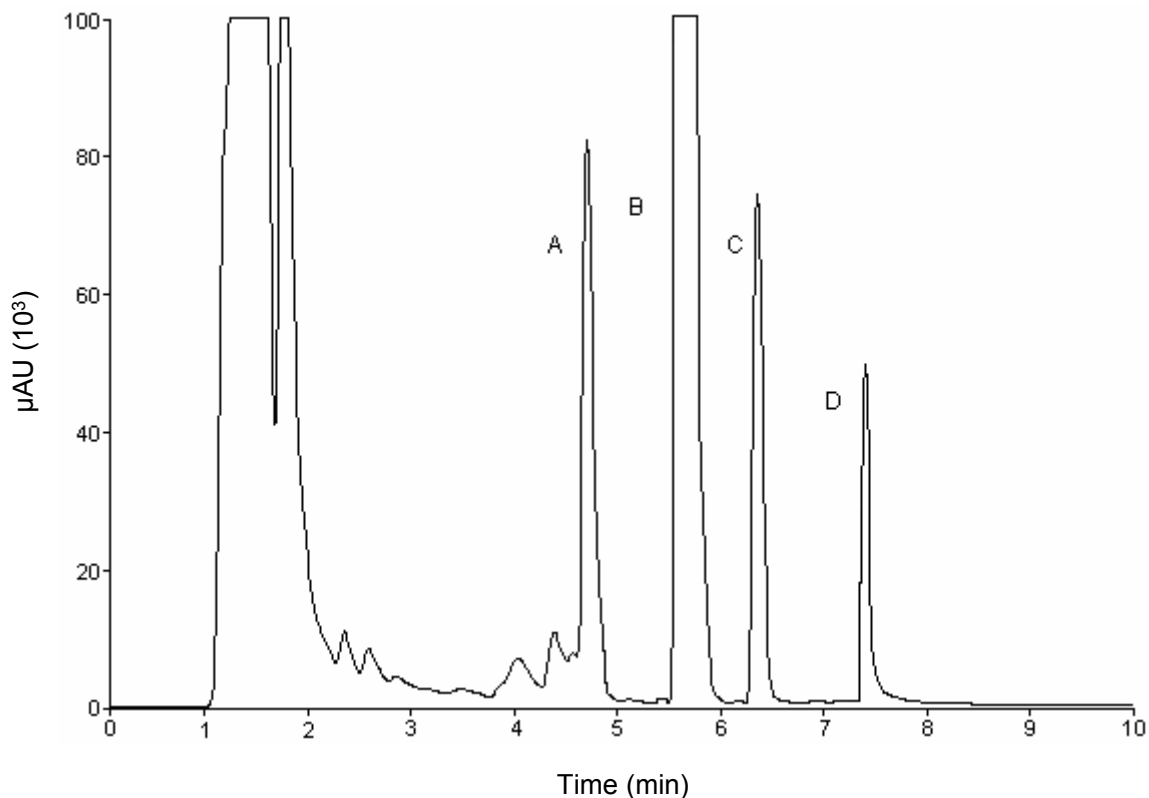
Figure 32 - Chromatographic record of incubation of *rac*-FLO with taurine in primary culture of hepatocytes, 24h-collection.
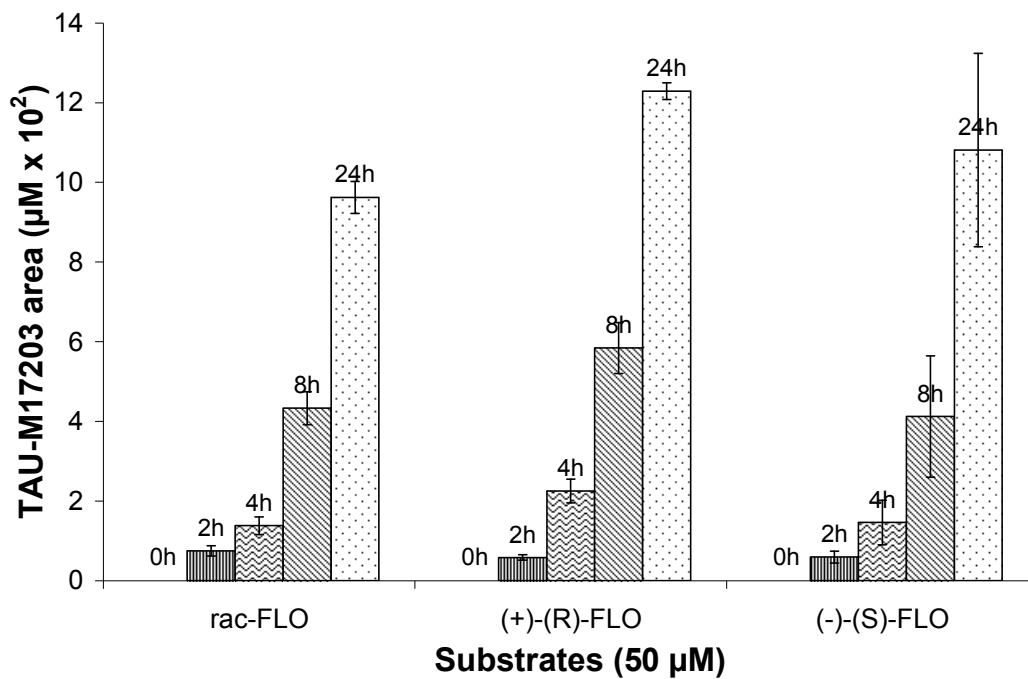
Figure 33 - Evaluation of the formation of M17203-TAU in primary culture of hepatocytes.

