In Vitro Metabolism of the Analgesic Bicifadine in the Mouse, Rat, Monkey, and Human

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ABSTRACT:
The in vitro metabolism of [14C]bicifadine by hepatic microsomes and hepatocytes from mouse, rat, monkey, and human was compared using radiometric high-performance liquid chromatography and liquid chromatography/tandem mass spectrometry. Two main metabolic pathways were identified in all four species. One pathway was an NADPH-dependent pathway in which the methyl group was oxidized to form a hydroxymethyl metabolite (M2). Its formation was inhibited in human microsomes only by quinidine, a CYP2D6 inhibitor. In incubations with individual cDNA-expressed human cytochromes P450, M2 was formed only by CYP2D6 and CYP1A2, with CYP2D6 activity 6-fold greater than that of CYP1A2. M2 was oxidized further to the carboxylic acid metabolite (M3) by hepatocytes from all four species. The second major metabolic pathway was an NADPH-independent oxidation at the C2 position of the pyrroline ring, forming a lactam metabolite (M12). This reaction was almost completely inhibited in human hepatic microsomes and mitochondria by the monoamine oxidase (MAO)-B-specific inhibitor selegiline. Clorgyline, a specific inhibitor of MAO-A, was less effective in inhibiting M12 formation. Other metabolic pathways of variable significance among the four species included the formation of carboxamoyl-O-glucuronide, hydroxymethyl lactam, and carboxyl lactam. Overall, the data indicate that the primary enzymes responsible for the primary metabolism of bicifadine in humans are MAO-B and CYP2D6.

Bicifadine [(±)-1-(4-methylphenyl)-3-azabicyclo[3.1.0]hexane hydrochloride; DOV 220,075 HCl; Fig. 1], an inhibitor of norepinephrine and serotonin uptake, is being developed for the treatment of acute and chronic pain. Bicifadine is a non-narcotic analgesic (Epstein et al., 1982) that is active in models of neuropathic pain with no evidence of abuse liability potential (Basile et al., 2007). Clinically, it has been shown to be effective in the treatment of acute dental pain (Stern et al., 2005) and bunionectomy pain (Riff et al., 2006). Bicifadine was also reported to be as effective as the standard of care in reducing chronic lower back pain (Stern et al., 2006).

To interpret the significance of preclinical and toxicological investigations to humans, it is important to understand the metabolism of bicifadine and to demonstrate that the toxicity species are exposed to the same metabolites that occur in humans. Consequently, in vitro metabolism studies using hepatic microsomes and hepatocytes from mice, rats, monkeys, and humans were conducted using [14C]bicifadine. The metabolic profiles of [14C]bicifadine were compared in the four species using hepatic microsomes and hepatocytes. Additionally, the principal enzymes responsible for the metabolism of [14C]bicifadine were identified to determine the potential for bicifadine to be involved in drug-drug interactions.

During the course of the investigations, evidence was generated demonstrating that the formation of the main metabolite, a lactam, involved the action of an MAO, primarily MAO-B. Data are presented demonstrating that liver microsomes, in addition to mitochondria, have a high capacity for this reaction.

Materials and Methods

Chemicals. [14C]Bicifadine (44 mCi/mmol; 254 μCi/mg) was supplied by Vitrax Company (Placentia, CA); its radiochemical purity was >99% by HPLC. A reference standard of bicifadine was supplied by DOV Pharmaceutical Inc. (Somerset, NJ). Clorgyline, selegiline, phthalazine, phthalazinone, and perphenazine were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were obtained from commercial sources, and they were of the highest quality available.

Enzyme Preparations. Hepatic microsomes from male CD-1 mice, male Sprague-Dawley rats, and male cynomolgus monkeys were obtained from BD Biosciences (Woburn, MA). Pooled hepatic microsomes from male and female humans were obtained from CellzDirect (Pittsburgh, NC) or from Tissue Transplantation Technologies (Edison, NJ). Pooled human liver mitochondria from three male and three female donors were obtained from Xenotech, LLC (Lenexa, KS). Isolated primary hepatocytes from male mouse, male rat, male monkey, and human were supplied by CellzDirect. The human donor was a 54-year-old nonsmoking female with no known history of exposure to hepatitis B or C or human immunodeficiency virus. Recombinant (cDNA-
expressed human cytochromes P450, MAO-A, and MAO-B (Supersomes, microsomes prepared from baculovirus-infected BTI-TN-5B1-4 insect cells) were obtained from BD Biosciences.

