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Quantitation of Human Cytochrome P450 2D6 Protein with Immunoblot and Mass Spectrometry Analysis

Ai-Ming Yu*, Jun Qu, Melanie A. Felmlee, Jin Cao and Xi-Ling Jiang

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,
University at Buffalo, The State University of New York, Buffalo, NY 14260-1200

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Corresponding Author: Dr. Ai-Ming Yu, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY 14260-1200, USA; Phone: 716-645-2842 ext. 256; Fax: 716-645-3693; E-mail: aimingyu@buffalo.edu

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Abbreviations: CYP, Cytochrome P450; CYP2D6, Cytochrome P450 2D6; LC, liquid chromatography; MS, mass spectrometry; CID, collision induced dissociation; ETD, Electron transfer dissociation.

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Abstract

Accurate quantification of cytochrome P450 (CYP) protein contents is essential for reliable assessment of drug safety including the prediction of in vivo clearance from in vitro metabolism data, which may be hampered by the use of uncharacterized standards and existence of unknown allelic isozymes. This study, therefore, aimed to delineate the variability in absolute quantification of polymorphic CYP2D6 drug-metabolizing enzyme and compare immunoblot and nano liquid chromatography coupled to mass spectrometry (nano-LC/MS) methods in identification and relative quantification of CYP2D6.1 and CYP2D6.2 allelic isozymes. Holoprotein content of in-house purified CYP2D6 isozymes was determined according to carbon monoxide difference spectrum and total protein was quantified with bicinchoninic acid protein assay. Holo-/total CYP2D6 protein ratio was markedly higher for purified CYP2D6.1 (71.0%) than that calculated for CYP2D6.1 Supersomes (35.5%), resulting in distinct linear calibration range (0.05-0.50 vs. 0.025-0.25 pmol) that was determined by densitometric analysis of immunoblot bands. Similarly, purified CYP2D6.2 and CYP2D6.10 and the CYP2D6.10 Supersomes all showed different holo-/total CYP2D6 protein ratios and distinct immunoblot linear calibration ranges. In contrast to immunoblot, nano-LC/MS readily distinguished CYP2D6.2 (R296C and S486T) from CYP2D6.1 by isoform-specific proteolytic peptides that contain the altered amino acid residues. Additionally, relative quantitation of the two allelic isozymes was successfully achieved with label-free protein quantification, consistent with the nominated ratio. Because immunoblot and nano-LC/MS analyses measure total CYP protein (holo- and apoprotein) in a sample, complete understanding of holoprotein and apoprotein contents in CYP standards is desired toward reliable quantification. Our data also suggest that nano-LC/MS not only facilitates CYP quantitation but also provides genotypic information.

Introduction

Cytochrome P450 (P450 or CYP) enzymes are the most important phase I drug-metabolizing enzymes responsible for the metabolic elimination of drugs in humans (Williams et al., 2004). Because of the striking species differences in CYP-mediated drug metabolism (Lin, 1998; Gonzalez and Yu, 2006; McLaughlin et al., 2008), there remains high interest in quantitative prediction of in vivo hepatic drug clearance from in vitro metabolism data acquired from human liver microsomes, hepatocytes and/or recombinant CYP enzymes according to physiology or population based models (Obach, 1999; Houston and Galetin, 2003; Barter et al., 2007). Per se, a good understanding of the abundance of individual CYP proteins in human liver and small intestine and CYP pharmacogenetics (e.g. CYP2D6 and CYP2C9) is required for successful extrapolation of in vitro metabolic data to in vivo clearance parameters.

Immunoblot analysis is a conventional method in protein quantitation, and it has been widely used in assessing the abundance of individual CYPs. Studies include the pioneering quantitative measurement of human hepatic (Guengerich and Turvy, 1991; Shimada et al., 1994) and intestinal (Paine et al., 2006) CYPs, and the systemic characterization of ontogeny and possible sexual dimorphism of individual CYPs in humans (Stevens et al., 2003; Wolbold et al., 2003; Stevens et al., 2008) and *CYP* transgenes in mouse models (Yu et al., 2005; Cheung et al., 2006; Felmlee et al., 2008). However, immunoquantification determines total protein, holoprotein (active protein with prosthetic group heme properly incorporated) and apoprotein (inactive protein lacking of heme or non-holoprotein), for individual CYPs in a given sample, whereas only holoprotein level is usually known for the standard and inevitably used for calibration. Certainly, the levels of holo- and apoprotein are largely variable due to distinct processes of the

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standards and samples, which causes the renowned discrepancy between CYP3A4 contents estimated with commercially available, unpurified recombinant enzymes as standards and those assayed with purified enzymes (Perrett et al., 2007).