5.2.3 Incubation with intact rat mitochondria

Fresh intact mitochondria were incubated with the substrates tested, acetyl-CoA and amino acid (taurine, glycine or glutamine). The conjugate was found only in incubates of M17203 with taurine (Figure 34). Other possible conjugates, with different amino acids (glycine and glutamine) as well as with the other possible substrates (FLO and DHF) were not detected under the used analytical conditions.
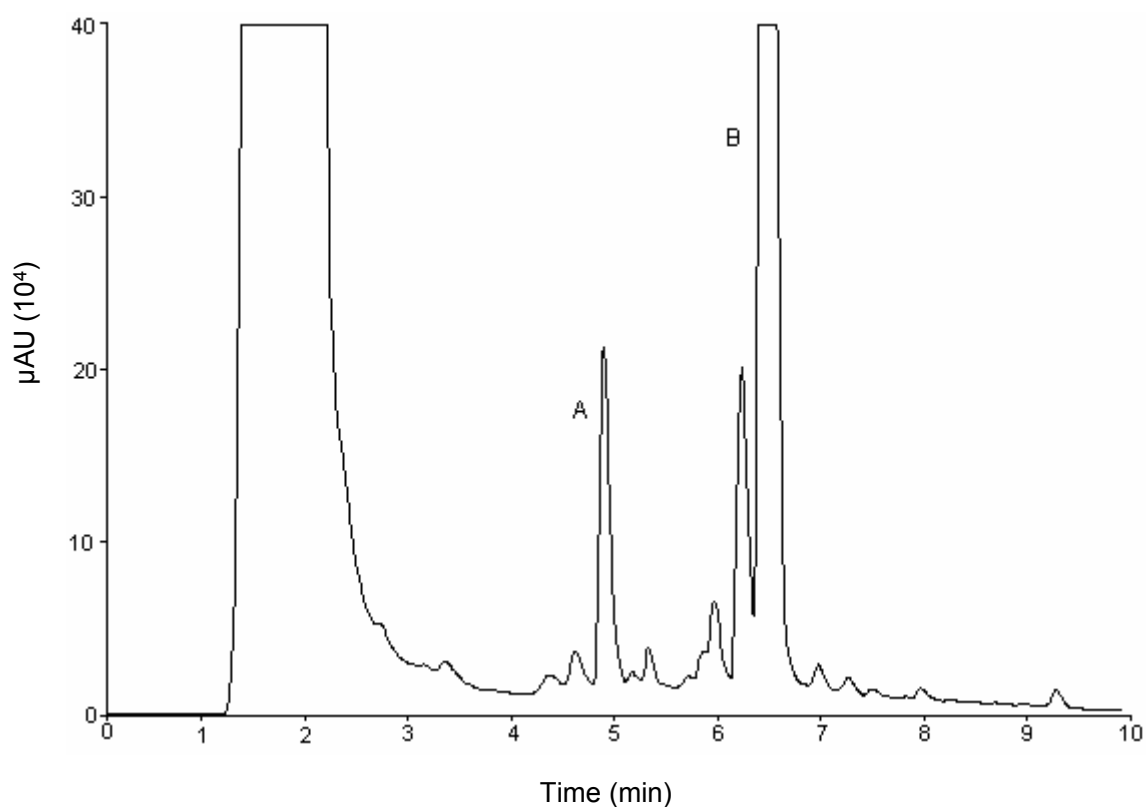
Figure 34 - Chromatographic record of mitochondrial incubation of M17203 with taurine. A: M17203-TAU; B: M17203.

5.2.4 Kinetics of M17203 conjugation with taurine

Seven concentrations of M17203 as substrate were used in the mitochondrial incubations: 0.020, 0.035, 0.050, 0.100, 0.200, 0.300 and 0.450 mM. It was determined that M17203 is conjugated with taurine. M17203-TAU was eluted within 4 to 5 min. Due to a lack of reference material, relative conjugation rates were determined as LC-UV peak areas. The best kinetic model of conjugation, a Michaelis-Menten plot, was found on the basis of F test comparison ($P < 0.05$) (Figure 35), and the kinetic parameters were $V_{max} = 10.66 \pm 0.43$ AUC/min/mg protein; $K_m = 71.0 \pm 8.8$ µM.
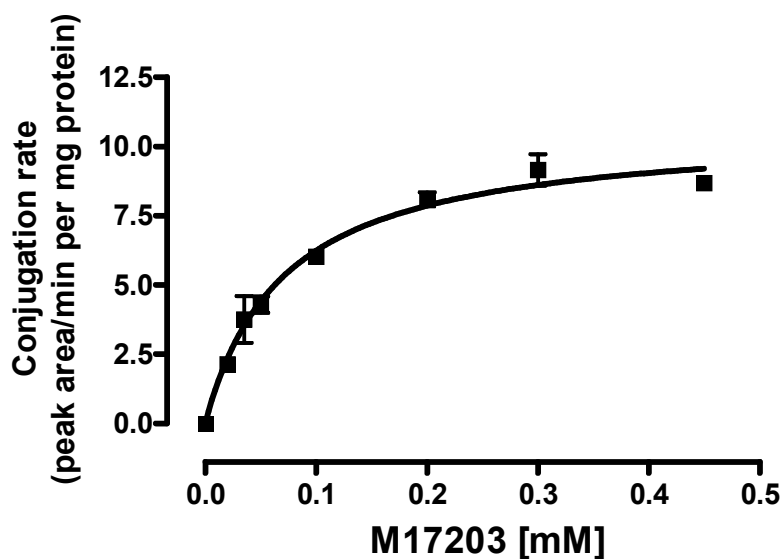
Figure 35 - Kinetic profile of TAU-M17203 formation in mitochondrial incubations. $V_{max} = 10.66 \pm 0.43$ AUC/min/mg protein; $K_m = 71.0 \pm 8.8$ µM; Values are given as mean $\pm$ S.D. from n = 4 to 6 experiments.

