IDENTIFICATION OF THE HUMAN LIVER ENZYMES INVOLVED IN THE METABOLISM OF THE ANTIMIGRAINE AGENT ALMOTRIPTAN

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ABSTRACT:

Almotriptan is a novel highly selective 5-hydroxytryptamine 1B/1D agonist developed for the acute oral treatment of migraine. In vitro metabolism of almotriptan has been investigated using human liver subcellular fractions and cDNA-expressed human enzymes, to study the metabolic pathways and identify the enzymes responsible for the formation of the major metabolites. Specific enzymes were identified by correlation analysis, chemical inhibition studies, and incubation with various cDNA expressed human enzymes. Human liver microsomes and S9 fraction metabolize almotriptan by 2-hydroxylation of the pyrrolidine group to form a carbinolamine metabolite intermediate, a reaction catalyzed by CYP3A4 and CYP2D6. This metabolite is further oxidized by aldehyde dehydrogenase to the open ring \( \gamma \)-aminobutyric acid metabolite. Almotriptan is also metabolized at the dimethylaminoethyl group by N-demethylation, a reaction that is carried out by five different cytochrome P450s, flavin monooxygenase-3 mediated N-oxidation, and MAO-A catalyzed oxidative deamination to form the indole acetic acid and the indole ethyl alcohol derivatives of almotriptan. The use of human liver mitochondria confirmed the contribution of MAO-A to the metabolism of almotriptan. Both, the \( \gamma \)-aminobutyric acid and the indole acetic acid metabolites have been found to be the major in vivo metabolites of almotriptan in humans. In addition, different clinical trials conducted to study the effects of CYP3A4, CYP2D6, and MAO-A on the pharmacokinetics of almotriptan confirmed the involvement of these enzymes in the metabolic clearance of this drug and that no dose changes are required in the presence of inhibitors of these enzymes.

Almotriptan\(^1\) (3-(2-dimethylaminoethyl)-5-(1-pyrrolidinylsulfonylmethyl)-1H-indole) is a new and selective 5HT\(_{1B/1D}\) agonist developed by Almirall Prodesfarma for the oral treatment of acute migraine attacks. The pharmacodynamic profile of almotriptan has been extensively investigated using in vitro and in vivo experimental models (Bou et al., 2000; Gras et al., 2000a). In human vessels almotriptan selectively contracts migraine-related arteries (meningeal vasculature) but is less effective with peripheral vessels (e.g., pulmonary artery) (Bou et al., 2001). Almotriptan also exhibited less spasmodogenic effect on cardiac arteries and therefore an improved vascular profile compared with the reference compound sumatriptan (Gras et al., 2000b). Oral almotriptan has a rapid onset of action and a significant headache relief is observed 0.5 h after administration of a 12.5-mg dose with efficacy sustained in most patients who respond by 2 h (Pascual et al., 2000; Cabarrocas et al., 2001; Dodick, 2001).

Examination of the urinary profiles following oral administration of almotriptan to healthy volunteers showed two major phase I metabolites corresponding to a carboxylic acid metabolite formed by pyrrolidine ring oxidation and opening and the indole acetic acid metabolite (unpublished data). The pathways of oxidation of almotriptan in man have been investigated in vitro to identify the enzymes involved in these reactions. The cytochrome P450-dependent monooxygenase system plays an important role in the metabolism of a wide variety of xenobiotics and therefore, we have used human liver microsomes, P450 selective inhibitors and cDNA-expressed P450s as tools for the identification of the isoenzymes involved in the metabolism of almotriptan (Wrighton and Stevens, 1992; Wrighton et al., 1993). Different receptor agonists closely related structurally to serotonin (5-hydroxytryptamine) such as sumatriptan, zolmitriptan, or rizatriptan, have been shown to be metabolized by oxidative deamination to their corresponding indole acetic acid derivatives by liver monoamine oxidase (Dixon et al., 1994; Wild et al., 1999; Vyas et al., 2000). Consequently, we have used human liver mitochondria to further characterize the contribution of MAO to the overall metabolism of almotriptan. In addition, zolmitriptan and rizatriptan have been shown to be metabolized by N-oxidation of their dimethylaminoethyl moiety and therefore, the involvement of flavin-containing oxidases in the metabolism of almotriptan has also been studied.

