Accelerated Communication

METABOLIC SCREENING USING ON-LINE ULTRAFILTRATION MASS SPECTROMETRY

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ABSTRACT:

An on-line mass spectrometric method has been developed to generate and identify drug metabolites formed by hepatic cytochromes P450. This method, pulsed ultrafiltration-mass spectrometry, may be used for rapid screening of drugs to determine their extent of metabolism by microsomal cytochromes P450 and to characterize the primary metabolites. Rat liver microsomes were trapped in a stirred ultrafiltration chamber fitted with a 100,000 molecular weight cut-off ultrafiltration membrane. A continuous flow of ammonium acetate buffer was pumped through the chamber and into an electrospray mass spectrometer. Substrates for cytochromes P450 including imipramine, chlorpromazine, and pentoxysresorufin were flow injected through the chamber along with the cofactor, NADPH, and metabolites were detected on-line by using electrospray mass spectrometry. Identical control experiments carried out using boiled microsomes or without NADPH showed no metabolite formation. Naringenin and quinidine, which are inhibitors of some isoforms of cytochrome P450 and are not known to be extensively metabolized, showed no major metabolites. For comparison, imipramine metabolites were also generated by standard batch incubation with microsomes and NADPH, followed by extraction and LC-MS analysis. Similar metabolites were obtained using the flow-through ultrafiltration method and the standard batch microsomal incubation. Tandem mass spectrometry was used to confirm structures of imipramine metabolites including 10-hydroxyimipramine, 2-hydroxyimipramine, imipramine N-oxide, and N-desmethylinipramine. Finally, the feasibility of using ultrafiltration mass spectrometry for high throughput metabolic screening was demonstrated by using on-line mass spectrometry for only 3 min per incubation instead of monitoring the entire elution profile. By carrying out multiple ultrafiltration experiments in parallel, efficient use of the mass spectrometric detector may be obtained with a throughput of at least 20 incubations per hour. Throughputs of up to 60 profiles per hour should be possible.

On-line ultrafiltration electrospray mass spectrometry offers a streamlined, higher-throughput method for in vitro formation and mass spectrometric characterization of microsomal drug metabolites.

Combinatorial chemistry, a new approach to the identification and optimization of drug leads in medicinal chemistry, has been enormously successful in synthesizing large number of compounds for pharmacological screening and testing (Gordon et al., 1996; Thompson and Ellman, 1996). In response, high throughput screening methods are being developed to rapidly identify lead compounds in combinatorial libraries that interact with specific receptors or show desirable pharmacological effects in bioassays (Loo, 1997). Among these new screening methods is pulsed ultrafiltration-mass spectrometry (van Breemen et al., 1997) that incorporates liver microsomes to streamline and increase the throughput of metabolic screening was demonstrated by using on-line mass spectrometry for only 3 min per incubation instead of monitoring the entire elution profile. By carrying out multiple ultrafiltration experiments in parallel, efficient use of the mass spectrometric detector may be obtained with a throughput of at least 20 incubations per hour. Throughputs of up to 60 profiles per hour should be possible.

On-line ultrafiltration electrospray mass spectrometry offers a streamlined, higher-throughput method for in vitro formation and mass spectrometric characterization of microsomal drug metabolites.

