QUANTITATIVE ANALYSIS OF CYTOCHROME P450 ISOZYMES BY MEANS OF UNIQUE ISOZYME-SPECIFIC TRYPTIC PEPTIDES: A PROTEOMIC APPROACH

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ABSTRACT:
A novel matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry method has been developed to quantitate cytochrome P450 (P450) isozymes based on their unique isozyme-specific tryptic peptides. It was shown that the molar ratio of P450 isozyme-specific peptides is linearly proportional to the mass peak area ratio of corresponding peptides not only in simple two-peptide mixtures, but also in complex digest mixtures. This approach is applicable both to in-gel (as shown for CYP2B1 and CYP2B2) and in-solution digests (as shown for CYP1A2, CYP2E1, and CYP2C19) and does not require introduction of stable isotopes or labeling with isotope-coded affinity tagging. The relative and absolute quantitation can be performed after developing corresponding calibration curves with synthesized P450 isozyme-specific peptide standards. The absolute quantitation of human P450 isozymes was performed by using CYP2B2 isozyme-specific peptide (1306.7 Da) as the universal internal standard. The utility of this approach was demonstrated for two highly homologous (>97%) rat liver CYP2B1 and CYP2B2 and three human P450 isozymes belonging to two different families and three different subfamilies: CYP1A2, CYP2E1, and CYP2C19. In summary, we have demonstrated that MALDI TOF-based peptide mass fingerprinting of different cytochrome P450 isozymes can provide not only qualitative but quantitative data, too.

The superfamily of cytochrome(s) P450 (P450) plays a key role in hepatic and extrahepatic drug metabolism, and qualitative and quantitative analyses of the P450 isozyme expression in a particular organ are critical in predicting a metabolic fate of a particular drug or in examination of the potential drug-drug interaction. The number of sequenced and named different P450 isozymes surpassed 3100 (dnelson.utmem.edu/CytochromeP450.html), and the degree of sequence homology, particularly among P450s belonging to the same subfamily, is high (Nelson et al., 1996). None of the existing research approaches to the analysis of individual P450 forms, which include specific P450 inhibitors (Halpert et al., 1994; Kobayashi et al., 2003) or substrates (Kobayashi et al., 2002; Stresser et al., 2002), antibody-based identification (Gelboin et al., 1999; Shou et al., 2000), and mRNA-based analysis (Chow et al., 1999; Zhang et al., 1999), is in a position to provide reliable quantitative and qualitative information on the individual P450 composition in a given type of microsomes. First, only a minority of known P450 isozymes is fully characterized by substrate specificity, and since they exhibit a broad, often overlapping substrate specificity, there is no known substrate or inhibitor that is absolutely specific for an individual P450 isozyme. Second, the high degree of sequence homology among members of the P450 superfamily confounds high specificity of antibody-based analysis, particularly among members of the same subfamily. Third, the application of a quantitative mRNA analysis for the evaluation of P450 isozyme expression, which once looked very promising, is questionable, too. It was shown that in many cases, correlation between protein abundances and mRNA levels for numerous hepatic and extrahepatic proteins is poor (Anderson and Seilhamer, 1997; Luss et al., 1997; Pradet-Balade et al., 2001; Chen et al., 2002; Huber et al., 2004). Most importantly, if an unknown or an unexpected P450 isozyme is expressed in the microsomes under investigation, none of these approaches will reveal it.

Over the last 10 years the mass spectrometry-based approach to the identification of proteins has become the method of choice for protein analysis in biological systems (Mann and Pandey, 2001). However, identification of different P450 isozymes represents quite a challenge even for proteomics. The main reason for this is that there is still no analytical method that can sufficiently separate different P450

ABBREVIATIONS: P450, cytochrome P450; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; MS, mass spectrometry; PMF, peptide mass fingerprinting; IS, internal standard; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; β-LGA, β-lactoglobulin A; BCA, bicinchoninic acid.
isozymes either by electrophoretic or by chromatographic means. Consequently, one has to deal with mixtures containing more than one P450, and this superfamily is the largest group of enzymes that share a high degree of similarity in protein sequence. Our laboratory has previously shown that peptide mass fingerprinting (PMF) can be successfully applied to the identification and differential analysis of closely related cytochrome P450 isozyymes in liver and brain microsomes from rat, mouse, and rabbit (Galeva and Altermann, 2002; Duzhak et al., 2003; Galeva et al., 2003). In this study, on the examples of rat and human P450s, we demonstrate that members of the P450 superfamily possess unique isoyme-specific tryptic peptide(s) that could be utilized for differential qualitative and quantitative analysis by MALDI-TOF mass spectrometry. By "unique" we mean peptide sequences generated by trypsinolysis that differ from any other tryptic peptide derived from any other identified to-date protein.