Materials and Methods

Chemicals. \(^{14}\)C-Almotriptan (32.5 mCi/mmol; thin layer chromatography radiochemical purity >98%) was synthesized by Huntingdon Life Sciences (Huntingdon, UK). Almotriptan and its metabolites M2 (\( \gamma \)-aminobutyric acid derivative, LAS-31911), M4 (N-desmethyl almotriptan, LAS-31612), M5 (almotriptan N-oxide, LAS-32195), M6 (indoleacetic acid derivative, EX01–
liver microsomes, 1 mM NADP and isovanillin were purchased from Sigma-Aldrich (St. Louis, MO). Clorgyline 5-hydroxytryptamine and benzylamine as substrates, respectively. Thoflavone and quinidine were purchased from Aldrich-Chemie (Steinheim, Germany). O-Naphthoflavone and quinidine, phenylmethylsulfonyl fluoride (PMSF), tetraethylthiuram disulfide (disulfiram), diethyliodihydrate (DDTC), menadione, allopurinol, dopamine hydrochloride, and isovanillin were purchased from Sigma-Aldrich (St. Louis, MO). C17-glycerol and R(-)-depenyl were purchased from RBI/Sigma (Natick, MA). Ketoneozone was provided by Impex Quimica (Barcelona, Spain) and (±)-mephenytoin was purchased from Ultrafine Chemicals (Manchester, UK). HPLC grade methanol and acetonitrile were purchased from Reactivos Scharlau (Barcelona, Spain). All other chemicals and reagents were of the highest commercially available quality.

Human Liver Subcellular Fractions. Pooled human liver S9 fraction and microsomes were supplied by Human Biologics Inc. (Phoenix, AZ) and XenoTech LLC (Kansas City, KS). Human liver microsomes from 14 individual donors were purchased from Human Biologics Inc. (HepatoScreen test kit). Microsomal preparations of 10 different recombinant human P450 isoforms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) were expressed in human B lymphoblastoid cell line AHH-1 and recombinant human liver flavin monoxygenase (FMO-3) were purchased from BD Gentest Corp. Human liver mitochondria were supplied by XenoTech LLC, which also provided the kinetic constants (Km and Vmax) for MAO-A and MAO-B using 5-hydroxytryptamine and benzylamine as substrates, respectively.

Incubation Conditions. Incubations of 14C-almotriptan with human liver microsomes and S9 fraction. Reaction mixtures (200 μl) containing human liver microsomes, 1 mM NADP+, 5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, 12 mM MgCl2, and 14C-almotriptan in 100 mM sodium phosphate (pH 7.4) were incubated at 37°C in a shaking water bath for 30 min. The reactions were stopped by the addition of 0.8 ml of 0.2M sodium acetate buffer (pH 4), and samples were ice-cooled for 10 min and centrifuged at 3000 g for 10 min. Clear supernatants were stored at −20°C until analysis. The effect of variations in the incubation time (10–240 min) and protein concentration (0.5–4 mg/ml) upon the extent of 14C-almotriptan metabolism were investigated. The concentration range for the study of almotriptan metabolism kinetics was 1 to 2000 μM. All the incubations throughout the study were carried out in duplicate.

Incubations of nonradiolabeled almotriptan with human liver microsomes and S9 fraction. Reaction mixtures (500 μl) containing 1 mg of protein (microsomal or S9 fraction), 1 mM NADP+, 5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, 12 mM MgCl2, and 14C-almotriptan in 100 mM sodium phosphate (pH 7.4) were incubated at 37°C in a shaking water bath for 30 min. The reactions were stopped by the addition of 1 ml of 0.2M sodium acetate buffer (pH 4), and the samples were centrifuged and stored at −20°C until analysis.