In terms of CYP2D6 quantitation, studies (Shimada et al., 1994; Paine et al., 2006) providing the basis for human hepatic and intestinal CYP “pie” all utilized unpurified CYP2D6 as standards, whose holoprotein/apoprotein levels were unfortunately unknown. Use of uncharacterized CYP standards including the purified proteins will certainly lead to inaccurate estimation of CYP contents in human tissues. Although the purified CYP2D6 enzymes from human liver microsomes have been shown to contain 6.3-38% of holoprotein (Gut et al., 1984; Distlerath et al., 1985), whether it represents the actual CYP2D6 holoprotein level in living human livers remains a question. First, substantial level of apoprotein may be removed or introduced during processing and purification. Second, polymorphic CYP2D6 consists of many different allelic isoforms that have significantly altered protein stability (Johansson et al., 1994), whereas the *CYP2D6* genotypes of those liver donors were unknown. Additionally, it is not clear if one antibody developed for one isoform (usually the wild-type) will react with another allelic isoform to the same degree in immunoblots.

Mass spectrometry (MS)-based methods not only reveal the identity of a protein analyte but also provide unparalleled sensitivity and selectivity for protein quantitation. Therefore, utilization of different MS techniques has become an indispensable approach for protein identification and quantification (Ong and Mann, 2005; Bantscheff et al., 2007). MS-based methods for absolute and relative quantification of CYP proteins are also emerging in recent years (Alterman et al.,

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2005; Jenkins et al., 2006; Duan et al., 2007; Lane et al., 2007). Recently, a nano-LC/MS method has been adopted for proteomic analysis in our laboratory, which provides exceptional sensitivity and wealthy information of peptide sequence (Qu and Straubinger, 2005). This technique may be employed to determine CYP2D6 abundance in a given sample and simultaneously identify allelic isoform-specific peptides, i.e. to provide genotype information.

This study, therefore, aimed to delineate the variability in immunoquantification of CYP2D6 caused by the use of different allelic isoforms from different sources, and to compare immunoblot and MS methods in relative quantification of CYP2D6.1 and CYP2D6.2 allelic isoforms. Our data showed that the immunoblot linear calibration ranges of purified and unpurified CYP2D6 allelic isozymes, which contain all levels of holoprotein and apoprotein, varied considerably. When CYP holoprotein content is only considered in the standard, level of the CYP in a sample will be markedly underestimated. In addition to the distinguishing of CYP2D6.1 from CYP2D6.2 that differ only in two amino acid residues, nano-LC/MS analysis successfully provided relative quantitation of the two allelic isoforms and the result was consistent with the nominal value calculated from bicinchoninic acid (BCA) assay and immunoblot analysis.

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Materials and Methods

Chemicals, Enzymes and Other Reagents. CYP2D6.1 and CYP2D6.10 Supersomes and pooled human liver microsomes were purchased from BD Discovery Labware, Inc. (Woburn, MA). Iodoacetamide, formic acid and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) were bought from Sigma (St. Louis, MO). Trypsin was purchased from Promega (Fitchburg, WI). In-house baculovirus-expressed CYP2D6.1, CYP2D6.2 and CYP2D6.10 were purified by Octyl-Sepharose, DEAE-Sepharose and ceramic hydroxyapatite column chromatographies, as described previously (Yu et al., 2002). All other chemicals utilized were of the highest analytical grade available.

Immunoblot Analysis. CYP2D6 samples containing 0.01-1.0 pmol of holoprotein were resolved by SDS-PAGE with a 12.5% resolving gel and proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Membranes were first probed with monoclonal antibodies against human CYP2D6 (MAB-2D6, BD Discovery Labware, Inc., Woburn, MA) then incubated with peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO), followed by enhanced chemiluminescence detection (Pierce Chemical Co., Rockford, IL) as described previously (Felmlee et al., 2008). Densitometric analysis was conducted using a Kodak Image Station (New Haven, CT). All immunoblot analyses were conducted in duplicate or triplicate, and all data were reproducible.

Determination of CYP2D6 Holoprotein and Total Protein Contents. Total protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL) following the manufacturer's instructions. Holoprotein contents of CYP2D6 Supersomes were

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obtained from the manufacturer, BD Discovery Labware, Inc. CYP2D6 holoprotein level of the purified CYP2D6 allelic isozymes was determined according to the carbon monoxide (CO) difference spectrum method (Omura and Sato, 1964), and total CYP2D6 concentration (holoprotein plus apoprotein; in molarity) was calculated according to its corresponding molecular weight after correction with the purity of CYP2D6 protein in each sample (90% for CYP2D6.1 and CYP2D6.2; 70% for CYP2D6.10) (Yu et al., 2002). Total protein levels were assayed in triplicate and holoprotein contents were determined in duplicate, and mean values were obtained (Table 1).