5.2.5 Identification of M17203-TAU by LC-MS/MS

The ESI mass spectra of the peak typically formed after incubation of M17203 with taurine in rat liver mitochondria, after incubation of primary culture of rat hepatocytes with *rac*-FLO, (+)-(*R*)-FLO and (-)-(*S*)-FLO, and SPE treated urine collected after administration of *rac*-FLO, showed a deprotonated molecule at *m/z* 354 indicating the molecular weight of the metabolite as 355 corresponding to M17203 conjugate with taurine. The single product ion at *m/z* 124 corresponding to deprotonated taurine was observed in the product ion spectrum of *m/z* 354 (Figure 36).
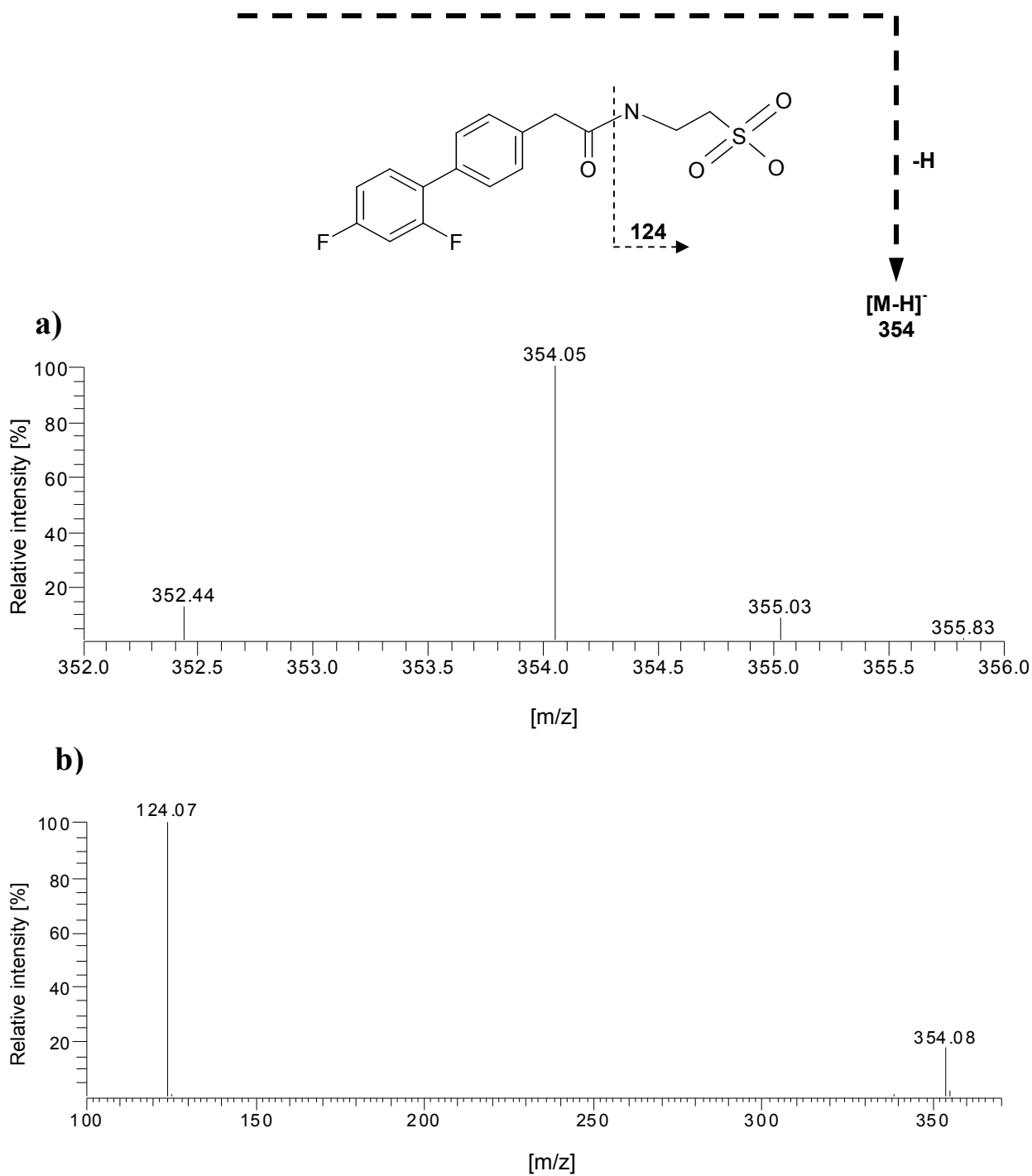
Figure 36 - Product ion spectra and proposed fragmentation scheme of the [M-H]⁻ ion at *m/z* 354 of M17203-TAU; a) ESI-MS spectrum and b) ESI-MS/MS spectrum.

6. DISCUSSION

6.1 Glucuronidation of Flobufen and M17203 *in vitro*

The stereoselectivity of glucuronidation *in vitro* of 2-arylpropionic acids has been previously studied and concluded that the enantioselectivity was both substrate and species dependent. For instance, considering naproxen, the *S*-enantiomer is preferentially glucuronidated in rabbits, opposite to rat and monkey, where the *R*-enantiomer was selectively conjugated. In humans, the ratio of *S/R*-naproxen glucuronidation was close to 1 (El Mouelhi *et al.* 1987, 1993). Other authors have reported that the formation of *S*-glucuronide of different profens *in vivo* (benoxaprofen, carprofen, flunixaprofen) was more than two times higher than the formation of the *R*-glucuronide (Spahn *et al.* 1989, Iwakawa *et al.* 1991).

