Short Communication

A PROTEOMIC APPROACH TO THE IDENTIFICATION OF CYTOCHROME P450 ISOFORMS IN MALE AND FEMALE RAT LIVER BY NANOSCALE LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-TANDEM MASS SPECTROMETRY

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ABSTRACT:
Nanoscale reversed-phase liquid chromatography (LC) combined with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) has been used as a method for the direct identification of multiple cytochrome P450 (P450) isoforms found in male and female rat liver. In this targeted proteomic approach, rat liver microsomes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by in-gel tryptic digestion of the proteins present in the 48- to 62-kDa bands. The resultant peptides were extracted and analyzed by LC-ESI-MS/MS. P450 identifications were made by searching the MS/MS data against a rat protein database containing 21,576 entries including 47 P450s using Sequest software (Thermo Electron, Hemel Hempstead, UK). Twenty-four P450 isoforms from the subfamilies 1A, 2A, 2B, 2C, 2D, 2E, 3A, 4A, 4F, CYP17, and CYP19 were positively identified in rat liver.

The cytochromes P450 (P450s) are one of the largest known gene families and carry out a wide range of biological oxidation and reduction processes important in the metabolism of a large number of drugs, xenobiotics, and endogenous compounds. P450 enzymes are characterized into families and subfamilies by their sequence similarities; there are over 280 different families of P450s and currently more than 1925 sequenced and named isoforms (drnelson.utmem.edu/CytochromeP450.html). In the rat, a species traditionally important in drug development studies, there are about 50 P450 genes as identified from the UniGene database (drnelson.utmem.edu/UNIGENE.RAT.html; Wheeler et al., 2004). Traditional methods for the detection of P450 proteins have relied on immunodetection of protein, activity assays, or on the detection of P450 mRNA (Patterson and Murray, 2002). These techniques have significant limitations. Immunoblotting, although very sensitive, relies on the availability of isoform-specific antibodies, and it is necessary to preselect which P450s are to be analyzed and to identify each isoform in turn. Activity assays that are geared to investigate the activity of a P450 isoform invariably require multiple analysis techniques, and different assays must be developed for different target substrates; even then, they may not be totally isoform-specific. Measurements at the mRNA level are fraught with uncertainty, since the presence and abundance of a particular type of mRNA do not necessarily indicate a similar presence and abundance of the corresponding protein (Anderson and Seilhamer, 1997; Chen et al., 2002; McFadyen et al., 2003). Mass spectrometry is an alternative method for the analysis of expressed proteins, uniquely offering the ability to detect low levels of multiple proteins in a single analytical run. To date, there have been relatively few reports of the analysis of P450 proteins by mass spectrometry; the majority of these have been activation-based, concentrating on the observation of substrates and metabolites involved in P450 reactions rather than the P450s themselves. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry has been used to determine the molecular masses of closely related CYP2B1 and 2B2 (Lewis et al., 1993). MALDI-TOF analysis of peptides generated by cyanogen bromide cleavage of CYP3A4 (He et al., 1998; Lightening et al., 2000) and trypsin proteolysis of CYP2E1 (Cai and Guengerich, 2001) have been described. With regard to multiple protein identification, two-dimensional gel electrophoresis followed by MALDI-TOF and peptide mass fingerprinting (PMF) has become a principal approach for the proteomic profiling of various in vitro and in vivo biological systems (Henzel et al., 1993). A major drawback of the two-dimensional gel electrophoresis approach is its low performance in the separation of membrane proteins including P450s (Galeva and Alterman, 2002). In recognition of this, SDS-PAGE (one-dimensional gel) separation of microsomal proteins followed by MALDI-TOF and PMF was used to separate and analyze the endoplasmic reticulum proteins from rat and rabbit livers. Using this one-dimensional gel separation, up to eight P450s were positively identified (Galeva et al., 2003). However, PMF is most reliable when samples containing only a few proteins are analyzed because the presence of multiple proteins within an SDS-PAGE band may lead to spurious results. This is particularly the case when proteins are present in low abundance, and their tryptic peptide sequence coverage is low (Clauser et al., 1999; Fenyo, 2000; Huang et al., 2002). Liquid chromatography (LC) -electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) has also been applied to the analysis of P450s
Very recently, Kislinger et al. (2003) described a multidimensional LC-ESI-MS/MS method for the identification of mammalian proteins. They applied this method to analyze liver microsomes from female mice and were able to detect 20 P450 isoforms. The multidimensional LC-ESI-MS/MS or multidimensional protein identification technology method (Link et al., 1999) does not require prefractonation of the protein sample but rather relies on chromatographic separation of the very complex mixture of peptides generated by proteolysis of unseparated protein mixtures. Although the multidimensional protein identification technology approach may hold advantages in the unbiased analysis of protein mixtures, in the current study we wish to target our analysis to the identification of P450 isoforms. In this regard we have used SDS-PAGE to separate endoplasmic reticulum proteins into discrete bands according to molecular weight and then selected the bands in the P450 molecular weight range for further analysis. Proteins within bands in the 48- to 62-kDa range were digested with trypsin, and the resulting peptides were separated and analyzed by nanoscale LC-ESI-MS/MS. The MS/MS spectra provide amino acid sequence information that, in combination with peptide mass data, provides a secure identification of proteins. Male and female rats were investigated to illustrate the use of this technique in the identification of gender differences in multiple P450 protein expression.