Proteolytic Digestion. 10 μ L of CYP2D6.1 or CYP2D6.2 (total protein concentration 0.60 μ g/ μ L for each sample) was diluted with 40 μ L of Tris buffer (50 mM, pH = 8.6). Then Tris 2-carboxyethyl phosphine solution was spiked to a final concentration of 1 mM. The solution was heated at 95°C for 5 min to denature the protein, and then cooled to room temperature. For alkylation of the cysteine residues, 1.8 μ L of freshly-prepared iodoacetamide solution (100 mM) were added. Then the mixture was incubated in darkness at room temperature for 30 min. TPCK-treated trypsin was stored in 0.5% acetate acid and was activated by adding 3 volumes of 50 mM Tris buffer prior to the digestion. Activated trypsin was added to achieve a substrate/enzyme ratio of 20:1, and the reaction mixture was incubated at 37°C for 16 h. Proteolysis was stopped by the addition of 2 μ L of formic acid. After centrifugation at 13,000 rpm for 5 min, the supernatants of triplicate digestions were subject to nano-LC/MS analysis separately.

Nano-LC/MS and Protein Identification. A high-resolution nano LC system including two MDLC nano pumps (GE Healthcare, NJ), a Spark Endurance autosampler (Emmen, Holland), a

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lab-made valveless RP-trap and nano-LC flow path, and two Vici10-port low-dead-volume valves was used for protein separation. Programming of the nano-LC runs were performed on a Unicorn (GE Healthcare, NJ) coding platform. A lab-made dynamic-flow nanospray ion source was used to couple the nano-LC system to the LTQ XL linear ion trap tandem MS with an Electron transfer dissociation (ETD) device attached (Thermo Fisher Scientific, San Jose, CA). Specifically, the nano column was connected down-stream of a metal Tee, on which the high voltage was applied (1.7-2.2 kV), and liquid junction approach was used to form the ESI Taylor-cone on the uncoated spray tip. The MS was operated under data dependent mode, in which one micro scan cycle was comprised of a MS1 survey scan followed by one zoom MS1 scan with higher resolution and then by six sequential dependent MS2 scans with collision induced dissociation (CID) and ETD as activation method alternatively. The reaction time for ETD was set at 130 milliseconds and the chemical ionization source temperature was 230°C. Targeted value for negative ions was 6×10^5 , and supplemental activation that uses a short CID of the charge-stripped radical ion was used to further fragment the doubly-charged precursors.

Tryptic samples were loaded on a reversed-phase peptide trap (5 mm x 300 µm I.D.) at a flow rate of 10 µL/min, and washed with 3% of acetonitrile in 0.1% formic acid for 3 minutes to remove salts and other hydrophilic buffer components. Then the trap was switched online with a lab-packed reversed-phase nano C-18 column (3-µm-particle-size, reversed-phase particle packed in a 20 cm long, 360 µm O.D., 75 µm I.D. and fused silica capillary ended with a non-coated 2 µm tapered tip). A shallow multi-steps gradient was used to resolve the samples, which included Buffer A (3% acetonitrile in 0.1% formic acid) and Buffer B (84% acetonitrile in 0.1% formic acid). 100% Buffer A was used for trap loading and the initial nano-LC

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equilibration. Then Buffer B was increased linearly to 23% in 95 min, to 55% in the following 40 min and to 73% in another 20 min, followed by 100% Buffer B to flush the column for 15 min. On-column flow rates ranged from 180-260 nL/min. For CID, the raw data was converted to DTA files with ZSA and Combolon filter algorithms to remove low-quality DTA files. The filtered DTA files was searched against the preindexed tryptic peptide database that was coded with known CYP2D6.1 sequences, with a number of possible post-translation modifications on specific amino acids. The resultant data was grouped into a SRF file, and the peptide probability scores were calculated and a stringent probability filter (< 0.01) was used to remove false-positive identification. To ensure the credibility of the result, a secondary group of filters was applied to the result: $X_{\text{corr}} > 2$ for $z = 1$; $X_{\text{corr}} > 2.5$ if $z = 2$; $X_{\text{corr}} > 3$ if $z = 3$. Processing of ETD data was performed by Charger (Thermo Fisher Scientific) software and then searched for C and Z fragments using SEQUEST. The filtering standard was the same as for CID as described above, except that a Sf score filter (> 0.85) was used. For each of the identified peptides, a manual examination of the fragment pattern was performed to eliminate database searching artifacts.