Imipramine is a tricyclic antidepressant drug that functions by inhibiting the re-uptake of norepineprine and dopamine by neurons, thereby increasing their concentration at synapses (Nogrady, 1988). The metabolism of imipramine has been studied extensively and involves two major pathways: N-demethylation and hydroxylation (Gram, 1974). N-Desethylinipramine, an active metabolite also used in clinical practice, is formed by action of cytochrome P450 3A4 and 1A2 (Lemoine et al., 1993). The major hydroxylated product, 2-hydroxyimipramine, is formed by aromatic ring hydroxylation by cytochrome P450 2D6 (Brosen et al., 1991). These major metabolites may be further metabolized to the common secondary product, 2-hydroxydesimipramine (Chiba et al., 1988). Additional minor pathways of imipramine metabolism include hydroxylation of the aliphatic...
(central) ring to form 10-hydroxyimipramine, oxidation of the side chain to form imipramine-N-oxide, double demethylation to form desmethyliimipramine, and elimination of the side chain to form iminodibenzyl. Oxidation products of imipramine are usually excreted as glucuronides. Other substrates for cytochromes P450 that will be investigated include chlorpromazine and 7-pentoxyresorufin. Like imipramine, chlorpromazine is an antidepressant drug that undergoes extensive hepatic metabolism (DeVane, 1995). Pentoxyresorufin is a standard substrate used in the assay of cytochrome P450 2B-2-catalyzed O-dealkylation activity (Burke et al., 1985), and enzymatic O-dealkylation results in the loss of the pentoxy group to form resorufin. This new on-line ultrafiltration mass spectrometric method to study drug metabolism will be applied to the metabolic screening of these well-characterized substrates for cytochromes P450 with an emphasis on the drug imipramine.

Materials and Methods

**Hepatic Microsomal Incubations.** Male and female Sprague-Dawley rats (180–200 g) were obtained from Sasco Inc. (Omaha, NE). Dexamethasone treatments were carried out according to the following protocols: rats were given intraperitoneal injections of 100 mg/kg in corn oil on days 1–3 and were sacrificed on day 4. For both treated and untreated animals, food was removed 15 hr before death. Microsomes were prepared from rat liver, and protein and cytochrome P450 concentrations were determined as described previously (Thompson et al., 1987). The microsomes were diluted (typically 3-fold) with 50 mM ammonium acetate buffer at pH 7.4 immediately before use to make the protein concentration approximately 10 mg/ml. An aliquot of approximately 100 μl of the diluted microsomes was injected into the magnetically stirred ultrafiltration chamber, which had a volume of 1.10 ml, so that the final concentration of microsomal protein was 1.0 mg/ml. Before being connected on-line to the mass spectrometer, the microsomes were washed for 30 min at 70 μl/min with 50 mM ammonium acetate buffer at pH 7.4 to remove low molecular weight contaminants. This washing step may be accelerated by using a higher flow rate, but care should be taken to avoid excessive pressure and rupture of the ultrafiltration membrane.

The ultrafiltration chamber was built in-house out of polysulfone and contained a Teflon-coated (Dupont, Wilmington, DE) magnetic stirring bar and O-ring, which formed a seal around the ultrafiltration membrane. The methacrylcellulose ultrafiltration membrane was purchased from Amicon (Beverly, MA) and had a molecular weight cut-off of 100,000. Ultrafiltration membranes with smaller pore sizes (i.e., 10,000 molecular weight cut-off) were investigated but could not be used without clogging. Chromatography tubing and fittings were made from polyetheretherketone (PEEK, Upchurch Scientific, Oak Harbor, WA).

NADPH and drug substrates were purchased from Sigma Chemical Company (St. Louis, MO). All solvents were HPLC grade. Into a mobile phase consisting of 50 mM ammonium acetate, pH 7.4, and 0.1 mM NADPH at a flow rate of 70 μl/min, chlorpromazine (3 μg) in 50 μl buffer was injected into the ultrafiltration chamber. Protanolated molecules of chlorpromazine, cofactor NADPH, and the microsomal metabolic products were recorded continuously using positive ion electrospray mass spectrometry. Initially, a wide mass range was scanned by the mass spectrometer to identify the major metabolites, and subsequent analyses used selected ion monitoring to follow the appearance of oxidized chlorpromazine, monitor the consumption of NADPH, and monitor the profile of unreacted chlorpromazine. In another experiment, 1 μg of pentoxyresorufin was injected into the ultrafiltration chamber containing hepatic microsomes as described above. Negative ion electrospray mass spectrometry was used to monitor the appearance of resoru-fin, the O-dealkylated product, at m/z 212. In subsequent experiments, NADPH was injected simultaneously with substrate instead of being added to the mobile phase. For example, imipramine and cofactor NADPH were injected simultaneously to give a maximum chamber concentration of 1.53 μg/ml (5.46 μM) imipramine and 0.46 mM NADPH. The imipramine metabolite profiles were obtained in scan mode instead of selected ion monitoring to illustrate that prior knowledge of the metabolism of the substrate is not necessary for successful screening. Identical control experiments for all substrates were carried out either without NADPH or with heat-inactivated microsomes.