Incubations with pooled human liver mitochondria. Almotriptan (50, 100, and 400 μM) was incubated with human liver mitochondria (0.5 mg/ml) in a final volume of 200 μl of 10 mM potassium phosphate buffer (pH 7.4). Samples were incubated at 37°C in a shaking water bath for 90 min. The reactions were stopped by the addition of 1 ml of 0.2M sodium acetate buffer (pH 4) containing the internal standard LAS-31936 at a concentration of 200 μM. PMSF, disulfiram, and (−)-mephenytoin were added to the incubation mixture (final concentration, 1% methanol). DDTC and troleandomycin were preincubated in the presence of the NADPH-generating system and microsomes for 10 min at 37°C before adding the substrate. The following inhibitors and concentrations were studied: proadifen (P450 non-specific; 100 and 200 μM), O-naphthoflavone (CYP1A2; 0.1, 0.5, 2, and 10 μM), sulfaphenazole (CYP2C9/10; 0.5, 2, 10, and 50 μM), (±)-mephenytoin (CYP2C19; 5, 20, 100, and 500 μM), clorgyline (CYP2D6; 0.2, 1, 5, 20 μM), DDTC (CYP2E1; 0.5, 2, 10, and 50 μM), ketoneozone (CYP3A4; 0.05, 0.2, 1, and 5 μM), troleandomycin (CYP3A; 0.5, 2, 10, and 50 μM), clorgyline (MAO-A; 2, 20, 100, and 200 μM) and deprenyl (MAO-B; 2, 20, 100, and 200 μM).

The involvement of molybdenum hydroxylases and alcohol and/or aldehyde dehydrogenases in the metabolism of almotriptan was studied using nonradio-labelled almotriptan and the following inhibitors and concentrations: PMSF (200 μM), disulfiram (2–500 μM), DDTC (2–500 μM), menadione (200 μM), allopurinol (200 μM), and isovanillin (200 μM). PMSF, disulfiram, and menadione were dissolved in ethanol and dispensed into clean test tubes with a microsyringe. The solvent was then removed under a stream of nitrogen and the residue resuspended in phosphate buffer. DDTC, allopurinol and isovanillin were dissolved directly in phosphate buffer.

The effect of monoamine oxidase inhibitors on the metabolism of almotriptan by human liver mitochondria was studied by incubation of different concentrations of the inhibitors and three concentrations of almotriptan (50, 100, and 400 μM). For the reversible metabolism inhibition study, human liver mitochondria (0.5 mg/ml) were incubated for 90 min at 37°C in the presence of dopamine (50, 100, 500, and 1000 μM) and almotriptan. For the irreversibly-independent inhibition, human liver mitochondria were preincubated at 37°C in incubations containing clorgyline (0.001, 0.01, 0.1, 0.25, 0.5, 1, and 5 μM) or deprenyl (0.1, 0.25, 0.5, 1, and 5 μM). After 20 min almotriptan was added, and another incubation was carried out at 37°C for 90 min.

Correlation Study. The correlation of the formation of 14C-almotriptan metabolites with P450 isoform-specific activities in the microsomes from a panel of 14 human livers (HepatoScreen test kit, Human Biologics Inc.) was studied at a substrate concentration of 100 μM. Statistical analysis was performed using Instat v2.01 (GraphPad Software Inc., San Diego, CA). The correlation parameter used was Spearman correlation coefficient (r). Two-tailed Student’s t test for paired data were used to calculate p values.

Sample Preparation and HPLC Analyses. Almotriptan and metabolites were extracted from the incubation samples using solid phase cartridges AASP-C18 (Varian SP Products, Harbor City, CA). Cartridges were activated with 1.8 ml of methanol and 1.8 ml of water, not allowing the cartridge to dry out. Samples were then loaded and the cartridge finally rinsed with 1.8 ml of water. Cassettes containing 10 cartridges were then loaded in the AASP system for automated injection.