Relative Quantification Based on Peptide Intensities by LC/MS. A label-free relative protein quantification approach was employed to determine the relative level of the two CYP2D6 allelic isoforms. A commercial protein quantification software package, the DeCyder MS (GE Healthcare, NJ), was used to compare in triplicate the extracting ion currents (XICs) of the multiply-charged peptide ions from CYP2D6.1 and CYP2D6.2. To facilitate the analysis, the retention times for XICs of the 6 runs were aligned prior to quantification. The background noise was subtracted by a uniform noise assumption, and any signal with $S/N > 20$ was investigated for possible peptide candidates. For a MS1-matched peptide, its 3-D signal intensity (vs. m/z and

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retention time) was determined. That is, the volume under the surface (VUS) was obtained for the matched peptide by considering all charge states after it was identified by both m/z calculation and XIC match. Of particular note, only frames (putative peptide signals) identified in all 6 samples (triplicate digestions of CYP2D6.1 and CYP2D6.2) were used for integration. After quantification, the DTA files derived from the MS2-dependent scans to the quantified precursors were exported and searched against protein database, as described above, for confirmatory identification of quantified peptides.

Results

Immunoblot Analysis Revealed A Marked Difference in Linear Calibration Range for Individual CYP2D6 Allelic Isoforms from Different Sources. To investigate the source variability on the absolute quantitation of CYP2D6 protein, immunoblots of purified CYP2D6.1 vs. CYP2D6.1 Supersomes and CYP2D6.10 vs. CYP2D6.10 Supersomes were compared side-by-side, respectively (Fig. 1). Difference in band density is obvious between purified CYP2D6.1 and the same amount (based on CO difference spectrum) of unpurified CYP2D6.1 Supersomes. Quantitative densitometric analysis revealed that the two standards also showed distinct linear calibration ranges, 0.05-0.5 pmol for purified CYP2D6.1 vs. 0.025-0.25 pmol for CYP2D6.1 Supersomes (Table 1). Similarly, the linear calibration ranges are markedly different between purified CYP2D6.10 (0.01-0.1 pmol) and CYP2D6.10 Supersomes (0.025-0.25 pmol). The results also indicate that different allelic isoforms (e.g. CYP2D6.1 or CYP2D6.10) from the same source (e.g. commercially available) could have distinct calibration curves. Depending on the standard selected, an over- or underestimation of CYP2D6 protein content is expected.

To further examine possible variability in CYP2D6 quantitation affected by CYP2D6 allelic isoforms, we employed high-purity (> 90% according to electrophoresis and LC), high-activity (≥ 5 pmol/ μ L according to CO difference spectrum or > 8.0 pmol/mg protein) CYP2D6.1 and CYP2D6.2 for comparative immunoblot analysis (Fig. 2). It revealed that the same amount of CYP2D6.1 and CYP2D6.2 did exhibit similar band intensity, resulting in identical linear calibration ranges (Table 1). This indicates that immunoblot responses to the same monoclonal antibody do not differ between the two allelic isoforms, and suggest that it is valid to quantify

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CYP2D6.2 and CYP2D6.1 with the same antibody. However, it does not exclude the possibility that other CYP2D6 allelic isoforms may respond differently from the wild-type CYP2D6.1.

Considerable Variability in Holoprotein and Apoprotein Levels of Individual CYP2D6

Allelic Isoforms from Different Sources. To examine the causes of difference in immunoblot response of different CYP2D6 standards, we thoroughly determined CYP2D6 holoprotein and total protein contents for the purified CYP2D6 samples using CO difference spectrum method and BCA protein assay, respectively. According to the actual CYP2D6 total protein level, we further calculated the percentage of holoprotein for each sample (Table 1). It revealed that the holoprotein represents 71.0% and 51.4% of total (holo- plus apoprotein) CYP2D6 in purified CYP2D6.1 and CYP2D6.2 samples, respectively. Based on the comparative quantitative immunoblots (Fig. 1), we further estimated the total CYP2D6 level and the holo-/total CYP2D6 ratios for the CYP2D6.1 and CYP2D6.10 Supersomes. As expected, CYP2D6.1 Supersomes has much lower holo-/total CYP2D6 protein level (35.5%). In contrast, CYP2D6.10 Supersomes has relatively higher holo-/total CYP2D6 protein ratio than in-house CYP2D6.10 (3.16% vs. 1.27%), which is presumably due to the denaturation of CYP2D6.10 during purification (Yu et al., 2002). Indeed, CYP2D6.10 has been known for its protein instability (Johansson et al., 1994) because of the change of *N*-terminal amino acid residue (P34S) critical for microsomal binding. Nevertheless, the large variability in holo-/apoprotein levels may explain the marked difference in immunoblot response of different allelic isoforms from different sources (Table 1).

