CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
Department of Biochemical Sciences

IN VITRO BIOTRANSFORMATION STUDY OF FENOFIBRIC ACID

Diploma thesis

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Location, day *Signature*

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This study is dedicated to my father, who had always believed but left this world too early and never managed to see.

ABSTRACT

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Title of diploma thesis: In vitro biotransformation study of fenofibric acid

Fenofibric acid is a hypolipidemic agent that acts through PPARα and contributes to treatment of many types of dyslipidemias. It is the active metabolite of fenofibrate, but can be also administrated by itself. Concerning its metabolism, the majority of fenofibric acid is conjugated with glucuronic acid, while a minor amount yields a reduced metabolite. Reduced fenofibric acid is also an active substance. The identity of the enzymes participating in the reducing metabolic process has not been revealed yet. The current study investigated this carbonyl reduction in human liver subcellular fractions and by the use of nine recombinant cytosolic carbonyl-reducing enzymes of the AKR and SDR superfamilies. Enzymatic activity toward fenofibric acid reduction appeared in both cytosol and microsomes and was found that affinity of cytosol is greater while velocity in microsomes is higher. Of the nine tested enzymes, five reductases were identified to play role in the reduction of fenofibric acid. The highest activity was exhibited by CBR1, followed by AKR1C3, AKR1C2, AKR1C1 and AKR1B1. Our finding of significant contribution of microsomal fraction to the carbonyl reduction of fenofibric acid stimulates further investigation on microsomal reducing enzymes.

ABSTRAKT

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Název diplomové práce: In vitro biotransformační studie fenofibrové kyseliny

Fenofibrová kyselina je hypolipidemikum, které působí prostřednictvím PPARα, a používá se k léčbě různých typů dyslipidemií. Je to aktivní metabolit fenofibrátu, ale může být také podáván přímo. Co se týče metabolismu, většina fenofibrové kyseliny je konjugována s glukuronovou kyselinou zatímco malé množství je přeměněno na redukovaný metabolit. Redukovaná fenofibrová kyselina je také aktivní látka. Enzymy účastnící se tohoto redukčního metabolického procesu nebyly dosud identifikovány. Předkládaná studie zkoumala karbonylovou redukci v subcelulárních frakcích z lidských jater a za použití cytosolických karbonyl-redukujících enzymů z nadrodiny AKR a SDR. Enzymatickou aktivitu způsobující redukci fenofibrové kyseliny vykazoval jak cytosol tak mikrosomy a bylo zjištěno, že cytosol má větší afinitu zatímco rychlost redukce je větší v mikrosomech. Z devíti testovaných enzymů byla u pěti reduktas nalezena aktivita k redukci fenofibrové kyseliny. Největší aktivitu vykazoval CBR1 následován AKR1C3, AKR1C2, AKR1C1 a AKR1B1. Naše zjištění o významném přispění mikrosomální frakce ke karbonylové redukci fenofibrové kyseliny podněcuje další studium v oblasti mikrosomálních karbonyl redukujících enzymů.

CONTENTS

1. INTRODUCTION

Fenofibric acid is the active metabolite of fenofibrate. It is a lipid-lowering drug that acts through the binding to nuclear peroxisome proliferator activated receptor alpha (PPARα). The activation of PPARα leads to the antihyperlipidemic effect through many pathways in the lipoprotein metabolism (Rang et al., 2012).

Nowadays, WHO states the cardiovascular diseases as the number one cause of death in developed countries (2014). Although statins are thought to be the first line drug for this purpose, when they are combined with fibrates, the therapeutic results are more potent (Tenenbaum and Fisman, 2012; Saha et al., 2007). It is true that the combination of statins and fibrates can increase their common side effect of rhabdomyolysis, however, reports have shown that in case of use of fenofibric acids this is much lower (Jones and Davidson, 2007).

It has also been shown that fibrates have a positive impact on reducing cardiovascular diseases, mainly by prevention of coronary artery disease (Jun et al., 2010). Moreover, it has been found that the prolonged therapy with fibrates lowers to a great degree the incidence on nonfatal myocardial infarction (Corsini et al., 2005; Saha et al., 2007). In 2009, the FIELD study has also presented the reducing effect of fenofibrate on microvascular complication of diabetes mellitus II (DM II), representing the superior positive action of fenofibrate in patients with metabolic syndrome (2005). The ACCORD-lipids study has also pointed this need especially for patients with DM II, increased triacylglycerols and low HDL (2010). Additionally, the predominant benefits were related to lowering of hypertriglyceridemia (FIELD study investigators, 2005; Krysiak, 2010).

It is worth mentioning, that fenofibric acid do not exhibit many drug - drug interactions due the avoidance of the inhibition of CYP450 allowing it to be administrated in multitherapy. However, this does not concern all fibrate compounds in the same degree (e.g. gemfibrozil) but undoubtedly, fibrates are important and promising pharmaceutical compounds (Alagona, 2010).

The mechanism of action of fibrates started to be understood in the beginning of 1990s. However, less is known about their metabolism. Fenofibrate is hydrolyzed in the liver to the active form of fenofibric acid by esterases. The majority of the amount of fenofibric acid is excreted in urine after the conjugation with glucuronic acid. The

rest of fenofibric acid is firstly metabolized to reduced fenofibric acid, and then follows glucuronidation and excretion *via* urine. Moreover, *in vivo* studies have shown that the oxidative metabolism of fibrates and fenofibric acid does not occur at a remarkable level (Abbott Laboratories, 2010). On the contrary, no further information is yet available for the enzymes that participate in the reductive metabolism of fenofibric acid.

2. THEORETICAL PART

2.1 Metabolism

Metabolism is the sum of chemical reactions and biological processes that occur in an organism and result in the modification of a molecule. It includes a variety of metabolic pathways. Each of them begins with an initial compound that after a cascade of reactions is transformed to its metabolite. Based on the result of this modification, the reaction may be anabolic or catabolic.

Enzymes are required to catalyze the metabolic reactions. Enzymes can be found in many sites in the organism (e.g. cytosol, mitochondria, smooth and rough endoplasmic reticulum, peroxisomes), and their location within the cell facilitates ordered metabolism (Cambel and Reece, 2004). Eukaryotes use organelles to compartmentalize metabolic pathways allowing different metabolic chemical reactions to occur in specific location.

The study of metabolism and various metabolic pathways can be accomplished *in silico* or/and by the use of pluripotent *in vitro* experimental methods (Testa et al., 2012). In that way, it is possible to use liver preparations to examine the possible metabolic fate of the compounds, to compare the differences of transformation of compounds in different species and predict the corresponding metabolism in human body.

2.2 Biotransformation of xenobiotics

The term “xenobiotic” has its meaning in the Greek root of the word “xenos” ξένος, which means “stranger”. Apart from drugs, xenobiotic is any substance that is not native to the body, such as food, chemicals, pollutants etc. The study of xenobiotic biotransformation has been an interest topic since 1950s, and concerns many scientific fields, such as chemistry, biology, pharmacology and toxicology (Testa et al., 2012).

Xenobiotics are usually characterized by high lipophilicity that enables them to penetrate membranes and other non-polar barriers, and at the same time they can avoid the elimination. The purpose of biotransformation steps on this. The aim is to

decrease lipophilicity and increase hydrophilicity in order to allow the substances to be excreted by urine or bile (Coleman, 2005).

During the biotransformation of a xenobiotic the produced metabolite may be the active compound, so we can call the process as activation or on the contrary, it can produce a less active derivate or not active at all, where it is called detoxification. Moreover, biotransformation allows us the production and use of prodrugs that can be metabolized to pharmacologically active compounds after reaching the target.

If the biotransformation of a drug is not possible, its molecule would remain capable of crossing the barriers for longer time than acceptable, leading to accumulation of the active compound, hence a toxic and harmful result. A metabolite of a xenobiotic compound can also be a toxic compound. It is of major importance and concerning during the drug discovery and design the investigation of the metabolism of a compound that can lead to toxification (Testa et al., 2012).

Biotransformation of xenobiotics is a biphasic process (Williams, 1959), known as phase I and phase II. The major organs that participate in these phases are liver, lungs and kidneys. An overview of the biotransformation phases of xenobiotics are presented in Figure 1.

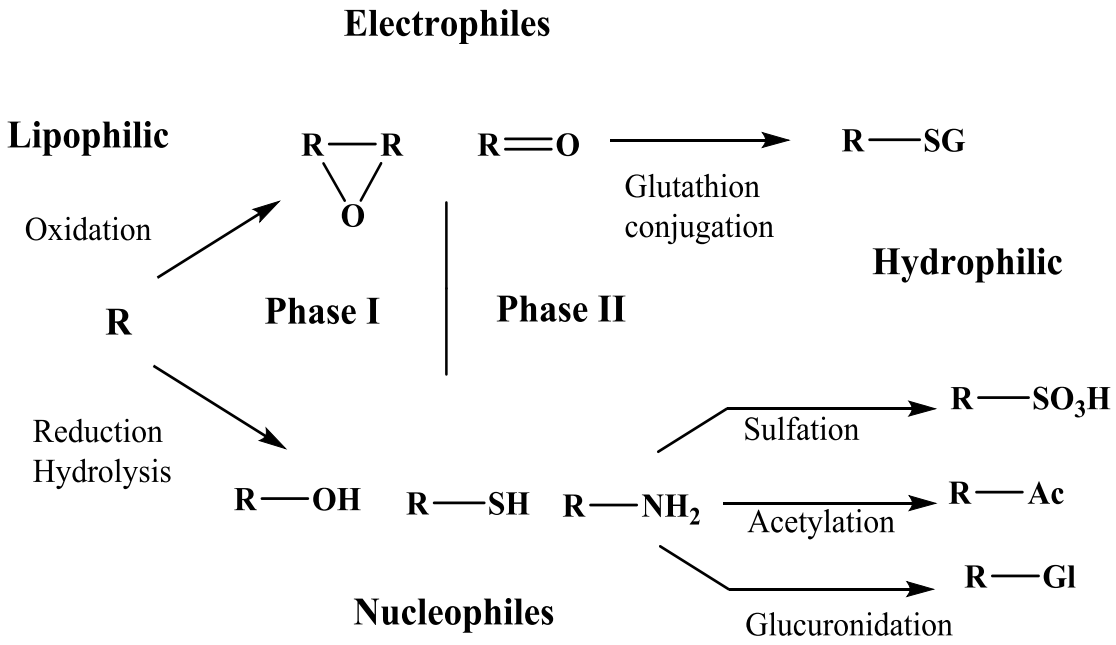


Figure 1. The two phases of drug metabolism of a lipophilic compound
Obtained from Wikipedia; https://encrypted-tbn3.gstatic.com/images?q=tbn:ANd9GcSFeJjzRdXALv3ab4A-R8aU1gnaG6gX_oTauW-cN10U_PdUFLDL1g.

2.2.1 Phase I reactions

The first phase of biotransformation is known as the functionalization. In this phase the purpose is to increase the polarity of the substances by addition or uncovering of a functional group. Most common are –COOH, -OH, -NH_2 and –SH. The main reactions of this phase are hydrolysis, reduction, and oxidation catalyzed by the respective enzymes. The enzymes and their place of action are shown in the Table 1.

Table 1. Main phase I reactions, enzymes, and the location of action.

Adapted from Parkinson, 1996; Penner et al., n.d.

Reaction	Enzyme	Localization
Oxidation/ Reduction	Cytochrome P450	Microsomes
	Monoamine oxidase	Mitochondria
	Flavine monooxygenase	Microsomes
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Cytosol, mitochondria
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Carbonyl reductases	Cytosol, microsomes, blood
	Nitro-reductase	Microsomes, microflora
	Azo-reductase	Microsomes, microflora
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol

The most common and known enzyme in phase I is the cytochrome P450 (CYP450). In fact, it is not a single enzyme, but a superfamily consisting of many subfamilies and enzymes that are responsible for the majority of the xenobiotic biotransformation (Nelson, 2004; Guengerich, 2008; Zanger & Turpeinen, 2008; Zanger & Schwad, 2013). Almost 60% of the drugs today are metabolized by isoforms of CYP family (Testa, 2012).

Apart of the phase I enzymes, another component required for biotransformation is often a co-factor. In most cases it is nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate ($NAD(P)^+$or NAD(P)H). These compounds are electron carries and therefore, enable the electron transfer.

Reductive metabolism and especially carbonyl reducing enzymes will be discussed in chapter 2.3.1.

2.2.2 Phase II reactions

The second part of the biotransformation is known as conjugation reactions. It is an anabolic stage where the formed compound of phase I accepts a substituent group that transforms it into an inactive compound. This conjugation results in attachment of small, polar and ionizable endogenous molecules to phase I product, or to the parent drug itself, resulting in more hydrophilic compounds ready for excretion.

Predominant organ of phase II is liver and the main enzymes of this phase are mainly transferases, as shown in Table 2.

Table 2. Main biotransformation reaction phase I, xeno-enzymes, and the location of action. Adapted from (Parkinson, 1996; Penner et al., n.d.)

Reaction	Enzyme	Localization
Conjugation	Uridine diphosphoglucuronosyl transferase	Microsomes
	Sulfotransferase	Cytosol
	Methyl transferase	Cytosol, microsomes
	N-acetyl transferase	Cytosol, mitochondria
	Amino acid conjugation enzyme	Mitochondria, Microsomes
	Glutathione S-transferase	Cytoplasm

All of these reactions require, apart from the enzyme, a high energy molecule, such as glucuronic acid and glutathione, that are attached on an electron rich atom of the substrate (i.e. O, S, N).

All of the transferases, have a wide spectrum of binding, that enables them to be active on many drugs, xenobiotics or even endogenous compounds.