The chromatographic system consisted of a model 1050 high pressure gradient pump (Hewlett Packard Analytical Direct, Wilmington, DE) or a model 515 HPLC pump (Waters, Milford, MA), an AASP solid phase injector (Vian Medical Systems, Pax Alto, CA). Model 753 LC tunable absorbance detector (Kontron Instruments, Zürich, Switzerland), a model Ramona-D radioactivity (4H/14C) detector (Raytest, Straubenhardt, Germany) and an Alpha Server 1000 4/266 computer (Compaq, Houston, TX) with Acces® Chrom software (Perkin Elmer Nelson Systems Inc., San Jose, CA) for data acquisition and processing.

Separations of 14C-almotriptan and metabolites were carried out on a Spherisorb ODS-2 analytical column (5-μm particle size; 4.6 × 250 mm; Tracer, Teknokroma, Spain) protected with a Guardpak μBondapak CN guard-column (Waters). The mobile phase consisted of solvent A (50 mM, pH = 4 sodium phosphate buffer, containing 0.2% triethylamine) and solvent B (acetonitrile/solvent A; 1:1; v/v). After 10 min of isocratic separation at 30%
radioactivity. Human liver microsomes converted 14C-almotriptan to six different metabolites: M2, M3, M4, M5, M6, and M7. The chemical structures of the metabolites are shown in Fig. 1. The oxidation of the pyrrolidine moiety proceeds further to the open ring γ-aminobutyric acid metabolite M2, a reaction that was inhibited by the same compounds that inhibit the hydroxylation pathway and by DDTC (57%) and sulfaphenazole (30%). On the other hand, the formation of metabolite M4 (N-desmethyl almotriptan) was partially inhibited by several inhibitors suggesting that various P450 isoforms can catalyze this reaction.

The oxidative deamination of 14C-almotriptan to the indoleacetic acid derivative M6 was inhibited up to 70% by ketoconazole, but not by troleandomycin, suggesting that an enzyme different from CYP3A4 was involved in this pathway. The formation of the indole ethyl alcohol metabolite M7 was also inhibited by ketoconazole.

Results

Metabolism of 14C-Almotriptan by Human Liver Microsomes.

Human liver microsomes converted 14C-almotriptan to six different metabolites, designated M2, M3, M4, M5, M6, and M7 based on their HPLC retention times. All these metabolites had been previously identified by mass spectrometry and nuclear magnetic resonance after isolation from urine (M2, M4, M5, and M6) or in vitro samples (M3 and M7). The chemical structures of the metabolites are shown in Fig. 1. The formation of metabolites M6 and M7 was not dependent on the presence of the NADPH-generating system, suggesting that an enzyme system different from cytochrome P450 or nonenzymatic catalysis was involved.

Preliminary experiments on the effects of variations in the incubation conditions (e.g., protein concentration, NADPH-generating system composition and incubation time) were carried out to optimize the rates of formation of the different metabolites. The formation of M2 and M6 was linear up to 4 mg/ml of microsomal protein concentration and 120 min of incubation. By contrast, for the other metabolites an incubation time of 30 min and a protein concentration of 2 mg/ml was a good compromise between linearity and metabolite final concentrations.

Figure 2 shows the effect of substrate concentration on the rate of 14C-almotriptan metabolism by human liver microsomes. The formation of the metabolites M4, M5, M6, and M7 followed monophasic Michaelis-Menten kinetics, suggesting that a single enzyme is predominantly involved in its formation. By contrast, the rates of formation of metabolites M2 and M3 are rapid at first but then decline at high substrate concentrations (1–2 mM). This observation could be explained by substrate inhibition of the enzyme(s) responsible for the formation of M3. However, the relevance of this effect was considered minor since the Cmax of almotriptan at the therapeutic dose of 12.5 mg is ca. 0.15 μM. The kinetic parameters for the formation of the different metabolites are summarized in Table 1. Nonlinear regression analysis of the untransformed data were used to calculate the apparent Kmax and Vmax values.