The assays performed in this study determined that, with the exception of DHF, all the other substrates are conjugated *in vitro* with glucuronic acid. However, but interestingly, the conjugation of the different substrates followed different kinetics, determining not only stereoselective glucuronidation of the different enantiomers, but suggesting also a possible implication of different UGT isoforms catalyzing the glucuronidation of the different substrates.

(−)-(*S*)-FLO displayed a typical Michaelis-Menten kinetics, whereas (+)-(*R*)-FLO exhibited substrate inhibition kinetics. This type of deviation is not so uncommon, although its mechanism is still unclear. Several models of substrate inhibition have been proposed, which include allosteric mechanisms (LiCata & Allewell 1997), enzymatic chemical oscillations (Shen & Larter 1994) and the Recovery Model (Kuhl 1994). Nevertheless, the experimental data obtained did not fit to any of the known models and it was necessary to derive an equation that could interpret the data.

The generated equation (equation 4) combines a Hill equation with substrate inhibition model.

When truncating the data for (+)-(*R*)-FLO to the lower concentrations, it was possible to verify that this part of the plot assumed a sigmoidal shape, i.e., followed the Hill equation. This equation is a useful mathematical tool to describe the degree of sigmoidicity of the substrate concentration/enzyme activity relationship. It was originally proposed to provide an indication of the number of subunits in a multimeric enzyme that bound successive ligands in a cooperative manner (Williams *et al.* 2002). The Hill coefficient value for the UGT isoform responsible for glucuronidation of (+)-(*R*)-FLO in the current set of experiments was around 4.0. Some authors have demonstrated that some isoforms of UGT exist as tetramers (Peters & Jansen 1986) and as dimers (Meech & Mackenzie 1997). Moreover, other studies suggest that some isoforms possess more than one active site (Rios & Tephly 2002). Based on these studies, it can be assumed that the UGT isoform implicated in (+)-(*R*)-FLO glucuronidation behaves as a cooperative ligand-binding multisubunit enzyme. When analysing the set of data as a whole, in opposition to a cooperative behaviour for lower substrate concentrations, substrate inhibition was observed for higher concentrations of substrate.

As it was not in the scope of this work, the empirical model here exposed needs further investigations in order to better explain the mechanisms of this particular type of substrate inhibition.

Analysing the kinetic parameters values for both enantiomers of flobufen, and considering only the truncated fitted values for (+)-(*R*)-FLO, it should be said that the *R*-enantiomer is the preferential substrate for glucuronidation, at lower concentrations, as its S_{50} (analogous to K_m) is about 9-fold higher than the K_m for the *S*-enantiomer. However, the overall view clearly suggests (−)-(*S*)-FLO as the elected substrate for glucuronidation, as for higher concentrations, the *R*-enantiomer exhibits substrate

inhibition, as already referred. The fitted kinetic values for *rac*-FLO are, in fact, about the average of the kinetic values of both enantiomers.

Glucuronidation of M17203 occurs to a lesser extent when compared to the other substrates studied, nevertheless it can be assumed that another UGT isoform different from the ones catalyzing the enantiomers of flobufen is implicated in this reaction, as it follows the Hill model with *nH*=1.6. The same explanation earlier given for the kinetics at lower concentration of (+)-(*R*)-FLO can be extended to the kinetics of M17203.

6.2 Taurine conjugation of M17203 *in vivo* and *in vitro*

A conjugate of M17203 with taurine was identified as the sole conjugate excreted after oral administration of *rac*-FLO. Moreover, *in vitro* incubations showed that M17203 is readily conjugated with taurine in intact mitochondria and in the primary culture of rat hepatocytes. The formation of this conjugate was not stereoselective as no significant difference was found after incubations with *rac*-FLO, (+)-(*R*)-FLO or (-)-(*S*)-FLO.

The kinetics of M17203-TAU formation was also studied: mitochondrial incubations with different concentrations of substrate were performed and fitted a Michaelis-Menten plot.

Conjugation of M17203, a biphenylacetic acid derivative, with an amino acid was not presumed as the major outcome in Flobufen Phase II biotransformation. Taurine conjugation is an important metabolic route for xenobiotic carboxylic acids; however, it usually represents only a small amount of the total metabolism in rats (Kanazu & Yamaguchi 1997). It has also been considered that bulky substituents close to the carboxy groups of aromatic and arylacetic acids cause steric hindrance of the attack on the carboxyl group by ATP or acetyl-CoA and consequently, to a failure of undergoing amino acid conjugation (Dixon *et al.* 1977). The same is referred to biphenylacetic acids, which in literature are said to unlikely be conjugated with amino acids, being glucuronidation the major pathway of biotransformation (Idle *et al.* 1978, Casarett & Doull 2001). In light of what was mentioned earlier, it had been supposed that a glucuronide should be the major way for excreting FLO, but the results show us that the taurine conjugate is the main metabolite.

The pathway of amino acid conjugation involves 2 steps: in the first step, the substrate is activated to an acyl-CoA thioester. This reaction requires ATP and is

catalyzed by acyl-CoA synthetase. In the past years, medium chain acyl-CoA synthetases have been isolated and characterized, which are thought to be implicated in amino acid conjugation (Vessey & Hu 1995, Kasuya *et al.* 1996, Kasuya *et al.* 1999). The second step is catalyzed by acyl-CoA:amino acid *N*-acetyltransferases, which transfers the acyl moiety of the xenobiotic to the amino group of the acceptor amino acid. The enzyme acyl-CoA:glycine *N*-acetyltransferase is known to occur in two distinct forms that have been purified to homogeneity and characterized extensively (Webster *et al.* 1976, Nandi *et al.* 1979, Kelley & Vessey 1992, Kelley & Vessey 1993).