2.3 Reductive metabolism

The reductive pathway of the phase I of the biotransformation can occur when compounds contain carbonyl, azo or nitro groups. Other compounds that can undergo reduction are epoxides, halogenated hydrocarbons and heterocyclic ring compounds. During reduction, carbonyl groups are reduced to alcohols, while azo and nitro group to respectively amino derivatives. Since these reactions are reductive, a source of electrons is required. In most cases, NAD(P)H holds this role (Gibson and Skett, 2001). Reduction is less common metabolic reaction in human living cells than oxidation.

2.3.1 Carbonyl reduction

This part concerns in detail the carbonyl reduction, and the enzymes that participate in this process. Xenobiotic compounds are reduced at the carbonyl moiety of their molecule. Carbonyl group is a group where a carbon atom is double-bonded to an oxygen atom: -C=O. Compounds that have this bond in their structure are aldehydes (RCHO), ketones (RCOR`), carboxylic acids (RCOOH), esters (RCOOR`), amides (RCONR`R``) and quinones. Carbonyl groups can be found in acyclic, aromatic, or aliphatic molecules.

The bond between oxygen and carbon is covalent but the difference of electronegativity between these atoms allows oxygen to be slightly negatively charged. This is what attracts H^+, an electrophile ion, to be attached to oxygen, thus reducing it.

In case of reduction of asymmetrical ketone compound to secondary alcohol the result is the creation of a chiral centre, thus the formation of enantiomers. In such cases, it is demanding determine the configuration of the metabolites, along with examination of enzyme stereospecificity (Skarydova and Wsol, 2012).

Because a reduction reaction may be the opposite of the oxidation reaction, both reactions are sometimes catalyzed by the same enzyme. For example, reduction of many aldehydes and ketones to primary and secondary alcohols, respectively, and the opposite reaction, oxidation, is catalyzed by the enzyme alcohol dehydrogenase (Weinner and Flynn, 1988). Alcohol dehydrogenase is a non-specific soluble

cytosolic enzyme that exists predominantly in liver cells. In human species, it has been found that there are approximately nine different isoenzymes of alcohol dehydrogenase, which are encoded by seven different genes (Davis et al., 1996). The oxidation reaction with alcohol dehydrogenase requires the reduction of the co-factor NAD^+. Carbonyl reduction, similarly as other phase I reactions, may result in either bioactivation or detoxification of initial compound (Hoffman and Maser, 2007). Studies have shown evidence for cases as activation of the drug (e.g. warfarin) or of the prodrug (e.g. propanolol), detoxification (e.g. quinine) or even toxification (e.g. daunorubicin) (Maser, 1995).

2.3.2 Carbonyl-reducing enzymes

Although the participation of carbonyl-reducing enzymes (CREs) in phase I metabolism was first mentioned in 1980s it is still largely undescribed (Oppermann & Maser, 2000). The existence of CREs has been found not only in humans, but in many other species of animals and plants, so they are ubiquitous.

Main CREs can be divided into two superfamilies, according to their protein structure; the aldo-keto reductases (AKR) and short-chain dehydrogenase reductase (SDR) superfamily. Recent development of genetic has allowed investigating these enzymes and starting to understand their influence on metabolism of drugs.

Recently, a review has been published about drugs metabolized by carbonyl-reducing enzymes. The drugs were divided in three groups, as shown in Table 3, according to the degree of knowledge about their metabolism by CREs. Additionally information of this table attached separately, are xenobiotics that have already been presented in previous review (Malatkova and Wsol, 2014).

Table 3. Drugs undergoing carbonyl reduction. Adapted from Malatkova and Wsol, 2014

Group 1	**Group 2**	**Group 3**	**Barski at al. (2008)**	
Benfluron	Nabumetone	Fenofibrate/	Acetohexamide	Loxoprofen
Dimefluron	Bupropion	Fenofibric acid	Befunolol	Nafimidone
Boceprevir	Methylnatrexone	Naftazone	Daunorubicin	Naloxone
Glucocorticoids	Eperisone	Pentoxyfylline	Dolasetron	Naltrexone
Tibolone	Tolperisone	Oxcarbazepine	Doxorubicin	Oracin
Norethynodrel	Warfarin	Ophthalmic β-blocker drugs	Ethacrynic acid	E-10/Z-Oxonortriptyline
Wortmannin	Metyrapone		Haloperidol	S-1360
			Ketoprofen	Timiperone
			Ketotifen	

Within contrast to CYP450 that exists only in microsomes, CREs do exist in both cytosol and microsomes. However, the cytosolic forms of CREs are much more known and investigated than the microsomal ones (Skarydova and Wsol, 2012).

2.3.2.1 Aldo-keto reductase superfamily

AKR is the biggest superfamily of NAD(P)(H) – dependent oxidoreductases in humans. This superfamily contains more than 190 members divided into 19 families. In humans have been found around 15 enzymes so far (Penning, 2015).

The enzymes form AKR superfamily have common protein structure. Their structural fold called $(\alpha/\beta)_8$ barrel consists of 8 parallel α-helixes that are external and include the 8 parallel β-strands. It is also called as TIM-barrel fold on the basis of the structure of triose-phosphate isomerase enzyme. Conserved sequence of amino acids that is required for the oxidation of NAD(P)H is Asp-Tyr-Lys-His. Tyrosine conserved residue is thought to play the most important role in the acid-base catalytic action of these enzymes. Many variations exist in either α-helixes, β-strands or the amino acids residue among the enzymes that allows the subdivision of this

superfamily as well as the differentiation of human AKRs from other mammalians AKRs (Barski et al., 2008). The reaction that AKRs catalyze has bi-bi ordered mechanism, meaning that there are two reactants and two products, where the cofactor needs to be bounded first to initiate the reduction and is unbound after termination of the reaction (Oppermann and Maser, 2000; Barski et al., 2008).

Another characteristic of AKRs is their gene polymorphisms. Gene polymorphisms occur either in coding, or in most cases in non-coding parts of these genes. The diversity of variants of particular gene influences the enzymatic activity in biotransformation of xenobiotics, but may be also involved in disease induction (Jin and Penning, 2007).

Mammalian members of this superfamily are active as monomers and predominate in cytosol. They act as aldehyde reductase, aldose reductase, and dihydrodiol dehydrogenase (Opermann and Maser, 2000; Mindnich and Penning, 2009).

Nomenclature of AKRs has been used the last 12 years. It follows the common system for naming enzymes, as well as in case of CYP enzymatic superfamily. The first three letters of all the enzymes are AKR meaning Aldo-Keto Reductases, defining the enzymatic catalytic reducing activity on aldehydes and ketones. Next to this prefix is a number of arabic system corresponding the family, then another capital letter representing the subfamily; A for aldehyde reductases, B for aldose reductases and C for hydroxysteroid dehydrogenases. In the end, a number for the respectively sequence of the one and only enzymes. Details and historical information are found in the database of the University of Pennsylvania in the following link: http://www.med.upenn.edu/akr/nomenclature.shtml. Figure 2 presents members of these subfamilies and its nomenclature.

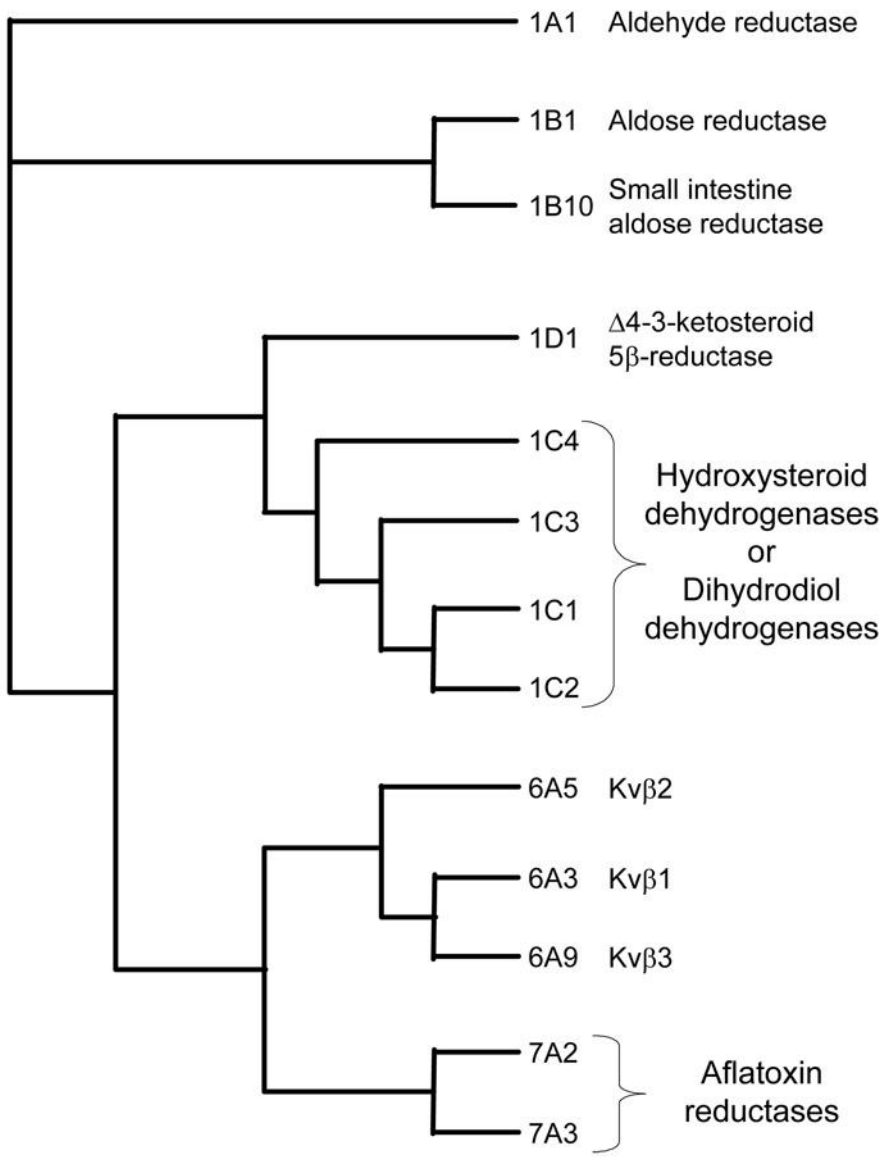


Figure 2. Phylogenetic tree of human AKRs enzymes. Adapted from Barski et al, 2008.

Another enzyme database system that participates in nomenclature of enzymes is BRENDA, found at www.brenda-enzymes.org. This scientific page provides a lot of information for the most enzymes, along with the Enzyme Commission (EC) number. EC number is a short numerical sequence based on the chemical reaction of any enzyme that is able to catalyze.

The ability of studying the enzymatic action of AKRs enzymes *in vitro*, is facilitated by the use of their recombinant forms, e.g. expressed in *Escherichia coli* (Jin and Penning, 2007).

Aldehyde reductase

Aldehyde reductase (AKR1A1, EC 1.1.1.2) is a cytosolic NADPH dependent reducing enzyme. It is one of the first AKRs that have been described, in concrete in 1961 in rat liver (Barski et al., 2008). It has been also found that AKR1A1 exist in other mammals, except humans and rodents.

The reducing catalytic activity of aldehyde reductase favors carboxyl group in substrates that are negatively charged, even though its wide spectrum of activity allow also aromatic, steroid and smaller aldehydes to be desirable substrates for AKR1A1 (Barski et al., 2008).

The prior importance of AKR1A1 has been found in the anabolism of ascorbic acid, where in the rodents is its participation essential. In humans, it is also the reducing enzyme of D-glyceraldehyde to glycerol and of melvadate towards the regulatorof cholesterol synthesis and triglycerides, mevalonic acid.

Concerning the action in a drug metabolism, it has been yet found only the role of AKR1A1 in reduction of antitumor agent doxorubicin to cardiotoxic metabolite doxorubicinol (Mindnich and Penning, 2009).

Aldose reductases

There are two members of the subfamily AKR1B; aldose reductase AKR1B1 (EC 1.1.1.21), and the small intestine aldose reductase AKR1B10 (EC 1.1.1.21). Both proteins are cytosolic NADPH dependent reductases.

AKR1B1 is the rate-limiting enzyme in the polyol pathway, where it reduces glucose to sorbitol, which makes this protein involved in pathogenesis of diabetes mellitus. Hence, up-regulation of this enzyme, leads to sorbitol accumulation. This information has led to use aldose reductase inhibitors for either prevention or treatment of hyperglycemic complication (Liu et al., 2009). While in animals studies diabetic retinopathy was related to AKR1B1, this has not been confirmed in humans (Abhary et al., 2010).

It was also found that many fibrate agents inhibit AKR1B1 in both directions of its redox actions. In particular, fenofibrate show in reduction direction pure non-competitive inhibition, and in oxidation direction competitive inhibition. Among all fibrates, fenofibrate was more effective in inhibiting aldose reductase action. This set the question how AKR1B1 can influence the metabolic processes of fibrates (Belendiran and Rajkumar, 2005). Moreover, AKR1B1, as well as AKR1A1, participates in the metabolism of lipid aldehydes (Penning and Drury, 2007).

Another drug, where AKR1B1 participates in the biotransformation, is cytotoxic cyclophosphamide. In reality, AKR1B1 catalyzes the reduction of cyclophosphamide's metabolite produced by CYP450, acrolein, that is responsible for the unwanted side effects of the chemotherapeutic therapy. Reduction of acrolein leads to minimization of hemorrhagic cystitis (Jin and Penning, 2007).

AKR1B10, is also an aldose oxidative sensitive protein, and is from 70% identical to the amino acid sequence of AKR1B1 (Cao et al., 1998). It is mainly expressed in intestine and colon cells, and so is called small intestine aldose reductase.

AKR1B10 has been suspected for existence of many mutations and pseudogenes. In particular, investigation of the influence of mutations of AKR1B10 on inhibition action by fibrates showed that reduced metabolite of fenofibric acid is less potent than fenofibrate, while it is the only that can induce pure non-competitive inhibition against DL-glyceraldehydes reduction. On the contrary, fenofibrate demonstrates mixed non-competitive inhibition at the reducing action of AKR1B10 in this reaction (Balendiran et al., 2009).