**Incubations. Microsomal P450 activity.** Hepatic microsomal incubations were performed in 100 mM phosphate buffer containing 1 mM EDTA and 3 mM MgCl₂, pH 7.4, at a final assay volume of 1 ml and microsomal protein concentration of 0.25 mg/ml. Incubations were performed at 37°C, and they contained 1 mM NADPH and 10 μM bicinefedited. CYP1A2 and [14C]bicinefedited were combined to maintain radioactivity levels at approximately 1 × 10⁶ dpm/ml. Incubations were initiated by the addition of 10 μl of a 100-fold concentrated solution of [14C]bicifadine. Incubations were terminated at 0, 5, 10, 20, and 30 min by the addition of 2 ml of ice-cold acetonitrile. Control incubations were performed as described above at 0 and 30 min in the absence of NADPH. Heat-inactivated microsomes were prepared by heating in boiling water for 10 min. Terminated incubation samples were mixed, and the protein was removed by centrifugation at 1400 g for 10 min at 4°C. The supernatants were stored at −20°C until analysis. Control incubations were conducted at all time points by incubating the appropriate concentrations of [14C]bicifadine in Williams’ E medium in the absence of hepatocytes.

**HPLC Analysis.** Acetonitrile extracts from microsomal or hepatocyte incubations were mixed, concentrated to dryness using a stream of nitrogen, and reconstituted with 500 μl of 0.1% formic acid/acetonitrile [9:1 (v/v)]. The samples were then mixed, sonicated for 20 to 25 min, and analyzed by HPLC. Analysis was performed on an Alltech Prevail C18 column (4.6 × 150 mm, 5-μm particle size; Alltech Associates, Deerfield IL) preceded by a Phenomenex SecurityGuard C18 guard column (4 × 3 mm; Phenomenex, Torrance CA). The column was interfaced with a Packard Series A-500 radioactivity detector equipped with a 0.5-ml TRLSC flow cell (PerkinElmer Life and Analytical Sciences, Meriden, CT). The liquid scintillation flow rate was 3 ml of Ultima Flo M/min (PerkinElmer Life and Analytical Sciences). For most experiments, mobile phase A was 0.05% formic acid in water, mobile phase B was acetonitrile, and the flow rate was 1 ml/min. For metabolite identification, the initial mobile phase of 97:3 A/B was increased linearly to 90:10 A/B from 5 to 7 min and held constant until 25 min. The mobile phase was then increased linearly to 5:95 A/B from 25 to 36 min; after another 9 min, it was re-equilibrated to 97:3 A/B. For experiments using P450 inhibitors and cDNA-expressed enzymes, the initial gradient was 95:5 A/B; after 2 min, it was increased linearly to 50:50 A/B at 25 min. The mobile phase was increased to 10:90 A/B to remove any residual material, and then it was re-equilibrated to 95:5 A/B.

**LC/MS/MS Analysis.** For metabolite identification, the LC/MS/MS studies were conducted using a Shimadzu LC system controller and pumps (models SCL-10A VP and LC-10AD VP, respectively; Shimadzu, Kyoto, Japan). A Phenomenex Luna C18 HPLC column was used with the same mobile phases A and B and acetonitrile gradient as used for metabolite separation described above. After passing through a column switcher, the HPLC column effluent was split, with approximately 30% of the flow diverted to the mass spectrometer (Micromass Quattro II with an ESP Z-Spray source in the positive ion mode; Micromass Co., Manchester, UK) and 70% to the radiometric detector (2.1 ml of Packard Ultima-Flo M LSC cocktail/min). MS settings were as follows: cone voltage, 10 to 28 V; mass range, 40 to 600 amu; scan time, 0 to 40–45 min; source block temperature, 130°C; desolvation temperature, 300°C; electrospray ionization nebulizer gas, 15 l/H (N₂); and bath gas, 400 l/H (N₂). The product ion LC/MS/MS analyses used the same instrument conditions and conditions as for the full-scan LC/MS analyses with the following exceptions to the MS conditions: cone voltage, 22 V; collision energy, 15 to 24 eV, and collision gas, argon.

