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STANOVENÍ ISOENZYMŮ CYTOCHROMU P450 POMOCÍ UHPLC-MS/MS V MODIFIKOVANÝCH BUNĚČNÝCH LINIÍCH C3A, CACO2 A V LIDSKÝCH JATERNÍCH MIKROSOMECH

DIPLOMOVÁ PRÁCE

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HRADEC KRÁLOVÉ, 2015 **LENKA REJŠKOVÁ**

CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

UNIVERSITY OF EASTERN FINLAND

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UHPLC-MS/MS ABSOLUTE QUANTIFICATION OF CYTOCHROME P450 ENZYMES IN C3A, CACO2 MODIFIED CELL LINES AND IN HUMAN LIVER MICROSOMES

DIPLOMA THESIS

Supervisor: doc. Ing. BARBORA SZOTÁKOVÁ, Ph.D.

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HRADEC KRÁLOVÉ, 2015 **LENKA REJŠKOVÁ**

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„Prohlašuji, že tato práce je mým původním autorských dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci jsou řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu.“

“I declare that this thesis is my original author piece. Literature and other sources that were used during the writing process are all listed in References and are properly cited throughout the work. The thesis was not previously used to acquire a Master’s or any other degree.”

V Hradci Králové 22.4.2015

Lenka Rejšková

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Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

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Název diplomové práce: **Stanovení isoenzymů cytochromu P450 pomocí UHPLC-MS/MS v modifikovaných buněčných liniích C3A, CACO2 a v lidských jaterních mikrosomech**

Isoenzymy cytochromu P450 (CYP) hrají podstatnou roli v metabolismu léčiv. Mohou být zodpovědné za selhání terapie, vedlejší nebo toxické účinky a za interakce léčiv. Modifikované C3A a CACO2 buněčné linie s exprimovaným konstitutivním androstanovým receptorem (CAR) a pregnanovým X receptorem (PXR) by mohly být použity pro *in vitro* biotransformační a absorpční studie místo klasických primárních buněčných linií. Isoenzymy CYP by v těchto liniích měly být neustále exprimovány. Tato studie je zaměřena na vytvoření kvantitativní metody pro stanovení nejběžnějších isoenzymů CYP u člověka: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1, CYP3A5 a CYP3A4 v uvedených buněčných liniích a v lidských jaterních mikrosomech. Absolutní kvantitativní stanovení isoenzymů CYP bylo provedeno na UHPLC přístroji spojeném s tandemovým hmotnostním detektorem pracujícím v dynamickém MRM módu. Software Skyline 2.6 byl použit pro vyhodnocení dat. V modifikovaných buněčných liniích CACO2 a C3A nebyly isoenzymy CYP detekovány. V lidských jaterních mikrosomech byly isoenzymy CYP detekovány. Průměrné hodnoty jsou od 0.6 pmol/mg do 21.5 pmol/mg celkového proteinu v mikrosomech. Nejnižší detekovaná množství proteinů CYP ve stokrát naředěném vzorku lidských jaterních mikrosomů byly 0.006 – 0.210 pmol/mg celkového proteinu v mikrosomech. Tyto výsledky poukazují na možnost, že koncentrace isoenzymů CYP v modifikovaných C3A a CACO2 buněčných liniích byly zřejmě pod limitem detekce.

Klíčová slova: Cytochrom P450, CACO2 a C3A modifikované buněčné linie, Lidské jaterní mikrosomy, Standardy peptidů, UHPLC-MS/MS, MRM, Software Skyline 2.6

3. ABSTRACT

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Title of Diploma thesis: **UHPLC-MS/MS absolute quantification of cytochrome P450 enzymes in C3A, CACO2 modified cell lines and in human liver microsomes**

Cytochrome P450 (CYP) enzymes play a crucial role in drug metabolism. They can be responsible for the failure of treatment, adverse and toxic effects or drug-drug interactions. Modified C3A and CACO2 cell lines with constitutive androstane receptor (CAR) and pregnane X receptor (PXR) might be used for *in vitro* biotransformation and absorption studies instead of primary cell lines. CYP enzymes should be expressed continuously in these modified cell lines. This study aimed to establish the quantitative method for the analysis of the most common CYP enzymes in human: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1, CYP3A5 and CYP3A4 in the above mentioned cell lines and in human liver microsomes. Absolute quantifications of CYP enzymes were carried out by UHPLC in line coupled with tandem mass spectrometry working in scheduled MRM mode. Data assessment was conducted by Skyline 2.6 software. CYP enzymes were not detected in CACO2 and C3A modified cell lines. However, these enzymes were found in human liver microsomes. Average values were ranging from 0.6 pmol/mg to 21.5 pmol/mg of microsomal protein. The lowest detected amounts of CYP protein were 0.006 – 0.210 pmol/mg of microsomal protein in a hundred times diluted human liver sample. These findings point out that CYPs protein levels in modified C3A and CACO2 cell lines were apparently below the limit of detection.

Key words: Cytochrome P450, CACO2 and C3A modified cell lines, Human liver microsomes, Heavy labelled peptide standards, UHPLC-MS/MS, MRM, Skyline 2.6 software

4. INTRODUCTION

Lipophilic drugs and other xenobiotics cannot be eliminated directly from the body. Our body is highly composed from water thus also drugs and xenobiotics must be transformed to more hydrophilic compounds to be excreted. Cytochrome P450 (CYP) enzymes are essentially involved in this process (Wang et al. 2006; Seibert et al. 2009). With regard to pharmacokinetic and pharmacological importance, the expression levels of metabolizing enzymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1, CYP3A5 and CYP3A4 (Kawakami et al. 2011; Gröer et al. 2014; Seibert et al. 2009) were studied in this diploma thesis. Any changes in expression of CYP isoforms have an impact on the success of therapy, occurrence of adverse effects or even increased risk of cancer because CYP enzymes play a crucial role in metabolism of many ordinarily used drugs (Wang et al. 2006; Seibert et al. 2009). Furthermore, they can be involved in pro-drug activation and thus their changed activity can significantly contribute to the failure of anti-cancer treatment (Seibert et al. 2009).

To sum up, knowledge of expression levels and their susceptibility to be either induced or inhibited would be the basic tool for personalized therapy. Therefore, *in vitro* and *in vivo* experiments of CYP mediated metabolism is an essential part of the drug development and clinical research (Liu et al. 2014).

In vitro studies can be done with primary cells or cell lines. The use of primary cells is limited because of the rare availability of fresh human liver, complicated isolation procedure, short-term viability, hepatocellular functions, inter-individual differences and also the price (Küblbeck et al. 2010; Elkayam et al. 2006; Gerets et al. 2012). Conversely, cell lines are phenotypic stable and immortal (Gerets et al. 2012) but their CYPs levels are low because of decreased transcription of concrete genes. Low amount of CYPs presents a problem with regard to model *in vitro* studies of drug and xenobiotic metabolism. This problem might be solved by formation of so called turbo receptors which continuously force cell lines into production of CYPs. Turbo Constitutive Androstane Receptor (CAR; NR1I3) and turbo Pregnane X Receptor (PXR; NR1I2) are created by adding an activation domain from other transcription factor to the either amino-terminal or carboxy-terminal part of human CAR or PXR. PXR and CAR belong to the nuclear receptors (NR) and they regulate many genes whose proteins are connected with drugs biotransformation (Küblbeck et al. 2010) and efflux mechanism. It has been also suggested that CYPs and efflux transporters may cooperate in relation to the first-pass metabolism and the biotransformation. Hence, the *in vitro* model, which could be enriched of both mechanisms, would be very beneficial (Korjamo et al. 2006).

5. AIMS OF STUDY

- To set up quantitative UHPLC-MS/MS analytical method for detection of CYP enzymes.
- To quantify CYP enzymes in modified cell lines with turbo receptors and in human liver microsomes.
- To compare measured levels of CYP enzymes with results from various scientific articles.
- To evaluate the high sensitivity of the Triple Quadrupole 6495.

6. THEORETICAL PART

6.1 Cytochrome P450 Enzymes

Cytochrome P450 (CYP) enzymes are membrane proteins which are placed in the endoplasmic reticulum (ER) (Langenfeld et al. 2009). All CYP enzymes are included in a big superfamily which is divided into 18 families and 42 subfamilies. The division of CYP enzymes is according to the percentage of identity in protein sequences. It means that the first Arabic number refers to the family where more than 40% of identity in protein sequence occurs and the following letter is associated with subfamily where more than 55% of identity in protein sequence is found (Wang et al. 2006). In practice, this high percentage of identity among CYP isoforms makes the selection of proteotypic peptides difficult (Kawakami et al. 2011; Langenfeld et al. 2009). In relation to the richest occurrence and abundance of various CYP isoforms in the liver tissue, which is the main place of metabolism in general, CYPs are significantly involved in metabolism of endogenous (steroids, vitamin D3, fatty acids, bile acids) and exogenous (xenobiotics and drugs) compounds (Wang et al. 2006). However, CYPs are especially studied in connection with drug elimination and drug-drug interactions (Seibert et al. 2009).

6.1.1 Biotransformation

Elimination of drugs is a process where active form of drug is absolutely eliminated from the organism. Elimination includes the metabolism, also known as the biotransformation, and itself excretion (Lincová et al. 2007). The biotransformation of drugs is a chemical conversion of drug into more hydrophilic and usually less toxic and less active compound in order to be excreted from the organism the most often by urine, faeces, exhalation and sweat (Seibert et al. 2009; Lincová et al. 2007). Nevertheless, we should always consider the fact that the biotransformation may lead to the formation of toxic and more reactive compounds and thus severe adverse effects may occur (Wang et al. 2006; Seibert et al. 2009; Lincová et al. 2007). The biotransformation is divided into two phases. The Phase 1 provides an oxidation, a reduction and a hydrolysis in order to convert drug to more hydrophilic compound (Wang et al. 2006; Lincová et al. 2007). The following Phase 2 is rather synthetic and the conjugation is performed. The main enzymes participating in the Phase 1 belong to the monooxygenase superfamily CYPs which basically insert one atom of oxygen to the drug compound and the second atom is converted into water (Lincová et al. 2007). The drug metabolizing enzymes from CYP family (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1,

CYP3A5 and mainly CYP3A4) have the pharmacokinetic and pharmacological importance (Kawakami et al. 2011; Gröer et al. 2014; Seibert et al. 2009). In addition, the metabolism is highly influenced by many factors such as age, genetics (inter-individual differences), drug interaction (the inhibition or the induction), pathological condition, gender, diet and intestinal microbiota (Gröer et al. 2014; Langenfeld et al. 2009; Seibert et al. 2009; Lincová et al. 2007). CYPs expression levels are decreasing with the higher age but differences in CYPs amount among females and males are not significant (Kawakami et al. 2011). In terms of induction/inhibition, the very common example is related to the induction of CYP1A2 by a tobacco smoke (Table 1). On the other hand, other CYPs are decreased in the case of smokers (Liu et al. 2014). Additionally, CYP1A2 mediates the conversion of pro-carcinogens, included in the tobacco smoke, into carcinogens (Seibert et al. 2009).

Table 1*.Comparisons of average quantitative values (pmol/mg of liver microsomal protein) of CYP enzymes between smokers and non-smokers (Liu et al. 2014)*

Isoforms	Smokers	Nonsmokers
CYP1A2	35.7	29.9
CYP2B6	3.5	4.8
CYP2C9	8.9	10.1
CYP2C19	3.0	2.7
CYP2E1	8.1	9.5
CYP3A4	45.2	56.5
CYP3A5	3.7	7.7

6.2 Nuclear Receptors: CAR, PXR

The nuclear receptors are considered to be ligand-activated transcription factors which include homodimeric receptors such as glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and androgen receptor (AR). Heterodimeric receptors such as CAR and PXR are always associated with retinoid X receptor (RXR). Some of them are directly located in the nucleus whereas for instance GR and CAR are placed in the cytosol. They are translocated to the nucleus as a complex with ligand. In terms of function of CAR and PXR, they are bound with RXR to the hormone release element (HRE) in the promoter part of the DNA where they activate transcription of target genes. CAR and PXR are composed of amino-terminal DNA-binding domain (DBD) and carboxy-terminal ligand-binding domain (LBD) (Chen 2008) as it is shown in Figure 1. LBD is considered to be very flexible (Tolson and Wang 2010). In the case of absence of ligand, RXR and LBD bind the co-repressor and the transcription is switched off. After the binding of ligand to LBD, the co-repressor is replaced by the co-activator as a result of conformation changes and the transcription can start (Chen 2008).

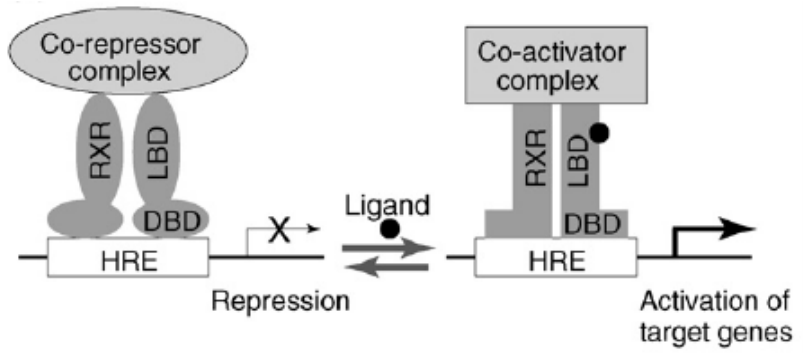

Figure 1. *The principle of CAR, PXR functioning*

When ligand binds to the LBD, conformation changes ensure the recruitment of the co-activator instead of the co-repressor and the transcription is started (Chen 2008).

It has been postulated that other various regulatory factors are involved in this process (Tolson and Wang 2010). Drug metabolizing enzymes of Phase II and I (UGT, SULT, CYPs, especially CYP3A4) (Chen 2008; Tolson and Wang 2010) and transporters (particularly MDR1, BCRP and MRP1) are the outcome of that activated transcription of target genes (Chen 2010). The range of ligands and final products of PXR and CAR activation is often overlapping and additionally the impact on these receptors may vary according to the dose of ligands. The inhibition or the induction might occur in both (Willson and Kliewer 2002; Tolson and Wang 2010).

Studies of CAR and PXR functions are important with regard to the drug development especially in the field of cancer treatment. The failure of anti-cancer therapy, resistant cancer in other words and subsequently cancer-related death is mainly caused by the high expression levels of efflux transporters and by the high activity of drug metabolizing enzymes. This fact is explained by the drug induction of PXR and CAR activated transcription (Chen 2008; Chen 2010). Especially PXR receptor is highly expressed in cancer-changed tissues (Chen 2008; Chen 2010) and additionally it is highly promiscuous, more than CAR receptor, in terms of the ligand-specificity (Willson and Kliewer 2002; Tolson and Wang 2010; Chen 2008). It means that many therapeutically used drugs induce transcription of PXR or CAR target genes and therefore their biotransformation and efflux increase (Willson and Kliewer 2002; Chen 2008).