Both aldose reductases have been target for modern approaches for anticancer therapies, since it is stated that their overexpression is related to many tumors, such as lung, breast, and colon tumors among others (Ramana and Srivastava, 2010). The decrease expression of AKR1B10 genes can be used as a diagnostic tool for colorectal cancer (Kropotova et al., 2009). Another common feature of AKR1B10 and AKR1B1, is their ability to reduce chemotherapeutic agent doxorubicin (Balendiran et al., 2009).

Hydroxysteroid dehydrogenases

Four members of the subfamily of hydroxysteroid dehydrogenases; AKR1C1 (EC 1.1.1.149), AKR1C2 (EC 1.1.1.213), AKR1C3 (EC 1.1.1.188) and AKR1C4 (EC 1.1.1.50); share over 80% homology in their genes (Chen and Zhang, 2012). They were named based on their participation in metabolism of steroid hormones, catalyzing their oxidation –reduction transformations. AKR1C4 exist almost only in liver, demonstrates the highest catalytic activity (Barski et al., 2008). Moreover, AKR1C1, AKR1C2 and AKR1C3 enhance progesterone metabolism (Hevir et al., 2011). AKR1C3 is in addition involved in the metabolism of prostaglandins, while AKR1C4 of bile acids (Barski et al., 2008). All of them participate in metabolism of xenobiotics, and are important in pathogenesis of many human diseases, especially cancers (Oppermann, 2007; Jin and Penning, 2007; Barski et al., 2008; Jin, 2013; Yun et al., 2015).

All of the enzymes of this subfamily share the reduction activity toward aldehydes and ketones to respectively primary and secondary alcohols, and the presence of NAD(P)H as cofactor. They are soluble proteins present in cytosol and active as monomers (Barski et al., 2008).

AKR1C1 activity has been studied in the metabolism of anticancer drugs, as the detoxification of cisplatin, and metabolism of methotrexate. The up-regulation of AKR1C3 protein was related to drug resistance to these drugs (Selga et al., 2008). Additionally, potential chemotherapeutic drug oracin is also reduced to both of its enantiomers by AKR1C enzymes. But, only AKR1C1 is able for the reduction to both of these isomers, while AKR1C2 and AKR1C4 only for one of them (Wsol et al., 2007). Befunolol, an antihypertensive agent, is another substrate for AKR1C1 and AR1C2 (Barski et al., 2008).

The first three isoforms of this subfamily of oxidoreductases (AKR1C1-C3), accompany the action of AKR1A1 and AKR1B1 in the detoxification of lipid aldehydes, PUFA's peroxidation products, that are involved then in to the expression of neurodegenerative diseases. Among all AKR1C enzymes, AKR1C1, showed the highest catalytic efficiency (Penning and Drury, 2007). Other common substrates of AKR1C isoforms is the antidepressant but also smoking cessation alleviating agent bupropion (Skarydova et al., 2014).

AKR1C3 regulation seems to be directly influenced by interleukin-6. Moreover, catalytic action of this enzyme is associated with cancers such as breast and prostate (Penning, 2015). Apart from the diseases, it also affects the chemotherapeutic resistance to methotrexate (Zhao, 2014).

Concerning the reducing catalytic activity of AKR1C3 toward xenobiotics it seems that in most cases it is not detected, apart from some neurological agents, such as naloxone, naltrexone, haloperidol and timiperone (Barksi et al., 2008).

Both AKR1C2 and AKR1C4 participate in the major metabolic pathway of the NSAID drug loxoprofen. On the contrary, reduction of ketoprofen is not the major metabolic pathway, but again AKRs participate in this reaction (Ohara et al., 1995). As well, oxcarbazepine is sufficiently reduced by all four isoforms of the human AKRs (Malatkova et al., 2014).

Another substrate of AKR1C1, AKR1C2, and AKR1C4 is nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK), a harmful constituent of tobacco. While the product of NNK metabolism by CYP450 is a tobacco-related cancer substance, the detoxification by those AKRs allows its glucuronosylation and excretion (Atalla et al., 2000).

2.3.2.2 Short-chain dehydrogenase/reductase superfamily

The second major superfamily of reducing enzymes is the short-chain dehydrogenase/ reductase superfamily. This superfamily counts more than 46000 members with great function diversity. Along with AKRs, they are considered to be the main enzymes catalyzing the oxidation – reduction reaction in a xenobiotics with carbonyl group (Hoffmann and Maser, 2007; Skarydova and Wsol, 2012). Nomenclature of those proteins was established, and more information is available on http://www.sdr-enzymes.org./.

SDRs are able to catalyze the reduction of aldehydes and ketones to respectively alcohols similarly to several AKRs. Those enzymes are NAD(P)H dependent oxidoreductases, while there have been favoring NADPH (Barski et al., 2008). Typical structure motif is Rossmann fold consisting of 6 parallel β-strands linked to two pairs of α-helixes in a specific order. Specific active site is made by amino

sequence Asn-Ser-Tyr-Lys, but it is the conserved Tyr amino acid that is responsible for the catalytic action. The reaction mechanism is bi-bi ordered (Oppermann et al., 1997). The majority of these superfamily members are either homodimers or homotetramers, but some exists also as monomers. SDRs are found as reductases and dehydrogenases (Hoffamnn and Maser, 2007; Skarydova and Wsol, 2012).

SDRs are involved in the metabolism of many endogenous and xenobiotic compounds, thus they can be located in many places within the cell, such as cytosol, microsomes, and mitochondria. 11β-Hydrosteroid dehydrogenase (11β-HSD) is a dimeric microsomal enzyme, and the most known of this superfamily. It consists of two isoforms, 11β-HSD1 and 11β-HSD2. Excellent substrates for 11β-HSDs are glucocorticosteroids and sex hormones along with their precursors (Skarydova andWsol, 2012). However, only 11β-HSD1 in involved in biotransformation of xenobiotics. Metyrapone, oracin and NKK are some of its favorable substrates (Malatkova and Wsol, 2013).

Several SDRs have another biological function apart from their enzymatic activity, a non-catalytic property. These multifunctional proteins are known as "moonlighting proteins". The multitasking activity of SDRs point to additional importance of those enzymes and give motivation for further research on CREs from this superfamily (Ebert et al., 2014).

Humans have been found to express three genes of cytosolic carbonyl reductases (CBRs) from the SDR superfamily; CBR1, CBR3, and CBR4 (Malatkova et al., 2010).

CBR1

CBR1 (EC 1.1.1.184) is a cytosolic monomeric enzyme of SDR superfamily, and it is widely distributed in human tissues (Maser and Hoffmann, 2007). An unusual and interesting characteristic of this enzyme, is that *in vivo* can be found in three slightly different forms, as a result of positional modification in Lys residue (Maser and Hoffmann, 2007; Malatkova et al., 2012).

Favorable endogenous substrates for CBR1 seem to be mainly quinones, prostaglandins and tetrahydrobiopterin, making this enzyme important in plenty of biochemical pathways (Malatkova et al., 2010). Quinones, either as endogenous or xenobiotic compounds are detoxified in two possible ways; one- and two-electron reduction, and in both cases CBR1 is the enzyme of major importance (Malatkova et al., 2010).

Besides AKRs reduction of NKK, as written previously, it is also CBR1 that detoxifies this compound, and in fact results in a more efficient biotransformation (Atalla et al., 2000). Alike, oxcarbazepine reduction metabolism by both CBR1 and CBR3 has showed interesting results *in vitro* but not further information for *in vivo* action is yet known (Malatkova et al., 2014). Benfluron, dimefluron, and wortmamnin have already shown evidence for carbonyl reduction by CBR1, while nabumetone not surely yet (Malatkova and Wsol, 2014). Flavonoids, menadion, indomethacin have been mentioned as inhibitors of CBR1 (Gonzalez-Covarrubias et al., 2008; Malatkova et al., 2010).

CBR1 and CBR3, concerning their sequences are very similar, but their activity differ a lot, since it is only CBR1 that is of high importance in reducing metabolism of endogenous and xenobiotic compounds (Malatkova et al., 2010). As a matter of fact, CBR3 a monomeric reductases, has not so far appear any significant catalytic activity, except for ortho-quinones and menadione (Hoffmann and Maser, 2007; Malatkova et al., 2010). CBR4 is sharing gene similarity with both CBR1 and CBR3 up to 23% and 22% respectively, but has not been mentioned to be involved in xenobiotic biotransformation at a significant level (Gonzalez- Covarrubias, 2008).

2.4 Carbonyl reduction in cytosol vs. in microsomes

Concerning metabolism, the two quantitatively most important subcellular localization are microsomes (endoplasmic reticulum) and cytosol. Different enzymes are present in either one or the other locations.

Liver microsomes are the metabolic active subcellular fraction of endoplasmic reticulum, and exists primarily as vesicles. Microsomal enzymes constitute the majority of oxidoreductases (e.g. CYP450, flavin containing monooxygenase), hydrolases, and so predominate in phase I of biotransformation reaction. Additionally, in microsomes can be found some enzymes that participate in phase II reactions, as uridine diphosphate-glucuronosyltransferase, methyl-transferase etc.

Cytosol is the soluble fraction of cytoplasm, and consists of many water-soluble enzymes. The enzymes found in cytosolic fraction, include hydrolases, AKRs and most of the conjugating enzymes, as glucuronidases, transferases, sulfotransferases etc., thus predominate in phase II reactions.

On the other hand, in phase II reaction, it is only one microsomal enzyme known, UDP-glucuronosyltransferase (UGT). UGT and its isoforms occur in microsomes, but they are participating in many biotransformation reactions.

Nevertheless, microsomal enzymes tend to be an important and promising part of the metabolism of both endogenous and exogenous compounds. However, there is a lot of information missing about microsomal enzymes, including microsomal carbonyl-reducing enzymes (Skarydova, 2011).

2.5 Fenofibric acid

Fenofibric acid (ABT 225) or according to its IUPAC name 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid is an antihyperlipidemic agent that is available in the market by many trade names in both delayed release capsules and tablets. In fact, the isopropyl ester of fenofibrate is available since 1976 and releases the active metabolite fenofibric acid. Lately, in the USA fenofibric has become also available in form of choline salt, under the trade name Trilipix©, by Abbott.

2.5.1 Pharmacological action

Mechanism of action of fenofibric acid can be characterized as pleiotropic, since this agent is able to influence metabolic processes in many ways.

Fenofibric acid acts by activation of peroxisome proliferator-activated receptors (PPARs). Three nuclear receptors have already been identified PPARα, PPARβ and PPARγ. Fenofibric acid is an agonist of PPARα that is found extensively in liver, muscle, kidney and heart, and can influence fatty acids degradation via β-oxidation in both peroxisomes and mitochondria. When PPARα is activated, its transcriptional activity is enhanced and results in expression of genes that are involved in lipoprotein metabolism and consequently lead to elevation of "good" high density lipoprotein (HDL). Moreover, this nuclear receptor affects many proteins and enzymes that are fat-regulating leading to reduced triacylglycerides by lower fatty acid synthesis and parallel increase of cellular uptake of fatty acids. Taken together, it is achieved increase of HDL (↑ 12-25%), and decrease of triacylglycerides (↓35-50%), LDL and total cholesterol (Chinetti et al., 2001; Kelly, 2001; Barbier et al., 2002; Tojcic et al., 2009).

Beyond the hypolipidemic effects of fenofibric acid, the activation of PPAR transcription factors, results in a cardioprotective and anti-inflammatory action due to prevention of atherosclerosis, decrease of the level of fibrinogen and elevation of C-reactive protein (Tziomalos and Athyros, 2006; Balakumar et al., 2011). Additionally, it is introduced in the therapy of patients with metabolic syndrome with accompanying dislipidemia and insulin resistance because it provides beneficial results in all targets (Wysocki, 2004). It is also a uricosuric agent (Achimastos et al., 2002).

2.5.2 Clinical uses

The pleiotropic effect of fenofibric acid provides the agent a wide spectrum of activity. It is a well-tolerated agent that can be used in monopharmacotherapy (Keating, 2011). First of all, it is used in mixed dyslipidemias, where we have to face both increased triglyceride and cholesterol serum levels. In same way, it is a safe option when this situation is accompanied by hyperuricemia (Rang et al., 2012). Furthermore, it is indicated in primary hypertriglyceridemia as a first line agent, and in less common type III dysbetalipoproteinemia (Staels et al., 1998). Of greatest interest is the indication in patients of metabolic syndrome with DMII, where the level of HDL is usually decreased. Additionally, the ACCORD-lipid study added evidence of an improvement in retinopathy of diabetic patients (ACCORD study group, 2001; MacKeage and Keating 2011). FIELD trial also mentioned the decrease in microvascular complications and the prevention role in non-fatal myocardial infarction (FIELD study investigators, 2005; Tziomalos and Athyros, 2006).Worth saying is that fenofibric acid, in contrary to other fibrates derivatives, is the only agent that has been approved for combination with HMG-CoA reductase inhibitors (statins) (Yang and Keating, 2009). This combination minimizes the complications of the other common combined therapy, muscle pain and myositis.

2.5.3 Metabolism

Both in case of prodrug fenofibrate, that requires first to be hydrolyzed in intestine to reveal the active metabolite, and in other, when fenofibric acid is administrated as choline salt and dissociation occurs in gastrointestinal track, the available administration is only *per os*. Concerning its distribution, once fenofibric acid enters the blood stream it is bounded to albumin in order to get to its target, PPARα.

Biotransformation takes place in the liver and kidneys. Fenofibric acid is able to be glucuronosylated immediately, before being reduced to its metabolite, and been detoxified (Chapman, 1987). *In vitro* study of the glucuronidation of fenofibric acid by recombinant isoforms of UGTs and liver microsomes, showed that this reaction can be performed by many isoforms of hepatic UGTs, but UGT2B7 and UGT1A9 holds the greatest role, followed by lower affinity for UGT1A3 and UGT1A6. Moreover, comparing to microsomes participation, it was suggested the high

variability among individuals, probably due to genetic factors (Gonzales-Covarrubias et al., 2008).