Materials and Methods

Chemicals. Acetonitrile, urea, and ammonium bicarbonate were purchased from Fisher Scientific Co. (Pittsburgh, PA); 0-ε-cyano-4-hydroxyxcinamic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI) and additionally purified by recrystalization; dithiothreitol was purchased from Bio-Rad (Hercules, CA); sodium iodoacetate (98% purity), β-lactoglobulin A from bovine milk, and bovine serum albumin were purchased from Sigma (St. Louis, MO); and sequencing grade modified trypsin was purchased from Promega (Madison, WI). Human recombinant cytochromes P450 were purchased from Pan-Vera/Invitrogen (Carlsbad, CA); CYP2C19 (lot number 26395B), CYP2E1 (lot number 12019A), and CYP1A2 (lot number 7166A).

Preparation of Microsomes. Heparinated microsomes from adult male Sprague-Dawley rats were prepared by differential centrifugation as previously described (Altermann et al., 1993).

Peptide Synthesis. Peptides, representing partial sequences of cytochromes CYP2B1 (F_1089SDLVPIVGVPQHPHR_1135, 1335.730 Da), CYP2B2 (F_1089/1437ADLAPILGHR_170, 1305.719 Da), CYP1A2 (Y_1100LPNALPQRA_1312, 1070.587 Da), CYP2C19 (G_1092HMPTYDAVHKHEVQ_1357, 1737.826 Da), and CYP2E1 (F_1091TLVPSNLHEAQR_1074, 1693.915 Da) were synthesized on an ACT 90 (Advanced ChemTech, Louisville, KY) instrument by means of a solid phase technique using Fmoc-protected amino acids. Peptides were purified by semipreparative HPLC performed on a Summit HPLC system (Dionex Corp., Sunnyvale, CA). The final peptide preparations were analyzed by MALDI-TOF MS and analytical reverse-phase HPLC, and were >99% pure. Stock solutions of each peptide at 10 mM were prepared with 50% acetonitrile/0.1% trifluoroacetic acid. The deposited samples crystallized within 30 min upon evaporation of the solvent at ambient temperature. For experiments performed on Voyager DE STR, we used 400-well Teflon-coated MALDI target plates, and in experiments executed on 4700 Proteomics Analyzer we used 192 well MALDI-TOF target plates.

MALDI-TOF Mass Spectrometry. Mass spectra were obtained on a Voyager DE STR Biospectrometry Workstation (MDS Sciex, Concord, ON, Canada) (in the case of CYP2B1 and CYP2B2 experiments) and a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) (in the case of CYP1A2, CYP2C19, and CYP2E1) MALDI-TOF mass spectrometers. The Voyager DE STR was operated in a positive reflector mode with the following parameters: acquisition mass range, 700-3000 Da; accelerating voltage, 20,000 V; grid voltage, 75%; mirror voltage ratio, 1.12; guide wire, 0.01%; low mass gate set at 700; extraction delay time, 150 ns; and the laser power attenuator set at 2200. To compensate for the heterogeneity of the analyte crystallization and to cover as much target area as possible, a spiral search pattern was used when a laser beam was moved from crystal area to crystal area manually, with five to six laser shots at each firing position (total 100 shots/spectrum). The 4700 Proteomics Analyzer was operated with a standard positive ion reflector acquisition program in the mass range 700 to 5000 Da (the focus mass was 1200 Da) and the laser power set at 4400. An automated spiral laser-firing pattern was used with four laser shots at each of 250 firing positions. The peak area values were determined by utilizing Applied Biosystems Data Explorer Version 4.5 for the 4700 Proteomics Analyzer and Data Explorer Version 4.0 for the Voyager DE STR. The mass peak list was exported and processed in Microsoft Excel (Microsoft, Redmond, CA). For both instruments the high voltage was turned on at least 40 min before the start of a data collection, and all samples were analyzed at the same laser power adjusted so that it would not produce saturated signals of analytes while producing analyze peaks with a signal-to-noise ratio >5.

