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An integrated strategy for the quantitative analysis of endogenous proteins: A case of gender-dependent expression of P450 enzymes in rat liver microsome

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ABSTRACT

Liquid chromatography mass spectrometry based methods provide powerful tools for protein analysis. Cytochromes P450 (CYPs), the most important drug metabolic enzymes, always exhibit sex-dependent expression patterns and metabolic activities. To date, analysis of CYPs based on mass spectrometry is still facing critical technical challenges due to the complexity and diversity of CYP isoforms besides lack of corresponding standards. The aim of present work consisted in developing a label-free qualitative and quantitative strategy for endogenous proteins, and then applying to the gender-difference study for CYPs in rat liver microsomes (RLMs). Initially, trypsin digested RLM specimens were analyzed by the nanoLC-LTQ-Orbitrap MS/MS. Skyline, an open source and freely available software for targeted proteomics research, was then used to screen the main CYP isoforms in RLMs under a series of criteria automatically, and a total of 40 and 39 CYP isoforms were identified in male and female RLMs, respectively. More importantly, a robust quantitative method in a tandem mass spectrometry-multiple reaction mode (MS/MS-MRM) was built and optimized under the help of Skyline, and successfully applied into the CYP gender difference study in RLMs. In this process, a simple and accurate approach named 'Standard Curve Slope" (SCS) was established based on the difference of standard curve slopes of CYPs between female and male RLMs in order to assess the gender difference of CYPs in RLMs. This presently developed methodology and approach could be widely used in the protein regulation study during drug pharmacological mechanism research.

1. Introduction

Cytochromes P450 (CYPs), the main enzymes involved in numerous oxidative reactions, play critical roles in xenobiotic metabolism and pharmacokinetics [1,2]. A dozen enzymes in 57 putatively functional human CYPs, which belong to the CYP 1, 2, and 3 families, are responsible for the biotransformation of a great majority of drugs in clinical use and are therefore of particular relevance to clinical pharmacology and toxicity [1–3]. In the past 20 years, the rat liver microsomes (RLMs) were frequently used to predict kinetics, toxicity and drug-drug interactions for single/multiple compounds as a practical in vitro metabolic incubation model since liver is the major organ involved in the biotransformation of various endogeneous compounds and xenobiotics [4–7]. According to the previous reports, some of CYPs in RLMs are regulated by sex hormonal and exhibit gender-dependent expression activity patterns [8,9]. For instance, the mRNA expression of CYP2B1 in male rat liver, for instance, is much higher than that in female rats [10]. CYP2C11, the major male specific androgen 2α- and 16α-hydroxylase of adult liver, is induced dramatically at beginning 4–5 weeks of age in male but not in female rats [11]. Besides, CYP2C13 is also male specific and expressed not only in liver but also in extra-hepatic tissues [12].

Several direct or indirect techniques for quantitative analysis of CYPs have been developed in the past decade years, including quantification of protein by western blotting, measuring the amount of messenger ribonucleic acid (mRNA) by amplification, and determining CYP activity by enzyme kinetic methods, *etc.* [13–16]. For western blotting method, specific antibodies are necessary. However, high sequence homology of CYPs makes specific antibodies preparation extremely difficult, particularly among the same sub-family. The drawbacks of amplification method

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are the high variability and poor relationship between mRNA and protein expression [17]. Enzyme kinetics assay requires different model substrates for each iso-enzyme which only characterizes enzyme activity rather than protein expression. In addition, all these techniques mentioned above are suitable for use in a single-protein analysis format only [18].

With the development of MS technique and data processing software, it is advantageous to measure protein expression differences of CYPs in male and female RLMs with high specificity and good sensitivity using quasi-proteomics approach based on mass spectrometry technique [19–21]. For instance, high resolution mass spectrometers have been proven to play a pivotal role in the field of proteomics research, and recent improvements in sensitivity and scan rates further widened their applications toward the study of biological molecules [22,23]. LTQ-Orbitrap XL, a hybrid high resolution mass spectrometry which incorporates Orbitrap and linear ion trap, enables to increase peptide identification rates and enhance proteome coverage [24,25]. More recently, Orbitrap MS combined with several popular search engines (e.g. SEQUEST, Maxquant and Mascot) was widely applied in discovering target proteins associated with disease, proteomic analysis of preclinical/clinical specimens, identifying post-translational modification sites of proteins, *etc.* [26–31]. In the past few years, isobaric tags for relative and absolute quantitation (iTRAQ) technology coupled with Orbitrap MS were frequently used to investigate the differentially expressed proteins in different groups of biological samples [32–34]. For instance, Liu et al. developed a proteome-derived approach in combination with stable isotope dilution assay to quantify CYPs in human liver microsomes [35]. Kummer et al. analyzed the liver specimens from different stages of the sublethal infection by quantitative peptide sequencing using 4-plex iTRAQ-labeling based on HPLC-Orbitrap Fusion system [36]. However, the average cost of a set of iTRAQ or Tandem Mass Tag™ 6-plex (TMTsixplex™) reagent would cost more than several hundred dollars due to the complex synthesis procedures required to produce these commercial reagents [37]. In general, triple quadrupole (QqQ) mass spectrometer is considered as the “gold standard” for absolute quantification. Modern triple quadrupole instrumentation has undergone significant technical improvements, and MRM-MS/MS has become prevalent as a highly useful analytical tool which is capable of precisely and quantitatively measuring large number of target peptides in complex biological samples [38,39]. One of the challenges of quantitative analysis of proteins based on MRM-MS/MS is the requirement of seeking characteristic peptides for the proteins. Besides, the determination of a set of general MS/MS parameters which applicable for the wide diversity of peptides is also a challenging task [40]. Skyline, a freely available open source software (http://proteome.gs.washington.edu/software/skyline), has been proven to be a reliable and flexible program for the targeted MRM-MS/MS analysis via preparing the transition lists and assisting in optimizing analytical conditions [41,42].