The drug development is focused on either inhibitors of transporters or antagonists of PXR, CAR (Chen 2010) with regard to the interspecies differences (Tolson and Wang 2010). Since PXR, CAR occurs also in healthy tissues (Tolson and Wang 2010; Willson and Kliewer 2002), both approaches often lead to the development of toxic drugs with many dangerous adverse effects. We have to always bear in mind that efflux transporters and drug metabolizing enzymes protect the body against harmful compounds and they keep a homeostasis (Chen 2008; Chen 2010). In addition, it has been postulated that PXR plays also role in physiological

hepatic lipogenesis, fatty acid β-oxidation, gluconeogenesis (Wada et al. 2009) and in the homeostasis of hepatic bile acids (Tolson and Wang 2010), even though none physiological ligand of PXR has been discovered yet (Chen 2008).

6.2.1 Cell Lines: CACO2, C3A

CACO2 cell line comes from the human colorectal carcinoma and it shows some morphological and biochemical properties which are highly similar to small intestinal enterocytes. These cells grow spontaneously in monolayer with some hydrolase enzyme activities which do not correspond to the enzymatic activities in the human adult intestine. However, CACO2 cell lines characteristics are quite easily influenced and changed by different cultivation conditions such as a seeding density, a cell culture medium or time of cultivation. The number of passages has an effect, for instance, on the mRNA levels of metabolizing enzymes or on the expression of transporters. Many CACO2 clones have been created thanks to different cultivation treatment. However, this fact makes the choice of the most suitable clonal cell line for the experiment difficult (Sambuy et al. 2005). CACO2 cell line is widespread used as an *in vitro* model for the intestinal transport and as drugs absorption model even though it is characterized by an inter-laboratory variability and a defective oxidative metabolism (Korjamo et al. 2006; Sambuy et al. 2005).

C3A is a clonal derivate of HepG2 which is the human hepatocellular carcinoma cell line. C3A cell line features strong inhibition of growth, high albumin production and growing ability in glucose deficient medium (C3A [HepG2/C3A, derivative of Hep G2 (ATCC HB-8065)] (ATCC® CRL-10741™) [online], cit. 2014-10-08). HepG2 cell line is capable of the biosynthesis similar to normal hepatocytes. CYP enzymatic system is partially preserved but highly influenced by cultivation conditions (Dehn et al. 2004). HepG2 cell line is broadly used for toxicological studies and the drug metabolism even though they generally express low amount of Phase I and Phase II enzymes (Hewitt N.J. and Hewitt P. 2004).

6.2.2 Turbo Cell Lines CACO2, C3A

Increase in CYPs mRNA expression levels can be done via ligand activation as it was described above or via transfection with NR expression vectors. It means without the essential presence of different ligands. C3A and CACO2 DNAs are exposed to the transfection just by one construct. Since the increase in mRNA levels is not sufficient enough to conduct other experiments, the transfection via strong activation domain from other transcription factor is

performed. In our case, p65 subunit of the nuclear factor κB was fused with the carboxy terminal part of CAR and PXR. Subsequently these turbo receptors are permanently active and they should provide higher mRNA levels of their products, with regard to our interest, mRNA levels of CYP enzymes (Küblbeck et al. 2010).

6.3 Targeted Proteomics Workflow

Proteomics deals with the structure and the function of all proteins in cells. The identification, modification, quantification and localization of proteins are involved. Proteins are the working units of genes ensuring all metabolic pathways, transportation, molecular signalling and physical interactions (Yates et al. 2009). Targeted proteomics workflow has recently emerged in the field of proteomics because it solves a problem of detection of low abundance proteins among high abundance proteins in the sample. Targeted proteomics workflow provides data of ions of interest instead of blind collecting data of all ions which are detectable. This method yields to increase the sensitivity and the reproducibility in the quantitative and qualitative analysis. This highly sensitive and highly reproducible measurement is achieved by using Multiple Reaction Monitoring (MRM) method which is explained in detail below (Deutsch et al. 2008). MRM method is always performed with High Performance Liquid Chromatography in line coupled with tandem mass spectrometry (HPLC-MS/MS) run (Picotti and Aebersold 2012).

6.4 (Ultra)High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a separation technique which is based on the continuous formation of the balance between the analyte and the stationary phase and between the analyte and the mobile phase. The solubility of the analyte in the mobile phase is the basic HPLC condition which is essential for the analysis. The mobile phase is usually composed from water and organic solvent whose mutual ratio has to be optimized in order to get good separation. These solvents are continuously pumped to the system at a constant flow rate and without the air which is, if it is necessary, removed by the vacuum degasser. The sample is injected into the running mobile phase with which is introduced to the column. Compounds included in the sample are separated in the column according to their polarity and subsequently detected in the detector on the basis of their physicochemical properties.

HPLC analysis can be conducted in different modes which depend on molecular weight, polarity, acidity, alkalinity and ionic character of compounds. In the terms of peptide or protein analysis, silica support particles of stationary phase are bonded to C18, C8, C4 alkyl chains. In

practice, it means that hydrophobic peptides are retained by the stationary phase until they are washed out by the hydrophilic mobile phase or until the polarity of mobile phase is changed due to the hydrophobic gradient. This mode is called Reversed Phase (RP). In the case of peptide separation, generally mobile phase consists of water with 0.1M formic acid in order to decrease the pH and keep the silanolic residues neutral (Si-OH) and acetonitrile is used for the hydrophobic gradient formation (LC/GC´s CHROMacademy [online], cit 2014-11-27).

Ultra High Performance Liquid Chromatography (UHPLC) is a variation of HPLC which works with miniaturized volumes at the high pressure environment. The size of particles in the column is decreased to sub-2µm and the column diameter is also reduced. As a response to the size reduction of particles, the back pressure is significantly increased but the separation efficacy and resolution are improved, the time of analysis is shortened and the sensitivity is enhanced thanks to limited band spreading (Nováková and Vlčková 2009; Yu et al. 2006).

In these days, the connection of UHPLC (HPLC) and mass spectrometer as a detector has become worldwide used fast-separation technique for large number of applications (Nováková and Vlčková 2009).

6.5 Mass Spectrometry

Mass spectrometry (MS) is one of the most developing technique of the analytical chemistry. It is based on the interaction between ions and the electric or magnetic field and it is favourably in-line connected to the High Performance Liquid Chromatography (LC-MS). This connection has performed the breakthrough in proteomic studies (LC/GC´s CHROMacademy [online], cit. 2014-09-01). LC-MS is a tool to gather information about the protein identification, expression, modifications and interactions. At the beginning of each new research it is necessary to consider which instrumentation, fragmentation method or analysis strategy should be used in order to get reproducible and accurate results (Han et al. 2008). The main process of MS detection consists from: the ionization (the formation of ions from analyte molecules), the mass analysis (the separation of ions according to their mass to charge (m/z) ratio), the detection (the amplification of electric signal in order to make it detectable) and the assessment of the mass spectrum (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Han et al. 2008).

Although molecules has to be in their vaporized form, using solids, liquids or gases samples are possible due to the presence of a heating coil at the beginning of the mass spectrometer. The occurrence of charged molecules is necessary because neutral molecules do not move in the

electric or magnetic field. Ions are formed by various processes but always there is either positive ion mode or negative ion mode. The type of ionization process also determines how the mass spectrum looks like and how it is analyzed and evaluated. Ionization is divided into Hard and Soft type. Hard techniques, including electron and chemical ionization, are not applicable to biological samples. Matrix Assistant Laser Desorption Ionization (MALDI) and Atmospheric Pressure Ionization (API) are two basic examples of Soft ionization techniques. API includes Electrospray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and Atmospheric Pressure Photo Ionization (APPI) (LC/GC´s CHROMacademy [online], cit. 2014-09-01). ESI and MALDI are the most often used ionization techniques for the structural characterization of peptides and proteins (Yates et al. 2009).

MALDI technique is based on the interaction of solid matrix containing the analyte with the UV laser beam in the vacuum. The UV laser beam´s energy kicks molecules of the sample out along with the little part of the matrix (LC/GC´s CHROMacademy [online], cit. 2014-09-01). Finally, molecules are charged by the proton transfer in the gas phase due to collisions with the matrix (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Steen and Mann 2004). MALDI does not provide multiply ions therefore it is usually used for the analysis of intact proteins and purified sample. (Yates et al. 2009).

6.5.1 Ionization Technique: Electrospray Ionization

Electrospray Ionization (ESI) is a soft ionization technique which can be used just for polar molecules or ions. ESI involves the main three parts: the nebulisation of the sample solution in nebulising gas, the ions releasing from microdroplets and the ions transportation from the atmospheric pressure chamber to the vacuum chamber of the mass analyser (Holčapek: Ionizační Techniky [online], cit. 2014-09-01). At first, the eluting analyte from the HPLC column goes to the electrospray needle whose end is provided by high voltage supply. The process of formation microdroplets is based on increased density of the same charges and thus repulsive forces are increasing too until the attainment of critical point called Coulombic explosion. Cuolombic explosion is associated with the overcoming the Rayleigh Instability Limit of surface tension. Rayleigh Instability Limit means that repulsive forces between charges are equal to the surface tension of droplet therefore the droplet can exist. In the process of Coulombic explosion, the round shape is changed into the conic one (Taylor Cone) in order that the charge repulsion is decreased and microdroplets are released. They would fall down without the presence of the potential difference which ensures their horizontal movement. This is called axial spray mode. Afterwards, evaporation of microdroplets in the so-called desolvation

zone leads to increasing the density of charges and the Coulombic explosion comes again. This process is repeated until the final formation of ions which are created by two possible ways (Figure 2). Ion Evaporation means that ion is kicked out by the superficial tension from the microdroplet. Charge Residue is the second way in which the solvent is evaporated from the charged microdroplet.

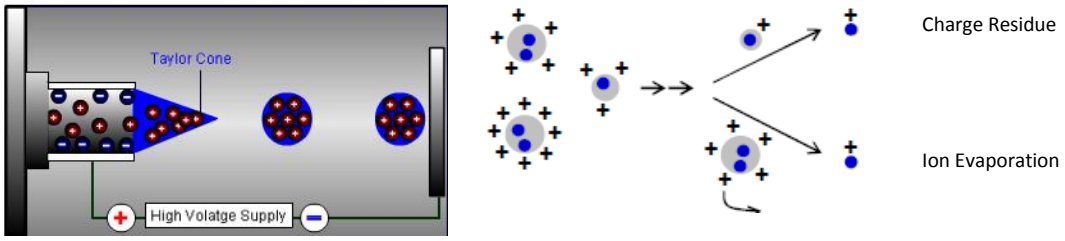

Figure 2. *The example of positive ion mode where microdroplets and subsequently ions are formed*
The process is described above (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Holčapek: Ionizační Techniky [online], cit. 2014-09-01).

The transportation of ions to the vacuum system is always connected with the risk of their cooling down and subsequently the formation of unwished clusters. Flowing nitrogen gas in the opposite direction or the ion source heating are two basic tools how to prevent from creating clusters. Generally, the presence of heating device is important to get more ions. Ions generated by ESI are usually doubly or more charged (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Holčapek: Ionizační Techniky [online], cit. 2014-09-01; Yates et al. 2009; Steen and Mann 2004).

6.5.2 Increased Collection of Ions to the Mass Analyser

Some enhancements have been developed to improve the collection of ions and their introduction to the mass analyser. Overheated electrospray enhances the evaporation, ionization and ions focusing near the MS inlet. Increased diameter of collection capillary or higher number of capillaries ensures the transportation of more ions to the MS instrument. And funnels help to focus ions and pump a residual gas out. To sum up, all these improvements lead to enter more ions to the first quadrupole of the mass analyser and therefore the sensitivity is increased and higher flow rate can be used. These tools are applied in triple quadrupole (Agilent 6495 Triple Quadrupole LC-MS System, Agilent Technologies, Santa Clara, CA, USA) which is used for measuring in this diploma thesis (Agilent 6490 Triple Quadrupole LC/MS System: Reach Ultimate Sensitivity with Breakthrough IFunnel Technology [online], cit. 2014-11-26).

6.5.3 Mass Analysers

The general aim of analysers is the separation of ions according to their mass to charge (m/z) ratio. There are various types of analysers which separate ions on the basis of different physical principles. The most common are Time Of Flight (TOF) analyser, Quadrupole analyser, Ion trap or Fourier-Transform Ion Cyclotron Resonance (FTICR) mass analyser (Han et al. 2008). Ionisation technique MALDI is usually connected with TOF analyser (MALDI-TOF) and similarly ESI usually works together with Quadrupole analyser. These combinations are preferentially used in proteomics (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Yates et al. 2009).

Time Of Flight (TOF) mass analyser is based on the time in which ions reach the detector. Kinetic energy is the same for all ions as well as the distance. Since kinetic energy is the function of the mass thus heavier ions fly slower than lighter ones. As the result of applied kinetic energy, lighter ions get to the detector sooner. TOF analyser always analyses the same type of charge (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Steen and Mann 2004).

6.5.4 Mass Analyser: Quadrupole Analyser

Quadrupole analyser consists from four poles (or electrodes, rods) which are arranged to the shape of a rhombus. They create the top, the bottom and two sides. Direct Current (DC) voltage and alternating Radiofrequency voltage (RF) are put on these electrodes. Charged electrodes carry the same charge in the horizontal level and in the vertical level. Therefore, ions move in this field on the basis of repulsing and pulling forces but they never mix up. After this first separation, they are secondly separated according to their mass. Certain ion with specific value of m/z ratio can go through the tube in the specific time because in this case the oscillations of this ion are stable and the ion can reach the detector. It is called non-collisional or stable trajectory of that particular ion whereas the rest of ions are caught by electrodes. Nevertheless, all ions are gradually allowed to reach the detector by continuous changes of DC voltage and RF amplitude so that their ratio is still constant. Collisional or unstable trajectory means that if the amplitude of oscillation exceeds, ion is discharged or passed out of the mass analyser (LC/GC´s CHROMacademy [online], cit. 2014-09-01; Holčapek: Hmotnostní Analyzátory [online], cit. 2014-09-01).