Few reports concerning conjugation of profens with amino acids have been published. The majority of them are related with taurine, and these conjugates have been found as a metabolite for suprofen (Sakai *et al.* 1984, Mori *et al.* 1985), and for trans-hydroxyloxoprofen (Tanaka *et al.* 1983) in dog urine. Shirley *et al*. (1994) reported a taurine conjugate with ibuprofen in human urine. Egger *at al.* (1982) is among the few authors that reported a taurine conjugate in rat and mouse urine, in this case, a conjugate of pirprofen pyrrole.

7. CONCLUSIONS

The outcome of the experiments carried out in this project can be summarized as follows:

1. Flobufen is conjugated with glucuronic acid, *in vitro*, in rat microsomes, which structure was resolved by LC-MS. The other possible phase II reactions were shown not to occur with Flobufen. Urine and faeces samples did not reveal the excretion of any conjugate of flobufen;

2. It was demonstrated that flobufen, like many other known NSAIDs, is selectively glucuronidated in rat *in vitro*. The study was performed with pure individual enantiomers employing achiral chromatographic conditions because no chiral inversion was observed when using chiral chromatography. Furthermore, a unique feature was found as the individual enantiomers of flobufen followed different kinetic profiles; while (−)-(*S*)-FLO displayed Michaelis-Menten type of kinetics, (+)-(*R*)-FLO followed a particular type of substrate inhibition model. The fitted kinetic values suggest that, for lower concentrations, (+)-(*R*)-FLO is the primary substrate for glucuronidation and following the increase of its concentration, where inhibition is exhibited, (−)-(*S*)-FLO is taken as substrate;

3. M17203 was proved to be conjugated with glucuronic acid, *in vitro*, in rat microsomes. Moreover, *in vitro* experiments performed on mitochondria and primary culture of hepatocytes revealed yet another phase II metabolite, a conjugate with the amino acid taurine. Also, we have identified the conjugate of M17203 with taurine as the prime phase II metabolite of Flobufen in rat *in vivo* as it was the sole metabolite excreted in urine after *p.o.* administration of

flobufen. Both phase II metabolites of M17203 were isolated and identified by

LC-MS. No phase II metabolites of DHF were detected.

4. The complete pathway of Flobufen biotransformation is depicted in Figure 37.
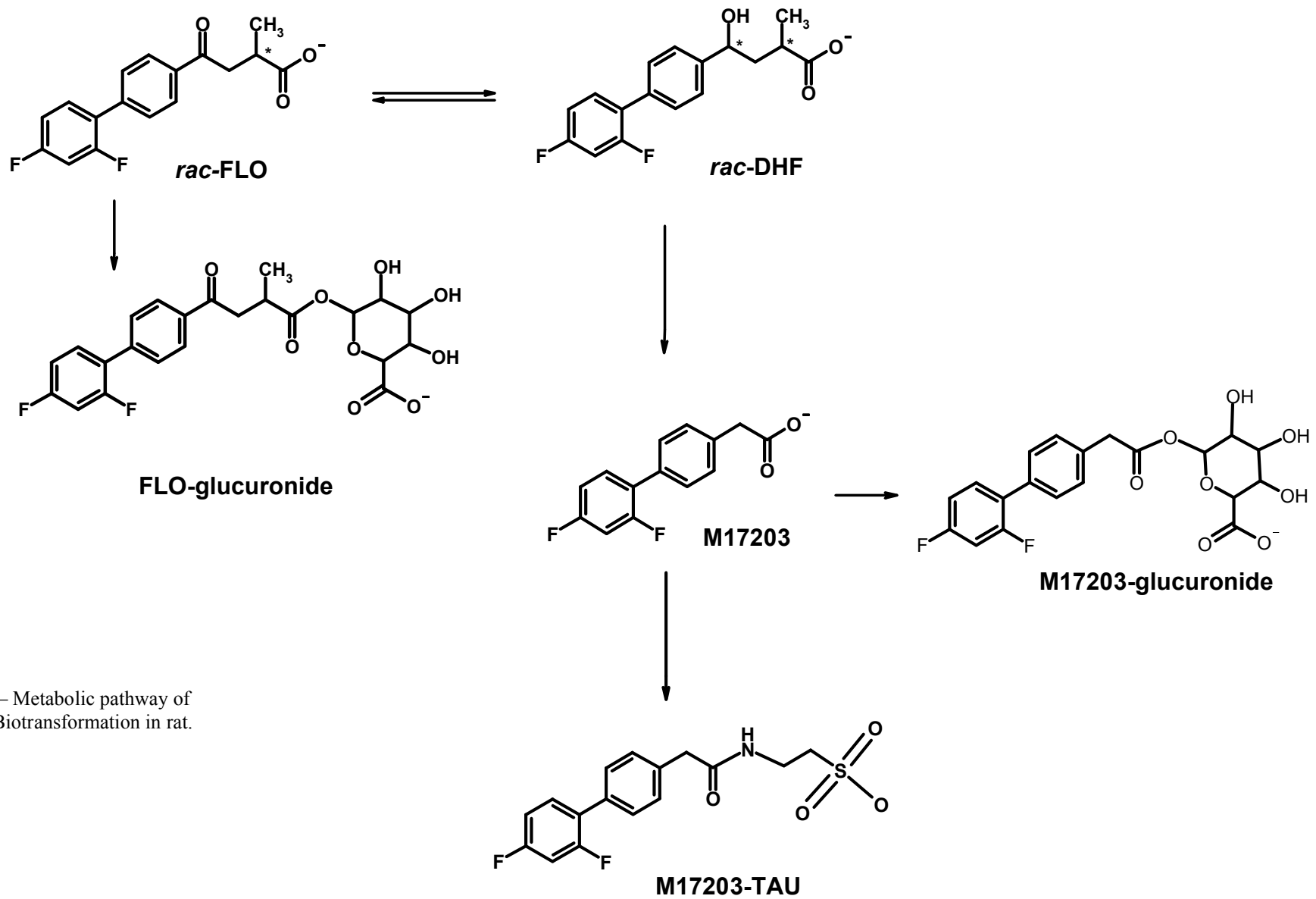
Figure 37 – Metabolic pathway of Flobufen Biotransformation in rat.