Reduced fenofibric acid, is the active product of the reduction of fenofibric acid. According to molecular structure of fenofibric acid, it is clear that the compound is reduced at the ketone moiety. An article published by FDA about available fenofibrate agents that are metabolized in liver and small intestine to fenofibric acid declares that: "*In vivo* metabolism data indicate that neither fenofibrate nor fenofibric acid undergo oxidative metabolism (e.g., CYP450) to a significant extent" (2008). Moreover, it is known that only 5% avoids the glucuronidation and undergo reduction (Chapman, 1987). On the contrary, the reductive product of fenofibric acid predominates in the biotransformation in rats and dogs (Caldwell, 1989). Additional *in vivo* study with rat hepatocytes has found apart from reduced fenofibric acid a small amount of another product due to methylation of fenofibric acid (Cornu-Chagnon et al., 1995). Noteworthy, no enzyme has been identified to date that is responsible for the reduction of fenofibric acid.

The benzhydrol product of the carbonyl reduction of fenofibric acid undergoes glucuronidation as well, in order to be excreted in urine. The excretion of glucuronosylated products in human occurs mainly *via* urine (~60%) and the rest leaves the organism through feces. In feces it was found that it is excreted fenofibric acid and other unidentified polar compounds (Weil et al., 1990). On the contrary, in the case of other mammals, as dog and rats, the excretion takes place in the greatest degree through the bile (80%) (Caldwell, 1989).

The following reaction scheme (Figure 3) shows the metabolism pathways of fenofibric acid.

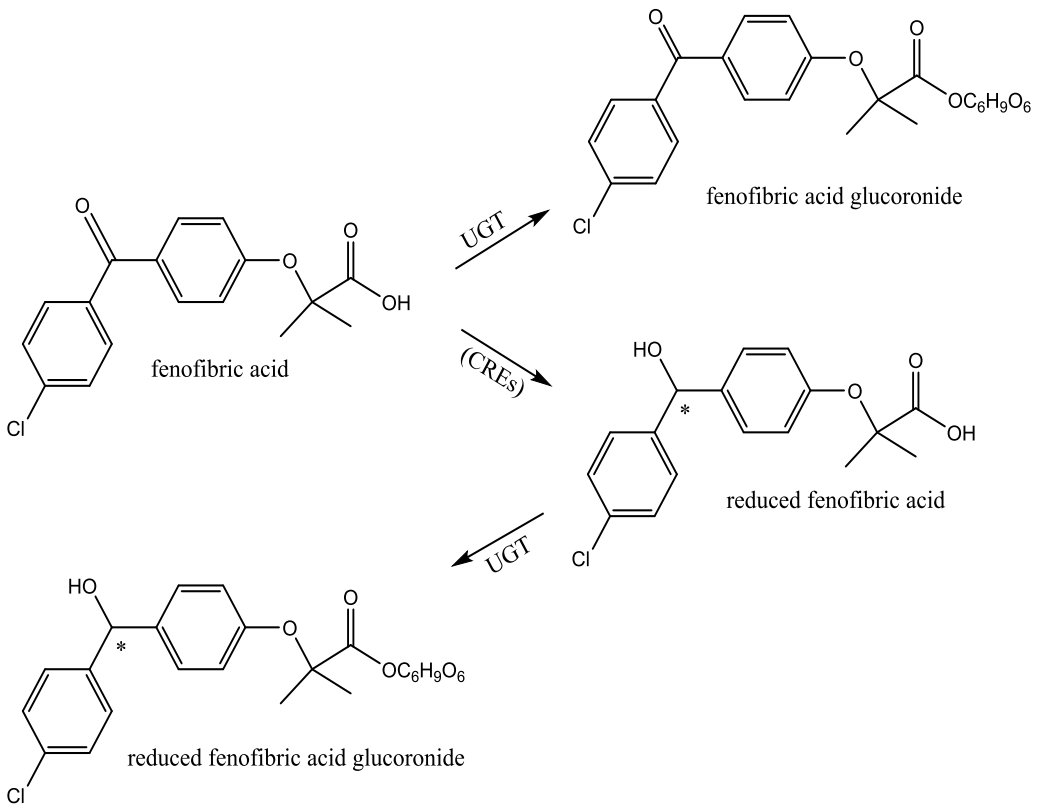


Figure 3. Reaction scheme of fenofibric acid. UGT= Uridine 5`- diphospho-Glucuronosyl Transferase, CREs= Carbonyl Reducing Enzymes. *= carbon chiral center.

3. AIM OF THE WORK

The aims of the present diploma thesis are:

- To examine the optimal extraction method for the reduced metabolite of fenofibric acid.
- To compare the activity of two human liver fractions, i.e. cytosol and microsomes, toward reduction of fenofibric acid.
- To investigate the ability of a variety of cytosolic enzymes to reduce fenofibric acid.
- To determine the enzymatic activity of the most active cytosolic enzymes at a various concentrations of fenofibric acid.
- To examine the influence of methanol on the reduction activity of cytosol toward fenofibric acid.
- To evaluate and quantify reduced fenofibric acid by the use of HPLC.

4. MATERIALS

4.1 Chemicals

Acetonitrile	Sigma-Aldrich, St. Luis, USA
Ammonia 23-25%	Penta, Czech Republic
Distilled water	Department of Biochemistry, Faculty of Pharmacy, Hradec Kralove
Ethyl acetate	Sigma-Aldrich, St. Luis, USA
Fenofibic Acid	Aldrich Chemistry, Milwaukee, USA
Glucose-6- Phosphate (6mM)	Sigma-Aldrich, St. Luis, USA
Glucose-6-Phosphate dehydrogenase(35U/I)	(from yeast), Boehringer Mannheim, (GmBH) Germany
Hydrochloric acid	Penta, Czech Republic
Methanol	Fluka analytical, Sigma-Aldrich, USA
$NADP^+$ (0,8mM)	SERVA, Germany
n- Hexane	Merck

4.2 Buffers and Solutions

Na- phosphate buffer 0,1M Na_2HPO_4, pH=7,4

The phosphate solution was prepared by mixing solutions of 0.1M $Na_2HPO_4.12H_2O$ (3.58g/100ml), and 0.1M $Na_2HPO_4.2H_2O$ (0.39g/25ml).

Regenaration system:

$NADP^+$ and glucose-6-phosphate were weighted out and diluted in $MgCl_2$ and Na-phosphate buffer. In the end, glucose-6-phosphate dehydrogenase was added. For the amounts see Table 4.

Table 4. NADPH Regeneration System recipe.

Substance	10 samples	Final amount
$NADP^+$	2mg	0.8mM
Glucose-6-phosphate	6mg	6mM
$MgCl_2$	100µl	3mM
Na-phosphate buffer	100µl	
Glucose-6-phosphate dehydrogenase	5µl	35U/1 sample

$MgCl_2$ (3mM) MgCl.6 H_2O 1.017g/50ml H_2O

0.1 M Na-phosphate buffer pH=7.4 see above

Mobile Phase:

Details will be presented in prepared paper by Nobilis et al.

4.3 Biologicals

Cytosol	c = 8μg/μl, c = 10μg/μl
Microsomes	c = 3.90μg/μl, c = 5μg/μl

The solutions of purified recombinant enzymes prepared at the Department of Biochemistry, Faculty of Pharmacy, Hradec Kralove

AKR1A1	c = 1.90mg/ml (09/11/2012)
AKR1B1	c = 1.25mg/ml (31/03/2014)
AKR1B1 BB (bugbuster)	c = 0.56mg/ml (09/01/2015)
AKR1B1 SON (sonication)	c = 0.79mg/ml (09/01/2015)
AKR1B10	c = 1.61mg/ml (08/03/2012)
AKR1C1	c = 1.84mg/ml (19/12/2013)
AKR1C2	c = 1.96mg/ml (12/12/2013)
AKR1C3	c = 1.92mg/ml (06/02/2014)
AKR1C4	c = 1.77mg/ml (06/08/2013)
CBR1	c = 2.11mg/ml (28/03/2014)
CBR3	c = 1.93mg/ml (28/03/2014)

4.3 Equipment

Analytical balance	Sartorius / Scaliter SBC22
Centrifuges	Mini spin plus, Eppendorf, Germany
	Biofuge Stratos, Herdeus HIM2812, Kendro Laboratory Product, Germany
HPLC column	HALO.5®, C18, 5µm, 4,6mm X 250mm, Advanced Materials Technology, USA
Concentrator	5301, Eppendorf, Germany
Disposable hypodermic needle	Braun (0.90 x 40 mm)
HPLC	Shimadzu® UFLC CTO-20AC, Japan
Incubator	Broer, Mixing Block MB102
	Thermomixer compact, Eppendorf
Microtubes	Eppendorf
Multipipettes	Eppendorf
pH meter	Sigma Instruments, India
Pipettes	Eppendorf, Biohit
Syringe filter	PTFE membrane Polypropylene
	(4mm, 0.2µm) Whatmann
Syringe without needle	1mL, Terumo, Philippines
Ultrasonic bath	Manufacture Expert
Vortex	VG3, IKA Werke GmbH & Co, Germany

5. METHODS

5.1 Preparation of liver fractions

The subcellular liver fractions (cytosol and microsomes) that were used in the present work were prepared corresponding to the Czech legislation and have been obtained by Cadaver Donor Program of the Transplant Center of the Faculty of Medicine in Hradec Kralove (Czech Republic). The tissue samples were prepared in order to obtain the subcellular fraction as it has been already described (Skarydova et al., 2013).The liver fractions were used at concentrations 100μg/20μl and 50 μg/ 20μl. Dilutions from the stock solution were formed in Na-phosphate buffer, pH 7.4, and the total amount of the liver fractions added to each reaction mixture was 20μl.

5.2 Preparation of recombinant enzymes

The preparation of the forms of the recombinant cytosolic enzymes that were tested in the present diploma thesis, i.e. AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, CBR1 and CBR3, were obtained by expression in *Escherichia coli*, by the means of the standard techniques as mentioned (Skarydova et al., 2014). The recombinant enzymes were used at concentration 20μg/20μl. Dilution from the stock solution was formed in Na-phosphate buffer pH 7.4, and the total amount of added to each reaction mixture was 20μl.

5.3 Incubation

Incubation procedure is necessary in order to investigate the enzymatic activity, and evaluate the specific activity. In any case, a variety of concentration of fenofibric acid was prepared (1mM, 2mM, 3mM, 5mM, 7mM, and 10mM) by dilution of stock solution (50mM in methanol) with 0.1 M Na-phosphate buffer pH=7.4. Fraction/ enzymes were also diluted to final amount of protein (20, 50, or 100μg in 20μl) with 0.1 M Na-phosphate buffer pH=7.4. NADPH regeneration system was always prepared fresh according to the number of tested samples (see composition in Buffers and Solutions). The microtubes were placed into the ice, and the solutions were added in the following order: 0.1 M Na-phosphate buffer pH=7.4, NADPH regeneration system, fraction/ enzyme/ phosphate buffer. Solution mixture was mixed, and shortly

centrifuged down in centrifuge. Final volume of reaction mixture including the substrate was 100µl, as shown in Table 5.

Table 5. Composition of reaction mixture

Solution	Volume [µl]
0.1M Na-phosphate buffer (pH=7.4)	50
NADPH Regeneration system	20
Enzyme/ fraction/ phosphate buffer	20
Fenofibric acid	10
Final volume:	100µl

Thermo block was set to the temperature of 37^{o}C. Pre-incubation of reaction mixture was performed for 5 minutes. Reaction was started by addition of fenofibric acid, and stopped after 30 minutes by addition of 40µl ammonium solution (25%), or 1M HCl, and tubes were transferred to the ice. If needed, 30µl of internal standard was added. Then, extraction was performed as described below. Every incubation was performed at least in three independent determinations for each experiment.

5.4 Preparation of samples

5.4.1 Optimization of Extraction methods

After stopping the enzymatic reaction, ethyl acetate was added and either one or two fold extraction was performed. In case of one fold extraction, 1000µl of ethyl acetate was added in each tube. The tubes were shaken for 15 minutes using shaker. Then, they were centrifuged for 2 minutes at 13.000 rpm. The upper organic phase was transferred into the new eppendorf tubes and the samples were evaporated to dryness into the concentrator under vacuum at 30^{o}C. In case of the two fold extraction, after the organic upper layer was transferred to a new tube, the same procedure was repeated with the down phase. So, in the end the extraction was completed by 1000µl + 1000µl ethyl acetate. Another extraction method that was tested was by 1000µl of

mixture of n-hexane and ethyl acetate (90:10, v/v) followed by the same procedure as in the case of ethyl acetate. The filtration method was also investigated instead of the liquid-liquid extraction. In this case, the incubation reaction was ended by addition of 300μl of ice-cold methanol , 30μl IS was added and the samples were stored in ice for 10 minutes. Then, samples were centrifuged at 10.000g at 4^{o}C for 15 minutes. Finally, they were filtered through a filter by the use of syringe, transferred in fresh tubes and evaporated in vacuum at 30^{o}C for approximately 3 hours.

5.4.2 Preparation of samples for HPLC analysis

The residue of the examined samples, was diluted in 200ul of mobile phase. Solutions were allowed to dissolve in the ultrasonic bath, mixed by vortex and shortly centrifuged, and finally transferred to vials with 400μl inserts.