6.5.5 Triple Quadrupole – Collision Induced Dissociation

Triple quadrupole is the example of very common used mass analyser where Collision Induced Dissociation (CID) is applied so as to get other ion fragmentation. Triple quadrupole consists of

two quadrupoles and one collisional cell which is in the middle. The collisional cell contains a collisional gas for instance nitrogen, argon or helium. The gas pressure here ensures high energy leading to other ions fragmentation. With the increasing gas pressure the degree of fragmentation is increasing too. There are so-called precursor ions (also parent ions) in the quadrupole Q1 and after the passing through the collisional cell there are product ions (or daughter ions) in the quadrupole Q3. The collisional cell allows the transportation of all product ions. Triple quadrupole shows just the unit resolution unlike TOF analyser which provides us four decimal values of m/z ratios. Therefore, elemental composition of the compound is more likely achievable by TOF analyser which is usually characterized by higher resolution and lower sensitivity capabilities in contrast to the triple quadrupole (LC/GC´s CHROMacademy [online], cit. 2014-09-01).

6.5.6 Tandem Mass Spectrometry

Mass spectrum MS^1 shows peaks of all ionized molecules in the background of signal to noise ratio. The peaks height is the function of intensity or abundances of that particular ion. The m/z ratio is represented by the axis x. However, this m/z value is not enough to identify peptides respectively proteins from which peptides come from (Pisitkun et al. 2007). Tandem mass spectrometry (MS/MS or MS^2) brings us the solution. It contains two mass analysers (the same or different type) which select one certain ion and fragment it in order to obtain structural information and to achieve better selectivity and sensitivity for the quantitative analysis (Figure 3). Characteristics of Triple quadrupole make it highly suitable for Tandem MS. Two mass spectra are the outcome of using Tandem MS. MS^1 spectrum of precursor ions and MS/MS spectrum of product ions (usually it is a spectrum of the most abundant peaks found in the MS^1 spectrum) (LC/GC´s CHROMacademy [online], cit. 2014-09-01). Peptide bonds between amino acids are broken in the collisional cell and b-ions and y-ions are generated. B-ions carry charge on the amino-terminal part of the peptide in contrast to y-ions whose charge is in the carboxy-terminal part of the peptide. The y-ions production predominates in the triple quadrupole or the quadrupole-TOF whereas both y-ions and b-ions can be found equally in the ion trap mass analyser (Steen and Mann 2004). If we consider that precursor ion is doubly charged (the most preferable mode) then product ions carry just one charge and their m/z value is considerably higher than of the precursor ion. In this case the difference between m/z values (or mass) of an adjacent peaks equals the mass of the certain amino acid in MS/MS spectrum. This identification is called de-novo sequencing (Steen and Mann 2004; Pisitkun et al. 2007). However, if the difference of m/z values of precursor and product ions is

considerably small, it means that quite common neutral-losses of water and ammonium happened in the collisional cell (Han et al. 2008). Different and simpler approach of the identification is comparison of measured MS/MS spectra with the database of theoretical peptide spectra thanks to specific software. These databases are generated from *in silico* tryptic digestion. Generally, the identification of protein is based on the occurrence of two or more specific peptides which are unique for that concrete protein (Steen and Mann 2004; Pisitkun et al. 2007).

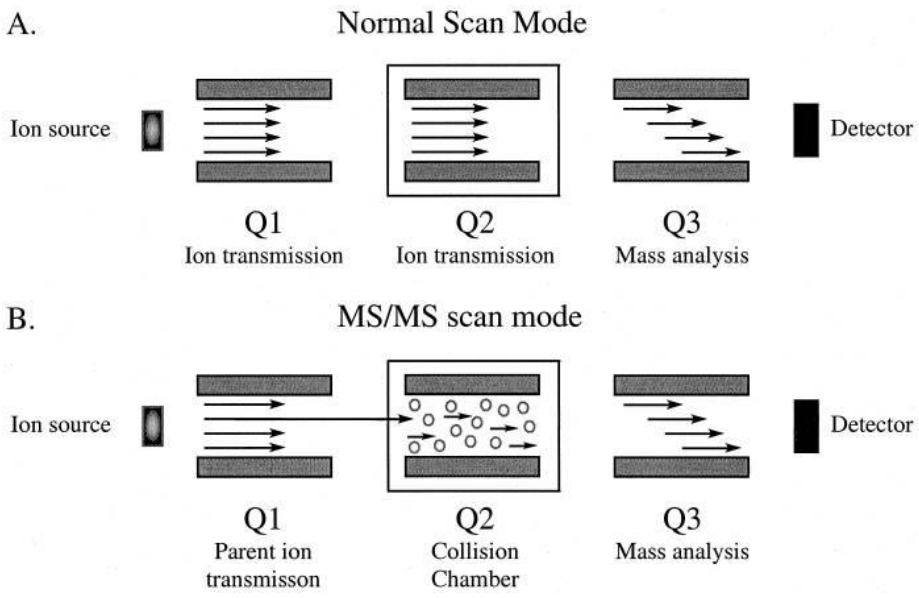

Figure 3. *The conventional and tandem MS mode of the analysis in the triple quadrupole*
In the normal scan mode, all ions generated in ionization chamber are allowed to pass the detector. In contrast, MS/MS scan mode presents ions selections in Q1 and subsequent fragmentation in the collisional cell (Graves and Haystead 2002).

6.6 Protein Identification

The first step in the proteomic analysis is the preparation of the sample which is usually a complex mixture of intact proteins. Generally, proteins undergo the denaturation, reduction and alkylation in order to get stable primary structure of proteins (Uchida et al. 2013; Kawakami et al. 2011). Other processing of the sample depends on the selected approach. In the Bottom-up approach proteins are enzymatically digested usually by trypsin and therefore peptides are analysed. Conversely, Top-down approach presents the analysis of intact proteins (Han et al. 2008).

In terms of Top down approach, the whole intact proteins are ionized and fragmentized during the MS run (Han et al. 2008). Although Top down methods could distinguish specific proteins isoforms and posttranslational modifications and the protein abundances could be measured

directly, this method is not so widespread used due to technological limitations (Yates et al 2009; Han et al. 2008). The potential application of this technique is the analysis of single protein (purified) or simple mixtures (Yates et al. 2009).

6.6.1 Bottom-up Approach (Shotgun Proteomics)

Bottom up approach brings us the possibility to identify and even quantify proteins via tryptic peptides which are unique for that concrete protein. Some of the advantages of bottom up approach are better sensitivity of mass spectrometer for peptides than for proteins and better handling with peptides due to the loss of some proteins properties like stickiness and variable solubility under the same conditions (Steen and Mann 2004). Especially membrane proteins are known as low soluble and high aggregable because of many hydrophobic regions (Hammarlund-Udenaes et al. 2014). There is an assumption that the quantity of protein equals the quantity of unique peptide (Steen and Mann 2004). However, in practice, errors may be in each step of the whole process. Firstly, peptides can stick to the surface of the test tube or to the pipette tips (Uchida et al. 2013), they might be differently modified and mutations may occur as well. Secondly, the solubilisation and tryptic digestion process may not be efficient enough (Uchida et al. 2011; Hoshi et al. 2013). Conversely, target peptides might be also produced by immature or insufficiently digested protein and thus the amount of target peptide is higher than it should be (Hoshi et al. 2013).

6.6.2 Sequencing Grade Modified Trypsin, TPCK Trypsin

Trypsin is the most common enzyme used in proteomics because it is stable and aggressive serine protease whose digestion is very conservative. Each peptide formed by tryptic digestion contains the amino acid Lysine (Lys) or Arginine (Arg) at the carboxy-terminal part of the peptide (Steen and Mann 2004). However, if the amino acid Arg or Lys is followed by amino acid Prolin, the cleavage here is not feasible (Uchida et al. 2013). The digestion is also limited in relation to the presence of acidic amino acids close to the place of the possible cleavage. Unmodified trypsin yields to the auto-proteolysis which brings two main undesirable effects. Fragments may interfere with LC-MS analysis. And the production of pseudotrypsin with the similar specificity in digestion as chymotrypsin may occur. This problem was solved by the preparation of Sequencing Grade Modified Trypsin by the reductive alkylation. In this manner modified trypsin is resistant to auto-proteolysis and more stable. The optimal value of pH is ranging from 7 to 9. Conversely, trypsin is reversible inactivated below the value of pH 4 (Certificate of Analysis: Sequencing Grade Modified Trypsin [online], cit. 2014-10-11).

Subsequently, trypsin undergoes the treatment with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK). This further modification ensures the inhibition of chymotrypsin activity but the activity of trypsin is kept (TPCK Trypsin and Immobilized TPCK Trypsin [online], cit. 2014-10-11).

6.7 Protein Quantification

The protein quantification is an indispensable part of global proteomics because it helps us to understand the protein kinetics and the molecular metabolism of endogenous and exogenous compounds (Snider: Quantitative proteomics [online], cit. 2014-09-02). The proteins quantification is carried out by relative or absolute quantitative methods (Steen and Mann 2004; Yates et al. 2009; Han et al. 2008).

6.7.1 Relative Quantitative Methods

Relative quantitative methods are based on the comparisons of areas under the extracted ion chromatogram curve. Extracted ion chromatogram curve means that areas under the peaks (currents) are integrated over the time (Pisitkun et al. 2007; Steen and Mann 2004). The first approach of relative methods is label-free quantification where peptide peaks intensities coming from two or more MS or MS/MS runs are compared. This approach is very demanding for the high mass accuracy because two or more separated runs of mass spectrometer are compared. Isotope Stable Labelling is the second type of relative quantification methods and it is based on different masses of chemically identical molecules that usually vary just in the number of neutrons (Pisitkun et al. 2007; Steen and Mann 2004). The first sample is labelled with light tag composed from natural isotopes of all atoms whereas heavy tag, consisting from heavy isotopes of all atoms, is put into the second sample. These two samples are mixed and then introduced to LC-MS/MS which distinguish two adjacent peaks. The quantification is made in the basis of MS^1 spectrum where preferentially areas of light and heavy samples under the extracted ion chromatogram curve are compared (Pisitkun et al. 2007). Stable isotope labelling can be conducted enzymatically, chemically or metabolically. The example of chemical labelling is Isotope Coded Affinity Tag (ICAT) which labels free Cysteines (Cys) of intact proteins. The absence of Cys in many tryptic peptides is the biggest limitation of this approach because in that case peptide is not quantified. Enzymatic labelling uses ^{18}O which is added to peptides or proteins. Metabolic method called Stable Isotope Labelling by Amino acids in Cell culture (SILAC) is an example of *in vivo* labelling which is more time consuming. Isotopic labels such as heavy essential amino acids (especially heavy Arg, Lys labelled with ^{13}C

and ^{15}N) are put into the cell culture media. These tags are then incorporated into cell proteins during natural cell culture growing. As a result of tryptic digestion, all peptides contain at least one labelled amino acid (Pisitkun et al. 2007; Yates et al. 2009; Steen and Mann 2004). Quantification is made from MS^1 spectrum in all these three methods whereas the interpretation of isobaric Tags for Relative and Absolute Quantification (iTRAQ) is done at MS^2 level (Pisitkun et al. 2007). iTRAQ can be used for absolute or relative measurements and it uses amine-reactive isobaric tags which reacts with Lys and with amino-terminus part of tryptic peptides (Pisitkun et al. 2007; Han et al. 2008).

6.7.2 Absolute Quantitative Methods

Absolute measurement of protein is carried out by using stable isotope-labelled synthetic peptides (internal standards) which are added to the sample in known concentration (Pisitkun et al. 2007). Light and heavy peptides (Snider: Quantitative proteomics [online], cit. 2014-09-02; Picotti and Aebersold 2012) have the same physico-chemical properties (retention time, peak shape, and ability to be ionized and fragmentized) but they differ in the mass (Hammarlund-Udenaes et al. 2014). They are usually labelled by ^{13}C and ^{15}N at the carboxy-terminus of the tryptic peptide (Steen and Mann 2004; Liebler and Zimmerman 2013) and they should be synthesized with >95% purity and quantified by amino acid analysis before adding to the real sample (Liebler and Zimmerman 2013). The quantification is then counted as a ratio between the ion-current of the naturally formed tryptic peptide and the corresponding ion-current of stable isotope labelled peptide (MRM [online], cit. 2014-09-02; Seibert et al. 2010; Deutsch et al. 2008). Absolute quantification is usually performed by Selected Reaction Monitoring (SRM) or Multiple Reaction Monitoring (MRM) which is considered to be a synonym of SRM (Picotti and Aebersold 2012; Han et al. 2008; Deutsch et al. 2008).

6.7.3 Multiple Reaction Monitoring

Multiple Reaction Monitoring (MRM) is one of the main approaches in Quantitative Targeted Absolute Proteomics (QTAP) which ensures selective detection and quantification of predetermined analytes. The advantage of MRM is very rapid analysis with high sensitivity, specificity, high mass precision and multiplexing capability which allows the analysis of dozens to hundreds of peptides in one run (MRM [online], cit. 2014-09-02; Deutsch et al 2008; Liebler and Zimmerman 2013). The biggest disadvantage is low resolution of Triple quadrupole which is the most often used analyser (Liebler and Zimmerman 2013; Uchida et al. 2013). The triple quadrupole mass analyser is preferably used because of its manner of ions selection and

because of its significant properties such as the high sensitivity and wide dynamic range (Uchida et al. 2013). Q1 quadrupole is the place of the first ion´s selection which is then fragmentized in the collision cell (Collision Induced Dissociation, CID) and Q3 quadrupole selectively allows pre-defined product ions to pass to the detector (Figure 4).

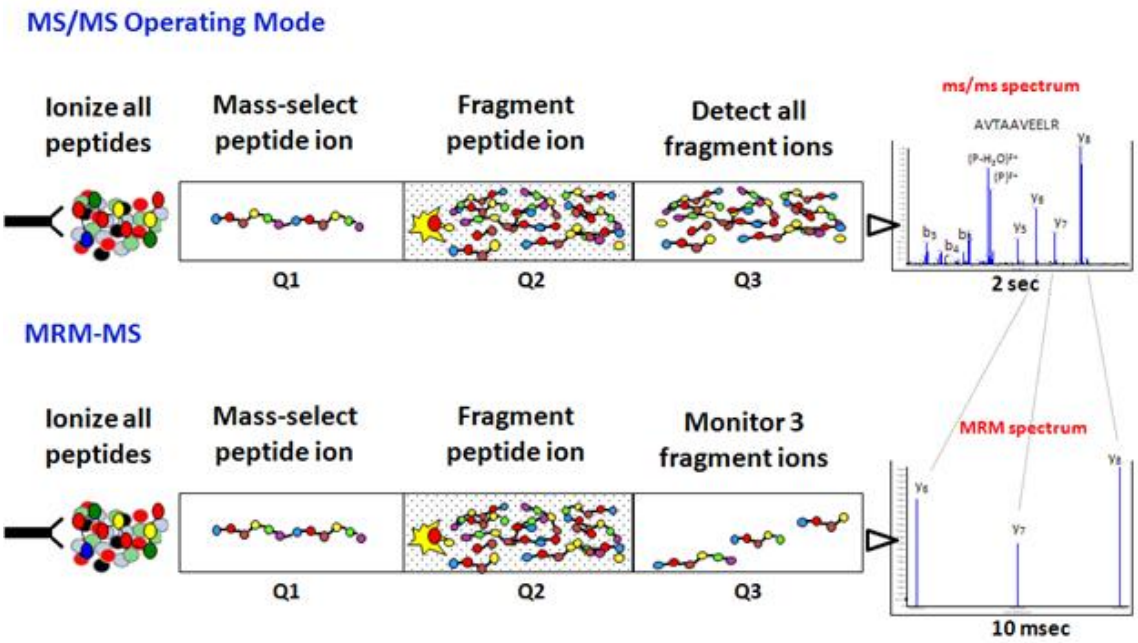

Figure 4. *The difference among the process of Tandem MS and MRM mode and between their spectra*

All ions generated in collisional cell continue to the detector in the case of tandem MS. Conversely, in MRM-MS mode dual selection is carried out in Q1 and Q3. Hence, the MS^2 spectrum is simpler and easily legible (MRM [online], cit. 2014-09-02).