Materials and Methods

Preparation of Rat Liver Microsomes. Fresh liver (9–10 g) from male and female outbred 10-week-old Wistar rats (Bantin and Kingman, Hull, UK) was washed with cold isotonic saline (0.9% sodium chloride, 4°C) to remove blood. Connective tissue was excised. Liver was homogenized using an Ultra Turrax T25 (Janke and Kunkel, IKA Laborertechnik, Staufen, Germany) in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 15% glycerol, and 0.67 mM phenylmethanesulfonyl fluoride. Microsomes were prepared using differential centrifugation as follows: an initial centrifugation at 2400 g for 10 min was used to sediment the cell debris, nuclei, and unbroken cells. The supernatant was centrifuged at 12,000 g for 20 min at 4°C. Supernatant from this step were centrifuged at 180,000 g for 1 h at 4°C. The resultant microsomal pellets were suspended in 0.1 M Tris-HCl, containing 15% glycerol and 1 mM EDTA, pH 7.4, and then centrifuged at 180,000 g for 1 h. The final pellet was resuspended in 0.1 M Tris-HCl, containing 15% glycerol and 1 mM EDTA, pH 7.4, and stored at −80°C. Microsomal protein was determined using the Bradford assay (Bradford, 1976).

SDS-PAGE. One-dimensional SDS-PAGE was performed using standard methods on the Hoefer Mighty Small gel system (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Microsome samples were boiled at 95°C for 8 min in a solubilization buffer (2% SDS, 20% glycerol, 0.01% bromphenol blue, and 50 mM Tris-HCl, pH 6.8) and 0.1 M dithiothreitol. Microsomal protein (20 μg) was resolved on a 10% acrylamide gel. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid (1 h), and destained overnight with the same solvent.

In-Gel Tryptic Digestion and Peptide Extraction. The molecular weight region on the SDS-PAGE gel between 48 and 62 kDa was divided into five approximately equal bands, and each band was excised with a scalpel. Bands were washed in distilled water until the pH was neutral and completely destained using 50 mM NH₄HCO₃ in 40% ethanol. Bands were cut into fine pieces to increase the surface area then dried with acetonitrile and then in a SpeedVac (Thermo Savant, Holbrook, NY) for 30 min. Digestion was carried out using sequencing-grade modified trypsin (approximately 75 ng/μl) (Promega, Southampton, UK) in 25 mM NH₄HCO₃. Sufficient trypsin solution was added to swell the gel pieces, which were kept on ice for 30 min and then covered with 25 mM NH₄HCO₃ and incubated at 37°C overnight. Peptides were desorbed from the gel pieces with acetonitrile.

![Fig. 1. MS/MS spectrum of the tryptic peptide GTAVLSTSLSVLHDSK [M + 2H]⁺ ion of m/z 815.4, identified to originate from CYP2C12. Shown in the inset is the peptide’s amino acid sequence. y and b ions are formed by peptide bond cleavage with charge retention on the C terminus and N terminus, respectively.](image-url)
were extracted from the gel pieces with ultrasonication, using sequential washes with a solution of 5% tris(hydroxymethyl)aminomethane (TRIS) in 50% acetonitrile. The extracts were combined and dried in a SpeedVac to complete dryness. Samples were stored at −80°C and reconstituted in 0.1% TFA before analysis.