Results and Discussion

Application of MALDI-TOF MS for qualitative analysis is well documented and is the basis for many current developments in proteomics (Gevaert et al., 2001). At the same time, the number of examples of the direct use of MALDI-TOF MS without introduction of stable isotoxes for quantitative analysis of biomolecules is limited (Walker et al., 2000; Hlongwane et al., 2001; Bucknall et al., 2002; Helmke et al., 2004). Working with minute amounts of biological sample obtained from liver, brain, kidney, or any other organ would benefit from a simpler quantitative approach. Application of a stable isotope-based internal standard is one such approach (Mirgorodskaya et al., 2000, 2004). Another approach is to measure relative quantitation by comparing the mass peak areas or intensities of structurally similar compounds like tryptic peptides from different proteins without using an internal standard. Several recent studies explored the applicability of such approaches to the liquid chromatography/MS electrospray ionization technique (Bondarenko et al., 2002; Chelius and Bondarenko, 2002).

With no existing general analytical approach capable of a reliable relative or absolute quantitation of a particular P450 isoyme in a complex biological sample, we decided to explore whether PMF based on MALDI-TOF MS could be used as the basis for the development of a global analytical approach for quantitative analysis of P450 isoymes. Two very closely related cytochrome P450 isoymes, CYP2B1 and 2B2, were chosen for proof-of-concept experiments.

Proteomic Analysis of CYP2B1 and CYP2B2. CYP2B1 is the major form of cytochrome P450 induced in liver of adult rats after exposure to phenobarbital. Phenobarbital also induces CYP2B2, although it still is unclear to what extent. These two proteins are highly homologous (>97%), differing in only 14 amino acids out of 491.
Their theoretical tryptic digests differ in five pairs of peptides, and four pairs of those peptides fall within the optimal MALDI TOF working range of 800 to 2500 atomic mass units (Table 1). The first pair of peptides originates from the N terminus (positions 1–21) and was very rarely found in experimental digests of purified P450s or microsomal fractions (Fig. 1). One of the peptides in the second pair (positions 317–323) has a molecular weight that differs in 1 atomic mass unit from one of the self-digest fragments of trypsin (842.439 versus 841.502) and cannot be a reliable indicator because of the overlap of resolved isotopomers. The third pair of peptides presents an interesting case. The CYP2B2 sequence contains Arg followed by Pro, and as a result, there is no cleavage in this position. In CYP2B1, Arg is followed by Leu and then by Pro, creating a more accessible cleavage site. However, in many experiments, we found a 1964.01 peak corresponding to missed cleavage (data not shown). Finally, a fourth pair of tryptic peptides appears to match the requirements for isozyme-specific tryptic peptides and was selected for further experiments. Since selected peptides originate from the same part of the molecule, position 359–370 (Fig. 1, inset), there should not be any doubt regarding their “equal accessibility” to tryptic digest, and we do not see any peptides with missed cleavages in position 370, either in CYP2B1 or in CYP2B2 digests. It should be emphasized that both of those peptides are unique in the sense that there are no similar tryptic peptides in any of the proteins listed to date either in the Swiss-Prot or NCBI (National Center for Biotechnology Information) databases (over 140,000 entries). The mass peak intensities of these peptides were always among the strongest in more than a hundred rat liver microsomal digests that we have performed to date, and their identity was confirmed by tandem MS (data not shown). The selected peptides were synthesized, mixed in different ratios, and analyzed by MALDI-
TOF MS. Figure 2 shows that the molar ratio of isozyme-specific tryptic peptides is linearly proportional to the mass peak area ratio of corresponding peptides.