For the P450 reaction phenotyping experiments with bicifadine lactam, analyses were performed using a Shimadzu LC system controller and pumps (models SIL-HTC and LC-10AD VP, respectively). The HPLC columns, mobile phases, and acetonitrile gradient were the same as used for metabolite separation described above. After passing through a column switcher, the HPLC column effluent was split, with approximately 30% of the flow diverted to the mass spectrometer (Micromass Quattro II with an ESP Z-Spray source in the positive ion mode) and 70% to the radiometric detector (2.1 ml of Packard Ultima-Flo M LSC cocktail/min). MS settings were as follows: cone voltage, 10 to 28 V; mass range, 40 to 600 amu; scan time, 0 to 40–45 min; source block temperature, 130°C; desolvation temperature, 300°C; electrospray ionization nebulizer gas, 15 l/H (N₂); and bath gas, 400 l/H (N₂). The product ion LC/MS/MS analyses used the same instrumentation and conditions as for the full-scan LC/MS analyses with the following exceptions to the MS conditions: cone voltage, 22 V; collision energy, 15 to 24 eV, and collision gas, argon.

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**Results**

**Comparative Metabolism of [14C]Bicifadine.** [14C]Bicifadine was incubated with hepatic microsomes and hepatocytes from mouse, rat, monkey, and human. There were 16 radiolabeled peaks other than [14C]bicifadine that were observed among the incubations. Discrete metabolic peaks are labeled on radiochromatograms according to their order of elution. Representative radiochromatograms displaying metabolite profiles for the microsomes and hepatocytes are presented in Figs. 2 and 3, respectively, and the percentages of total radioactivity attributed to each of the metabolites are listed in Tables 1 and 2. Due
to the high specific activity of the [14C] used in the experiments, the protonated molecular ion and the radiolabel-containing product ions were 2 amu higher than that of the nonradioactive compound.

A potential metabolite (M13), constituting 4 to 14% of the profiled radioactivity among the incubations, was present in approximately equal amounts at 0 and 30 min of incubation. Based on LC/MS/MS analysis (m/z 204 of protonated molecular ion), it was putatively identified as N-formyl [14C]bicifadine. Since formic acid was a constituent of the sample resuspension solution and M13 formation was time- and NADPH-independent, it was considered to be an artifact formed during sample preparation.

Microsomes. In hepatic microsomal incubations, two principal metabolites of [14C]bicifadine were detected among the four species, M2 and M12. A major metabolite from all four species, M12, was identified as [14C]bicifadine lactam, formed by oxidation at the 2-position of the pyrrolidine ring. It constituted approximately 5 to 8% of the profiled radioactivity in rat and human microsomes, 18% in mouse microsomes, and 36% in monkey microsomes (Table 1). The formation of M12 did not require the presence of NADPH, and it formed equally well in incubations with or without NADPH. It had a protonated molecular ion at m/z 190, 16 amu higher than that of [14C]bicifadine. The product ion mass spectrum for m/z 190 gave ions at m/z 173 (–NH₃), 172 (–H₂O), 143, 134, 105, 98, and 57. The ion at m/z 105 indicated that the toluene moiety was not modified, whereas the ions at m/z 57 and 134 indicated that an oxidation had occurred at the 2-position of the pyrrolidine ring. [14C]M9 had the same product mass spectrum as an authentic standard of the compound (DOV 255,833), with the exception of the ions that were 2 amu higher due to the presence of the 14C-atom with a high specific activity (Supplemental Data Fig. 2). The second principal metabolite, M2, was approximately as abundant as M12 in rat and human, and it was identified as hydroxymethyl [14C]bicifadine. Its formation was NADPH-dependent. M2 had a protonated molecular ion at m/z 192, 16 amu higher than that of [14C]bicifadine. The product ion mass spectrum for m/z 192 gave ions at m/z 175 (–NH₃), 162, 157, 149 (–C₃H₃N), 145 (m/z 175 – C₂H₄), 130, and 121 (m/z 149 – C₂H₄). The presence of the ions at m/z 149 and 121 indicated that hydroxylation had occurred on the methyl group.