To investigate the potential for high throughput metabolic screening using pulsed ultrafiltration mass spectrometry, analyses of imipramine, pentoxyresorufin, quinidine, and naringenin were carried out as described above, except that the ultrafiltration chamber was connected to the mass spectrometer for only 3 min per incubation during a period when a high concentration of metabolites and substrate elute (26–29 min after injection of the substrate). The transfer line between the ultrafiltration chamber and the mass spectrometer was flushed for 1 min, then mass spectra were recorded for 2 min. Control experiments were identical except that the microsomes had been inactivated by boiling. Naringenin and quinidine were used as negative controls, since they are not known to be extensively metabolized by cytochromes P450, and naringenin is known to inhibit cytochrome P450 3A4 (Guengerich and Kim, 1990).

Off-line microsomal incubations were carried out at room temperature for 30 min using liver microsomes containing 1.14 mg/ml protein, 6.48 μg/ml imipramine, and 0.35 mM NADPH. Experiments were carried out either in 50 mM ammonium acetate buffer, pH 7.4, or potassium phosphate. Each reaction was stopped by freezing the reaction mixture at −20 °C. Imipramine metabolites were extracted from the off-line microsomal incubation mixture according to the method of Sequeira and Strobel (1995) except that chloroform was used instead of ether. The extract was evaporated to dryness under vacuum and redissolved in 200 μl methanol/water/acidic acid solution (75:24:1; v/v/v). For direct comparison, an on-line ultrafiltration experiment was carried out using identical conditions and amounts of microsomes, substrate, and cofactor. After 30 min, the mobile phase was switched to 90% methanol at 0.2 ml/min and the eluate was collected for 2 min. Aliquots (20 μl each) of the on-line and ultrafiltration incubation mixtures were analyzed using liquid chromatography-mass spectrometry (LC-MS) as described below.

**Mass Spectrometry.** An Applied Biosystems (Foster City, CA) 140A dual syringe pump with a Rheodyne 8125 injector was used for all analyses. Mass spectra were acquired using either a Micromass (Manchester, UK) Quattro II triple quadrupole mass spectrometer equipped with an electrospray ionization source or a Hewlett-Packard (Palo Alto, CA) 5989B quadrupole mass spectrometer with an Analystica (Branford, CT) electrospray ion source. Both instruments were tuned to a peak width of 0.6 mass units over the entire mass range. During MS/MS, collision-induced dissociation was carried out using a collision energy of 25 eV and argon collision gas pressure of 2.7 μbar. During single quadrupole scanning, the scan range was m/z 230–330 at 3 sec/scan.

Liquid chromatography-mass spectrometry (LC-MS) was carried out using a Hypersil (Hewlett Packard Co., Wilmington, DE) BDS C18 column (2 × 250 mm) with the triple quadrupole mass spectrometer as detector. The solvent system consisted of a gradient from 75% water (containing 0.5% acetic acid, adjusted to pH 3.5) to 70% methanol in 50 min and then to 90% methanol over an additional 10 min at a flow rate of 180 μl/min. The entire HPLC eluate was analyzed by electrospray mass spectrometry without splitting. LC-MS analyses were carried out on 20-μl aliquots from imipramine incubations obtained from either the 90% methanol eluate from the ultrafiltration chamber (400 μl total) or from the reconstituted extract of an off-line incubation (200 μl total).