Identification and Relative Quantification of CYP2D6 Allelic Isoforms by Nano-LC/MS. To verify immunoblot quantitation, we employed the nano-LC/MS for relative quantification of the

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two highly-purified CYP2D6 allelic isoforms, CYP2D6.1 and CYP2D6.2. First, we identified isoform-specific tryptic peptides that distinguish CYP2D6.1 and CYP2D6.2 (R296C and S486T) from each other (Fig. 3). Because the majority of tryptic peptides from CYP2D6 allelic isoforms are rather polar, an optimized, shallow nano-LC gradient using low-organic mobile phase compositions was employed to obtain a relatively evenly-distributed peptide peaks within the initial 90-min elution window (data not shown) for comprehensive peptide identification. Two complementary techniques, CID and ETD, were used alternatively for data-dependent fragmentation of the tryptic peptides to achieve higher sequence coverage. Consequently, 92% of the predicated amino acid residues were identified for CYP2D6.1 with high confidence and 89% for CYP2D6.2. Two expected tryptic peptides from CYP2D6.2 (Fig. 3), which contain the altered amino acid residues (C296 and T486), were readily identified by either CID or ETD. It is notable that the C296 residue in CYP2D6.2 precluded tryptic cleavage at C-terminal of this position, in contrast to the R296 in CYP2D6.1. As a result, a longer tryptic peptide containing 46 amino acid residues were produced. Due to the larger number of residues, this peptide was highly charged and thus not suitable for CID analysis due to a possible mobile proton impediment mechanism. Indeed, this peptide was not identified by CID but detected by ETD (Fig. 3C) that is especially efficient for long and highly-charged peptide ions. In contrast, the other three isoform-specific tryptic peptides were identified not only by CID (Fig. 3A, 3B and 3D) but also by ETD (data not shown).

Label-free protein quantification, which was performed with the DeCyder MS software package, was then conducted to compare the relative level of CYP2D6 in CYP2D6.1 and CYP2D6.2 samples. To ensure the accuracy of the quantification, the MS1 ion channel for quantification

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was obtained by a stand-alone high resolution LTQ scan (FWHM=0.1 Th), which greatly improved the success of ion match by DeCyder MS. Second, a 3-D integration was used to calculate the VUS of individual signals (intensity vs. the retention time and m/z) that include all isotopic forms and at all charge states from the putative peptide precursor ions of CYP2D6.1 and CYP2D6.2. A representative relative quantification of the two isozymes using a matched peptide (FGDIVPLGVTHMTSR) is shown in Fig 4. The data indicate that the relative amount of total CYP2D6 protein between the purified CYP2D6.1 and CYP2D6.2 is around 1:1.2 (Table 2), in agreement with the nominal ratio (1:1) derived from BCA protein assay and selective CYP2D6 immunoblots (Fig. 2).

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Discussion

Although human hepatic and intestinal CYP contents are readily quantified by immunoblot analysis with form-selective antibodies, discrepancy is obvious between the use of purified and unpurified CYP standards, which may hamper accurate quantitative prediction of in vivo drug clearance from in vitro metabolic data. Current study, therefore, compared the difference in immunoblot response and linear calibration range between in-house purified CYP2D6 allelic isoforms and commercially available CYP2D6 Supersomes, which are basically governed by the total CYP2D6 content including both holoprotein and apoprotein. While nano-LC/MS analysis provided accurate quantitation of CYP2D6.1 and CYP2D6.2 allelic isoforms, it readily proved the identity of each protein by delineation of form-specific tryptic peptides.

Immunoquantification of a CYP determines both holo- and apoprotein of the CYP, i.e. total CYP protein. In contrast, not the total CYP protein but holoprotein level is usually known for the CYP standards, especially those commercially available membrane preparations. Disparity has been noted that CYP3A4 contents assayed with purified CYP3A4 standards are significantly higher than the levels determined with unpurified standards (Perrett et al., 2007). It is notable that CYP quantitation is more complex than that assumed, even if the holoprotein/apoprotein ratios were the same in a standard and a sample. The holoprotein level of each purified CYP from human livers summarized by Perrett et al. (2007) truly indicates the holoprotein level of corresponding CYP in the purified sample or represents protein stability and difficulty in processing, rather than the actual level of CYP (e.g. CYP2D6) holoprotein in living human tissues. Less or unstable CYP isozymes may be partially denatured or inactivated as processed. On the other hand, due to distinct lipophilicity or binding affinity, some denatured CYP protein (apoprotein) may be

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separated from holoprotein on certain columns during purification. Therefore, whether all or partial of the translated CYP enzymes in living human tissues are holoproteins is subject to further investigation.