MRM scheduling gives us a chance to measure more peptides (proteins) in one LC-MS run. Scheduling is put through the retention time (RT). Dwell time is needed time to detect one transition (a pair of parent ion and product ion of the peptide). Cycle time is a sum of dwell times for that concrete peptide. Setting the dwell time as long as possible provides the higher signal-to-noise ratio and the possibility to detect low abundance peptides. Scheduling is based on the detecting selected transitions per peptide in a small time window in which the expected elution time is centralized (Picotti and Aebersold 2012). Each transition is unique for the particular peptide (Picotti and Aebersold 2012). If just one transition per peptide is measured, there is a high risk of unreliability of quantitative data because of very complex sample. Therefore, the occurrence of not target peptide´s signal is possible in the area of target peak. In all, it is always recommended to measure at least three transitions per peptide to increase the reliability and reproducibility (Kawakami et al. 2011; Liebler and Zimmerman 2013). The first selection prevent from ions competition in the collisional cell to be fragmentized. As a

result of this regulation, MRM saves time (Pisitkun et al. 2007). Furthermore, there are just peaks of selected ions (transitions) in MS^2 spectrum instead of full scan of all product ions (Figure 4) (Deutsch et al. 2008). From this point of view, MRM enables to analyse low abundant peptides (respectively proteins e.g. transporters, receptors, enzymes) for which the detection is usually very difficult because of high background noise (Uchida et al. 2013; Kamiie et al. 2008). However, we definitely must know in advance what we will look for (Deutsch et al. 2008).

6.7.4 Quantification of CYP Enzymes

CYP enzymes can be quantified by different methods. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) provides the level of mRNA which often does not correlate to the level of the concrete protein. The disadvantage of Western blotting is the low resolution ability of antibodies among highly similar sequences of CYP isoforms. CYPs quantifications by measuring of their enzymatic activities cannot be carried out until specific substrates are found. Nowadays, (U)HPLC-MS/MS connection has become the most convenient approach to analyze CYPs among other proteins (Seibert et al. 2009; Liu et al. 2014). MRM mode of reversed phase UHPLC in-line coupled with tandem MS was applied method in this diploma thesis. CYPs levels measured by different scientific groups vary a lot (Table 2) except for CYP2B6, 2C8, 2C19, 2D6 and 3A5. However, in the case of CYP1A2, 2C9, 2E1 and 3A4 CYPs values differ 2 times, even 8 times, 7 times and 2 times, respectively.

Table 2. *Comparisons of average quantitative values (mean±SEM) of the most abundant CYPs in human liver microsomes samples (pmol/mg of microsomal protein)*

Isoforms	Kawakami et al. 2011	Gröer et al. 2014	Liu et al. 2014
CYP1A2	17.7±0.6	12.8±0.17	39.3
CYP2A6	49.2±1.7		
CYP2B6	6.86±0.44	9.59±0.38	4.3
CYP2C8	29.3±0.6	26.9±0.54	
CYP2C9	80.2±1.4	37.3±2.50	10.3
CYP2C19	3.64±0.22	2.18±0.18	3.0
CYP2D6	11.5±0.3	9.34±0.15	
CYP2E1	51.3±0.9	65.3±1.5	9.3
CYP3A4	64.0±1.9	32.6±0.38	54.0
CYP3A5	3.54±0.28	1.96±0.05	4.6

Seibert et al. (2009) conducted 1D (2D) electrophoresis followed by LC-MS/MS analysis of CYP1A2 and 2E1 in human liver microsomes (HLM) where average protein levels were 222.9±56.2 and 140.8±29.8 pmol/mg (mean±standard deviation), respectively. The same approach was used also by Wang et el. (2006) whose measured amount of CYP2E1 was 100 pmol/mg of microsomal protein in HLM. The research of Langenfeld et al. (2009) was focused

on CYP2D6 isoform and it was carried out also by the connection of 1D electrophoresis and LC-MS/MS. The average absolute protein amount was 0.364 pmol/mg in HLM. The research carried out by Wang et al. in 2008 was aimed at CYP3A4 and CYP3A5 protein amount in HLM. Their levels were following 67±7.4 pmol/mg and 30±17 pmol/mg HLM protein for CYP3A4 and 4±27 pmol/mg and 4.7±25 pmol/mg HLM protein for CYP3A5 (mean±coefficient of variation %). In light of these results, CYP protein amount is apparently influenced by the history of the sample and by the approach of the analysis.

6.7.5 Workflow of Quantitative Targeted Absolute Proteomics

According to previous studies, projects, scientific literature or prior knowledge we select the target proteins and then target unique peptides per protein (Figure 5) (Picotti and Aebersold 2012). Selection of target peptides is a real learning curve because not all unique peptides might be observable in MS spectra and additionally some multiple proteins or protein isoforms might include the same peptides.

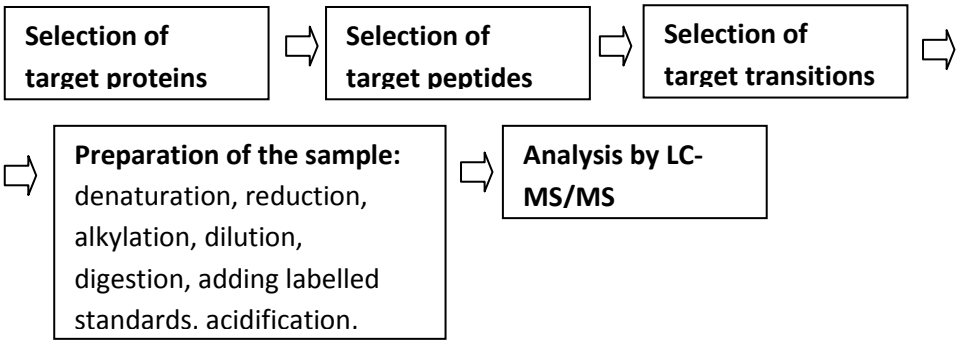

Figure 5. *Illustrative scheme of Quantitative Absolute Targeted Proteomics (QTAP) workflow*

The hydrophobicity, charge, energetic and structural properties have to be considered in the process of selection peptides. Short hydrophilic and long hydrophobic peptides are not preferable whereas the length ranging approximately from eight to ten amino acids (AA) should be the best. The carboxy-terminal part of tryptic digested peptides has to carry Arg or Lys but none of these two amino acids have to be following AA or to be in the middle of peptides. The amino acid Prolin is not allowed to occur as the following AA after the Arg or Lys residues because of inefficient tryptic digestion. The amino acids Methionine (Met) and Cystein (Cys) should be avoided totally because of the easy oxidation of Met to Met-sulfoxide and easy formation of disulfide bonds due to SH group of Cys. In addition, any posttranslational modifications are not acceptable (Deutsch et al. 2008; Picotti and Aebersold 2012; Uchida et al. 2013). Exclusion of Aspartic acid-Glycin pairs (deamidation), N-terminal

Glutamin (pyroglutamic acid formation) and N-terminal Asparagin (deamidation) are other tools which may reduce the potential risk of artificial modification and increase the reliability of unique peptides (Liebler and Zimmerman 2013; Langenfeld et al. 2009). The important part of peptides selection is using online data repositories such as PeptideAtlas or Global Proteome Machine Database (GPMD) which gather peptides, spectra, transitions that have been measured before. Peptides, which have not been measured yet, can be predicted by ESP Predictor or PeptideSieve (Liebler and Zimmerman 2013; Picotti and Aebersold 2012). Uniqueness of peptides is then checked out by BLAST search (http://www.uniprot.org/blast/) against human protein UniprotKB database (Kawakami et al. 2011; Langenfeld et al. 2009). Finally we select best transitions per peptide usually the most intense and most reproducible fragment ions which are unique for the peptide (Deutsch et al. 2008). Online libraries, predictors or MRM analysis of synthetic peptide standards are the tools for decision which transitions are the most suitable (Liebler and Zimmerman 2013; Picotti and Aebersold 2012). Higher m/z values of fragment ions are always preferable because the potential interference of noise background is noticeably reduced (Liebler and Zimmerman 2013). The selection of three transitions is essential for increasing confidence of specific assay (Kawakami et al. 2011; Liebler and Zimmerman 2013).

6.8 Software Skyline 2.6

Skyline is free available and Windows acceptable software (MacLean et al. 2010) which was introduced for the first time in 2009 (Liebler and Zimmerman 2013) and it has been widely used for academic and commercial purposes since this time due to the high performance of results, easy installation and using Skyline at ease (MacLean et al. 2010). Skyline is software that provides a platform for theoretical MRM analysis (selection of target peptides, transitions, optimization of parameters such as collision energy, retention time before the analysis) and assessment of experimental data (peak integration, the quantification and identification of proteins). Data might be uploaded in the form of the list of peptides sequences, proteins or transition list in Excel format, the whole protein sequences in the Fasta format (Liebler and Zimmerman 2013; MacLean et al. 2010) or direct experimental data import from Agilent, Applied Biosystems, Thermo Fisher Scientific and Waters triple quadrupole instruments. Conversely, Skyline provides export of various selected information usually in csv. format.

Skyline supports building MRM method (MacLean et al. 2010). It means an optimization of peptide and transition settings, (Liebler and Zimmerman 2013) *in silico* digestion of uploaded protein sequences or the formation of transitions list (MacLean et al. 2010). Skyline also

provides information about expected retention time according to Sequence Specific Retention Calculator algorithm (SSRCalc). SSRCalc is a retention time predictor on reversed-phase LC systems which is based on amino acids sequences and their molecular weight (Krohkin et al. 2004). Therefore, scheduled MRM analysis with known retention times can be set up by Skyline.

Skyline qualitative analysis is based on the co-elution of heavy and light peptides therefore they share the same retention time in one chromatogram. Skyline automatically integrates peak currents and provides quantitative values which are obtained from the ratio between the light peptide and the heavy one.

Skyline uses up created MS/MS libraries but simultaneously Skyline contributes to update these libraries or to create a new one.

Skyline can process and update large amount of data without latency. Skyline tree, chromatogram graph pane and other useful information in different panes can be displayed simultaneously in the Skyline window. To sum up, the architecture of Skyline software is easy understandable, easy handling even though it can gather large amount of information. In addition, Skyline has become a useful tool in theoretical phase of the research as well as in the experimental phase with high reliable output (MacLean et al. 2010).

7. EXPERIMENTAL PART

7.1 Materials and Methods

7.1.1 Chemicals

- Acetonitrile, Hi Perv Solv Chromanorm for HPLC (VWR Prolabo BDH, EC)
- Bio-Rad Protein assay, Dye reagent concentrate (Bio Rad, München, Germany)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, Missouri, USA)
- Formic acid 98%, eluent additive for LC-MS (Fluka Analytical, Germany)
- K_2-EDTA (Ethylenediaminetetraacetic acid Dipotassium salt $C_{10}H_{14}K_2N_2O_8$ * H_2O) (Fluka Analytical, Germany)
- Protein Preparation Kit 100Assay (AB Sciex Pte. Ltd., USA)
    - CYP450 Peptide Standards Extended Panel Human Induction: 3 peptides for each isoform: 2C9, 2C19, 2E1 (AB Sciex Pte. Ltd., USA)
    - Cysteine-Blocking reagent: 200mM methyl methane-thiosulfonate (MMTS) in isopropanol (AB Sciex Pte. Ltd., USA)
    - Denaturant: 20% (w/v) n-octyl glucoside (OGS) (AB Sciex Pte. Ltd., USA)
    - Digestion buffer: 0.1M Tris[hydroxymethyl] aminomethane hydrochloride (TRIS), 4mM Calcium Chloride (AB Sciex Pte. Ltd., USA)
    - P450 Peptide Standards Human Induction: 3 peptides for each isoform: 1A2, 2B6, 3A4, 3A5 (AB Sciex Pte. Ltd., USA)
    - Peptide Dilution Solution: 20% ACN, 0.1% TFA (AB Sciex Pte. Ltd., USA)
    - Reducing Reagent: 50mM tris-(2-carboxyethyl)-phosphine (TCEP) (AB Sciex Pte. Ltd., USA)
- Sequencing Grade Modified Trypsin, Porcine (Promega, Madison WI USA)
- Tris-HCl ultra pure (MP Biomedicals, LLC, France)
- Tris ultra pure (MP Biomedicals, Inc., France)

7.1.2 Instrumentation

- Agilent 1290 Infinity LC System, UHPLC (Agilent Technologies, Santa Clara, CA, USA)
- Agilent 6495 Triple Quadrupole LC-MS System (Agilent Technologies, Santa Clara, CA, USA)
- Beckman Coulter AvantiTM J-30I centrifuge (Beckman Coulter, Inc., USA)
- ELx 800 UV (BIO-TEK Instruments, Inc., USA)
- Eppendorf AG Centrifuge 5415D (Eppendorf AG, Hamburg, Germany)
- MSE Soniprep 150 Ultrasonic Disintegrator (MSE Scientific Instruments, England)
- Potter-Elvehjem homogenizer (Heidolph, W-Germany)
- Sorvall WX Ultra Series (Thermo Electron Corporation, USA and Germany)
- Titramax 1000 (Heidolph, Germany)
- TS – 100 Thermo Shaker (Biosan Ltd., EU)
- USF ELGA Maxima Water Purification system (USF ELGA Maxima, England)
- Vortex – Genie, Bohemia N.Y. 11716 (Scientific Industries, Inc., USA)

7.1.3 Samples

Wild Type (WT) cell lines were both originally from American Tissue Culture Collection (ATCC). C3A WT and CACO2 WT were used for the comparative purposes.

CACO2 (ATCC HTB-37) cell line was obtained from human colon of male patient who died of colorectal carcinoma in the age of 72 years. These epithelial cells are tumorigenic and undifferentiated (Caco-2 [Caco2] (ATCC® HTB-37™) [online], cit. 2014-10-08).