Other metabolites detected in radiochromatograms from microsomal incubations were M3 and M8. M3 was identified as carboxyl [14C]bicifadine, and it was present in hepatic microsomal incubations from all four species, although in human it was detectable by LC/MS only. The protonated molecular ion of M3 was at m/z 206, 30 amu higher than [14C]bicifadine. The product ion mass spectrum for m/z

FIG. 2. Radioflow HPLC analysis of [14C]bicifadine metabolites generated by hepatic microsomes from the mouse, rat, monkey, and human. [14C]Bicifadine (10 μM) was incubated with hepatic microsomes for 30 min at 37°C, and the suspensions were extracted and analyzed as outlined under Materials and Methods.
206 gave ions at \( m/z \) 189 (\(-\text{NH}_3\)), 171, 163 (\(-\text{C}_2\text{H}_3\text{N}\)), 145 (\(-\text{CO}_2\)), 135 (\(-\text{C}_2\text{H}_4\)), 130, 128, and 117. The ions at \( m/z \) 163 and 135 indicated that the methyl group had been oxidized to the carboxylic acid. M8 was identified as hydroxymethyl \([^{14}\text{C}]\)bicifadine lactam, and it was present in mouse and monkey incubations, although in mouse it was detectable by LC/MS only. The protonated molecular ion of M8 was at \( m/z \) 206, also 30 amu higher than \([^{14}\text{C}]\)bicifadine. The product ion mass spectrum for \( m/z \) 206 gave ions at \( m/z \) 188 (\(-\text{H}_2\text{O}\)), 174, 159 (\(-^{14}\text{CH}_3\text{NO}\)), 150, 129, 121, 105, 93, 84, and 57. The ion at \( m/z \) 121 indicated that hydroxylation had occurred on the methyl group. The differences in the way that M8 fragmented with respect to \([^{14}\text{C}]\)bicifadine indicated that a modification had occurred on the pyrrolidine ring. Specifically, ions at \( m/z \) 57 and 150 indicated that an oxidation had occurred at the 2-position of the pyrrolidine ring.

Three minor metabolites (MA, MB, and MC) were detected only in microsomal incubations in low amounts, and their formation was neither time- nor NADPH-dependent. Their protonated molecular ions are \( m/z \) 190, \( m/z \) 299, and \( m/z \) 215 for MA, MB, and MC, respectively; their identities are uncertain.

**Hepatocytes.** In hepatocyte incubations using 10 \( \mu \text{M} \) \([^{14}\text{C}]\)bicifadine, the principal metabolites, depending on the species, were M2, M3, M9, and M12. In all species, unchanged \([^{14}\text{C}]\)bicifadine was a minor component after 180 min of incubation (Fig. 3). In mice, two of the main metabolites were M3 and M12. The other

![Graph](https://example.com/graph.png)
The formation of the products was measured using HPLC with radioflow detection. The protonated molecular ion was at m/z 192, 16 amu higher than that of [14C]bicifadine. The major metabolite in monkey hepatocytes was M9, with a protonated molecular ion of m/z 159, 144, 133 (base peak, -C2H6), 130, 128, 117. The presence of the ion at m/z 135 indicated that elucidation had occurred at the C2 position of the pyrrolidine ring. The product mass spectrum of an authentic standard of M12 (DOV 255,828) had the same fragmentation as [14C]M12 with the exception of the ions due to the presence of the radiocarbon atom, which increased the amu by 2 (Supplemental Data Fig. 3).

In rat hepatocyte incubations, the major metabolite was M12, with lesser percentages of M3 and M9. Two minor metabolites, M5 and M6, had very similar mass spectra. Based on their protonated molecular ion (m/z 262) and fragmentation, both structures were deduced to be N-acetylated M9. The difference in their retention times may be due to the position of the acetyl group relative to the plane of the pyrrolidine ring. The immediate precursor to M5 and M6 could not be determined based on the present results.

The major metabolites in monkey hepatocytes were M9, M3, and M2. Also detected as significant metabolites in monkey hepatocytes were M8 and its O-glucuronide conjugate (M7). A glutathione adduct of [14C]bicifadine (M4) was detected only in incubations from monkey hepatocytes. The protonated molecular ion was at m/z 481, 305 amu higher than that of [14C]bicifadine; the specific site of attachment could not be determined.