When compared to purified CYP2D6.1, the same amount of active CYP2D6.1 Supersomes that are often used for quantification of CYP2D6 (Paine et al., 2006; Stevens et al., 2008) shows stronger response to the same monoclonal antibody (Fig. 1) and thus different linear calibration range in immunoblot quantitation (Table 1). This indicates that the abundance of CYP2D6 will be markedly underestimated when CYP2D6.1 Supersomes is used as the standard and its apoprotein level is not considered. Agreeing with what found by Perrett et al. (2007), the reason is that the CYP2D6.1 Supersomes consists of much lower level of holoprotein (holo-/total CYP2D6 protein, 35.5%) than the in-house purified CYP2D6.1 (71.0%). In contrast, we have found that the holoprotein levels are also largely variable (Table 3) in purified recombinant CYP2D6 enzymes that were reported in the literature (Gillam et al., 1995; Kempf et al., 1995; Imaoka et al., 1996; Modi et al., 1996; Yu et al., 2002; Ng et al., 2003; Rowland et al., 2006). Most of them do show relatively higher holoprotein levels (CYP2D6 holoprotein/total protein, >50%), whereas the others have comparable ratios (14-40%) as CYP2D6.1 Supersomes (36%) or purified CYP2D6 from human tissues (6.3-38%) (Gut et al., 1984; Distlerath et al., 1985). Of particular note, those CYPs purified from human donors would be superb quantitation standards when they are characterized properly.

With the advances of MS in relative and absolute protein quantitation, some MS methods have also been developed for quantitative analysis of CYP isozymes (Alterman et al., 2005; Jenkins et

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al., 2006; Duan et al., 2007; Lane et al., 2007). Our nano-LC/MS method has proved to provide excellent sensitivity in protein quantification (Qu and Straubinger, 2005). In current study, the ratio of total CYP2D6 between CYP2D6.1 and CYP2D6.2 samples determined by label-free quantification of form-matched peptides (Table 2; Fig. 4) separated by nano-LC is close to the nominated value calculated from total protein assay (Table 1) and selective CYP2D6 immunoblots (Fig. 2). While MS methods usually provide excellent sensitivity (e.g. amol to fmol), they share the same limitation as immunoblot analyses in determining the total (active and inactive) level of the analyzed protein. Therefore, use of uncharacterized CYP standards, whose holoprotein levels are determined only, would not provide reliable quantitation of the CYP in human tissues.

In addition to relative quantification of CYP proteins, Lane et al. (2007) have nicely shown the application of nano-LC/MS to peptide identification for individual CYPs. While the majority of CYPs of interest was unambiguously identified, that method was unable to distinguish two highly-homologous isozymes, CYP2A4 and CYP2A5, from each other. To identify CYP2D6.1 and CYP2D6.2 allelic isozymes that differ only in two amino acid residues, our nano-LC/MS method employs two complementary fragmentation techniques, CID and ETD, and an optimal peptide separation condition. In turn, higher sequence coverage (92% for CYP2D6.1 and 89% for CYP2D6.2) has been achieved. Among them, the CYP2D6.2 form-specific tryptic peptide derived due to the C296R change consists of 46 amino acids and may not be suitable for CID, whereas it has been readily identified by ETD (Fig. 3). Our finding suggest that CYP2D6 genotyping, which are usually accomplished by the analysis of human DNA or mRNA samples

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(Chou et al., 2003; Meijerman et al., 2007), may be also achieved by direct analysis of CYP2D6 protein in human biopsy using appropriate MS methods.

In summary, MS-based methods not only provide accurate quantification of CYP proteins but also identify allelic isoform-specific peptides. Because immunoblot and MS analyses determine both inactive and active protein for a CYP enzyme, it is essential to use fully characterized CYP standards toward better understanding of CYP contents in humans and reliable prediction of in vivo clearance from in vitro metabolic data.

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Footnotes

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Address correspondence to:

Dr. Ai-Ming Yu

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences,
University at Buffalo, The State University of New York, Buffalo, NY 14260-1200, USA

Phone: 716-645-2842 ext. 256

Fax: 716-645-3893

E-mail: aimingyu@buffalo.edu

Figure Legends

Figure 1. Immunoblot analysis (A) of unpurified (Supersomes) and purified (in-house) CYP2D6.1 and CYP2D6.10 reveals distinct quantitative densitometric responses for CYP2D6.1 (B) standards from different sources, and for CYP2D6.10 (C). 0.01, 0.025, 0.05, 0.1, 0.25 or 0.5 pmol holoprotein of individual allelic isozymes were loaded into each well and proteins were separated by SDS-PAGE. Immunoblot analyses were conducted using a selective antibody against CYP2D6.

Figure 2. Immunoblot (A) and densitometric analysis (B) of highly-purified CYP2D6.1 and CYP2D6.2 suggests that CYP2D6 total protein levels are similar in the two samples. 0.1, 0.25, 0.5, 1.0 or 2.0 pmol holoprotein of each isozyme was loaded into each well and proteins were separated by SDS-PAGE. Immunoblot analyses were carried out using a selective antibody against CYP2D6.