5.5 High performance liquid chromatography

The HPLC machine was SHIMADZU® and the column was HALO.5® C18, of size 5μm, length 250 and diameter 4mm. The analysis was performed at isocratic flow with the mobile phase as stated before. The flow rate of the pump was 1 ml/min, the pressure was 110-111 bars, and the column was kept at the temperature 25^{o}C. UV-VIS detection was performed at 229nm, 288nm at and 292nm, with elution time 5 minutes, 8.55 minutes and 10.720 minutes for detection of reduced fenofibric acid, fenofibric acid and internal standard, respectively. The duration of analysis was 15 minutes per tested sample. Peaks, areas and graphs of the analysis were evaluated using the LC solution software by SHIMADZU®.

The samples were injected automatically into the chromatographic system in the volume of 100μl. Degasser is responsible for removing the gas from the mobiles phase, while the pump was set to provide it in the column constantly by 1 ml/min. The oven was set to maintain the proper temperature. The photodiode-array detector was used to monitor the signal of the eluent.

All details regarding the analytical HPLC method as well as the selection of internal standard (IS) and chromatographic conditions will be presented in prepared paper by Nobilis et al.

The HPLC provide us the opportunity of both qualitative and quantitative analysis. In this case it allowed us to examine if the enzymes had reduced the fenofibric acid to its metabolic product, but also to calculate the amount of that product.

The following Figure 4 shows the HPLC SHIMADZU® that was used for the analysis.

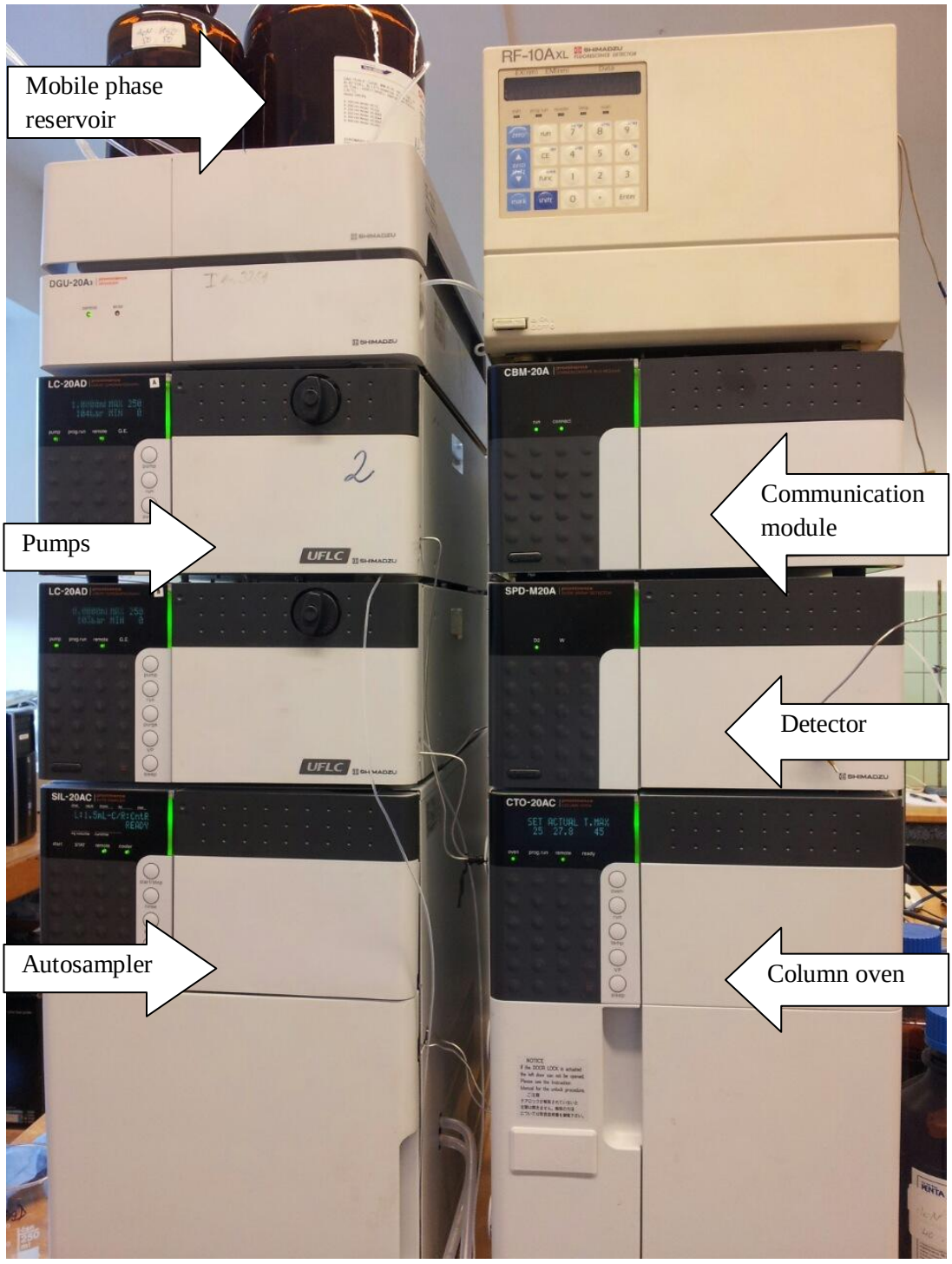


Figure 4. SHIMADZU® HPLC and its parts.

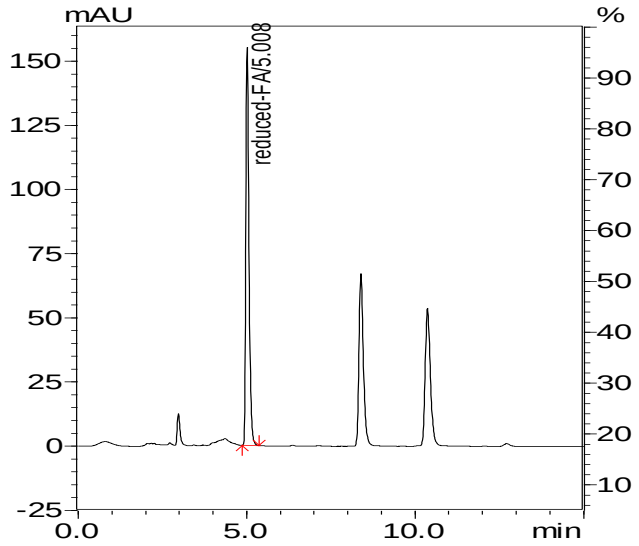
5.6 Determination of enzyme kinetics

Cytosol, microsomes and the carbonyl-reducing enzymes that exhibited reductase activity toward fenofibric acid were used to determine the kinetic parameters. Incubations were performed as previously described with a final concentration of fenofibric acid in the range of 100 to 1500 μM. The apparent kinetic parameters were calculated using GraphPad Prism ® 6.0 computer software. The formation of reduced fenofibric acid was fitted to both the Michaelis-Menten hyperbola and allosteric sigmoidal kinetic models. The best-fit model was determined based on the ranking of the coefficient of determination (R^2) and Hill slope (h). Enzymatic activity was expressed as the specific enzyme activity that represents pmol or nmol of formed reduced fenofibric acid per mg of protein in 1 min. Internal clearance (CL_{int}) represents enzymatic efficiency and is defined as V_{max}/K_m. The values were given as the mean ± S.D. from n = 3–8 experiments.

6. RESULTS AND DISCUSSION

6.1 High performance liquid chromatography

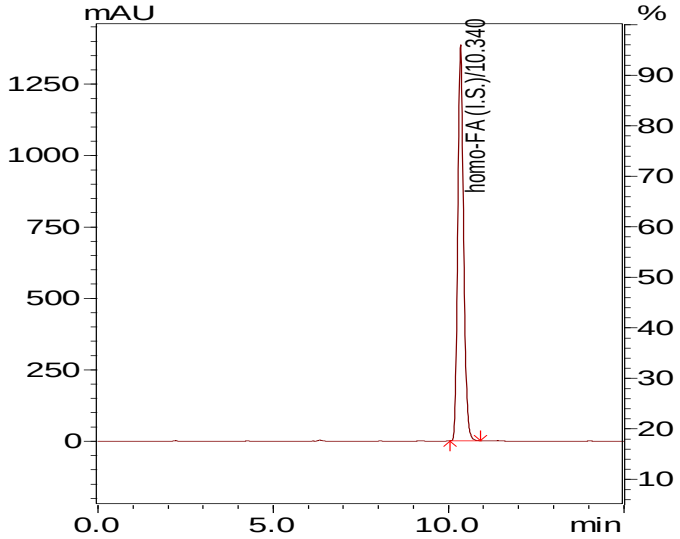
The detection of the reductive product was achieved by the use of the standard of reduced fenofibric acid (10^{-5} M). The volume of injected samples and standards was 100 µl.



Graph 1. Representative chromatogram of the standard of reduced fenofibric acid.

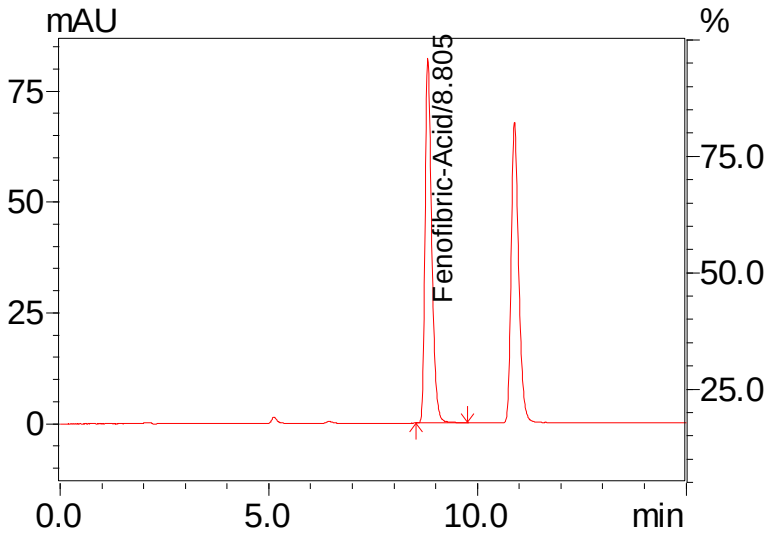
The area of the standard of reduced fenofibric acid was around 923700 corresponding to 320.77ng. Elution time of reduced fenofibric acid was 5.000 minutes (±- 0.200) as depicted in Graph 1.

Graph 2 represents the internal standard (IS) that was used in these analyses. The concentration of this standard injected was again 10^{-5} M and the detection was achieved at 292nm with elution time at 10.720 minutes (±0.400).



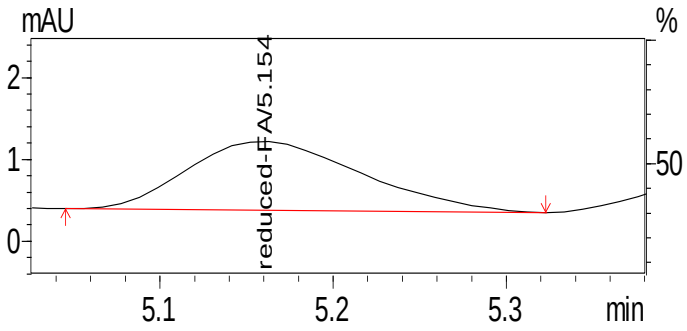
Graph 2. Representative chromatogram of standard of IS.

Finally the standard of our investigated drug, fenofibric acid (injected at the concentration 10^{-5} M) was detected at 288nm at 8.55 minutes (±0.300). Graph 3 shows the peak of fenofibric acid.



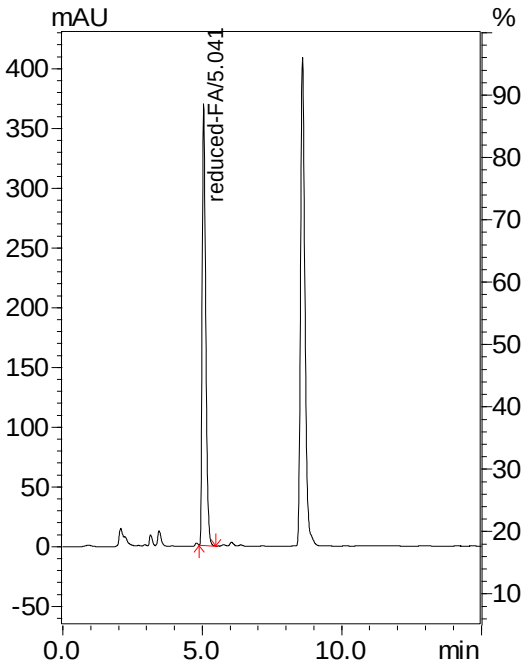
Graph 3. Representative chromatogram of standard of fenofibric acid.

The following graphs belong to the examined cytosolic fraction and cytosolic enzymes, which were analyzed for their ability to reduce fenofibric acid.



Graph 4. Representative chromatograph of reduced fenofibric acid by cytosol at 500µM FA.

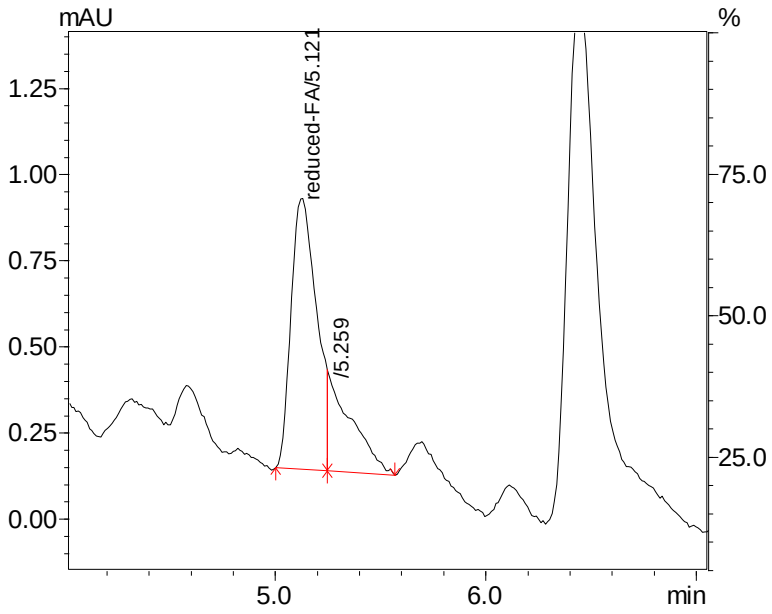
Graph 4 shows the area of reduced fenofibric acid after incubation of cytosol with 500µM fenofibric acid. The depicted area is 5896, that corresponds to 2.49 ng of reduced fenofibric acid.