C3A (ATCC CRL-10741) is a clonal derivate of HepG2 (ATCC HB-8065) which was obtained from human liver of male patient who died of hepatocellular carcinoma in the age of 15 years. Epithelial cells are non-tumorigenic and differentiated (C3A [HepG2/C3A, derivative of Hep G2 (ATCC HB-8065)] (ATCC® CRL-10741™) [online], cit. 2014-10-08).

Samples C3A PXR7, C3A CAR28, CACO2 PXR and CACO2 CAR were created by the fusion of strong activation domain p65 with human PXR or CAR at their carboxy-terminus (Küblbeck et al. 2010).

The sample of Human liver microsomes (HLM) was used for verification of method and suitable transitions. The piece of liver was kindly donated from the University Hospital of Oulu in Finland as a surplus from cadaver kidney transplantation donor. The patient´s history of drug use was dexamethasone, nizatidine, phenytoin 24 hours before death from intracerebral haemorrhage.

Microsomes were prepared by differential ultracentrifugation and protein concentration was determined by Bradford method. Subsequently, samples were undergone tryptic digestion.

7.1.4 Preparation of Homogenization Buffer

The homogenization buffer (100 mM Tris-HCl buffer pH 7.4, 1 mM K_2EDTA) was prepared from one litre of 0.1 M Tris-HCl and from 200 ml of 0.1 M Tris. The pH 7.4 of Tris-HCl was adjusted with Tris solution. Corresponding amount of K_2EDTA (0.4045 g) was dissolved in one litre of the Tris-HCl pH 7.4 buffer.

7.1.5 Isolation of Microsomes from Modified Cell Lines

Seven or five tubes of dried cell lines ($30x10^6$ – $100x10^6$ cells) were suspended in the total volume 5 ml of homogenization buffer (100 mM Tris-HCl buffer pH 7.4, 1 mM K_2EDTA) and they were pooled. The suspension was sonicated 4 times 10/10 seconds and homogenized with Potter-Elvehjem homogenizer 5 times. Samples must have been always cooled down in the ice container. The homogenate was subsequently centrifuged 20 minutes with the speed 10 000 g, at a constant temperature +4 °C. Mitochondria, nuclei and other heavy particles went down the bottom of centrifuge tubes. The resulting supernatant was ultra-centrifuged for one hour with the speed 100 000 g, at the constant temperature +4 °C. Microsomes went down the bottom of the tube and they created pellet. Microsomal pellets were homogenized with small pestle in 0.5 ml of the homogenization buffer and frozen at -80 °C.

7.1.6 Bio-Rad Protein Determination

Bio-Rad Protein assay is a simple method of Bradford for measuring concentration of solubilised proteins. Different protein concentrations cause colour changes after the addition of an acidic dye (Coomassie brilliant blue G-250) which preferentially binds to the basic and aromatic amino acids residues of proteins. Absorbance is measured by spectrophotometer or microplate reader at 595 nm. Total concentration of proteins in the sample is found out from

the calibration curve of Bovine Serum Albumin standards (Bio-Rad Protein Assay [online], cit. 2014-10-12).

Bovine Serum Albumin (BSA) was diluted with deionized water to make calibration standards of concentration 0.35, 0.25, 0.20, 0.15, 0.10, 0.05, 0.025 mg/ml. Blank (buffer and deionized water were in the ratio 1:80), calibration standards and diluted microsomes (ratio with deionized water was 1:4 and 1:16) were pipetted in the volume of 10 µl to 96 wellplate in duplicate. Bio-Rad solution was prepared by dilution with deionized water in the ratio of 1:5. Bio-Rad solution was added in the volume of 200 µl to wells by multi-channel pipette. After 15 minutes of incubation at the room temperature and mixing by microplate mixer, samples were measured by microplate reader at 595 nm. Concentration of proteins in the sample was calculated from the calibration curve with regard to the dilution.

7.1.7 Preparation of Sample for LC-MS/MS Analysis

The samples were prepared according to the CYP450 Protein Assay – Human Induction Kit Protocol (CYP450 Protein Assay - Human Induction Kit [online], cit. 2014-10-11). Briefly, 100 µl of sample were taken and denatured by adding 5 µl of Denaturant (20 % n-octyl glucoside). Basically, after the each step, samples must have been mixed with vortex and briefly spinned. Disulfide bonds were reduced by 10 µl of Reducing Reagent (50 mM tris-(2-carboxyethyl)-phosphine). After the 1 hour lasting incubation at the temperature 60 °C, the Cysteine Blocking Reagent (200 mM methyl methane-thiosulfonate in isopropanol) was added in the volume of 5 µl. Samples were mixed and incubated at the room temperature for 10 minutes.

The vial of Sequencing Grade Modified Trypsin contains 20 µg of lyophilized trypsin (Certificate of Analysis: Sequencing Grade Modified Trypsin [online], cit. 2014-10-11). According to the CYP450 Protein Assay – Human Induction Kit Protocol, the recommended amount of trypsin for digestion is ranging from 4 to 50 µg. Dried trypsin was reconstituted by 50 µl of deionized water and then mixed and spinned. Before the adding 10 µl of freshly reconstituted trypsin, samples were diluted with 100 µl of Digestion Buffer (0.1 M Tris[hydroxymethyl] aminomethane hydrochloride, 4 mM Calcium Chloride). Samples were again mixed and spinned. For trypsin digestion, samples were incubated at 37 °C for 4 hours.

Two vials of different peptide standards (P450 Peptide Standards: CYP1A2, 2B6, 3A4, 3A5 and CYP450 Extended Panel Peptide Standards: CYP2C9, 2C19, 2E1) were both reconstituted with 200 µl of Peptide Dilution Solvent (20 % ACN, 0.1 % TFA). Since the MS instrument is at least 50-folds more sensitive, the amount of added peptide standards must have been decreased.

5 µl of both were pipetted and 40 µl of Peptide Dilution Solvent were added in order to prepare 1:10 dilution. Or in the case of 1:5 dilution, just 15 µl of Peptide Dilution Solvent were added. After the digestion, samples were splitted up to the two equal volumes and 2 µl of those diluted peptide standards were added. After the mixing and spinning, samples were either frozen or directly submitted for LC-MS/MS analysis. The total amount of heavy labelled peptide standards in samples were 716.9 or 1433.8 fmol/ml in the cases of 1:10 or 1:5 dilution, respectively.

7.2 LC-MS/MS Conditions and Method Development

Quantitative analysis was performed by the UHPLC system (Agilent 1290 Infinity LC System, Agilent Technologies, Santa Clara, CA, USA) which was on-line connected with an ESI-triple quadrupole mass spectrometer (Agilent 6495 Triple Quadrupole LC-MS System, Agilent Technologies, Santa Clara, CA, USA). Samples were injected onto an AdvanceBio Peptide Mapping column C18 (250 mm x 2,1 mm ID, 2,7 µm particles, Agilent Technologies, Santa Clara, CA, USA).

Table 3. *The examined gradients*

The first gradient			**The second gradient**		
Time (min)	**A (%)**	**B (%)**	**Time (min)**	**A (%)**	**B (%)**
0	95	5	0	90	10
20	30	70	30	60	40
20.1	95	5	31	20	80
25	95	5	34	20	80
			35	90	10
			40	90	10

Two different gradients were tested during the method development (Table 3). As mobile phases, water with 0.1 % formic acid (FA) (solvent A) and acetonitrile with 0.1 % FA (solvent B) were applied at a flow rate of 300 µl/min.

Time scheduling was three times changed. We started with non-scheduled method where all transitions were measured continuously. However, the dwell time must have been shortened and the cycle time was increased with this method disadvantageously. Subsequently, the time segments were created according to the measured retention time of the heavy labelled peptide standards.

Finally, Dynamic (scheduled) MRM method with defined retention times was applied and the value of Delta retention time of 2 min was used in the case of the retention time shift. The following parameters of MS/MS run, described in the Table 4 were finally applied.

Table 4. *Applied MS/MS parameters*

Parameter	Value	Parameter	Value
Ion mode	Positive	Nebulizer	40 psi
Dwell time per MRM	20 ms	Sheath gas temperature	250 °C
Collision energy	25 eV	Sheath gas flow	11 l/min
Flow rate	0.3 ml/min	Capillary voltage	3000 V
Injection volume	20 µl	Nozzle voltage	1500 V
Gas temperature	200 °C	Chamber current	0.98 µA
Gas flow	15 l/min		

7.3 Contribution of Skyline 2.6 and Calculations

We knew target peptides from CYP450 Protein Assay – Human Induction Kit Protocol (CYP450 Protein Assay - Human Induction Kit [online], cit. 2014-10-11). The list of peptides with corresponding proteins was inserted to the Skyline software which subsequently generated the list of transitions in the basis of settled parameters, described in the Table 5.

Table 5. *Skyline settings*

Peptide Settings		Transition Settings	
Enzyme	Trypsin (KR/P)	Precursor and Product ion mass	Monoisotopic
Structural modification	Carbamidomethyl Cystein	Precursor charges	2, 3
		Ion charges	1, 2
Isotope labelled type	Heavy	Ion types	y
		Ion match tolerance	0.5 m/z
Isotope modification	Label 13C(6)15N(4) (C-term R)	Min m/z	50
	Label 13C(6)15N(2)(C-term K)	Max m/z	1500
		Method match tolerance m/z	0.055

Detailed description of settled parameters is described in the Workflow Guide called Agilent Triple Quadrupole LC/MS Peptide Quantitation with Skyline (Agilent Triple Quadrupole LC/MS Peptide Quantitation with Skyline [online], cit. 2014-10-15). According to the acquired Skyline list of transitions we chose doubly and triply charged precursor ions and product ions whose m/z values were generally higher than m/z values of precursor ions (Table 6). These values with peptide sequences were inserted to the Data acquisition list of Agilent MassHunter Workstation Data Acquisition B.07 and Dynamic MRM method was run. Obtained data from Agilent 6495 Triple Quadrupole instrument were imported to Skyline which recognized data for corresponding transitions. Unidentified transitions were removed to produce simplified target list.

All transitions were integrated and panes of Peak areas and Retention times were displayed in the Skyline window (Figure 7). Retention times pane presents the retention times conformity for the same peptide in different samples. All transitions belonging to one peptide must have

the same retention time in order to be identified and quantified. The Peak areas pane shows the mutual ratios of areas of individual transitions for one precursor ion. As soon as the light peptide was co-eluted together with the heavy one, thus they shared the same retention time, we could have identified that peptide. Retention times and Peak areas panes helped us to find and defined the correct peak boundaries. Skyline enabled us also to quantify CYP enzymes by exporting the report. The report included the name of protein, peptide sequences, the name of the sample, retention time and area ratio light to heavy peptide (Figure 8) $A_{(light)}/A_{(heavy)}$.

The concentration of heavy labelled peptide standards in the sample was calculated due to the known amount of the peptide standards (100 ng per each peptide standard) and due to the monoisotopic protein mass (Da) with regard to the dilution. The concentration of light peptides was obtained by using formula (1). Finally, peptide concentration was divided by the known total amount of microsomal protein in the sample which was found out by the Bradford protein determination.

$$C_{(light)}/C_{(heavy)} = A_{(light)}/A_{(heavy)} \quad (1)$$

C.....Concentration

A.....Area under the curve

Table 6. *Sequences and MRM transitions of CYP isoforms*

			MRM Transitions (m/z)			
Isoform	**Sequence**	**Q1**	**Q3-1**	**Q3-2**	**Q3-3**	**Q1Charge**
CYP1A2	YLPNPALQR	536.3	795.4	698.4		2
	YLPNPALQR*	541.3	805.5	708.4		2
	YLPNPALQR	357.9	795.4	584.4	487.3	3
	YLPNPALQR*	361.2	805.5	594.4	497.3	3
	ASGNLIPQEK	352.9	614.4	501.3		3
	ASGNLIPQEK*	355.5	622.4	509.3		3
	DITGALFK	432.7	636.4	535.3	478.3	2
	DITGALFK*	436.8	644.4	543.3	486.3	2
	DITGALFK	288.8	535.3	478.3	407.3	3
	DITGALFK*	291.5	543.3	486.3	415.3	3
CYP2B6	ETLDPSAPK	319.8	499.3	402.2	315.2	3
	ETLDPSAPK*	322.5	507.3	410.2	323.2	3
	FSVTTMR	421.2	607.3			2
	FSVTTMR*	426.2	617.3			2
	FSVTTMR	281.1	607.3	508.3	407.2	3
	FSVTTMR*	284.5	617.3	518.3	417.2	3
	IAMVDPFFR	365.9	681.3	566.3	469.3	3
	IAMVDPFFR*	369.2	691.3	576.3	479.3	3
CYP3A4	VWGFYDGQQPVLAITDPDMIK	1197.1	1312.7	603.3	506.3	2
	VWGFYDGQQPVLAITDPDMIK*	1201.1	1320.7	611.3	514.3	2
	VWGFYDGQQPVLAITDPDMIK	798.4	1312.7	603.3	506.3	3
	VWGFYDGQQPVLAITDPDMIK*	801.1	1320.7	611.3	514.3	3
	LSLGGLLQPEKPVVLK	846.0	909.6	555.4	458.3	2
	LSLGGLLQPEKPVVLK*	850.0	917.6	563.4	466.3	2
	LSLGGLLQPEKPVVLK	564.4	909.6			3
	LSLGGLLQPEKPVVLK*	567.0	917.6			3
	EVTNFLR	293.5	650.4	549.3	435.3	3
	EVTNFLR*	296.8	660.4	559.3	445.3	3
CYP3A5	GSMVVIPTYALHHDPK	883.0	1178.6	633.3	496.3	2
	GSMVVIPTYALHHDPK*	887.0	1186.6	641.3	504.3	2
	GSMVVIPTYALHHDPK	589.0	1178.6	633.3	496.3	3
	GSMVVIPTYALHHDPK*	591.6	1186.6	641.3	504.3	3
	SLGPVGFMK	312.5	678.4	581.3	482.2	3
	SLGPVGFMK*	315.2	686.4	589.3	490.3	3
	DTINFLSK	313.2	608.3	494.3	347.2	3
	DTINFLSK*	315.8	616.4	502.3	355.2	3
CYP2C9	GIFPLAER	301.5	585.3	488.3	375.2	3
	GIFPLAER*	304.8	595.3	498.3	385.2	3
	SLVDPK	220.1	571.3	458.3	359.2	3
	SLVDPK*	222.8	579.4	466.3	367.2	3
	SHMPYTDAVVHEVQR	590.3	1413.7	668.3	531.3	3
	SHMPYTDAVVHEVQR*	593.6	1423.7	678.4	541.3	3
CYP2C19	GTTILTSLTSVLHDNK	850.5	626.3	513.2	376.2	2
	GTTILTSLTSVLHDNK*	854.5	634.3	521.3	384.2	2
	GTTILTSLTSVLHDNK	567.3	626.3	513.2		3
	GTTILTSLTSVLHDNK*	570.0	634.3	521.3		3
	SNYFMPFSAGK	624.8	606.3	509.3	362.2	2
	SNYFMPFSAGK*	628.8	614.3	517.3	370.2	2
	SNYFMPFSAGK	416.9	606.3	509.3	362.2	3
	SNYFMPFSAGK*	419.5	614.3	517.3	370.2	3
	GHFPLAER	309.5	585.3	488.3	375.2	3
	GHFPLAER*	312.8	595.3	498.3	385.2	3
CYP2E1	FSLTTLR	279.8	603.4	490.3	389.3	3
	FSLTTLR*	283.2	613.4	500.3	399.3	3
	GIIFNNGPTWK	623.8	588.3	531.3	434.2	2
	GIIFNNGPTWK*	627.8	596.3	539.3	442.3	2
	GIIFNNGPTWK	416.2	588.3	531.3		3
	GIIFNNGPTWK*	418.9	596.3	539.3		3
	FITLVPSNLPHEATR	848.0	1121.6	710.4	613.3	2
	FITLVPSNLPHEATR*	853.0	1131.6	720.4	623.3	2
	FITLVPSNLPHEATR	565.6	1121.6	710.4	613.3	3
	FITLVPSNLPHEATR*	569.0	1131.6	720.4	623.3	3

Asterisk indicates amino acid residues labelled with heavy ^{13}C and ^{15}N. Underlined peptides are not 100% suitable because of the presence of Met (M), Aspartic acid-Glycin pair (D-G) and acidic AA (D, E, N, Q) close to the possible cleavage.