The aim of the present study was to exploit an integrated technique platform and strategy to qualitative and quantitative analysis of endogenous proteins in biological specimen. As model proteins, CYPs in female and male RLMs were identified and quantitatively analyzed based on nanoLC-LTQ-Orbitrap MS/MS and QqQ MS/MS combined with Skyline software. Firstly, the peptide samples digested from the male and female RLMs were analyzed by the nanoLC-LTQ-Orbitrap MS/MS, and a total of 40 and 39 CYP isoforms were identified in the male and female RLMs, respectively. After screening by Skyline according to a series of criteria, CYP1A2, CYP2B3, CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP2C23, CYP2C70, CYP2D10, CYP2D26, CYP2E1 and CYP3A2 were confirmed as the main CYP isoforms which could be quantified by MRM-MS/MS. In order to investigate the linearity of CYPs in RLMs, a series of protein aliquots of male and female RLMs were prepared and determined by LC-MS/MS. The differences of the standard curve slopes between female and male RLMs could accurately represent the gender differences in CYP protein expression since each point in the standard curve could be used to calculate the relative expression of CYPs in female and male RLMs. Then, a novel approach ‘Standard Curve Slope” (SCS), which was independent of specific authentic standard and isotope labeling, was proposed to assessing the gender difference of CYP expression in RLMs. The presently developed techniques and approach would find its wide applications in the study of mechanisms and targets of new drugs.

2. Experimental section

2.1. Chemicals and reagents

Sequencing grade-modified trypsin, trifluoroacetic acid (TFA), D,L-dithiothreitol (DTT), iodoacetamide (IAM) and sodium cyanoborohydride were purchased from Sigma-Aldrich Corporation (Sigma Aldrich, St. Louis, MO, USA). Oasis ® HLB 1cc/30 mg extraction cartridges were purchased from Waters Corporation (Waters, Milford, MA, USA). LC-grade acetonitrile and methanol were purchased from Merck Company (Merck, Darmstadt, Germany). Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. (Thermo Fisher, Waltham, MA, USA). Deionized water, prepared by Milli-Q system (Millipore Corporation, Billerica, MA, USA), was used throughout the study. All other reagents were of analytical grade.

2.2. Preparation of RLMs and protein digestion

2.2.1. Animals

Male and female Sprague-Dawley rats (200 ± 20 g) were purchased from the Shanghai Super – B & K laboratory animal Corporation Limited (B & K, Shanghai, China), and were housed in standard cages under controlled environment conditions of temperature (23+2 ℃) and humidity (60%) with free access to laboratory food and water throughout the test period. All animal management procedures were conducted in accordance with the Laboratory Animal Management Regulations (State Council of the People's Republic of China, 2010), and the protocol was approved by the Animal Ethics Committee of China Pharmaceutical University (Nanjing, Jiangsu, China). Ethical procedures were conducted under Reduction, Replacement and Refinement (the 3 Rs rule).

2.2.2. Preparation of rat liver microsome

The RLMs were prepared and characterized as previously described assay with slight modifications [43–45]. Rat livers were obtained from 6 pairs of male and female Sprague–Dawley rats by cervical dislocation and decapitation under anesthesia. Wash the livers with cold homogenization buffer (HB, 4 °C) which consisted of 0.25 M sucrose, 50 mM of Tris–HCl, pH 7.4, 1 mM of EDTA, 100 mM of sodium chloride, and 0.1 mM of DTT. Male or female rat liver (10g) was allowed to thaw in 5-fold the volume of HB solution, and microsomes were prepared by the following centrifugation procedures: an initial centrifugation at 2400g for 20 min at 4 ℃ in a Sorvall refrigerated centrifuge (Thermo Biofuge Stratos, Waltham, Massachusetts, USA) was used to sediment the cell debris, nuclei and unbroken cells. The supernatant was centrifuged at 23,000g for 15 min, and the supernatant was further centrifuged at 180,000g (Beckman L5–65, Fullerton, CA, USA) for 1 h at 4 °C. The microsomal pellet was suspended in 50 mM of Tris–HCl (pH 7.4) containing 15% glycerol, 1 mM of EDTA, and was then re-centrifuged at 180,000g for 1 h. The final pellet was re-suspended in 0.1 M of Tris–HCl buffer (pH 7.4) containing 15% glycerol, 1 mM of EDTA and then stored at −80 °C. The re-suspended solution was the microsomal fraction and its protein concentration was determined using the Pierce BCA protein quantification assay.

2.2.3. Protein digestion

A series of protein aliquots of female and male RLMs (20, 50, 100, 200, 300, 500 and 750 μg protein) were added in Eppendorf Protein LoBind tubes (Eppendorf, Hamburg, Hamburg, GER), followed by adding 10-fold volume of acetonitrile to precipitate protein. After