Figure 3. LC/MS/MS identification of the difference in amino acid residues between CYP2D6.2 (R296C and S486T) and CYP2D6.1 allelic isozymes. (A) CID MS identification of AKGNPESSFNDENLR from CYP2D6.1. (B) CID MS identification of PSHHGVFAFLVSPSPYELCAVPR from CYP2D6.1. (C) ETD MS identification of GNPESSFNDENLCIVVADLFSAGMVTSTTLAWGLLLMILHPDVQR from CYP2D6.2. (D) CID MS identification of PSHHGVFAFLVTPSPYELCAVPR from CYP2D6.2.

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Figure 4. Illustration of relative quantification of CYP2D6.2 vs. CYP2D6.1 by DeCyder MS using a matched tryptic peptide (FGDIVPLGVTHMTSR; in the highlighted areas) existing in both isozymes. Upper panels: zoom view of paired MS1 signals (m/z vs. retention time); lower panels: the 3-D integration of the representative peptide at all charge states.

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Table 1. Difference in CYP2D6 immunoblot linear calibration range and holo-/total CYP2D6 protein ratio between CYP2D6 Supersomes and in-house purified CYP2D6 allelic isoforms. Values represent mean of duplicate or triplicate measurements. Total protein concentration was determined using the BCA Protein Assay, and CYP2D6 holoprotein content was determined according to the CO difference spectrum. Total CYP2D6 concentration for in-house purified CYP2D6 isozyme was calculated according to its average molecular weight (MW).

Sample	MW (Da)	Total protein (mg/mL)	CYP2D6 holoprotein (pmol/μL)	Total CYP2D6 (pmol/μL)	holo-/ total CYP2D6 protein (%)	Immunoblot linear range (pmol)
CYP2D6.1 Supersomes	55,769.5	3.20	1.00	2.82 ^a	35.5 ^a	0.025-0.25
CYP2D6.10 Supersomes	55,773.4	1.10	1.00	31.6 ^a	3.16 ^a	0.025-0.25
CYP2D6.1 (in-house)	55,769.5	0.60	6.90	9.72 ^b	71.0 ^b	0.05-0.50 (Fig. 1) 0.10-1.0 (Fig. 2)
CYP2D6.2 (in-house)	55,730.4	0.60	5.00	9.72 ^b	51.4 ^b	0.10-1.0
CYP2D6.10 (in-house)	55,773.4	11.3	1.80	142 ^{b,c}	1.27 ^{b,c}	0.01-0.10

^a Estimated after compared with corresponding in-house CYP2D6 allelic isoforms (Fig. 1); ^b Values corrected with the purity of CYP2D6; ^c Low level of CYP2D6.10 holoprotein due to enzyme denaturation during purification (Yu et al., 2002).

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Table 2. Relative level of total CYP2D6 between the purified CYP2D6.2 and CYP2D6.1 calculated from representative matched peptides by DeCyder MS. After characterization with BCA protein assay, CO difference spectrum and immunoblot analysis, equal amount (holoprotein + apoprotein) of CYP2D6.2 and CYP2D6.1 was used for the analysis.

Peptide ID ^a	Retention time (min)	Peptide sequence	CYP2D6.2/CYP2D6.1 ratio	X _{corr}
130	34.2	R.MTWDPAQPPR.D	1.22	3.14
556	43.2	R.FGDIVPLGVTHMTSR.D	1.11	3.38
473	43.3	K.DEAVWEKPFR.F	1.23	3.31
253	50.2	R.LLDLAQEGLK.E	1.34	2.61
214	52.1	R.VQQEIDDVIGQVR.R	1.39	5.25
205	60.0	R.FGDIVPLGVTHMTSR.D	1.22	2.69
476	63.1	K.AVSNVIASLTCGR.R	1.45	2.79
112	87.2	K.GTTLITNLSSVLK.D	1.28	4.20
141	93.3	R.PPVPIQILGFGPR.S	1.19	2.61
Mean ± S.D.			1.27 ± 0.11	

^a An unique ID for each matched peptide assigned by the DeCyder MS “Pepmatch” module.

Table 3. CYP2D6 holoprotein levels (percentage of total protein) in the purified recombinant CYP2D6 enzymes reported in the literature. Average molecular weight (MW) of each protein was calculated according to its corresponding amino acid sequence described in that paper unless otherwise indicated.