In humans, the major metabolite was M12, with lesser amounts of M3. As in the rat, human hepatocytes produced a small amount of M10, with a protonated molecular ion of m/z 192, 16 amu higher than that of [14C]bicifadine. The presence of the ions at m/z 105 and 91 indicated that the hydroxylation had not occurred on the toluene group, whereas ions at m/z 59 and 134 indicated that oxidation had occurred at the C2 position of the pyrrolidine ring.

All four incubations produced limited amounts of the carbamoyl-O-glucuronide of [14C]bicifadine (M11). Its protonated molecular ion was 220 amu higher than that of the parent compound, and it had a number of similarities with that of [14C]bicifadine. The presence of the ions at m/z 105 and 91 indicated that the hydroxylation had not occurred on the toluene group, whereas ions at m/z 59 and 134 indicated that oxidation had occurred at the C2 position of the pyrrolidine ring. The proposed metabolic scheme for [14C]bicifadine is presented in Fig. 5.

**Reaction Phenotyping of [14C]Bicifadine Metabolism in Humans.** When 1 μM [14C]bicifadine was incubated with pooled human liver microsomes, M2 (Rt = 8.0 min) and M12 (Rt = 23.9 min) were the main metabolites formed (Fig. 6). Their identities were confirmed by LC/MS/MS. The absence of NADPH in the incubation mixture did not change the rate of formation of M12, and neither metabolite was formed by heat-deactivated microsomes (data not shown).

To determine which enzyme(s) were responsible for the formation
of the NADPH-dependent metabolite M2, experiments were performed using pooled microsomes with P450-selective inhibitors. Quinidine, a selective inhibitor of CYP2D6, was the only P450 inhibitor that decreased microsomal M2 formation rates (Fig. 7). This observation was confirmed using cDNA-expressed isozymes. cDNA-expressed CYP2D6 formed M2 at a rate of 0.053 pmol/min/pmol P450. CYP1A2 also produced M2, but at a much lower rate (0.006 pmol/min/pmol P450). No detectable formation of M2 was observed with cDNA-expressed CYP2A6, 2B6, 2C8, 2C9, 2C19, 2E1, or 3A4 (data not shown).

The oxidation of [14C]bicifadine to M12 in the absence of NADPH indicated that this reaction was catalyzed by neither P450 nor flavin-containing monooxygenase. Experiments were performed to investigate whether MAO could be involved in its formation. After preincubation of human liver microsomes with increasing concentrations of the MAO-B inhibitor selegiline, the formation of M12 declined by >90% from 37.7 to 2.35 pmol/min/mg protein (Table 4). CYP1A2 also produced M2, but at a much lower rate (0.006 pmol/min/pmol P450). No detectable formation of M2 was observed with cDNA-expressed CYP2A6, 2B6, 2C8, 2C9, 2C19, 2E1, or 3A4 (data not shown).

The kinetics of M12 formation was determined in pooled human hepatic microsomal incubations containing increasing concentrations of [14C]bicifadine (Fig. 8). The data suggested biphasic kinetics, although it was not possible to accurately estimate the low-affinity kinetic phase due to the range of concentrations tested. The high-affinity phase had apparent $K_m$ and $V_{max}$ values of 3.72 μM and 129 pmol/min/mg protein, whereas the values for the low-affinity phase were 328 μM and 1390 pmol/min/mg protein. The intrinsic clearance values ($V_{max}/K_m$) for the high- and low-affinity enzymes were calculated to be 34.7 and 4.2 μl/min/mg protein, respectively.

MAO is considered to be present mainly in the mitochondrial fraction of the cell. Therefore, [14C]bicifadine was incubated with pooled human mitochondria and increasing concentrations of the two MAO inhibitors. Similar to microsomal incubations, 1 μM clorgyline had little effect on the formation of M12 by mitochondria, whereas 1 μM selegiline decreased mitochondrial M12 formation rates from 17.3 to 2.67 pmol/min/mg protein (Table 4). As a confirmation of this observation, [14C]bicifadine was incubated with cDNA-expressed MAO-A and MAO-B and M12 was also formed at rates of 5.5 ± 1.1 and 2.6 ± 1.3 pmol/min/mg protein, respectively.