Graph 5. Representative chromatograph of reduced fenofibric acid by CBR1 at 300µM FA.

Graph 5 represents the area (3225689) of the chromatograph for the analysis of reduction of fenofibric acid (300µM) by CBR1. This area corresponds to 1365.5ng of produced reduced fenofibric acid.

Finally, Graph 6 shows a peak with the area 6709 for reduction of fenofibric acid (100μM) by AKR1C3 that corresponds to 2.84ng of reduced fenofibric acid.



Graph 6. Representative chromatograph of reduced fenofibric acid, by AKR1C3 at 100μM FA.

The repetition of experiment helped us to investigate a problem with the evaluation of the HPLC analysis for enzyme AKR1C3. It was found that in many cases separation of the reduced fenofibric acid was not clear. In many cases there were present "peak tailing" as shown in the Graph 6, or "shoulder peaks". The examination was repeated again after washing of column and pumps of the HPLC, but these problems remained. Worth saying is that this situation concerned only the enzyme AKR1C3. Since contamination was excluded, suggestions for avoiding this poor chromatography resolution are either to change the flow rate, or the mobile phase.

6.2 Optimization of extraction

In this test, we examined the optimal extraction method. The test of the extraction was performed using standard incubation with fenofibric acid at two concentrations (100 and 500 μM) and 38,4μg of the enzyme AKR1C3. The methods used for extraction were filtration, extraction by ethyl acetate or by mixture of n-hexane: ethyl acetate (90:10, v/v). In each method, 10 μg of internal standard were added after the enzymatic reaction was stopped. The efficiency of extraction method was calculated for the internal standard.

Table 6. Extraction efficiency of internal standard using various extraction methods.

Sample	Final conc. FA [μM]	Amount of redFA [ng]	Amount of FA [ng]	Amount of IS [ng]	Extraction efficiency of IS [%]
C3-1/f	100	0	25.8	61.2	0.6 (±0.05)
C3-2/f	500	0	148.1	57.9	0.6 (±0.03)
C3-1/e	100	3.07	2395.4	10814.1	109.0 (±11.7)
C3-2/e	500	5.51	12870.3	9149.4	92.2 (±1.9)
C3-1/nhe	100	4.62	2244.1	9743.9	98.2 (±1.6)
C3-2/nhe	500	4.72	12703.4	95.3	96.0 (±1.4)

F= filtration, e= one fold extraction with ethyl acetate, nhe= extraction with n-hexane:ethyl acetate (90:10), FA = fenofibric acid, redFA = reduced fenofibric acid.

According to the results of the extraction efficiency of internal standard, as shown in the Table 6, the most efficient extraction at the lower concentration of fenofibric acid was one fold extraction with ethyl acetate (extraction efficiency 109%), followed by extraction with the mixture of n-hexan and ethyl acetate (90:10, v/v) (extraction efficiency 98.2%). On the other hand, at higher concentration of fenofibric acid, the extraction efficiency was slightly higher with the mixture n-hexan and ethyl acetate (extraction efficiency 96%) than with ethyl acetate (extraction efficiency 92.2%). The extraction efficiency of internal standard using filtration method was at both concentration of fenofibric acid almost zero (0.62% and 0.58%). Moreover, the analyzed amount of both fenofibric acid and reduced fenofibric acid was very low or

even zero, respectively. Therefore, filtration is not an appropriate method for the extraction of the fenofibric acid and its metabolite or for internal standard.

Taking a further look at the facts shown in the Table 6, can be observed that the amount of the produced reduced fenofibric acid in case of 100μM of fenofibric acid is higher using the extraction method with mixture of n-hexane and ethyl acetate (90:10, v/v) than by ethyl acetate (4.62 ng vs. 3.07, respectively). For the higher concentration, 500μM fenofibric acid, the amount of the reduced product is slightly higher in the extraction method by ethyl acetate than mixture of n-hexane and ethyl acetate (5.51 vs. 4.72 ng reduced fenofibric acid, respectively). The amount of extracted fenofibric acid was somehow higher using extraction by ethyl acetate than mixture of n-hexane and ethyl acetate at both concentrations of fenofibric acid.

Correspondingly, as represents Table 7, calculated specific activity for the samples shows that the specific of the samples extracted by mixture of n-hexane and ethyl acetate was higher than ethyl acetate extraction (12.5 vs. 8.3 pmol/min/mg, respectively). However, the specific activity for the second concentration, 500μM fenofibric acid, was greater using the ethyl acetate extraction method (15.00 vs. 12.7 pmol/min/mg).

Table 7. Specific activity for reduction of fenofibric acid using various extraction methods.

Extraction	Reaction stopped	Final conc. FA [μM]	Specific activity [$pmol.min^{-1}.mg^{-1}$]
Ethyl acetate	HCl	100	8.3
Ethyl acetate	HCl	500	15.0
n-Hexane:Ethyl ac.(90:10)	HCl	100	12.5
n-Hexane:Ethyl ac.(90:10)	HCl	500	12.7
Filtration	MetOH	100	0.0
Filtration	MetOH	500	0.0

FA = fenofibric acid

Because the extraction efficiency by ethyl acetate and the mixture of n-hexane and ethyl acetate was comparable for all tested compounds but n-hexane is more toxic than ethyl acetate, ethyl acetate was chosen as extraction reagent for further experiments.

The following part of the search for the optimal extraction method was to compare extraction efficiency of one fold and two fold extraction with ethyl acetate. Again the examined enzyme was AKR1C3, but in amount of 20μg in 100μl of the reaction mixture. The extraction efficiency of both one and two fold ethyl acetate extraction was calculated for fenofibric acid using the method where fenofibric acid was added into the incubation mixture after the reaction was terminated by the addition of the HCl, which was considered to provide 100% of fenofibric acid. The following Table 8 contains the results.

Table 8. Extraction efficiency of fenofibric acid.

Sample	**Final conc. FA[μM]**	**Amount of FA detected (ng)**	**Extraction efficiency of FA (%)**
C3-1/e	100	2834.7	93.2 (±3.67)
C3-2/e	500	20121.1	103.7 (±2.54)
C3-1/ee	100	2787.5	91.6 (±1.13)
C3-2/ee	500	17944.3	92.4 (±6.15)

FA was added after the termination of the reaction, e=one fold extraction with ethyl acetate, ee=two fold extraction with ethyl acetate, FA = fenofibric acid.

At both tested concentrations of the drug, the highest extraction efficiency of fenofibric was with one fold extraction with ethyl acetate. Interestingly, the extraction efficiency of one fold extraction was higher at 500μM fenofibric acid than at 100μM fenofibric acid, giving the extraction efficiency 103.7% and 93.2%, respectively. The two fold ethyl acetate extraction at both concentrations of fenofibric acid showed similar extraction efficiency with values 91.6 % and 92.4% at lower and higher concentrations of fenofibric acid respectively.

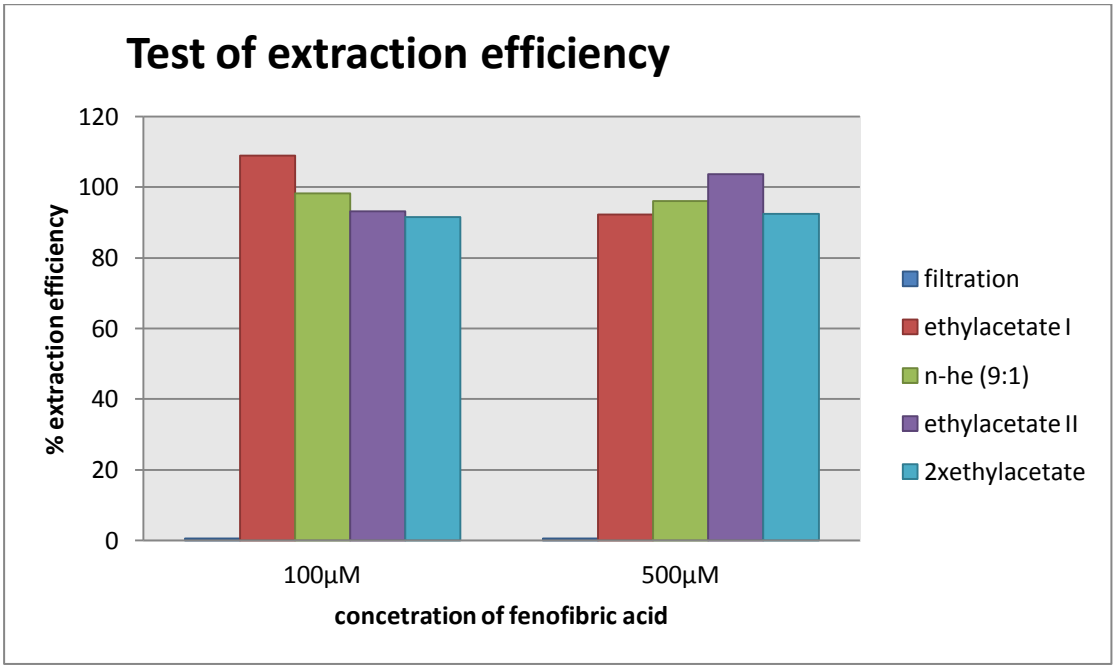
In the Table 9 are written the specific activities calculated for both extraction methods.

Table 9. Specific activity for reduction of fenofibric acid using various extraction methods.

Extraction	**Reaction stopped**	**Final conc. FA [µM]**	**Specific activity [pmol.min^{-1}.mg^{-1}]**
1x ethyl acetate	HCl	100	35.9
1x ethyl acetate	HCl	500	67.4
2x ethyl acetate	HCl	100	18.4
2x ethyl acetate	HCl	500	21.2

FA = fenofibric acid

All the extraction methods and their percentage efficiency results of are shown in the Graph 7.

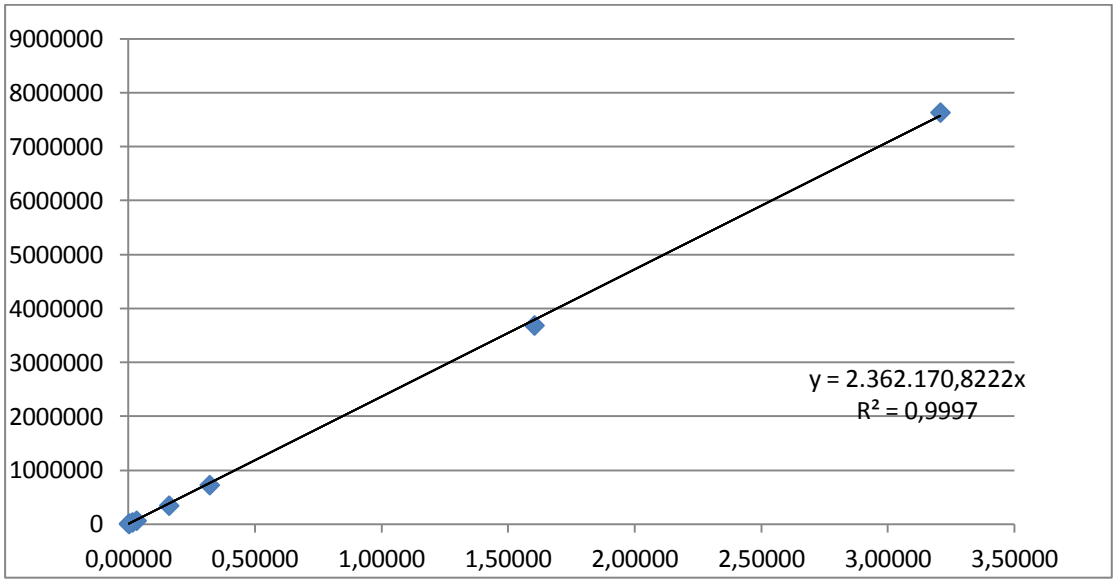


Graph 7. Results of % extraction efficiency of all tested methods. Ethyl acetate I and n-he – extraction efficiency was calculated for internal standard. Ethyl acetate II and 2x ethyl acetate I - – extraction efficiency was calculated for fenofibric acid.

According to extraction tests that were performed using the enzyme AKR1C3, the most efficient extraction method was the one fold extraction with ethyl acetate.

6.3 Calibration curve

For the quantification of the metabolized product of the fenofibric acid by the tested enzymes, i.e. reduced fenofibric acid, it was used the calibration curve. For the construction of calibration curve eight concentrations of fenofibric acid in the range of 0.05 - 10.00 μM were used. In detail, the concentrations were: 0.00002μg/μl, 0.00003μg/μl, 0.00016μg/μl, 0.00032μg/μl, 0.00160μg/μl, 0.00321μg/μl, 0.01604μg/μl, and 0.03208μg/μl. The volume injected was 100μl.



Graph 8. Calibration curve of reduced fenofibric acid

Important parameter that characterizes the calibration curve is the linearity of the method, which arises from the value of the R^2. The closer to the 1 the R^2 is, the better the linearity. In this case the R^2 equals to 0.9997. This shows that the obtained calibration curve is accurate and can be used for calculations.

6.4 Test of influence of methanol

In order to achieve complete dissolution of fenofibric acid, the stock solution was prepared in methanol. Methanol is an organic solvent that is toxic and can inhibit the activity of many enzymes (Vuppugalla et al., 2007; Behere et al., 2014). The final amount of methanol in the incubation mixture was in the range of 0.2 to 3%. At the concentration of fenofibric acid of 500μM, the amount of methanol was 1%.