**Results and Discussion**

For the development of an on-line mass spectrometric method for the study of drug metabolism, compounds were chosen that were known to be substrates for hepatic cytochrome P450 isozymes. Although the concentrations of enzyme, cofactors, and substrate were selected to be similar to traditional microsomal incubation experiments, a volatile mobile phase buffered with ammonium acetate was used to prevent buffer contamination of the ionization source of the mass spectrometer. Since off-line microsomal incubations of imipramine in either ammonium acetate buffer or phosphate buffer showed identical results using LC-MS, the use of a volatile buffer did not alter the metabolic profiles. An ultrafiltration membrane with a 100,000 molecular weight cut-off was the minimum pore size membrane that was compatible with this system. When smaller pore size membranes...
were used (i.e. 10,000 molecular weight cut-off), membranes became clogged and ruptured because of excessive back pressure. The flow rate through the ultrafiltration chamber (70 μl/min) was a compromise between high flow rates for rapid analysis and low flow rates which would facilitate thorough mixing and reaction of substrate, cofactor, and microsomes before unreacted substrates and metabolites were flushed out of the chamber into the mass spectrometer. Higher flow rates would mean more rapid analysis but lower yields of metabolites.

The feasibility of on-line formation and detection of drug metabolites using pulsed ultrafiltration mass spectrometry is demonstrated in fig. 1A for the metabolism of chlorpromazine by microsomal cytochrome P450. The protonated molecule of unreacted chlorpromazine at m/z 319 was observed along with an overlapping peak of oxidized chlorpromazine, [MH+16]+ at m/z 335, which probably corresponded to chlorpromazine sulfoxide and 7-hydroxychlorpromazine (Muralidharan et al., 1996) and possibly small amounts of other monooxygenated metabolites. During the elution of chlorpromazine metabolites, the concentration of NADPH decreased as it was being consumed during the on-line reaction. Although the identification of specific monooxygenated metabolites of chlorpromazine would require an additional chromatographic step to separate isomeric metabolites (as illustrated by the imipramine example below), this on-line experiment provides a means to carry out on-line screening to determine whether a drug is metabolized by cytochromes P450.

As an example of an O-dealkylation reaction, pentoxyresorufin was injected into the ultrafiltration chamber containing cytochromes P450. For comparison, a control incubation was carried out which was identical except for the omission of the NADPH cofactor. The negative ion electrospray mass chromatograms showing the elution profiles for the deprotonated molecules of resorufin at m/z 212, which was the O-dealkylation product, and the substrate pentoxyresorufin at m/z 282, are shown in fig. 1B. As expected, the control incubation showed no evidence of metabolism, but pentoxyresorufin was extensively converted to resorufin by cytochromes P450 in the presence of NADPH. Negative ion electrospray was chosen because it produced abundant [M-H]− ions of the metabolite resorufin even though its precursor, pentoxyresorufin, did not ionize efficiently under these conditions. In this example and that of chlorpromazine above, liver microsomes were used from a rat that had not been treated with any compounds to induce cytochrome P450 activity. The on-line observation of metabolites formed using uninduced rat cytochromes P450 illustrates the great sensitivity of this method and suggests that use of uninduced human microsomes should be practical.

Although NADPH may be dissolved in the mobile phase at a constant concentration as illustrated for the on-line metabolism of chlorpromazine and pentoxyresorufin, multiple experiments of this type would become expensive. Furthermore, this on-line method is incompatible with NADPH regeneration systems. Therefore, we investigated co-injection of NADPH with substrate instead of maintaining a constant concentration of NADPH in the mobile phase. In fig. 1C, elution of imipramine and its major metabolites was monitored following co-injection of NADPH with imipramine. From the mass spectra recorded during the on-line incubation, computer-recon-
structured mass chromatograms for the elution of the three species were extracted and shown in fig. 1C including the protonated molecule of unchanged imipramine at m/z 281, monooxidation products, [MH+16]^+ at m/z 297, and N-desmethylimipramine at m/z 267. The control incubation, which contained no NADPH, showed no metabolism of imipramine. Because similar metabolic profiles were obtained when NADPH was added to the mobile phase, all subsequent experiments used the more cost effective co-injection approach.