Among factors known to contribute to difficulties associated with quantitative application of MALDI-TOF MS, most significant are the heterogeneity of analyte crystallization (Cohen and Chait, 1996; Garden and Sweedler, 2000) and the ion suppression effect (Kratzer et al., 1998; Knochenmuss et al., 1999). To control reproducibility and to compensate for heterogeneity of analyte-matrix cocrytallization, we standardized and, to some degree, optimized the conditions for data acquisition (see Materials and Methods). The evaluation of the ion suppression effect was performed by spiking digests of bovine serum albumin (BSA) and β-lactoglobulin A (β-LGA) with synthesized CYP2B1 and CYP2B2 isozyme-specific peptides in various ratios. Figure 3 shows a representative MALDI-TOF mass spectrum of 2 pmol of the BSA digest containing CYP2B1 and CYP2B2 isozyme-specific tryptic peptides in a 1:1 ratio. In both cases, a linear response between the molar ratio and the corresponding mass peak areas was observed. Figure 4 illustrates such dependence for digests of BSA (Fig. 4A) and β-LGA (Fig. 4B) spiked with synthesized CYP2B1- and CYP2B2-specific peptides.

Next, we applied the developed method to the microsomal sample separated on SDS-polyacrylamide gel. Rat liver microsomes were obtained from untreated male rats. Previously, it was shown that such microsomes do not contain CYP2B1 and CYP2B2 (Galeva and Altermann, 2002; Galeva et al., 2003; Nisar et al., 2004). Twenty micrograms of total microsomal protein were electrophoresed on 10% SDS-polyacrylamide gel. Several bands with an apparent molecular mass of 50 to 60 kDa were excised and subjected to tryptic digest. The band containing CYP2D2 (sequence identity to CYP2B1 and CYP2B2, 41%) was chosen for further experiments. The tryptic digest of CYP2D2 was spiked with synthesized CYP2B1- and CYP2B2-specific tryptic peptides in different ratios to simulate digests of CYP2B1 and CYP2B2 and then analyzed by MALDI-TOF MS. To determine the relative amounts of CYP2B1 and CYP2B2, a calibration curve was developed using corresponding synthetic peptides (Fig. 5).

As is seen from Fig. 5, there was a good correlation between the experimental points (Fig. 5, open circles) and the calibration curve.

Proteomic Analysis of Human CYP1A2, CYP2E1, and CYP2C19. Based on the results obtained from experiments with CYP2B1 and CYP2B2, we decided to explore whether the PMF MALDI TOF-based quantitative approach is applicable to other P450 isozymes and particularly to human P450s. The human genome encodes 57 cytochrome P450 genes. Thirty-five of these genes encode P450s belonging to families 1 to 4 (Danielson, 2002). P450s associated with families 1 to 3 are the key enzymes of phase I in human drug metabolism, whereas members of the CYP4 family are mainly in-

Fig. 2. A linear dependence between molar ratio of CYP2B1/CYP2B2-specific tryptic peptides and corresponding monoisotopic peak areas. Each data point represents the average ± S.D. of data collected in six experiments.

Fig. 3. A representative MALDI-TOF mass spectrum of BSA tryptic digest spiked with synthesized CYP2B1- and CYP2B2-specific tryptic peptides (peak 1306.735 is the CYP2B2-specific peak and 1336.739 is CYP2B1-specific).
involved in fatty acid and arachidonic acid metabolism. The remaining 14 P450 families for the most part are implicated in steroid metabolism.

To begin with, considering the large number of human P450s and the high degree of homology between members of P450 subfamilies, we needed to establish whether all of the human P450s possess unique isozyme-specific tryptic peptides. To this end, we undertook a database search for unique isozyme-specific tryptic peptides of human P450s. The following set of requirements was considered in this search. Suitable tryptic peptide candidates should not have any similar counterparts (homologs), preferably in any organism, or, at least, in humans; should have a mass between 900 and 1900 Da, to achieve the best possible accuracy and resolution in the MALDI TOF spectrum; ideally should have an Arg at the C terminus, since Arg-ending peptides produce much stronger MS signals in MALDI-MS than in Lys-ending MS; and do not contain any missed cleavages.