LC-ESI-MS/MS. Nanoscale LC was performed using an LC Packings UltiMate capillary high-performance liquid chromatography system with FAMOS autosampler (Dionex, Camberley, Surrey, UK). A separate UltiMate Micro Pump (Dionex) was used as a loading pump. The sample (1 μl) was injected via a sample loop (using 0.1% TFA in water as carrier solvent) onto a 1 mm × 300 μm PepMap C18 guard column (5 μm) (LC Packings, Sunnyvale, CA). The sample was washed with 0.1% TFA for 3.5 min on the guard column before being switched onto a 15 cm × 75 μm PepMap C18 column (5 μm) (LC Packings) equilibrated with 95% mobile phase A (5% acetonitrile containing 0.1% formic acid) and 5% mobile phase B (80% acetonitrile containing 0.1% formic acid), at a flow rate of 200 nl/min. Five minutes after sample injection the proportion of mobile phase B was increased linearly to 50% over 30 min and then stepped to 95% and maintained at this level for 10 min (wash phase). The column was then re-equilibrated for 20 min with 95% mobile phase A, 5% mobile phase B. The column effluent was continuously directed into an LCQduo mass spectrometer fitted with a nano-ESI source (Thermo Electron, Hemel Hempstead, UK), and spectra were recorded. Mass spectrometer conditions were optimized using in-solution tryptic digests of purified recombinant P450 isoforms 1A2, 2E1, and 3A4, obtained from PanVera Corp. (Madison, WI).

ESI was performed under the following conditions: positive ionization mode; spray voltage, 1.8 kV; capillary voltage, 28 V; capillary temperature, 180°C; and no sheath or auxiliary gas was used. Data were collected in the full-scan and data-dependent MS/MS modes; three microscans were performed with the maximum ion injection time of 200 ms. In the full-scan mode, ions were collected in the m/z range of 400 to 2000. The MS/MS collision energy was set to 35%.

Protein Identification. MS/MS spectra were searched using Sequest Browser software (Thermo Electron) (Eng et al., 1994; Yates et al., 1995), against a rat protein database containing 21,576 entries including 47 P450s (Finnigan Xcalibur; Thermo Finnigan, San Jose, CA; revision 1.0 P/N XCALI-64012, July 2000). The aim of the Sequest approach is to find the peptide sequence in a database that best explains the fragment ions present in a spectrum. Candidate sequences are found in the database on the basis of intact peptide masses, and the complete or partial spectra expected to result from the fragmentation of these candidate peptides are generated and compared with the experimental spectrum. The final score assigned to each candidate peptide sequence is the Xcorr, a measure of how well the theoretical spectrum cross-correlates to the observed spectrum. Proteins that were matched by two or more peptides with Xcorr values ≥2.5 were considered conclusively identified, provided that the peptides were unique to that protein in the database (Ducret et al., 1998).

Results and Discussion

Initially, it was considered necessary to evaluate the performance of the SDS-PAGE LC-ESI-MS/MS procedure for the identification of P450s. This was achieved by loading known amounts of purified recombinant P450 isoforms 1A2, 2E1, and 3A4 onto the SDS-PAGE gel and proceeding through the analytical cycle to identify the proteins. It was found that P450s could be successfully identified down to approximately 1 pmol loaded onto the gel (results not shown). A successful identification was defined as one in which two or more unique peptides were found with Sequest Xcorr values ≥2.5.

Identification of P450s in Rat Liver Microsomes. Five bands of approximately equal size covering the molecular weight range of 48 to 62 kDa were cut out from SDS-PAGE and subjected to in-gel digestion with trypsin. The resultant peptides were extracted and analyzed by LC-ESI-MS/MS. To identify the proteins present, the MS/MS spectra were submitted to the Sequest algorithm. Sequest then identified the tryptic peptides by matching their MS/MS spectra against insilico generated theoretical spectra from the database. This is illustrated in Fig. 1, which shows the MS/MS spectrum of the doubly charged peptide of m/z 815.4. Sequest determined the amino acid sequence of the peptide to be GTAVLTSLTSVLHDSK, from the CYP19 P340 isoform (1A2, 2E1, and 3A4) (Ducret et al., 1998). Other proteins, which were identified with good sequence coverage (>15%), are: probable protein disulfide isomerase ER-60 precursor (42%), kinase (38%), aldolase dehydrogenase, microsomal (28%) rat ATP synthase β chain (26%), glutamate dehydrogenase precursor (24%), mitochondrial precursor (22%), microsomal epoxide hydrolase (21%), UDP-glucuronosyltransferase 2B12 precursor (16%), UDP-glucuronosyltransferase 2B3 precursor (18%), UDP-glucuronosyltransferase 2B1 precursor (19%), and F1-ATPase β subunit.