Although the action of MAO normally results in the cleavage of the carbon-nitrogen bond, the oxidation of the neurotoxin MPTP by MAO-B has been shown to stop at the formation of an imine (Chiba...
et al., 1985). Further oxidation to the MPTP lactam is due to the cytosolic enzyme AO (Yoshihara and Ohta, 1998). To check for residual activity in the cell fractions, the AO substrate phthalazine was incubated with liver cytosol, microsomes, and mitochondria in the presence and absence of \( \frac{1}{100} \)M perphenazine, an AO inhibitor. Cytosolic AO was inhibited 55% (Fig. 9), confirming the effectiveness of the inhibitor. Residual AO activity was also present in the microsomal and mitochondrial fractions, and it was inhibited 30 and 26%, respectively, by perphenazine. In contrast, the oxidation of \([14C]\)bicifadine to the lactam M12 was not affected by perphenazine, suggesting that AO is not involved in the formation of M12.

Although a minor metabolic pathway, P450 reaction phenotyping experiments were also conducted for the formation of M8 from M12. None of the P450-specific inhibitors were particularly effective against M8 formation. However, some degree of inhibition was observed with \( \alpha \)-naphthoflavone (30% inhibition, selective for CYP1A2), sulfaphenazole (9% inhibition, selective for CYP2C9), omeprazole (13% inhibition, selective for CYP2C19), and diethylthiocarbamate (14% inhibition, selective for CYP2E1). When M12 was incubated with a battery of cDNA-expressed P450s, M8 was formed to some extent by all of the enzymes examined except CYP2A6. However, the enzymes that most efficiently formed M8 were CYP2C19 (1.27 pmol/min/pmol P450), CYP1A2 (0.969 pmol/min/pmol P450), CYP2D6 (0.717 pmol/min/pmol P450), and CYP2E1 (0.367 pmol/min/pmol P450).

Discussion

The major metabolic pathways of \([14C]\)bicifadine were similar among the four species examined when the results using hepatic microsomes and isolated hepatocytes were compared. One of the two major metabolic pathways is initiated by hydroxylation of the methyl group to form M2. In human microsomes, this reaction is catalyzed predominately by CYP2D6, with minor involvement of CYP1A2. M2 is oxidized further in hepatocytes from all four species to the carboxylic acid metabolite M3. Based on the combined percentages of M2 and M3, approximately 15% of \([14C]\)bicifadine is metabolized via this pathway in rat and human hepatocytes, whereas 42% is metabolized by this pathway in the monkey. A clinical trial would be needed to determine whether concomitant administration of CYP2D6 inhibitors could affect the clearance of bicifadine.

Like bicifadine, M12 is also oxidized to a hydroxymethyl metabolite (M8). Thus, it was of interest to determine whether this reaction...
was also carried out by CYP2D6. A variety of cDNA-expressed human cytochromes P450 were capable of producing this metabolite, including CYP2D6. However, quinidine (selective for CYP2D6) was ineffective as an inhibitor of M8 formation in microsomal incubations, indicating that CYP2D6 is not involved in this reaction. Although CYP2D6 may have a limited capacity for M8 formation from M12, the enzymes primarily responsible for this reaction seem to be CYP1A2, CYP2C19, and CYP2E1.

The other major pathway for the metabolism of [14C]bicifadine is oxidation of the C2 carbon of the pyrrolidine ring to form the lactam metabolite M12. Approximately equal amounts were formed by microsomes in the presence and absence of NADPH from all four species, indicating that the oxidation was not due to either P450 or flavin-containing monooxygenase. The reaction is catalyzed mainly by MAO-B as determined by the addition of the specific inhibitor selegiline to incubations with microsomes and mitochondria. Formation of M12 declined by 94% in the presence of 1 μM selegiline compared with control incubations. MAO-A may be involved to a lesser extent since the formation of M12 declined approximately 11% when 1 μM clorgyline was added to the microsomal suspensions.