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WSOL V, KRAL R, SZOTAKOVA B, TREJTNAR F, FLIEGER F (2001). Stereospecificity and stereoselectivity of flobufen metabolic profile in male rats in vivo and in vitro: phase I of biotransformation. *Chirality* **13**, 754-759.

9. ATTACHMENTS

9.1 List of Publications and Presentations

9.1.1 Publications

BABÚ YN, NEMEC M, SOLICH P, WSÓL V (2008). Liquid chromatographic-electrospray mass spectrometric determination (LC-ESI-MS) of phase II metabolites of flobufen in rat liver microsomes - Chiral discrimination. *Talanta* **75**: 494-502.

KRÁL R, SKÁLOVÁ L, SZOTÁKOVÁ B, VELÍK J, SCHROTEROVÁ L, BABÚ YN, WSÓL V (2003). The stereospecificity of flobufen metabolism in isolated guinea pig hepatocytes. *BMC Pharm* 3:5.

SKÁLOVÁ L, KRÁL R, SZOTÁKOVÁ B, BABÚ YN, PICHARD-GARCIA L, WSÓL V (2003). Chiral aspects of metabolism of anti-inflammatory drug flobufen in human hepatocytes. *Chirality*, **15**: 433-440.

KRÁL R, SKÁLOVÁ L, SZOTÁKOVÁ B, BABÚ YN, WSÓL V (2004). Stereospecificity of flobufen metabolism in guinea pigs *in vitro* and *in vivo*: phase I of biotransformation. *Chirality*, **16**: 1-9.

9.1.2 Posters and Abstracts

VLADIMÍR WSÓL, YOGEETA BABÚ, LUCIE BIMKOVÁ, BARBORA SZOTÁKOVÁ AND LENKA SKÁLOVÁ. Phase II biotransformation of NSAID Flobufen in male rats. *In vivo* glucuronic acid and glutathione conjugation. Poster and Abstract In: The 6th International ISSX Meeting – Drug Metabolism Reviews. Munich, Germany, October 7-11, 2001, p.167.

YOGEETA BABÚ, VLADIMÍR WSÓL. Preliminary study of Phase II biotransformation of flobufen in male rats. Poster and Abstract In: XVIII Meeting of Czech and Slovak Societies for Biochemistry and Molecular Biology. Vysoké Tatry, Stára Lesná, Slovak Republic, September 10-13, 2002, p.323.

YOGEETA BABÚ, VLADIMÍR WSÓL. Conjugation of Flobufen and its metabolites in male rats. Poster and Abstract In: 18th European Workshop on Drug Metabolism. Valencia, Spain, September 16-20, 2002, p.59.

YOGEETA BABÚ, MICHAL NĚMEC, VLADIMÍR WSÓL. Phase II biotransformation study of Flobufen and its metabolites in male rats. Poster and Abstract In: XII Symposium of Xenobiochemistry. Smolenice, Slovak Republic, June 9-11, 2003.

YOGEETA BABÚ, RADÍM KRÁL, VLADIMÍR WSÓL. Metabolism of Flobufen in Man: a preliminary study. Poster and Abstract In: XIX Meeting of Czech and Slovak Societies for Biochemistry and Molecular Biology. Olomouc, Czech Republic. August 31- September 3, 2004.

BABÚ Y.N., WSÓL V. Enantioselective glucuronidation of Flobufen in rat liver microsomes. Poster and Abstract In: 11th International Symposium on Separation Sciences (ISS). Pardubice, Czech Republic, September 12-14, 2005.

9.2 Copy of Publications

Liquid chromatographic-electrospray mass spectrometric determination (LC-ESI-MS) of phase II metabolites of flobufen in rat liver microsomes - Chiral discrimination.

Yogeeta Nautamlal Babú, Michal Němec, Petr Solich, Vladimír Wsól. *Talanta* **75: 494-502 (2008).**

The stereospecificity of flobufen metabolism in isolated guinea pig hepatocytes.

Radim Král, Lenka Skálová, Barbora Szotáková, Jakub Velík, Ladislava Schröterová, Yogeeta N. Babú and Vladimír Wsól. BMC Pharmacology 3:5 (2003) (http://www.biomedcentral.com/1471-2210/3/5, open access).

Studies of the metabolism of FLO in rat determined DHF as its main metabolite, which is further metabolised into M17203, an acetic acid derivative. However, preliminary *in vivo* experiments conducted in Man revealed differences in FLO metabolites excreted by rat and Man, as in Man FLO metabolism ended with the formation of DHF.

Further inter-species investigation showed that guinea pig was the species more closely related to Man in terms of FLO metabolism, as *in vitro* studies on cytosol and microsomes of guinea pig determined DHF stereoisomers as the sole metabolites of FLO in these sub-cellular fractions.

The aim of this work was to describe the metabolism of FLO and its pure enantiomers *in vitro* in primary culture of guinea pig hepatocytes.