Because methanol can influence the activity of the enzymes (Vuppugalla et al., 2007; Dayanididhi et al., 2014), the influence of increasing amount of methanol (0.5, 1.0,

2.0, 3.0 and 5.0%) on the reducing activity of cytosol was tested. For this examination, samples were prepared with 50µg of cytosolic fraction and 500µM fenofibric acid and different amount of methanol. The experiment was repeated four times. The values were then edited in Microsoft Office EXCEL 2007, to calculate the specific activity of the cytosol, and ANOVA was applied to test if there is significant difference between the samples of different concentrations of methanol. Results are presented in Table 10.

Table 10. The influence of methanol on specific activity.

Protein [µg]	**Final conc. FA [µM]**	**methanol [%]**	**Specific activity [pmol.min^{-1}.mg^{-1}]**
50	500	0.5	7.16 (±0.82)
50	500	1.0	7.18 (±0.85)
50	500	2.0	8.83 (±0.85)
50	500	3.0	9.91 (±1.04)
50	500	5.0	7.55 (±0.77)

The values of the specific activity of cytosol in samples with different concentrations of methanol were examined with ANOVA (see Table 11), and the ρ value was equal to 0.01. Since $\rho < 0.05$, there were no statistically significant differences between group means. To the current test it means that at the concentration of 500µM fenofibric acid the amount of methanol in percentage from 0.5 to 5.0 % does not influence the enzymatic activity.

Table 11. ANOVA calculations

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	22,28087	4	5,570218	4,817631	0,010622	3,055568
Within Groups	17,34323	15	1,156215			
Total	39,6241	19				

6.5 Screening of liver fractions and enzymes

The purpose of the current test was to investigate cytosol, microsomes and a variety of cytosolic enzymes for their ability to reduce fenofibric acid. Liver fractions were added at the final concentrations 100 and 50 μg/100μl, and fractions of cytosolic enzymes at 20μg/100μl. Reaction was stopped in all incubations by HCl, and in many cases also by NH_3. All samples were extracted once by ethyl acetate. Findings of the screening are shown in the following Table 12.

Both cytosol and microsomes exhibited reductase activity toward fenofibric acid. At the lower concentration of fenofibric acid, the activity of cytosol was more than two times higher than that of microsomes. On the contrary, at concentration 500μM of fenofibric acid, the activity of cytosol was slightly higher than that of microsomes. Table 12 indicates where the reaction was stopped by NH3 and where by HCl. However, HCl was better reagent for termination of the reaction and was used in all following experiments.

The most active among all the enzymes was CBR1, an enzyme from the SDR superfamily, followed by an AKR enzyme, AKR1C3. Significant activity was also exhibited by the AKR enzymes AKR1C1, AKR1C2 and AKR1B1. Moreover, slight activity was detected with AKR1C4 but only at the lower concentration of fenofibric acid. On the other hand, the enzymes AKR1B10, AKR1A1 and CBR3 did not show any reductase activity toward fenofibric acid.

Taken together, the order of the reducing activity of the recombinant cytosolic enzymes was as follows: CBR1>>AKR1C3>AKR1C2≥AKR1C1>AKR1C4. In addition, the order of the reducing activity in human liver subcellular fraction was microsomes>cytosol.

The highest reducing activity of the recombinant enzymes tested was found for CBR1. CBR1 showed higher activity compared to cytosol and microsomes and its activity was 70 times higher than that of the second most active enzyme, AKR1C3. Therefore, CBR1 may be suggested as the main cytosolic enzyme responsible for the reduction of fenofibric acid.

Table 12. Screening of enzymes and fractions.

Fraction/Enzyme	protein [µg]	FA conc.[µM]	Specific activity [pmol.min^{-1}.mg^{-1}]	Reaction stopped	Extraction
Cytosol	100	100	5,00	NH3	Ethyl acetate
	100	500	25,39	NH3	Ethyl acetate
Pooled	50	100	25,94	HCl	Ethyl acetate
Pooled	50	500	47,31	HCl	Ethyl acetate
Microsomes	100	100	2,75	NH3	Ethyl acetate
	100	500	6,72	NH3	Ethyl acetate
Pooled	50	100	11,39	HCl	Ethyl acetate
Pooled	50	500	43,58	HCl	Ethyl acetate
CBR1	20	100	13,05	NH3	Ethyl acetate
	20	500	60,14	NH3	Ethyl acetate
	42,2	100	2780,30	HCl	Ethyl acetate
	42,2	500	12200,90	HCl	Ethyl acetate
AKR1C3	20	100	6,61	NH3	Ethyl acetate
	20	500	not determined	NH3	Ethyl acetate
	20	100	35,86	HCl	Ethyl acetate
	20	500	67,31	HCl	Ethyl acetate
AKR1C1	20	100	3,09	NH3	Ethyl acetate
	20	500	3,54	NH3	Ethyl acetate
	36,8	100	2,90	HCl	Ethyl acetate
	36,8	500	6,20	HCl	Ethyl acetate
AKR1C2	20	100	3,34	NH3	Ethyl acetate
	20	500	3,95	NH3	Ethyl acetate
	39,2	100	5,10	HCl	Ethyl acetate
	39,2	500	6,50	HCl	Ethyl acetate
AKR1B1	20	100	0,00	HCl	Ethyl acetate
	20	500	1,32	HCl	Ethyl acetate
	25	100	6,00	HCl	Ethyl acetate
	25	500	9,00	HCl	Ethyl acetate
AKR1B10	20	100	0	HCl	Ethyl acetate
	20	500	0	HCl	Ethyl acetate
AKR1A1	20	100	0	NH3	Ethyl acetate
	20	500	0	NH3	Ethyl acetate
AKR1C4	20	100	2,76	NH3	Ethyl acetate
	20	500	0	NH3	Ethyl acetate
CBR3	20	100	0	NH3	Ethyl acetate
	20	500	0	NH3	Ethyl acetate

Although AKRB1 was active on reduction of fenofibric acid, its activity was 7-times lower than the activity of the most active AKR enzyme, AKR1C3. Other AKR enzymes, AKR1C1 and AKR1C2 had similar activity toward fenofibric acid reduction but even lower than AKR1B1. Their activity was 10-times lower than that of AKR1C3. Additionally, AKR1C4 showed the lowest activity of all tested enzymes and therefore, this enzyme was not involved in further experiments.

It was also performed screening test for the enzyme AKR1B1 at the concentration of fenofibric acid of 500μM, in order to compare the activity of recombinant enzymes prepared by two different approaches. The one approach involved using Bug Buster for release of the overexpressed protein, and the second one sonication. As it is depicted in Table 13, the sample obtained from bug buster had higher reducing activity (59.87 vs. 48.46 pmol/min/mg).

Table 13. Results of AKR1B1 comparison

Enzyme	protein [μg]	Conc. FA [μM]	Reaction stopped	Extraction	Specific activity [$pmol.min^{-1}.mg^{-1}$]
AKR1B1	20	500	HCl	Ethyl acetate	46.29 ±16.10
AKR1B1_BB	16.8	500	HCl	Ethyl acetate	59.87±15.45
AKAR1BI_SON	16.8	500	HCl	Ethyl acetate	48.46±8.93

BB= bug buster, SON= sonication

6.6 Enzyme kinetics

After the initial screening of the subcellular fractions and the cytosolic enzymes it was determined the enzyme kinetics. For this purpose, it was measured the velocity of the reductase activity of the enzymes at various concentration of the substrate. In particular, it was measured at 7 concentrations of fenofibric acid; 100μM, 200μM, 300 μM, 500μM, 700μM, 1000μM and 15000μM. The incubation was performed with optimized conditions, i.e. 20μg of recombinant enzymes or 50μg of subcelullar fraction was incubated with fenofibric acid and the reaction was terminated by the addition of HCl followed by one fold extraction with ethyl acetate. The amount of produced reductive product was determined by the help of HPLC and the specific activity calculated in pmol per minute per mg of protein. Finally, kinetic parameters

were determined using the GraphPad Prism ® 6.0 computer software, as described in Methods.

The Figure 5 (A-C) presents three measurements that correspond to the kinetic of cytosol, since it was repeated in 3 different days and there was significant difference between them. While for microsomes, the kinetic was unambiguously fitted to Michaelis-Menten, as shown in the Figure 5D.

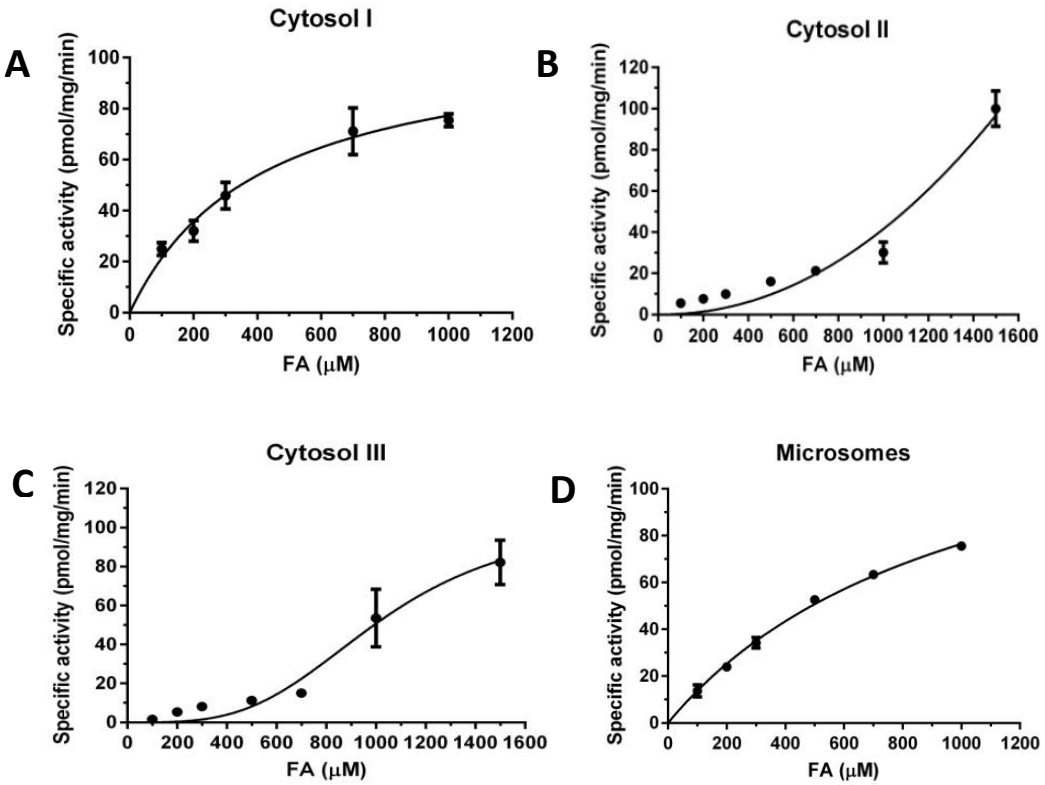


Figure 5. Kinetics of fenofibric acid reduction by human liver cytosol (A-C) and microsomes (D). A is according to Michaelis-Menten, B and C according to allosteric sigmoidal kinetics, D kinetics of microsomes according to Michaelis-Menten equation.

Parameters of the kinetics of the human liver fractions are written in the Table 14.

Table 14. Kinetic parameters of human subcellular liver fractions.

	V_{max} [pmol/mg/min]	**K_m or K_{half} [μM]**	***h* *[Hill slope]***	**R^2**	**CL_{int} [μl/mg/min]**	**Model**
C I	109±8.192	410.00±69.09		0.9307	0.265	M-M
C II	*~597372*	*~98246±1.74x10^8*	*2.087*±0.670	*0.9416*		*Allost.*
C III	*105*±20.84	*1020±149.6*	*3.464*±0.866	*0.9228*	*0.103*	*Allost.*
M	154.9±7.636	1027,00±82.72		0.9923	0.151	M-M

C = cytosol, M = microsomes, V_{max} is maximum enzyme activity, K_m Michaelis constant for Michaelis-Menten (M-M) equation, and h is the Hill slope, that characterizes the steepness of the curve and presence of cooperativity.

Comparing the measurements according to Michaelis-Menten equation, we can assume that V_{max}, expressed as specific activity, of the microsomes is 42.1% greater than that of cytosol (154.9 vs. 109 pmol/mg/min). However, Michaelis constant for microsomes is 150% higher than that for cytosol (1027 vs. 410 μM), meaning the greater affinity of cytosol over microsomes .

On the other hand, in the second measurement of cytosol kinetics, there was found better fit for allosteric sigmoidal equation. This can lead us to a though that probably, it can occur difference in kinetics between the individuals (Takahashi et al., 2008). It should be noted, that in all three kinetic measurements, it was used the same stock solution of cytosol. The tested cytosol was pooled and obtained from liver of eight different people.

Another parameter taken in consideration is the internal clearance CL_{int} expressed as V_{max}/K_m [l/mg proteins/min]. Comparing the Cl_{int} derived from the Michaelis Menten equation of cytosol and microsomes, we observe that the metabolism in cytosol is 70% higher than in microsomes (0.265 vs. 0.151 μl/mg/min).

Figure 6 and Table 15 show the kinetics of the enzymes that exhibited the highest specific activity during the screening experiment. Apart from the enzyme AKR1C3 for which the number of independent determinations was 8, for the rest of enzymes were performed 4 determinations. The selection of the equation of the enzymatic activity, Michaelis-Menten or allosteric sigmoidal, was according to best fit.