Next, a direct comparison was made between imipramine metabolites formed on-line using pulsed ultrafiltration and off-line using the traditional batch incubation. Instead of using on-line mass spectrometric detection, metabolites formed in the ultrafiltration chamber were rapidly eluted using a methanol/water solution and then loaded onto a reversed phase HPLC column for LC-MS analysis. An aliquot of an extract from an off-line incubation of imipramine was analyzed in the same manner. Fig. 2 shows the LC-MS computer-reconstructed mass chromatograms for the major imipramine metabolites formed during off-line and ultrafiltration incubations. Both incubation methods produced the same metabolites in similar yields and in similar ratios. Hydroxylated metabolites predominated, although N-desmethylimipramine and imipramine N-oxide were also detected in both systems. Metabolite identification was based on molecular weight determined during LC-MS and fragmentation patterns observed during tandem mass spectrometry using collision induced dissociation. A summary of the positive ion tandem mass spectra for all major metabolites is shown in table 1. The monooxygenated metabolites 10-hydroxyimipramine, 2-hydroxyimipramine, and imipramine N-oxide could be distinguished based on their tandem mass spectra. Imipramine N-oxide showed an abundant ion at m/z 102, which corresponded to the monooxygenated polar side chain after the loss of the ring system (see table 1). The site of oxygenation could be localized to the amino group by observation of an ion at m/z 236, which was formed by elimination of HN(CH3)2O from the protonated molecule. In contrast, 10-hydroxyimipramine and 2-hydroxyimipramine showed an abundant ion for the unmodified side chain at m/z 86 instead of 102. Loss of water was observed only in the tandem mass spectrum of

![Computer-reconstructed mass chromatograms of the positive ion electrospray reversed phase LC-MS analyses of imipramine metabolites formed by incubation with rat liver cytochromes P450 using A) off-line batch incubation, or B) flow-through ultrafiltration.](image-url)

Identical amounts of substrate, NADPH, and microsomes were used in each incubation, and similar quantities of each incubation mixture were injected onto the LC-MS. These results show that similar metabolic profiles are obtained whether the standard off-line batch mode or the flow-through ultrafiltration method is used.
10-hydroxyimipramine (table 1).

The chromatograms in fig. 1 show that after injection of a drug approximately 1 hr is required to completely elute the unchanged drug and its metabolites from the ultrafiltration chamber containing microsomes. Furthermore, the concentration of the eluting metabolites reaches a maximum between 20–30 min after injection of the drug. To achieve high throughput metabolic screening using ultrafiltration mass spectrometry, a scheme was devised using multiple ultrafiltration chambers arranged in parallel with a single HPLC injector/autosampler and one mass spectrometer detector (see scheme in fig. 3). High throughput is achieved by injecting each drug sample into a different ultrafiltration chamber in the parallel array and then recording mass spectra of the metabolite mixture eluting from each chamber at a fixed time after injection. Constant flow of incubation buffer is maintained through all chambers, but only one chamber at a time is connected to the mass spectrometer. Drug metabolite profiles are obtained by recording mass spectra over a period of up to 1 min (if screening 60 samples per hour) or up to 3 min (20 samples per hour) when the maximum concentration of metabolites is eluting (approximately 20–30 min after the drug is injected).

Metabolism of imipramine, chlorpromazine, quinidine, and naringenin was evaluated using the 20-analyses-per-hour format described above, and control assays were carried out using heat inactivated microsomes. As expected, background ions but no ions of metabolites were observed in the control experiments (i.e., fig. 4A and 4C). Metabolites of imipramine and chlorpromazine were observed (for example, see fig. 4B and 4D).