A list of isozyme-specific tryptic peptides was developed using PAWS software (Genomic Solutions, Inc., Ann Arbor, MI) to generate simulated tryptic digests and the ScanProsite search engine (http://au.expasy.org/tools/scanprosite) to scan protein sequences from Swiss-Prot, TrEMBL, and PDB (The Protein Database) with a user-entered pattern (in our case, candidate tryptic peptides). As it turns out, all human P450s have from 2 to 14 unique isozyme-specific tryptic peptides, and the complete list encompasses several hundred peptides. Table 2 shows predicted unique isozyme-specific tryptic peptides for three human P450s (CYP1A2, CYP2E1, and CYP2C19)

that were used in further experiments. As can be seen from Table 2, all of them have eight to nine unique isozyme-specific tryptic peptides. Clearly, not all of these peptides will show up in a tryptic digest and/or produce strong signal in the MALDI-TOF mass spectrum. To perform reliable quantitation, we needed to establish which one of the candidate peptides is consistently formed during trypsinolyis and generates the strongest MS signal. To this end, all three purified isozymes were subjected to tryptic digest (Fig. 6). It should be noted that we performed multiple digests differing in conditions (in-solution versus in-gel, with or without reduction and alkylation, 37°C overnight versus 58°C for 45 min) with each of these isozymes, and the results were consistent. For each P450 there was at least one isozyme-specific tryptic peptide that produced a dominant mass peak in the corresponding PMF MALDI-TOF mass spectrum (Fig. 6; bold text in Table 2). Sequences of these peptides were confirmed by tandem MS (data not shown). It should be emphasized that these major isozyme-specific tryptic peptides were conserved even in simplified digests performed without destaining of gel bands, reduction, and alkylation (cf. panels A and B, Fig. 6). Some other predicted unique isozyme-specific peptides were seen in the digest mass spectra, too, but their peaks were not as strong. The identified major isozyme-specific tryptic peptides were synthesized and used for quantitative analysis of human P450s. In all further experiments CYP2B2 isozyme-specific peptide (1306.7 Da) was used as an IS. The calibration curves for the absolute quantitation of P450 isoforms were generated using mixtures of four peptides (IS peptide plus three synthetic isozyme-specific peptides). Each MALDI target spot contained 20 pmol of IS peptide, and from 500 fmol to 70 pmol of the synthetic CYP1A2- and CYP2E1-specific peptides and from 500 fmol to 50 pmol of the synthetic CYP2C19-specific peptide. Linear regression analysis data presented in Fig. 7 indicate that for all three isozymes, the peak area ratios are linear with the amount of the synthesized isozyme-specific peptides. Subsequently, we prepared two mixtures of purified P450s with different molar ratios based on their concentrations, determined
spectrophotometrically by UV-visible spectra, and then spiked them with IS peptide and performed in-solution tryptic digest. A representative MALDI-TOF mass spectrum of a combined digest of all three P450 isozymes is shown in Fig. 8. The peak area ratios of isozyme-specific peptides to IS peptide was measured from MS spectra, and the concentrations of all three P450s in a given mixture were determined simultaneously using the developed calibration curves. The P450 isozyme concentrations measured by MALDI-TOF MS were generally higher than the concentrations measured spectrophotometrically in individual P450 stock solutions (Table 3). Somewhat elevated

<table>
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<th>P450 Isozyme</th>
<th>Start-End</th>
<th>Sequence</th>
<th>Monoisotopic Mass</th>
<th>Length (aa)</th>
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<tr>
<td>CYP1A2</td>
<td>80–90</td>
<td>IGSTPVVLVLSR</td>
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<td></td>
<td>297–306</td>
<td>ASQNLIPQEK</td>
<td>1055.561</td>
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<td></td>
<td>378–392</td>
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<td>DTTLNGFYTPK</td>
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**TABLE 2**
List of predicted unique tryptic peptides for CYP1A2, CYP2E1, and CYP2C19.