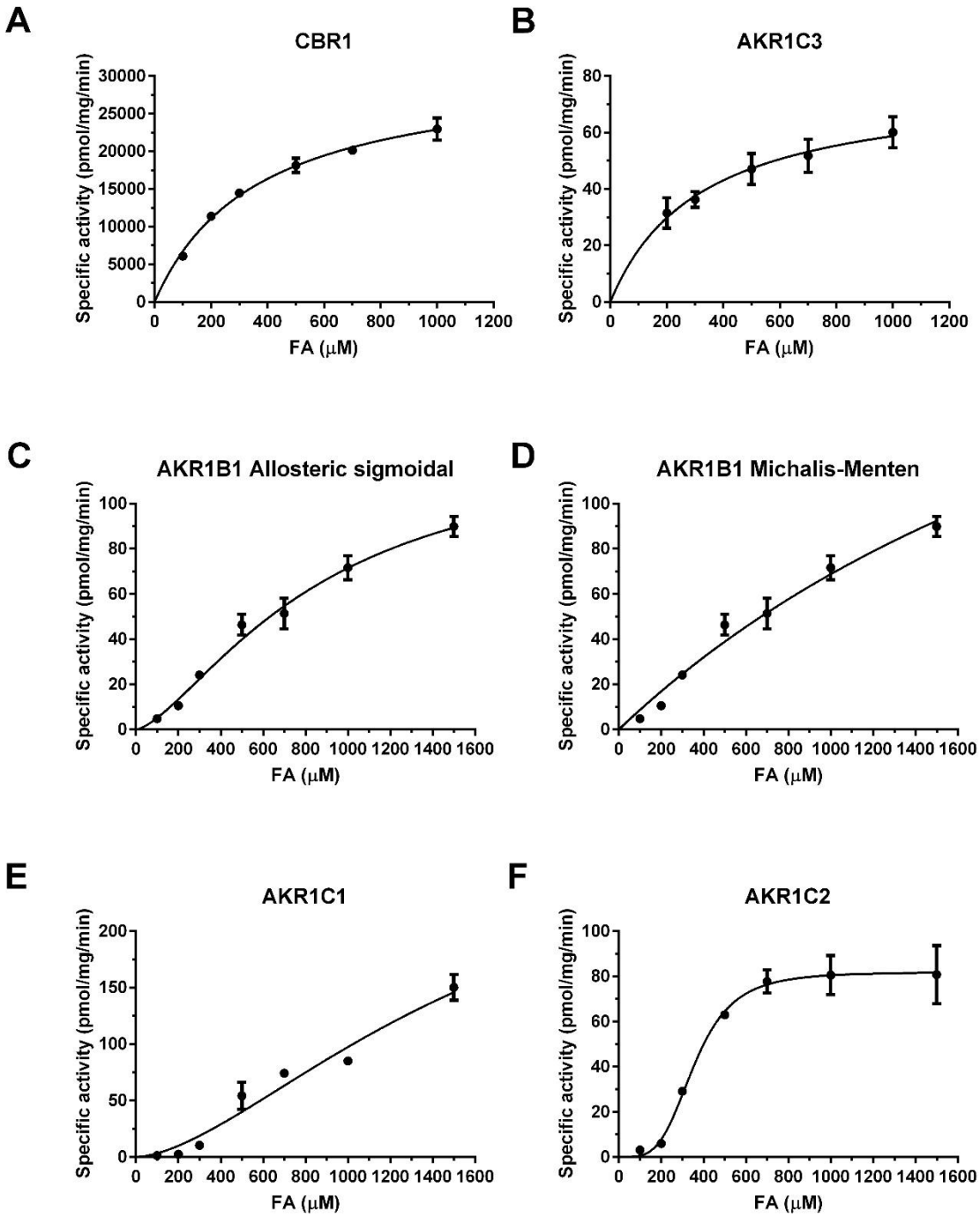


Figure 6. Kinetics of the fenofibric acid reduction catalyzed by the recombinant cytosolic enzymes. A) CBR1 according to Michaelis-Menten, B) AKR1C3 according to Michaelis-Menten, C) AKR1B1 allosteric sigmoidal kinetic, D) AKR1B1 according to Michaelis-Menten, E) AKR1C1 allosteric sigmoidal and F) AKR1C2 allosteric sigmoidal kinetics.

Table 15. Kinetic parameters of recombinant cytosolic enzymes.

	V_{max} [pmol/mg/min]	K_m[µM] or *Khalf*	*h [Hill slope]*	R^2	CL_{int} [µL/mg/min]	Model
CBR1	31183±979	362.7±27.13		0.9826	85.974	M-M
AKR1C3	77.56±4.332	320.5±47.5		0.8100	0.242	M-M
AKR1B1	296.7±68.81	3316±1007		0.9683	0.089	M-M
AKR1B1	*128.7*±17.85	*860.6±179.4*	*1.477± 0.1653*	*0.9783*	*0.150*	*Allost.*
AKR1C1	*312.6*±158.8	*1629±941.2*	*1.625±* 0.3342	*0.9539*	*0.192*	*Allost.*
AKR1C2	*81.89*±2.294	*357.2±13.48*	*3.895± 0.4197*	*0.9824*	*0.229*	*Allost.*

CBR1 and AKR1C3 were fitted to Michaelis-Menten (M-M) kinetics, AKR1B1 to both allosteric sigmoidal and Michaelis-Menten kinetics, AKR1C1 and AKR1C2 to allosteric sigmoidal kinetics. V_{max} is maximum enzyme activity, K_m Michaelis constant for Michaelis-Menten equation, K half for allosteric sigmoidal and h is the Hill slope, that characterizes the steepness of the curve and presence of cooperativity.

The enzymatic activity of the enzymes CBR1, AKR1C3 and AKR1B1 fitted to Michaelis-Menten equation. As shown in Table 15, the enzyme CBR1 is clearly the most active enzyme toward fenofibric acid reduction. V_{max} expressing the enzymatic activity of CBR1 is almost 400 times higher than that of AKR1C3, and 100 times higher than that of AKR1B1. Considering Michaelis constant, affinity of CBR1 and AKR1C3 is very similar and both have almost 10 times higher affinity than AKR1B1.

Nevertheless, the enzyme AKR1B1 fitted as well allosteric sigmoidal model of kinetics along with the enzymes AKR1C1 and AKR1C2. This result suggests the thought of the existence of multiple ligand binding sites. Hill coefficient reflects the degree of cooperativity among those binding sites. Therefore, AKR1C2 has a maximum number of interacting sites.

Additionally, the highest enzymatic efficiency expressed as CL_{int} is for CBR1 355 times higher than that for AKR1C3 (85.97 vs. 0.242 µl/mg/min). Concerning the rest of the tested enzymes, their CL_{int} is even much lower than that of CBR1, while for

AKR1C2 is similar as that for AKR1C3 (0.242 vs. 0.229 μl/mg/min). CL_{int} of AKR1C1 is only slightly lower than that of AKR1C3 (0.242 vs. 0.192 μl/mg/min). Finally, AKR1B1 shows the lowest CL_{int}, reaching almost 1000 times lower value than that of CBR1 (85.97 vs. 0.089 μl/mg/min).

So the order of the activity of the carbonyl-reducing enzymes toward fenofibric acid could be the following, from highest to lowest: CBR1>>AKR1C3>AKR1C2>AKR1C1>AKR1B1.

Up to now, examination of the metabolism of fenofibric acid has been taken place for rats, dogs, guinea pigs, cynomolgus monkey (Weil et al., 1988; Cornu-Chagnon, 1995; Liu et al., 2009a; Liu et al., 2009b; Yang et al., 2013).

In 1988, Weil et al. demonstrated that reduced fenofibric acid was the major elimination metabolite of fenofibrate excreted via urine, as a result of carbonyl reduction in rat, guinea pig and dog (Weil et al., 1988). Caldwell published in 1989 the biochemical pharmacology of fenofibrate, reporting the carbonyl reduction of fenofibric acid to its reduced metabolite in humans. It was also pointed the important quantitative difference in the metabolism of fenofibric acid among species (Caldwell, 1989).

In 1995, Cornu-Chagnon et al. while investigating primary cultured hepatocytes assessing peroxisomal β-oxidation by cyanide-insensitive palmitoyl CoA for metabolism of fenofibrate among other, also identified after a 24hours metabolization the formation of reduced fenofibric acid after addition of fenofibric acid. They also reported ester glucuronides of both fenofibric and reduced fenofibric acid (Cornu-Chagnon et al., 1995).

Unique additional findings of metabolites occurred so far in cynomolgus monkeys species, in which fenofibric and reduced fenofibric acid are conjugated with taurine (Liu et al., 2009). Those metabolites where also identified in Sprague-Dawley rats after plasma and urine analysis (Liu et al., 2009).

In vitro and *in vivo* study of the metabolism of fenofibric acid in beagle dog hepatocytes concluded that fenofibrate is more metabolized to reduced fenofibric acid than to fenofibric acid (Yang et al., 2013).

The Table 16 concludes all those data concerning the metabolism of fenofibrate.

Table 16. Summary of fenofibrate metabolites in different species.
Adapted from Yang et al., 2013.

Metabolites	**Human**	**Monkey**	**Guinea Pig**	**Rat**		**Dog**	
				In vivo	**Hepato-cytes**	***In vivo***	**Hepato-cytes**
FA	√	√	√	√	√	√	√
RFA	√	√	√	√	√	√	√
FAEG	√	√	√	√	√	√	√
RFAEG	√	√	√	√	√	√	√
FAT		√		√	√	√	√
RFAT		√		√	√	√	√
B		√		√	√	√	√
X		√		√	√	√	√
AR		√			√		
A	√						√

FA=fenofibric acid, RFA= reduced fenofibric acid, FAEG= fenofibric acid ester glucuronide, RFAEG= reduced fenofibric acid ester glucuronide, FAT= fenofibric acid taurine, RFAT = reduced FAT, metabolite B= 4-chloro-4'-isopropoxybenzophenone, ,compound X= 2-[4-(4-chloro-benzoyl)-phenoxy]-2-methylpropionic acid methyl ester, compound A= 4-chloro-4_-hydroxybenzophenone, compound AR= reduced compound.

Information for biotransformation of reduced fenofibric acid, do not exist further than the previous ones, taking in consideration that fenofibrate is rapidly hydrolyzed by esterases to fenofibric acid.

Another notable information about metabolism of fenofibric acid is the fact that fenofibric acid was initially reported as substrate for reduction by cytochrome P450 isoforms but it has been lately proven false (Prueksaritanont et al., 2002; Whitfield et al., 2011).

The finding of CBR1 as an efficient reducing enzyme toward fenofibric acid gives us the opportunity to guess and further investigate drug-drug interactions. CBR1 is one of the well-studied CREs, which has proofed its participation in many xenobiotic biotransformation pathways. E.g., nabumetone has been found to be *in vitro* reduced mainly by CBR1, but *in vivo* studies demonstrated lower activity than the expected results (Skarydova et al., 2013; Malatkova and Wsol, 2014). Benfluron and dimenfluron that are both antineoplastic agents are reduced by CBR1. This

information is of limited importance, though those agents failed to undergo further clinical trials.

Monotherapy of either fenofibric acid or statins is a rational pharmacotherapy for managing dyslipidemias. However, in many cases has been discussed a combination therapy with both of them. Concerning many fibrate derivatives, this combination therapy is contraindicated due to drug-drug interactions, e.g. competing glucuronidation by the enzymes UGT1A1 and UGT1A3. However, fenofibric acid undergo glucuronidation by enzymes UGT1A9 and UGT2B7 that do not metabolize statins. Additionally, fenofibric acid reduction is mediated by other enzymes than biotransformation of statins that is mediated through enzyme CYP3A4 (Corsini et al., 2005). Thus, it is feasible that the biotransformation of fenofibrate by carbonyl-reducing enzymes from SDR and AKR superfamilies does not lead to interaction with metabolism of statins.

7. CONCLUSION

Biotransformation of a drug is an important process leading to formation of compounds that either initiate or extend the pharmacological activity, or detoxify the parent xenobiotic.

Regarding the hypolipidemic agent fenofibric acid, it has been known that in human and other mammalians it is metabolized to reduced fenofibric acid, but without any further knowledge for the identity of involved enzymes. In our study it was confirmed that fenofibric acid is turned to its reduced metabolite as it undergoes carbonyl reduction, a reaction that can be catalyzed in both cytosolic and microsomal subcellular fractions. The present study has examined both subcellular liver fractions but only cytosolic recombinant enzymes. These cytosolic enzymes were from two superfamilies of carbonyl-reducing enzyme, the AKR and SDR superfamily. In particular, we found that the greatest enzymatic activity belongs to the SDR enzyme CBR1, followed by several AKR enzymes.

This is for the first time when an enzyme performing reduction of fenofibric acid was identified. The determination of the enzymes that are responsible for the metabolism of fenofibric acid adds one more important information for understanding the drug-drug interaction, especially with statins. The present diploma thesis proceeded only as an *in vitro* study. *In vivo* research will be definitely important because it may shed more light to the biotransformation of fenofibric acid and the current findings.

We believe that our findings stimulate the interest in further examination of the metabolism of fenofibric acid by carbonyl-reducing enzymes. Especially, the identification of microsomal enzymes responsible for carbonyl reduction of fenofibric acid is desirable. Additionally, another interesting area is the investigation of the chiral aspects of the production of reduced fenofibric acid by the carbonyl-reducing enzymes. Notably, both these directions of fenofibric acid research are in progress, performed by another diploma student.

8. LIST OF ABBREVIATIONS

11β-HSD	11β-Hydrosteroid dehydrogenase
AKRs	aldo-keto reductases
CBRs	carbonyl reductases
CREs	carbonyl-reducing enzymes
CYP450	cytochrome P450
DM II	diabetes mellitus II
HDL	high-density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coanzyme A
IS	internal standard
LDL	low-density lipoprotein
$NAD(P)^+$or NAD(P)H	nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate
NKK	nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PPARα	peroxime proliferator-activator alpha
PUFAs	polyunsaturated fatty acids
SDRs	short-chain dehydrogenase reductases
UGT	UDP-glucuronosyltransferase

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