8. RESULTS

8.1 Method Development and Data Evaluation

The first shorter gradient for LC-MS/MS run did not provide good resolution of adjacent peaks and chromatograms were not legible enough to identify or even quantify peptides. The time prolongation to 40 min and retention time scheduling has brought higher resolution power, sensitivity and all peptides were eluted till 30 min with that gradient (Figure 6). Dynamic MRM method limited the number of compounds measured in one time and it has presented optimized flow rate, gradient, retention time and the transition list. Hence, it could be used for LC-MS/MS analysis of CYP enzymes. We have obtained their quantitative values from HLM sample, presented in the Table 9.

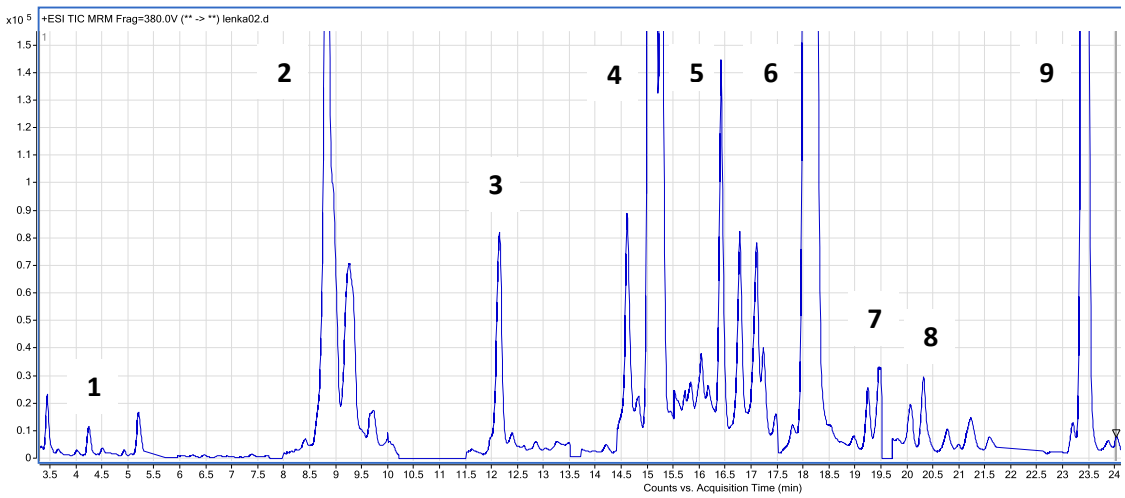

Figure 6. *Total Ion Current/Chromatogram (TIC) of HLM determined by Agilent MassHunter Qualitative analysis B.06*
Chromatogram is described in the table listed below.

No	Isoform	Peptide	Retention time	Q1	Charge	Q3
1	CYP2B6	ETLDPSAPK*	4.2	322.5	3	507.3
2	CYP2C9	SHMPYTDAVVHEVQR*	8.8	593.6	3	1423.7
3	CYP3A5	DTINFLSK*	12.1	315.8	3	616.4
4	CYP1A2	DITGALFK	15.1	432.7	2	478.3
5	CYP2C19	SNYFMPFSAGK*	16.4	628.8	2	614.3
6	CYP3A4	LSLGGLLQPEKPVVLK*	18.1	850	2	917.6
7	CYP2B6	IAMVDPFFR*	19.5	369.2	3	691.3
8	CYP2C19	GTTILTSLTSVLHDNK	20.1	850.5	2	376.2
9	CYP3A4	VWGFYDGQQPVLAITDPDMIK*	23.4	1201	2	1320.7

Asterisk indicates amino acid residues labelled with heavy ^{13}C and ^{15}N.

Data was evaluated using free available software Skyline 2.6. Skyline peptide tree of each CYP enzyme was constituted of three peptides, doubly and triply charged precursor ions (light and heavy) and from two to three transitions per each precursor ion (Figure 7). After the integration of all transitions, the peptide was identified due to the co-elution of all product ions belonging to the particular precursor ions of the peptide. The identification was simplified by taking an advantage of graphical display of the retention time conformity in different samples (Retention Times pane) and the mutual ratio of intensities coming from product ions and their precursor ion in different samples (Peak Areas pane).

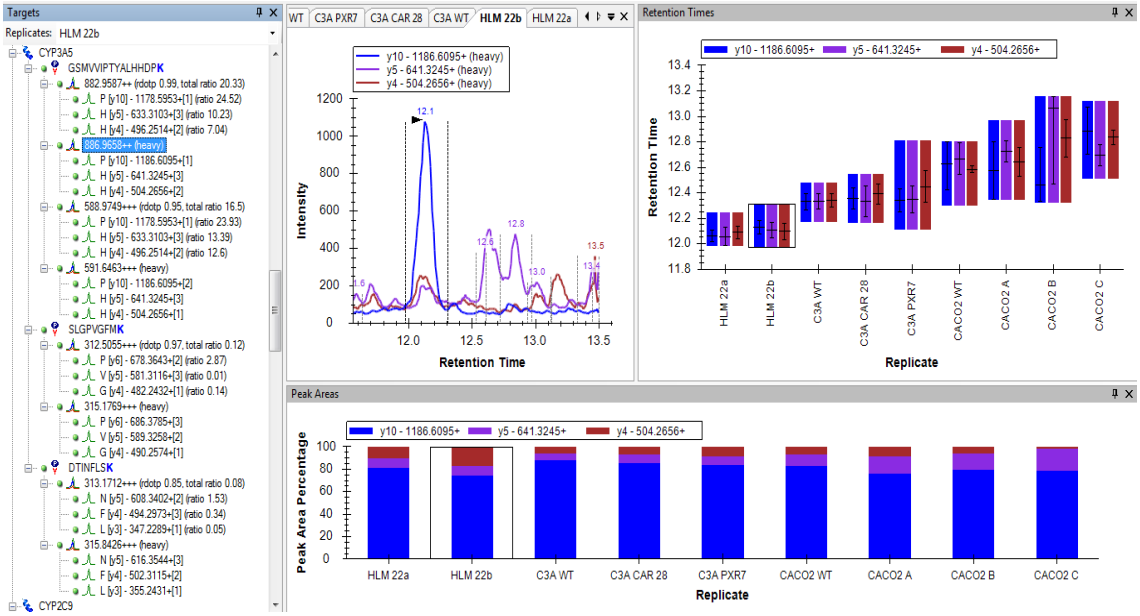

Figure 7. *Skyline window of CYP3A5 peptide (GSMVVIPTYALHHDPK) and it´s doubly charged heavy labelled precursor ion of m/z value 887 (highlighted)*

Skyline tree (on the left); chromatogram of the heavy labelled peptide standard (in the middle); Retention Times pane (on the right); Peak areas pane (in the bottom). Retention time of heavy labelled peptide standard is not the same in HLM and modified cell lines samples as it is clear from the Retention Times pane. The impurities contained in samples caused apparently the retention time shift. Peak area ratios of heavy transitions, belonging to the precursor ion, are almost the same in HLM and modified cell lines.

The quantification of CYP enzymes was done from the peak area ratio of co-eluted light and heavy peptides. The report was generated by Skyline (Figure 8). The retention time little bit differs in the same but diluted sample. Additionally, dilution also influenced the ratio light to heavy. Especially, the highest dilution provides different values.

	Protein Name	Peptide Sequence	Replicate Name	Peptide Retention Time	RatioLightToHeavy
2	CYP2B6	FSVTTMR	HLM 22	8.67	9.4428
3	CYP2B6	FSVTTMR	HLM 22 (10x)	8.88	11.1738
4	CYP2B6	FSVTTMR	HLM 22 (100x)	8.87	3.6675
5	CYP2B6	IAMVDPFFR	HLM 22	19.18	2.0146
6	CYP2B6	IAMVDPFFR	HLM 22 (10x)	19.29	0.675
7	CYP2B6	IAMVDPFFR	HLM 22 (100x)	19.81	1.4221
8	CYP3A4	VWGFYDGQQPVLAITDPDMIK	HLM 22	22.88	260.7467
9	CYP3A4	VWGFYDGQQPVLAITDPDMIK	HLM 22 (10x)	23.04	269.3316
10	CYP3A4	VWGFYDGQQPVLAITDPDMIK	HLM 22 (100x)	23.09	103.5759
11	CYP3A4	LSLGGLLQPEKPVVLK	HLM 22	17.67	253.713
12	CYP3A4	LSLGGLLQPEKPVVLK	HLM 22 (10x)	17.9	236.9841
13	CYP3A4	LSLGGLLQPEKPVVLK	HLM 22 (100x)	17.98	145.2817

Figure 8. *The Report from Skyline in csv.format*

8.2 Human Liver Microsomes

In order to verify selected transitions and suitability of Dynamic MRM method, sample of human liver microsomes was analyzed. As a result of this measurement, all desired CYP isoforms were identified and quantified. However, CYP enzymes were not unfortunately identified and quantified by all peptides and transitions which were mentioned in the Table 6. Some signals were very low and covered by background. Generally, this was the case of some triply charged ions whose m/z values of precursor ions were below 400 (Figure 9).

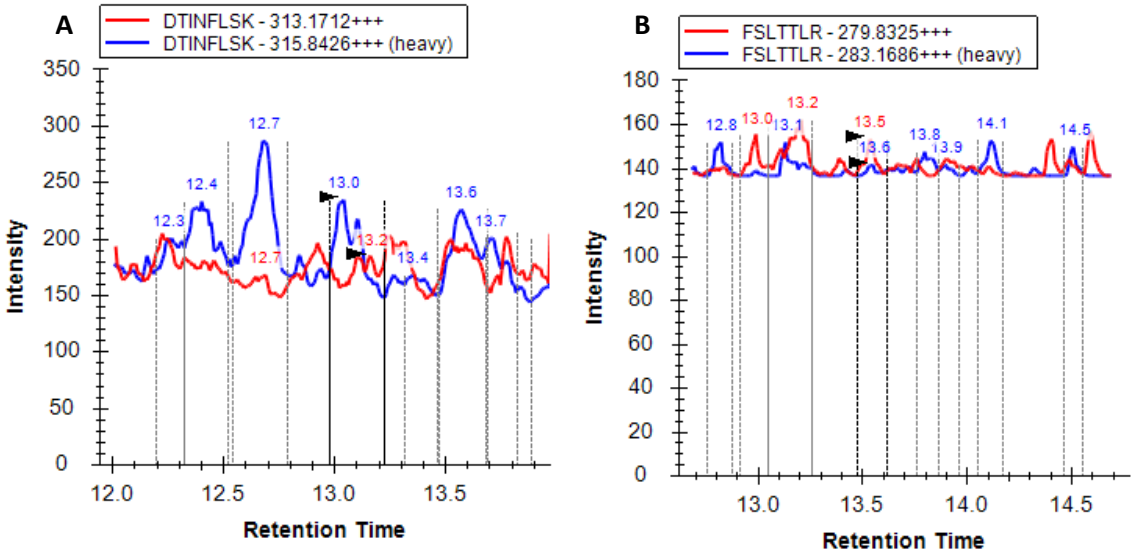

Figure 9. *Chromatograms of CYP3A5 (on the left) and 2E1 (on the right) from HLM sample*

Triply charged precursor ions with the m/z values below 400 did not provide clear peaks because of the background. Red line presents light peptides and the blue one presents heavy ones. (A) Chromatogram of CYP3A5 (DTINFLSK light red line and heavy blue line). (B) Chromatogram of CYP2E1 (FSLTTLR light red line and heavy blue line).

The average values were following 2.7±0.2, 3.4±0.3, 21.5±1.0, 1.4±0.1, 1.6±0.3, 0.6±0.1 and 3.9±0.4 pmol/mg for CYP 1A2, 2B6, 3A4, 3A5, 2C9, 2C19 and 2E1, respectively. The most abundant enzymes were CYP 3A4 (Figure 10), followed by 2E1 (Figure 11) and 2B6.

When raw data from Agilent 6495 Triple Quadrupole were uploaded to Skyline, majority of found transitions belonged to triply charged precursor ions. Hence, in the ionization chamber of MS instrument triply charged precursor ions were predominantly created instead of doubly charged ions. Some signals coming from triply charged ions were even higher than from doubly charged ions. The light violet ion y^6 provided the signal of $1.4x10^6$ of triply charged precursor ion (Figure 11). However, the same ion of doubly charged precursor ion performed just the signal of $40x10^3$ (Figure 13).

With regard to different intensities of CYP3A4 transitions, we can notice that intensity of violet light ion y^5 is about seven times higher than the intensity of the blue light one in chromatogram of triply charged precursor ions (Figure 10). Conversely, both ions have almost the same decreased intensity in chromatogram of doubly charged light precursor ions (Figure 12). Heavy transitions present the similar case where the difference between heavy y^5 ion and heavy y^{12} ion is about four times in chromatogram of triply charged precursor ions (Figure 10). And again both ions are of the same intensity in chromatogram of doubly charged precursor ions (Figure 12). The light and heavy brown y^4 ion gives bad signal in all chromatograms. The amount of CYP3A4 in HLM sample is higher than the amount of added peptide standard as it is obvious from the differences of intensities among light and heavy transitions in both types of charged precursor ions. Intensities of light transitions are higher than intensities of heavy ones (Figure 10, Figure 12).