Protein	MW (Da)	Theoretical CYP2D6 content (mg/mL)	Experimental CYP2D6 content (mg/mL)	Holoprotein level (%)	Expression system	Reference
CYP2D6m-Δ21	53,262.2	18.8	10.0	53.2	E.coli	Gillam et al., 1995
[His] ₆ -CYP2D6-Δ25	55,535.7	18.0	7.12 ^a 2.67 ^b	39.6 ^a 14.8 ^b	E. coli	Kempt et al., 1995
CYP2D6-Δ	48,500 ^c	20.6	12.2	59.2	Yeast	Imaoka et al., 1996
CYP2D6-Ext	56,235.1	17.8 ^d 20.0 ^c	18.3	- 91.5	Baculovirus (insect cell)	Modi et al., 1996
CYP2D6.1	55,769.5	17.9	11.5	64.2	Baculovirus (insect cell)	Yu et al., 2002
CYP2D6m	55,647.3	18.0	12.5	69.4	E.coli	Ng et al., 2003
CYP2D6m-Δ-[His] ₄	53,705.7	18.6	11.0	59.1	E.coli	Roland et al., 2006

^a Purified without detergent; ^b Purified with detergent; ^c Values reported in corresponding papers; ^d Value we calculated according to its MW.

Fig. 1

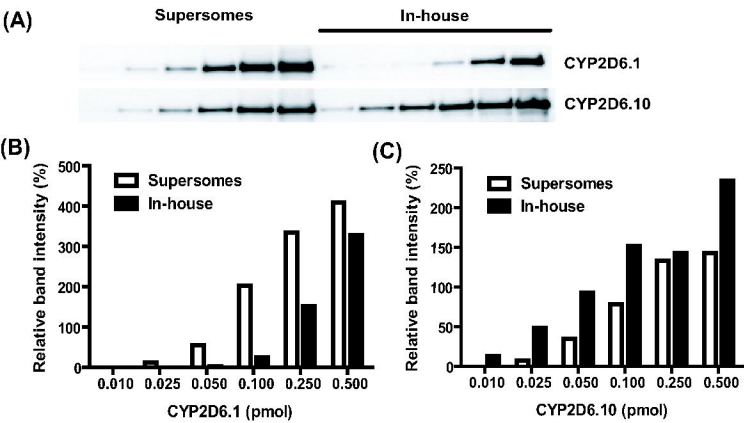


Fig. 2

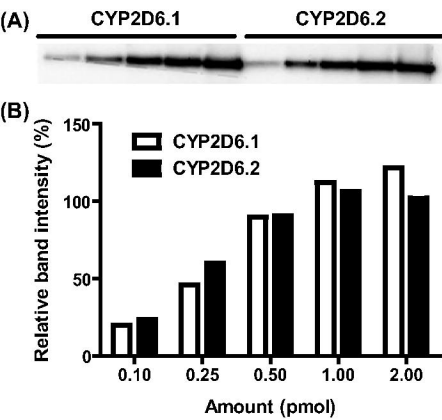


Fig.3

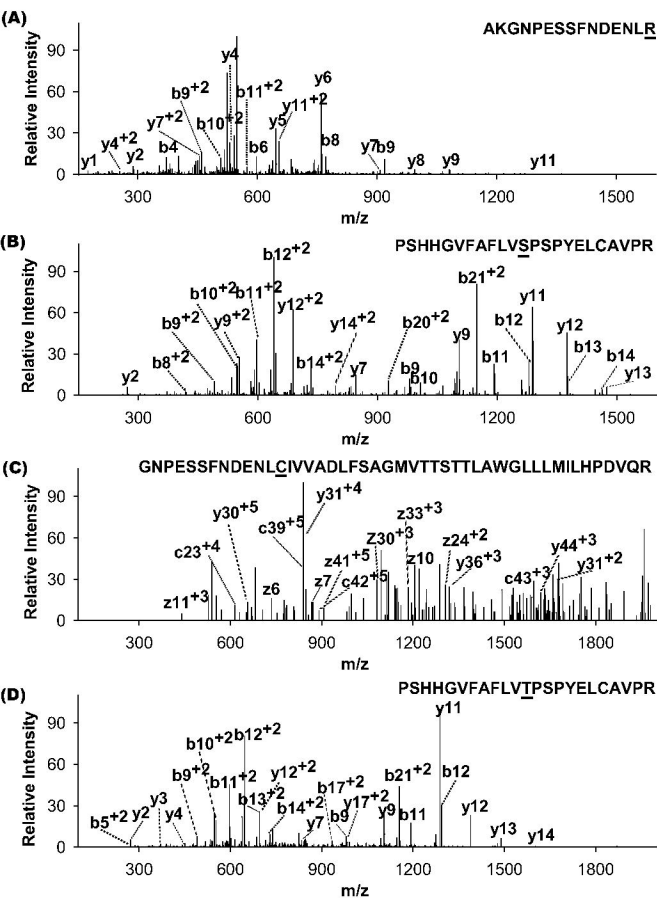


Fig.4