The biotransformation of FLO in primary culture of guinea pig hepatocytes differed qualitatively from the one in microsomes and cytosol. In addition to DHF stereoisomers, three other metabolites were found: M17203, UM-1 and UM-2. The exact structure of UM-1 and UM-2 is not well defined, but their UV-spectral properties confirm a relationship to structures close to M17203 and DHF. It is thought that these metabolites could be products of phase II biotransformation (conjugates) or compounds M17203-like or DHF-like but with the side carbon chain substituted in a distinctive manner. The metabolism of the structurally related compound ibuprofen adverts to hydroxylation of the side chain. On the other hand, the structures could also be derived

from fenbufen metabolites found in Man, as fenbufen is structurally the most related compound to FLO.

Rac-FLO, its pure individual enantiomers and all four individual DHF stereoisomers were used as substrates for incubations with primary culture of hepatocytes.

Results concerning incubations with *rac*-FLO determined (2*R*;4*S*)-DHF and (2*S*;4*S*)-DHF as the main DHF stereoisomers, originated from (+)-(*R*)-FLO and (-)-(*S*)-FLO, respectively. The amounts of the two other stereoisomers were negligible. Based in these data, it can be said that reducing enzymes form stereospecificaly DHF stereoisomers with *S* configuration at carbon 4.

(+)-(*R*)-FLO as substrate gave rise to (2*R*;4*S*)-DHF and to a higher extent when compared to incubations with *rac*-FLO, but also a considerable amount of (2*S*;4*S*)-DHF was formed. Due to these results, it can be assumed that may have occurred inversion of (+)-(*R*)-FLO to (-)-(*S*)-FLO or inversion of (2*R*;4*S*)-DHF to (2*S*;4*S*)-DHF, or both cases together.

Interestingly, when (-)-(*S*)-FLO was used as substrate, (2*R*;4*S*)-DHF remained the most produced DHF stereoisomer. Inversion of (-)-(*S*)-FLO to (+)-(*R*)-FLO or inversion of (2*S*;4*S*)-DHF to (2*R*;4*S*)-DHF or a combination of both should be taken into account.

Inversions between DHF stereoisomers became clear when incubations with the individual DHF stereoisomers were performed.

(2*R*;4*S*)-DHF as substrate was inverted predominantly to (2*S*;4*S*)-DHF and when (2*S*;4*S*)-DHF was used as substrate, an inversion to (2*R*;4*S*)-DHF was observed. In this way, an equilibrium between these two stereoisomers is established, although shifted in

the direction of (2*R*;4*S*)-DHF formation, thus explaining why this stereoisomer is the main metabolite arising after incubation with (-)-(*S*)-FLO.

(2*S*;4*R*)-DHF was converted into (2*S*;4*S*)-DHF and (2*R*;4*R*)-DHF was readily transformed into (2*R*;4*S*)-DHF. For this reason, the amounts of these stereoisomers were negligible in the incubations with the other substrates above mentioned.

Regarding to the formation of M17203, this metabolite was selectively formed from (-)-(*S*)-FLO. This result was confirmed by incubations of the individual stereoisomers, where it was observed that DHF stereoisomers formed from (-)-(*S*)-FLO were stereoselectively chosen for M17203 formation. In a similar fashion, UM-1 and UM-2 were produced mainly from (-)-(*S*)-FLO.

Liver homogenate was also used as a biological matrix in this work. Incubations of fresh liver homogenate with *rac*-FLO and *rac*-DHF did not lead to production of M17203 nor UM-1/UM-2, the same result after incubations with microsomes and cytosol. It can be concluded that the integrity of the hepatocytes is essential for the formation of M17203, UM-1 and UM-2.

Stereospecificity of Flobufen Metabolism in Guinea Pigs *In Vitro and In Vivo*: Phase I of Biotransformation.

Radim Král, Lenka Skálová, Barbora Szotáková, Yogeeta N. Babú and Vladimír Wsól. Chirality 16: 1-9 (2004).

Preliminary studies in Man and other animal species determined that guinea pig was the most related species to Man in regard to FLO metabolism.

This work aimed the investigation of phase I biotransformation of FLO in vitro and in vivo in guinea pigs.

In vitro studies concerned experiments with microsomes and cytosol. These sub-cellular fractions were incubated with *rac*-FLO, and its pure individual enantiomers, (-)-(*S*)-FLO and (+)-(*R*)-FLO.

For both sub-cellular fractions, it was possible to determine that after incubation with (+)-(*R*)-FLO, the only metabolites formed were (2*R*;4*S*)-DHF and (2*R*;4*R*)-DHF, and when (-)-(*S*)-FLO was the substrate, it gave rise solely to (2*S*;4*S*)-DHF and (2*S*;4*R*)-DHF. On the other hand, incubations with *rac*-FLO in microsomes formed mainly (2*R*;4*S*)-DHF and (2*R*;4*R*)-DHF, while in cytosol the principal metabolites were (2*R*;4*S*)-DHF and (2*R*;4*S*)-DHF. However, due to the different ratios of the stereoisomers formed in each case, it is possible to say that the microsomal reductases differ from the cytosolic reductases in their activity as well as in their stereospecificity. (2*R*;4*S*)-DHF was the main metabolite in the microsomes, whilst in the cytosolic fraction (2*S*;4*S*)-DHF predominated.