In the case of CYP2E1 chromatograms (Figure 11, Figure 13), doubly and triply charged precursor ions provide product ions of similar ratios of intensities. The amount of CYP2E1 in HLM sample is again higher than the amount of added peptide standard.

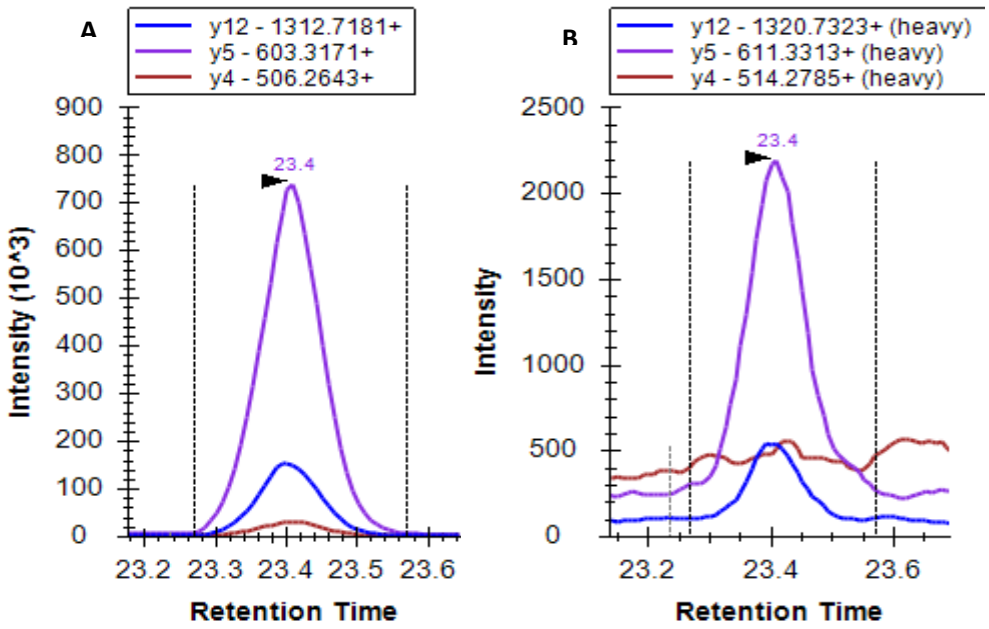

Figure 10. *Chromatograms of CYP3A4 from HLM sample. Signals come from triply charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (A) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK light), precursor ion (m/z 798.4) is triply charged. (B) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK heavy), precursor ion (m/z 801.1) is triply charged.

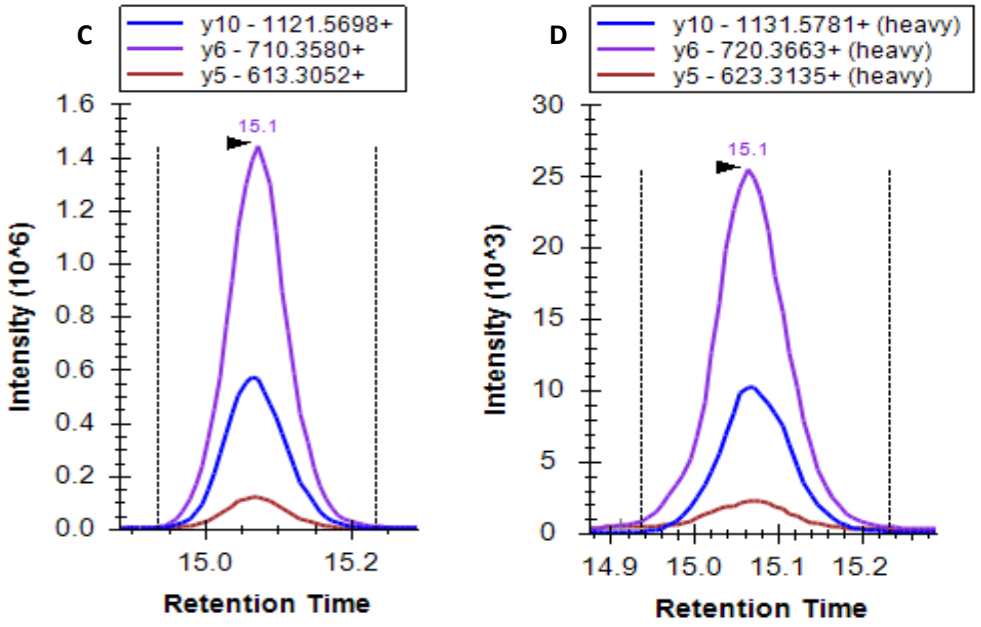

Figure 11. *Chromatograms of CYP2E1 from HLM sample. Signals come from triply charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (C) Chromatogram of CYP2E1 (FITLVPSNLPHEATR light), precursor ion (m/z 565.6) is triply charged. (D) Chromatogram of CYP2E1 (FITLVPSNLPHEATR heavy), precursor ion (m/z 567) is triply charged.

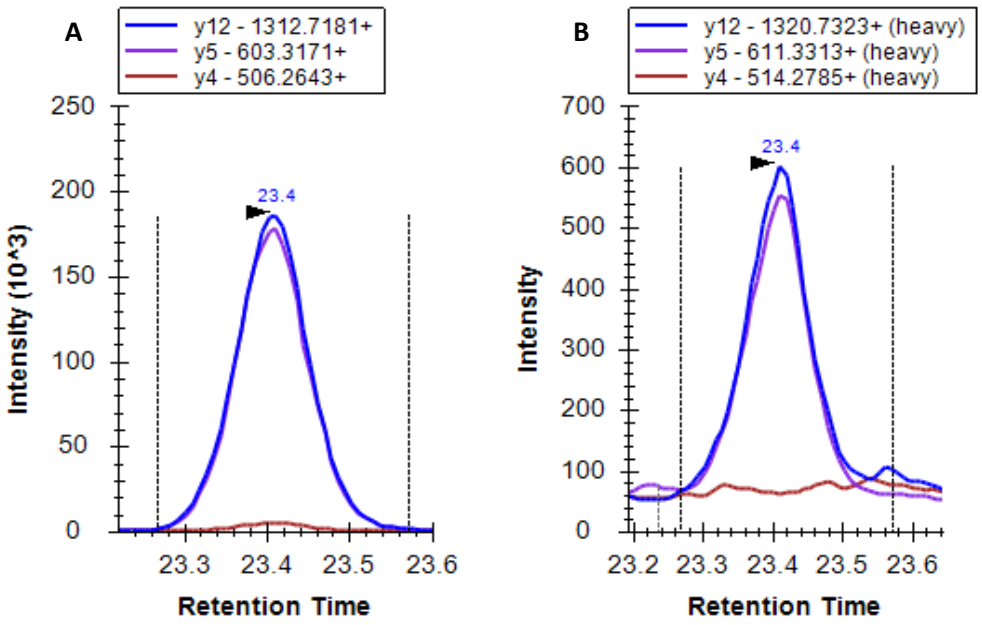

Figure 12. *Chromatograms of CYP3A4 from HLM sample. Signals come from doubly charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (A) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK light), precursor ion (m/z 1197.1) is doubly charged. (B) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK heavy), precursor ion (m/z 1201.1) is doubly charged.

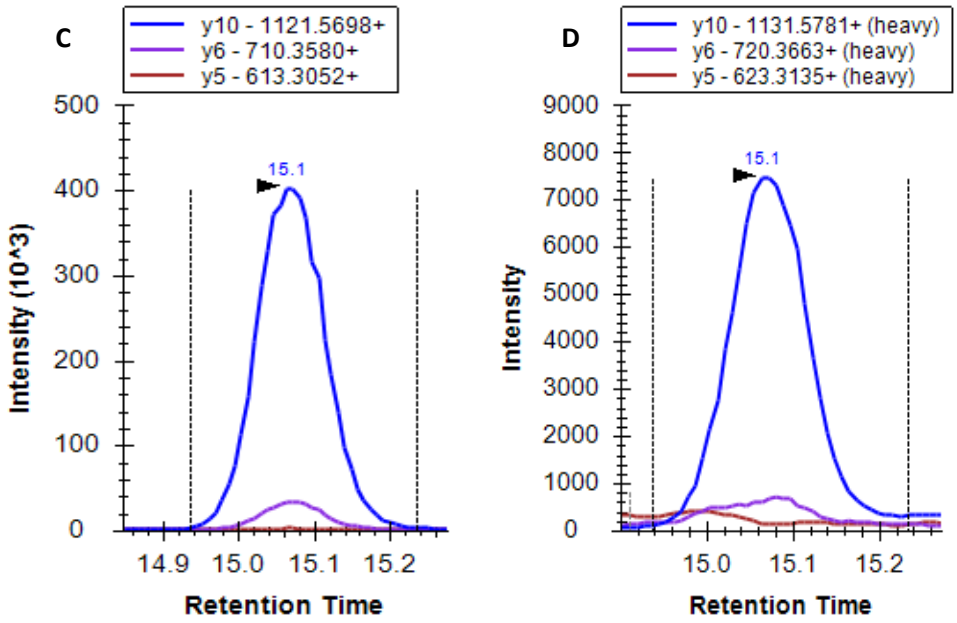

Figure 13. *Chromatograms of CYP2E1 from HLM sample. Signals come from doubly charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (C) Chromatogram of CYP2E1 (FITLVPSNLPHEATR light), precursor ion (m/z 848) is doubly charged. (D) Chromatogram of CYP2E1 (FITLVPSNLPHEATR heavy), precursor ion (m/z 853) is doubly charged.

8.3 CACO2 and C3A Cell Lines

The quantitative measurement of CYP enzymes by Dynamic MRM method has not revealed any CYPs in modified cell lines. Heavy labelled peptide standards were detected in chromatograms but light peptides were not found in samples (Figure 14, Figure 15). Since CYP enzymes were quantified in HLM sample, we should have seen them also in modified cell lines, provided that they were in the sample. On the other hand, their levels might have been apparently under the limit of detection which was calculated thanks to results of diluted HLM sample (Table 7).

Table 7. *The examples of detectable protein amount in a hundred times diluted HLM sample*

Isoform	Peptide	Charge	fmol/injection	pmol/mg
CYP1A2	YLPNPALQR	3	0.32	0.019
CYP3A4	VWGFYDGQQPVLAITDPDMIK	3	0.15	0.210
CYP3A5	GSMVVIPTYALHHDPK	2, 3	0.20	0.006
CYP2E1	FITLVPSNLPHEATR	2, 3	0.21	0.041

Those values (fmol/injection or pmol/mg of microsomal protein) present the lowest amount of peptides which can be still detected. 20µl were injected to the LC-MS/MS.

Despite of the fact that heavy labelled peptide standards were added in the same concentration (1433.8 pmol/l) to all samples, their intensities differed as it is shown in chromatogram of CYP3A4 in C3A CAR28 sample (Figure 14) where the signal of heavy violet product ion y^5 was about 8000. In contrast, the same heavy ion in the HLM sample (Figure 10) provided just the signal of 2000. However, in the case of CYP2E1 (Figure 15, Figure 11), CACO2 PXR and HLM samples contained approximately the same amount of peptide standards. Values of intensity are approximately the same. It is obvious that some error during pipetting may have occurred.

Retention time shift occurred quite often, as it is, for instance, obvious from Figure 14 and Figure 10 which show chromatograms of CYP3A4 in C3A CAR28 and in HLM samples. Retention time of the peptide (VWGFYDGQQPVLAITDPDMIK) belonging to CYP3A4 is 23.4 min in the HLM sample and it is 23.5 min in the C3A CAR28 sample. It was most likely caused by impurities and maybe also by other compounds which influenced the retention time.

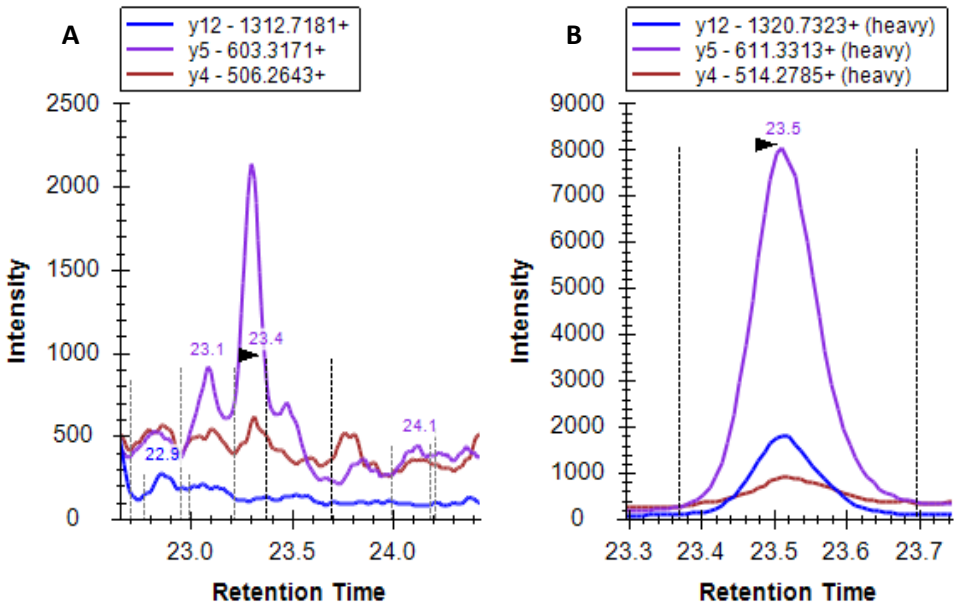

Figure 14. *Chromatograms of CYP3A4 from C3A CAR28 sample. Signals come from triply charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (A) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK light), precursor ion (m/z 798.4) is triply charged. The light peptide is not found in the sample C3A CAR28. (B) Chromatogram of CYP3A4 (VWGFYDGQQPVLAITDPDMIK heavy), precursor ion (m/z 801.1) is triply charged.