**FIG. 6.** Representative tryptic peptide mass fingerprinting MALDI-TOF mass spectra of isolated human P450s. Panel A, CYP1A2 digest (asterisks denote 1A2 tryptic peptides); panel B, CYP1A2 simplified digest without destaining, alkylation, and reduction; panel C, CYP2C19 digest (asterisks denote 2C19 tryptic peptides); panel D, CYP2E1 digest (asterisks denote 2E1 tryptic peptides).
values of P450 concentrations measured by MALDI-TOF MS compared with UV-visible measurement reflect the fact that the mass spectrometric method measures the apoprotein amount, whereas UV-visible measures holoenzyme (P450 molecules containing heme moieties). Since in cytochrome(s) P450 the prosthetic heme group is not covalently bound to the apoprotein (except CYP4As), part of the P450 molecules lose it relatively easily. All three P450 isozymes used in this study were recombinant proteins produced from over-expressed plasmid in *Escherichia coli*. According to the manufacturer’s certification (Panvera/Invitrogen), their specific content varied from 10 nmol of spectral P450/mg protein, in the case of CYP2C19, to 12 nmol/mg protein for CYP2E1, and 16 nmol/mg protein for CYP1A2. These values indicate the presence of heme-depleted P450s and/or the existence of some protein impurity. Indeed, the proteomic analysis identified the presence of β-galactosidase from *E. coli* in all preparations of these isozymes (data not shown). In line with these findings, we established that P450 concentrations calculated based on protein measurements in P450 isozyme stock solutions were consistently higher than MALDI-TOF MS-measured values (Table 3, CYP1A2 versus CYP2E1 versus CYP2C19).

Due to the low sequence similarity among the predicted isozyme-specific tryptic peptides, we did not design the IS peptide but, rather, decided to use CYP2B2-specific tryptic peptide (1305.7 Da) as the universal internal standard. However, it should be emphasized that isozyme-specific peptides for CYP2C19 and CYP2E1 originate from the same part of the P450 molecule as CYP2B1- and CYP2B2-specific peptides, whereas CYP1A2-specific peptide comes from a different part of the molecule. If this trend can be confirmed in further studies, then a single internal standard per P450 family/subfamily could be designed that, in turn, might increase the accuracy of this approach.

In summary, a novel MALDI-TOF MS method has been developed to quantify cytochrome P450 isozymes based on their unique isozyme-specific tryptic peptides. This approach is applicable both to in-gel (as shown for CYP2B1 and CYP2B2) and in-solution digests (as shown for CYP1A2, CYP2E1, and CYP2C19) and does not require introduction of stable isotopes or labeling with isotope-coded affinity tagging. The relative and absolute quantitations can be performed after developing corresponding calibration curves with syn-

![Fig. 7. Absolute quantitation standard curves: panel A, CYP1A2 standard curve; panel B, CYP2E1 standard curve; panel C, CYP2C19 standard curve. Each data point represents the average ± S.D. of data collected in six experiments.](image-url)
thesized isozyme-specific peptide standards. The absolute quantitation of human P450 isozymes was performed by using CYP2B2 isozyme-specific peptide (1306.7 Da) as the universal internal standard. The utility of this approach was demonstrated for two highly homologous (97%) rat liver CYP2B1 and CYP2B2 and three human P450 isozymes belonging to two different families and three different subfamilies: CYP1A2, CYP2E1, and CYP2C19. Furthermore, the data obtained point to the fact that sample preparation and data acquisition conditions need to be carefully controlled to obtain optimal results. In a more general context, our data, along with some other recent publications (Hlongwane et al., 2001; Bucknall et al., 2002; Mims and Hercules, 2003; Alterman et al., 2004; Helmke et al., 2004), suggest that application of MALDI-TOF mass spectrometry for relative or direct quantitation is a valid alternative to the stable isotope approach. Particularly advantageous in direct quantitation by MALDI TOF is the simplicity of the sample processing. The data presented, along with new findings involving CYP2A6 and CYP2A13 (manuscript in preparation), strongly suggest that this technique could become a universal method for the quantitative analysis of P450 isozyme expression. Studies involving relative and absolute quantitation of human P450 isozyme expression in liver and other organs are currently in progress in our laboratory.

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Chen G, Gharib TG, Huang C-C, Taylor IMG, Misek DE, Kardia SLR, Giordano TI, Iannettoni...
MALDI TOF-BASED QUANTITATION OF P450s


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MALDI TOF-BASED QUANTITATION OF P450s


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