In vivo experiments consisted in administration of *rac*-FLO or its individual enantiomers. In urine samples, together with the two known metabolites, DHF and M17203, an unknown metabolite was also detected, UM-2. The accurate chemical structure is not well defined although its UV-spectral properties resembles to the

structures of M17203 and DHF. This compound can be supposed to be a product of phase II biotransformation (conjugate) or a compound like M17203 or DHF, but with a different substituted side carbon chain. If the order of all determined compounds is considered, UM-2 should be the most lipophilic substance. The biotranformation of M17203 or DHF by some transferases (e.g., methyltransferases or acetyltransferases) could result in a compound with those properties. However, accordingly to a different theory, UM-2 could be deduced from the metabolism of structurally related compounds, such as ibuprofen, which can undergo a side chain hydroxylation. On the other hand, the structure of UM-2 can also be derived from fenbufen metabolites arising in Man, as this compound is structurally the most closely related to FLO. It is clear that UM-2 is the major metabolite in urine, exceeding the amount of excreted M17203 and DHF stereoisomers and it is produced to the highest extent after administration of (-)-(S)-FLO. Regarding to the excretion of DHF stereoisomers, (2R;4S)-DHF was the most prominent DHF stereoisomer detected after administration of *rac*-FLO and (+)-(R)-FLO. When (-)-(S)-FLO was administered, (2S;4S)-DHF was the major DHF stereoisomer excreted.

Faeces were also analysed and the metabolites excreted in faeces were qualitatively distinct from the urine samples. UM-2 and DHF were the sole metabolites detected, showing no trace of M17203. UM-2 was the predominant metabolite and in the same amount as in urine. Similarly to urine samples, excretion of UM-2 was higher after administration of (-)-(S)-FLO. In respect to DHF stereoisomers excretion, faeces samples also differed qualitatively from urine samples: (2R;4S)-DHF and (2S;4S)-DHF were the absolute majority of all stereoisomers detected in faeces and were excreted in the highest amounts after administration of *rac*-FLO. (2R;4S)-DHF was selectively

excreted after administration of (+)-(*R*)-FLO, while (2*S*;4*S*)-DHF was the prevailing metabolite after administration of (-)-(*S*)-FLO.

Chiral aspects of Metabolism of Antiinflammatory Drug Flobufen in Human Hepatocytes.

Lenka Skálová, Radim Král, Barbora Szotáková, Yogeeta N. Babú, Lydiane Pichard-Garcia and Vladimír Wsól. Chirality 15: 433-440 (2003).

Metabolism of FLO was preciously studied in different animal species. However, extrapolation of data obtained in these species is limited, and for this reason, the study of FLO biotransformation in human liver samples was initiated. Among the *in vitro* models used in drug metabolism studies, primary cultures of hepatocytes play a prominent role.

This work aimed the evaluation of the stereospecificity and stereoselectivity of FLO reduction and the chiral inversion of FLO enantiomers in primary culture of human hepatocytes.

Hepatocytes were obtained from livers of 4 donors, within the Cadaver Donor Programme of Transplant Centre of Faculty of Medicine of Charles University, Hradec Králové.

In human hepatocytes, FLO underwent only two types of metabolic transformations: reduction and chiral inversion. The reduction of FLO produced four stereoisomers of DHF. Due to the stereoselectivety and stereospecificity of the reductases, the amounts of the individual stereoisomers were different and their ratio changed depending on the incubation time. The application of pure enantiomers as substrates provided further information about the reduction and the reductases implicated in FLO biotransformation. Although comparison of the results obtained in hepatocytes from various donors showed some inter-individual variability, the principal routes of FLO reduction were maintained in all donors tested. Based on the results, it was assumed that at least three enzyme systems are involved in the reduction of FLO:

the first one selectively chose (+)-(*R*)-FLO as a substrate and directed the reduction into the S position on C4. The second enzyme, which reduces (-)-(*S*)-FLO to (2*S*;4*S*)-DHF showed a lower activity. The behaviour of both enzymes was comparable in all four cultures of hepatocytes. In hepatocytes of donor 4 and also partly of donor 3, it was possible to observe the higher activity of the third enzyme system, which reduced the substrate (+)-(*R*)-FLO to the *R* configuration at position C4. Interestingly, the use of the pure enantiomers instead of the racemate led only to a slight shift in the proportion of the DHF stereoisomers formed.

Chiral inversion was the second type of reaction observed in FLO metabolism. The chiral analysis of the residual substrate in the cultivation medium showed that (+)-(*R*)-FLO is converted into its respective antipode in hepatocytes. As a small amount of (+)-(*R*)-FLO was detected in hepatocytes from donor 4 incubated with (-)-(*S*)-FLO, chiral inversion of this enantiomer cannot be excluded. Together with the chiral inversion of FLO enantiomers, focused also on whether DHF could undergo chiral inversion or not. Therefore, human hepatocytes 2 and 4 were incubated with pure DHF stereoisomers. In both cultures, it was found a significant inversion of (2*S*;4*S*)-DHF to (2*R*;4*S*)-DHF and (2*R*;4*R*)-DHF to (2*S*;4*R*)-DHF. These inversions were practically unidirectional. The enzyme system catalyzing the chiral inversion of DHF used as substrates only those stereoisomers with identical configuration at the second and the fourth carbon atoms. The possibility of chemical (instead of enzymatic) inversion was excluded by the stability of DHF stereoisomers over 24 h in medium without hepatocytes.

Besides chiral inversion, it was also possible to observe partial oxidation of DHF stereoisomers to FLO in human hepatocytes. Chiral discrimination was also seen in the case of these oxidases. (2*S*;4*R*)-DHF was the selective substrate for formation of

(-)-(S)-FLO and (+)-(R)-FLO was preferably formed from ($2R$;$4S$)-DHF, although this reaction proceeded at a markedly lower rate than the formation of the S-enantiomer. The remaining stereoisomers were only slightly oxidized. ($2R$;$4S$)-DHF can be classified as the most metabolically stable substance, since it does not undergo any chiral inversion and is only negligibly oxidized to FLO.