Lactam formation from an aliphatic carbon–nitrogen bond has been shown to be due to the sequential action of MAO-B (Chiba et al., 1985), or P450 (Cashman et al., 1992), followed by oxidation of the resulting imine by cytosolic AO (Brandlänge and Lindblom, 1979; Yoshihara and Ohta, 1998; Vickers and Polsky, 2000). The presence of residual AO activity in the microsomal and mitochondrial fractions precludes eliminating the involvement of this enzyme in the formation of M12. But, the addition of the AO inhibitor perphenazine to the incubations had no effect on the oxidation of bicifadine to M12. Also, AO oxidizes quaternary azaheterocyclic imines to lactams, but it has little to no activity on tertiary azaheterocyclic imines (Beedham, 2002). The putative intermediate imine of bicifadine formed by MAO-B would have a tertiary structure. Although the present findings indicate that MAO is critical to the formation of the bicifadine lactam M12, the identification of the enzyme that catalyzes the oxidation of the putative imine intermediate to the lactam requires further investigation.

The inhibition of M12 formation in hepatic microsomes by MAO-B inhibitors was unexpected since this is considered a mitochondrial enzyme (Schnaitman et al., 1967). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is oxidized to MPDP+ predominantly by mitochondrial MAO-B; the microsomal fraction produced mainly the N-desmethyl and N-oxide metabolites but very little 1-methyl-4-phenyl-2,3-dihydropyridinium (Yoshihara and Ohta, 1998). The preparations used to demonstrate the conversion of milacemide to glycine by MAO-B were
enriched in mitochondria (Janssens de Varebeke et al., 1988). Formation of M12 is not due to the semicarbazide-sensitive amine oxidase because the reaction is sensitive to selegiline, and bicifadine is not a primary amine (Kinemuchi et al., 2004). Nonetheless, others have reported significant MAO activity in the microsomal fraction. For example, Salva et al. (2003) found almotriptan metabolism by MAO in both the microsomal and mitochondrial fractions of human liver. Yu et al. (2003) documented MAO activity for the formation of tryptophol from tryptamine in human liver microsomes. Goñi et al. (1988) demonstrated the presence of MAO-A and MAO-B activity in rat liver microsomes using enzyme-specific substrates and inhibitors and changes in temperature and pH sensitivity that were similar to those in mitochondria. Iwasa et al. (2003) found that MAO activity for the metabolism of rizatriptan was similar between the microsomal and mitochondrial fractions of Japanese liver donors. In the present study, microsomal MAO activity seems to be qualitatively similar to that in the mitochondrial preparation. However, unlike other examples of microsomal MAO activity, the formation of M12 from bicifadine is a major metabolic pathway.

M12 is oxidized further to M9, mainly by mouse and monkey hepatocytes, and it is a minor metabolite in rat hepatocytes; it is not detectable in human hepatocytes. The initial hydroxymethyl intermediate (M8) and its $O$-glucuronide (M7) were detected only in monkey hepatocyte incubations, but reaction phenotyping studies demonstrated a low capacity for M8 formation by human hepatic microsomes. Although the acid lactam M9 can potentially be formed from M2 or M3, the results from the mouse and monkey hepatocyte incubations indicate that M12 is the more likely precursor to this metabolite. Both M12 and M8 had much higher concentrations after 1 h of incubation compared with 3 h in these two species, whereas M9 increased 3- to 5-fold over the same period. These changes are indicative of a precursor–product relationship among M12, M8, and M9.

$^{14}$C]Bicifadine was also metabolized to the carbamyl-$O$-glucuro-
nide conjugate M11 by all four species. Similar glucuronides have been reported for tocainide (Elvin et al., 1980; Kwok et al., 1990), rimantadine (Brown et al., 1990), carvedilol (Schaefer, 1992), the benzazepine SK&F 86466 (Straub et al., 1988), and sertraline (Tremaine et al., 1989). This suggests to be a minor pathway for the metabolism of bicifadine in comparison with oxidation of the methyl group and the pyrrolidine ring.

In summary, there are two main metabolic pathways of bicifadine in mouse, rat, monkey, and human microsomes and hepatocytes. One pathway is the hydroxylation of the methyl group (via CYP2D6 in human) with subsequent oxidation to the carboxylic acid. The other pathway is oxidation of the pyrrolidine ring, initiated by MAO-B, to form the lactam metabolite. Furthermore, it seems that lactam formation is a major metabolic route for bicifadine in microsomes, being as active as P450 for [14C]bicifadine metabolism in humans.

References