<table>
<thead>
<tr>
<th>Cytochromes</th>
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<th>Number of Matched Peptides (percentage of sequence coverage by amino acid count)</th>
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<td>1A2</td>
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</tr>
<tr>
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<tr>
<td>CYP17</td>
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</tr>
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</table>

N.D., not detected.

Table 1: CYPs identified by MS/MS to be present in rat liver microsomes

Other proteins, which were identified with good sequence coverage (>15%), are: probable protein disulfide isomerase ER-60 precursor (42%), kinase (38%), aldolase dehydrogenase, microsomal (28%) rat ATP synthase β chain (26%), glutamate dehydrogenase precursor (24%), mitochondrial precursor (22%), microsomal epoxide hydrolase (21%), UDP-glucuronosyltransferase 2B12 precursor (16%), UDP-glucuronosyltransferase 2B3 precursor (18%), UDP-glucuronosyltransferase 2B1 precursor (19%), and F1-ATPase β subunit.
MS/MS of isolated proteolytic peptides, as in our LC-ESI-MS/MS approach.

As the goal of this preliminary study was to identify the P450 proteins in liver microsomes, it was decided not to pursue a stable-isotope dilution approach, such as the isotope-coded affinity tag method (Gygi et al., 1999), for relative quantification. With stable-isotope dilution methods, proteins isolated from different sources are differentially stable-isotope labeled, combined, digested, and then analyzed by LC-MS/MS. This approach requires dilution of one sample by the other and also the analysis of selectively labeled peptides. Such a procedure would inherently limit differentiation between P450 isoforms with high sequence similarity and may also elevate the P450 protein detection limit.

Although the results of the current study are of a qualitative nature, and the nonidentification of a protein in a sample does not necessarily equate its absence, at least to a first approximation, the greater the concentration of a protein within a given sample, the greater the probability of its identification. With this in mind, some comments can be made with regard to the identification of P450 proteins based on rat gender (see Table 1). Most of the known gender-related differences in compound toxicity in rats are due to gender-related differences in hepatic metabolism (Czerniak, 2001). Hormones and growth factors are strongly implicated in the gender-influenced expression of hepatic P450s (Waxman, 1992; Pampori and Shapiro, 1999; Kalsotra et al., 2002). Whether P450 expression is gender-specific rather than gender-predominant or -biased will depend on the absolute expression of protein in one sex compared with the other. It has been suggested that a P450 is gender-specific only if the relative expression is 10-fold or higher in one sex compared with the other; lower than this has been suggested to represent enriched P450 expression in one gender rather than sex specificity (Kato and Yamazoe, 1993). Our results show that CYP2C11 and 2C13 were found in male but not female rats, whereas CYP2C12 was found in females only, supporting previous work that indicated that these three isoforms are gender-specific/predominant (Agrawal and Shapiro, 2001). Our study shows also that other CYP2C members are gender-predominant; specifically, CYP2C22 was found only in male livers, whereas CYP2C23 and 2C24 were found only in female livers. Regarding female-predominant CYP2A1, 2C6, and 2C7 (Agrawal and Shapiro, 2001), these isoforms were identified in both genders by LC-ESI-MS/MS. CYP2D1, 2D2, and 2D3 were found in both sexes whereas CYP2D4 was female-specific and CYP2D5 was male-specific. Gender selectivity of some 2D family members was previously identified (Schulz-Utermoehl et al., 1999). CYP3A18 and 4A2 were observed in male but not female rat liver, which is supported by previous studies showing that most members of the CYP3A (including CYP3A18) and

*Different amino acid sequence

Highlighted sequences were identified for CYP2D2 and 2D3 using LC-MS/MS.

FIG. 2. Sequence alignment and coverage map for CYP2D2 and 2D3.
CYP4A families to be male gender-specific or -predominant (Waxman et al., 1995; Robertson et al., 1998; Holla et al., 2001; Mitchell et al., 2001; Anakk et al., 2003). CYP17 and CYP19, two enzymes that contribute to sex steroid synthesis, were identified in female but not male liver.

The sex-related differences indicated above suggest that the next step in the proteomic study of rat liver microsomal P450s should involve relative protein quantification between the sexes. Unfortunately, the straightforward use of stable-isotope dilution methods may not be applicable, as such methods will require isof orm-specific labeling, which will not be trivial for P450 isoforms with high sequence similarity.

In summary, in the current study 24 P450 isoforms have been conclusively identified by LC-ESI-MS/MS. The results for the majority of P450s found are consistent with previously published studies describing expression profiles of selected P450s in rat liver. Clearly, there is a considerable advantage in using LC-ESI-MS/MS in the identification of multiple P450s from complex tissues.

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References