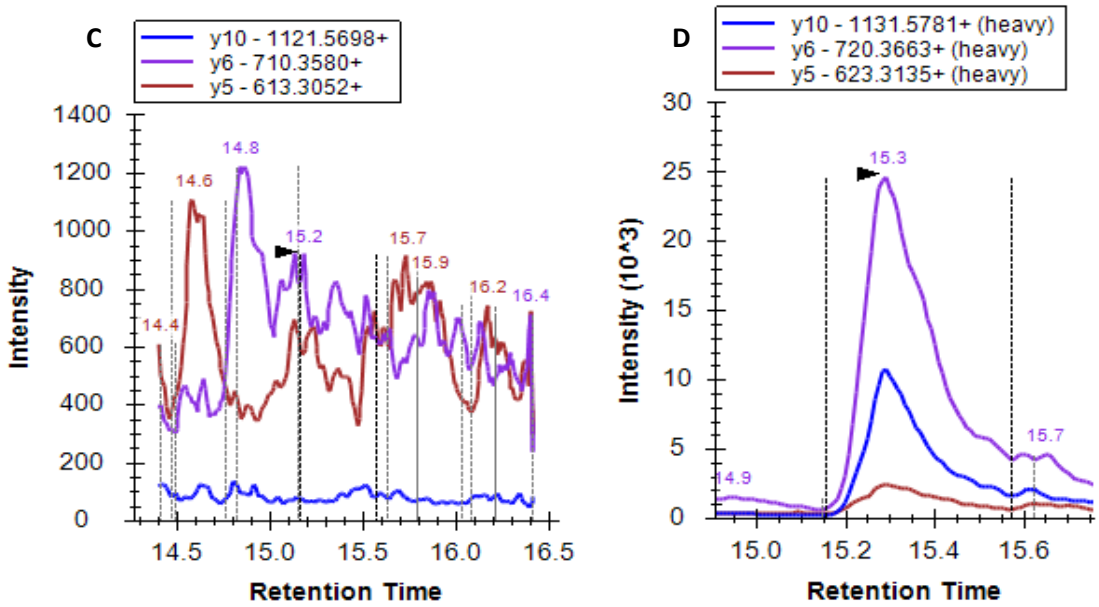

Figure 15. *Chromatograms of CYP2E1 from CACO2 PXR sample. Signals come from triply charged precursor ions*

Violet, blue and brown lines represent the signal of particular product ions which are described in the legend above the chromatograms. (C) Chromatogram of CYP2E1 (FITLVPSNLPHEATR light), precursor ion (m/z 565.6) is triply charged. The light peptide is not found in the sample CACO2 PXR. (D) Chromatogram of CYP2E1 (FITLVPSNLPHEATR heavy), precursor ion (m/z 567) is triply charged.

9. DISCUSSION

Modification with strong activation domain p65 should have increased protein levels of mainly CYP2B6, CYP2C9 and CYP3A4 (Küblbeck et al. 2010) because their genes are known targets for PXR and CAR (Korjamo et al. 2005). The function of NR genes might be increased either by their ligands or by the transactivation with strong activation domain (Küblbeck et al. 2010). According to the study made by Küblbeck et al. (2010), shown in the Table 8, C3A cell lines showed higher mRNA expression levels in modified cell lines than in C3A WT even without ligand activation. C3A CAR28 cell line contained higher mRNA levels of CYP3A4, 2B6, 2C9, at least ten times in comparison with wild type. In contrast, C3A PXR7 cell line contained less mRNA amount of CYP2B6 than in wild type. Generally, the mRNA expression levels of CYPs in C3A PXR7 cell line were lower than in C3A CAR28 (Küblbeck et al. 2010).

Table 8. *CYP mRNA expression levels in modified cell lines*

	Increase in mRNA (fold vs. WT) for			**Metabolism**
Cell line	**CYP3A4**	**CYP2B6**	**CYP2C9**	**CYP3A4**
C3A CAR28	15.0±1.0	18.0±0.4	142.1±5.9	7.9±1.0
C3A PXR7	4.6±0.2	0.01±0.002	5.1±1.2	6.9±1.3
CACO2 PXR	21.0±1.7	11.5±3.6	12.0±5.4	
CACO2 CAR	7.5±2.2	12.0±4.4	28.0±8.9	

All values were calculated as fold vs. WT. CYP mRNA expression levels and CYP3A4 mediated metabolism are presented as the mean±SEM (Küblbeck et al. 2010; unpublished data of CACO2 cell line obtained from Küblbeck J.).

CACO2 cell line has been characterised by very small expression of CYPs, especially CYP3A4, 1A2. In relation to the study conducted by Korjamo et al. (2006), protein levels in modified CACO2 cell line were higher but not as high as the levels after the ligand activation by vitamin D through the specific receptor. The mRNA levels after the transfection with turbo receptors were neglectably elevated (Korjamo et al. 2006). In the Table 8, mRNA levels of all measured CYPs in CACO2 PXR and CAR were increased, at least ten times in comparison with wild type except for CYP3A4 in CACO2 CAR cell line. In this case the increase was less than ten times in contrast to others.

In light of these results, we had expected increased levels of, at least, those earlier mentioned CYPs. However, all CYP isoforms levels were probably under the limit of detection. According to the results of CYP3A4 mediated metabolism, shown in the Table 8, at least CYP3A4 in C3A cell line should have been detected because metabolism is mediated by proteins. It has been postulated that, mRNA expression levels often do not correspond to the genuine levels of CYP proteins. The good correlation between mRNA levels and protein levels has been found mainly

in CYP3A4, 2B6 and 2C8. However, in the case of low expression levels, the correlation is not good even for these CYPs (Ohtsuki et al. 2011). This was also proved by the results of mediated metabolism in the Table 8. The mRNA levels of CYP3A4 in C3A CAR28 and PXR7 differed 3.3 times, approximately. However, CYP3A4 mediated metabolism in these cell lines differed just 1.1 times. Additionally, NR genes need many regulatory co-factors for their correct function. From this point of view, the lack of these regulators may have caused lower expression activity (Küblbeck et al. 2010; Korjamo et al. 2005). Cultivation conditions may have influenced protein expression too (Küblbeck et al. 2010). By the way, some metabolizing enzymes might be expressed just in differentiated cell lines (Korjamo et al. 2005). In relation to our negative results we cannot say if there is difference in protein expression levels between CACO2 and C3A samples which are from undifferentiated and differentiated wild type cell lines, respectively.

From the range of possible errors, which could cause negative results, we have to exclude incorrect LC-MS/MS conditions, incorrect method and insensitivity of the instrument because these were tested with the solution of standards. Nevertheless, we have to always take into account errors during sample preparation and the instability of proteins (Ohtsuki et al. 2011). Peptides could stick to the bottom of the test tube or to the pipette tips and different undesired modifications might have been performed during sample handling. The efficiency of tryptic digestion is the key factor in absolute quantification of proteins in general (Uchida et al. 2011; Hoshi et al. 2013). Some peptides underlined in the Table 6 contain AA which may cause decreased digestion efficacy. The assumption of low digestive efficacy might be supported by the fact that CYP protein levels in HLM were decreased in comparison with other studies (Table 9).

Table 9. *Comparisons of average quantitative values of the most abundant CYPs in human liver samples from different scientific articles and from this diploma thesis*

	Protein amount (pmol/mg microsomal protein)			
Isoforms	**Kawakami et al. 2011**	**Liu et al. 2014**	**Gröer et al. 2014**	**Diploma thesis**
CYP1A2	17.7±0.6	39.3	12.8±0.17	2.7±0.2
CYP2B6	6.86±0.44	4.3	9.59±0.38	3.4±0.3
CYP2C9	80.2±1.4	10.3	37.3±2.50	1.6±0.3
CYP2C19	3.64±0.22	3.0	2.18±0.18	0.6±0.1
CYP2E1	51.3±0.9	9.3	65.3±1.52	3.9±0.4
CYP3A4	64.0±1.9	54.0	32.6±0.38	21.5±1.0
CYP3A5	3.54±0.28	4.6	1.96±0.05	1.4±0.1

Each value represents mean±SEM.

Values of CYPs in different studies are very dissimilar, as it is illustratively shown in the Figure 16, apart from CYP2B6, 2C19 and 3A5 where differences are not as significant as in the case of other CYPs. It is obvious that CYPs expression levels highly vary in different HLM samples. It could be explained by their ability to be either easily induced or inhibited and therefore their levels are highly influenced by the history of the sample. However, we would need a much larger set of HLM samples for better comparison between them.

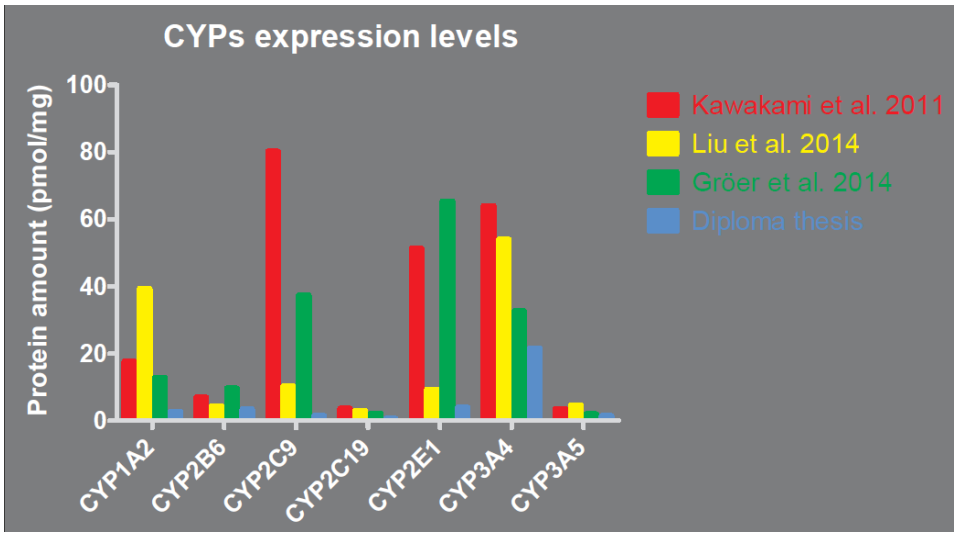

Figure 16. *Graph of CYPs expression levels in various scientific articles and in this diploma thesis*

In the Table 10 different instruments and approaches of these experiments mentioned before are described. Basically, conditions do not vary a lot except for length of the column and the method of determination. In terms of the diploma thesis, we used quite long column in comparison with others. However, our choice was based on the main properties of this column. It means high suitability for peptides identification and quantification.

Table 10. *Comparisons of various approaches of protein quantitative analysis in different scientific articles and in this diploma thesis*

	Kawakami et al. 2011	Liu et al. 2014	Gröer et al. 2014	Diploma thesis
Sample	Pooled HLM	HLM	Pooled HLM	HLM
Amount of protein in sample	50 µg	50-100 µg	50-100 µg	97 µg
Injection volume		20 µl	20 µl	20 µl
Analysis	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
HPLC	Agilent1100 system, Agilent (Santa Clara, CA, USA)	Agilent1100 Series Agilent Technologies (Palo Alto, CA, USA)	Perkin Elmer 200 LC system (PerkinElmer, Waltham MA)	Agilent 1220 Infinity LC System (Santa Clara, CA, USA)
Column	Agilent SB-C18 150 mm × 0.5 mm ID, 5 µm particles	C18 50 mm × 2.1 mm, 2.1 µm particles	Ascentis Express Peptide ES-C18 100 mm × 2.1 mm, 2.7 µm particles	AdvanceBio Peptide Mapping column C18 250 mm × 2.1 mm, 2.7 µm particles
Flow rate	50 µL/min	500 µl/min	300 µl/min	300 µl/min
Time	50 min			40 min
Dwell time	10 ms	50 ms		20 ms
MS	API5000, Applied Biosystems (Ontario, Canada) (Agilent Technologies, USA)	API4000, Applied Biosystems (Ontario, Canada)	4000 QTRAP Triple Quadrupole (AB Sciex, USA)	Agilent 6495 Triple Quadrupole LC-MS System (Agilent Technologies, CA, USA)
Determination	Analyst software version1.4.2 (Applied Biosystems, Ontario Canada); The average of quantitative values obtained from 3 or 4 MRM transitions with signal peaks over 5000 counts; Calibration curve	Analyst software version1.4.2 (Applied, Biosystems, Ontario, Canada); Calibration curve	Analyst 1.5 software (AB Sciex, USA); Calibration curve	Skyline 2.6

HLM – Human Liver Microsomes

The lower limit of quantification is varied from sample to sample due to the matrix effect.

10. CONCLUSION

To sum up, CYP enzymes were successfully analyzed in human liver microsomes as an evidence of method´s suitability. Our Dynamic MRM method can be used for the CYP analysis of biological samples. However, results show that up-regulation of CYP enzymes in modified cell lines CACO2 and C3A does not reach CYPs levels in human liver microsomes. Respectively, CYP enzymes levels were not detectable in modified cell lines. Application of turbo receptors to increase CYPs expression could be useful tool for biotransformation studies and for drugs absorption model. However, further studies have to be conducted to optimize cultivation conditions, presence of co-regulators and ligands to get modified cell lines with measurable CYP levels.

11. ABBREVIATIONS

AA	Amino Acid
ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
APPI	Atmospheric Pressure Photo Ionization
BCRP	Breast Cancer Resistance Protein
BSA	Bovine Serum Albumin
C3A	Cell line
CACO2	Cell line
CAR	Constitutive Androstane Receptor
CID	Collision Induced Dissociation
CYP	Cytochrome P450
DBD	DNA-Binding Domain
DC	Direct Current
EDTA	EthyleneDiamineTetraacetic Acid
ESI	Electrospray Ionization
FTICR	Fourier-Transform Ion Cyclotron Resonance
HLM	Human Liver Microsomes
(U)HPLC-MS	(Ultra)High Performance Liquid Chromatography-Mass Spectrometry
IS	Internal Standards
ICAT	Isotope Coded Affinity Tag
iTRAQ	isobaric Tags for Relative and Absolute Quantification
LBD	Ligand-Binding Domain
MALDI	Matrix Assistant Laser Desorption Ionization
MDR1	MultiDrug Resistance protein 1

MRM, SRM	Multiple Reaction Monitoring, Selected Reaction Monitoring
MRP1	Multidrug Resistance associated Protein 1
MS	Mass Spectrometry
MS/MS, MS^2	Tandem Mass Spectrometry
m/z	mass to charge ratio
NR	Nuclear Receptor
PXR	Pregnane X Receptor
QQQ	Triple Quadrupole
QTAP	Quantitative Targeted Absolute Proteomics
RF	RadioFrequency voltage
RP	Reversed Phase mode of HPLC
RT	Retention Time
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SILAC	Stable Isotope Labelling by Amino acids in Cell culture
SSRCalc	Sequence Specific Retention Calculator algorithm
TFA	TriFluoroAcetic acid
TIC	Total Ion Current/Chromatogram
TOF	Time Of Flight
TPCK	L-1-Tosylamido-2-Phenylethyl Chloromethyl Ketone
WT (CACO2, C3A)	Wild Type (of cell line)

12. LIST OF TABLES

13. LIST OF FIGURES

14. REFERENCES

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