### Table 1

<table>
<thead>
<tr>
<th>Metabolite (Retention Time, min)</th>
<th>[M+H]+</th>
<th>a1</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine (49.7)</td>
<td>281</td>
<td>86 (100)</td>
<td>58 (23.0)</td>
<td>236 (2.1)</td>
<td>208 (3.0)</td>
<td></td>
</tr>
<tr>
<td>Desmethyliimipramine (49.5)</td>
<td>267</td>
<td>72 (100)</td>
<td>58 (6.1)</td>
<td>252 (1.7)</td>
<td>224 (8.7)</td>
<td>209 (0.9)</td>
</tr>
<tr>
<td>2-Hydroxyimipramine (33.4)</td>
<td>297</td>
<td>86 (100)</td>
<td>58 (7.6)</td>
<td>[252-H2O]+</td>
<td>[224-H2O]+</td>
<td>[MH-H2O]+</td>
</tr>
<tr>
<td>10-Hydroxyimipramine (31.3)</td>
<td>297</td>
<td>86 (100)</td>
<td>58 (7.6)</td>
<td>234 (2.6)</td>
<td>206 (9.5)</td>
<td>279 (3.8)</td>
</tr>
<tr>
<td>Imipramine N-oxide (50.1)</td>
<td>297</td>
<td>102 (100)</td>
<td>236 (1.8)</td>
<td>208 (3.7)</td>
<td>72 (3.1)</td>
<td>84 (3.9)</td>
</tr>
</tbody>
</table>

1 Ion abundances (listed in parentheses) are normalized to the most abundant fragment ion in each set.
found in both the control and experimental incubations correspond to NADPH, naringenin is not extensively metabolized by cytochromes P450. Background ions m/z the ions of m/z 297 corresponding to monooxygenated imipramine in the control experiment shown in A), but extensive oxidation of imipramine is evident from the appearance of oxygenated metabolites at m/z 297 in B. For comparison, C) and D) show that naringenin is not extensively metabolized by cytochromes P450. Background ions found in both the control and experimental incubations correspond to NADPH, microsomes related compounds, and mobile phase contaminants.

The formation of metabolites is determined by comparing a control incubation (containing NADPH and inactive microsomes) to an incubation with active microsomes. As expected, no metabolites were observed in the control experiment shown in A), but extensive oxidation of imipramine is evident from the appearance of oxygenated metabolites at m/z 297 in B. For comparison, C) and D) show that naringenin is not extensively metabolized by cytochromes P450. Background ions found in both the control and experimental incubations correspond to NADPH, microsomes related compounds, and mobile phase contaminants.

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Ultrasound mass spectrometry has been demonstrated to be a new approach for the in-line investigation of microsomal drug metabolism. As a flow-through method, there is potential for automated high throughput screening to rapidly determine 1) whether a compound is a substrate for cytochromes P450, 2) how rapidly and extensively a compound is metabolized relative to other substrates, and 3) which cytochrome P450 isozyme is responsible for metabolic transformation of a substrate. Because mass spectrometric measurement of metabolites takes place immediately after enzymatic formation, there is also potential for this method to be useful for the identification of unstable, short-lived reactive metabolites that might not be stable enough to be observed after extraction from conventional microsomal incubations. Finally, ultrafiltration incubation procedures are sufficiently flexible that metabolites may be collected, extracted, and analyzed using conventional LC-MS and LC-MS-MS procedures.

Conclusions

Ultrafiltration mass spectrometry has been demonstrated to be a new approach for the on-line investigation of microsomal drug metabolism. As a flow-through method, there is potential for automated high throughput screening to rapidly determine 1) whether a compound is a substrate for cytochromes P450, 2) how rapidly and extensively a compound is metabolized relative to other substrates, and 3) which cytochrome P450 isozyme is responsible for metabolic transformation of a substrate. Because mass spectrometric measurement of metabolites takes place immediately after enzymatic formation, there is also potential for this method to be useful for the identification of unstable, short-lived reactive metabolites that might not be stable enough to be observed after extraction from conventional microsomal incubations. Finally, ultrafiltration incubation procedures are sufficiently flexible that metabolites may be collected, extracted, and analyzed using conventional LC-MS and LC-MS-MS procedures.

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