Chemical Inhibition. Troleandomycin and ketoconazole inhibited the hydroxylation of the pyrrolidine moiety of 14C-almotriptan to M3 by 90% (IC50 = 0.13 μM) and 80% (IC50 = 1.8 μM), respectively. Quinidine also inhibited the formation of M3 by 23% (IC50 < 0.2 μM). The oxidation of the pyrrolidine moiety proceeds further to the open ring γ-aminobutyric acid metabolite M2, a reaction that was inhibited by the same compounds that inhibit the hydroxylation pathway and by DDTC (57%) and sulfaphenazole (30%). On the other hand, the formation of metabolite M4 (N-desmethyl almotriptan) was partially inhibited by several inhibitors suggesting that various P450 isoforms can catalyze this reaction.

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solvent B, a linear gradient was programmed from 30 to 50% solvent B in 20 min, the final conditions were held for 10 min. The mobile phase flow rate was 1 ml/min, and the eluate was monitored by UV detection at 227 nm and 14C radioactivity.

Calibration curves of 14C-almotriptan were prepared over the range 0.2 to 100 μM. All calibration samples were extracted and analyzed as real samples. Validation of the analytical method was carried out to establish linearity, precision, and accuracy. Metabolite identification was carried out by cochromatography with authentic metabolite standards and by mass spectrometry.

Incubation mixtures of nonradiolabelled almotriptan and human liver microsomes were analyzed using a μBondapak analytical column (10-μm particle size, 3.9 × 300 mm; Waters), protected with a Guardpak μBondapak CN guard column (Waters). The mobile phase consisted of solvent A (10 mM ortho-phosphoric acid and 0.1% triethylamine adjusted to pH = 6.5 with sodium hydroxide) and solvent B (acetonitrile/solvent A, 80:20, v/v). A linear gradient (1 ml/min) was programmed from 20 to 60% solvent B in 30 min. The eluate was monitored at 227 nm.

Incubation samples of almotriptan and human liver mitochondria were analyzed by isocratic HPLC using a Spherisorb ODS-2 analytical column (5-μm particle size, 250 × 4.6 mm; Waters) protected with a precolumn Guard-Pak μBondapak CN (Waters). The mobile phase consisted of acetonitrile/sodium phosphate buffer containing 0.2% triethylamine (50 mM; pH 4.3) (22:78, v/v), the flow rate was 1 ml/min, and the eluate was monitored at 227 nm.

Figure 2 shows the effect of substrate concentration on the rate of 14C-almotriptan metabolism by human liver microsomes. The formation of the metabolites M4, M5, M6, and M7 followed monophasic Michaelis-Menten kinetics, suggesting that a single enzyme is predominantly involved in its formation. By contrast, the rates of formation of metabolites M2 and M3 are rapid at first but then decline at high substrate concentrations (1–2 mM). This observation could be explained by substrate inhibition of the enzyme(s) responsible for the formation of M3. However, the relevance of this effect was considered minor since the Cmax of almotriptan at the therapeutic dose of 12.5 mg is ca. 0.15 μM. The kinetic parameters for the formation of the different metabolites are summarized in Table 1. Nonlinear regression analysis of the untransformed data were used to calculate the apparent Kmax and Vmax values.

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Moreover, the formation of M6 and M7 was not dependent on the presence of the NADPH-generating system in the incubation medium. The addition of clorgyline (MAO-A inhibitor), completely blocked the formation of both M6 and M7 over the inhibitor concentration range (2–200 μM). Extensive inhibition was also observed in the presence of deprenyl (MAO-B inhibitor), although at the lowest concentration tested (2 μM), both metabolites could be detected.

Correlation with P450 Marker Activities. Table 2 shows the results obtained for the sample-to-sample variation in 14C-almotriptan metabolism by liver microsomes from a panel of 14 human livers. The formation of M3 correlated well with dextromethorphan N-demethylation (3A4) \( (r_c = 0.6999, p < 0.0062) \), testosterone 6β-hydroxylation (3A4/5) \( (r_c = 0.7423, p < 0.0024) \), and dextromethorphan O-demethylation (2D6) \( (r_c = 0.6440, p < 0.0129) \). By contrast, the oxidation of M3 to M2 only correlated with CYP2D6 marker activity \( (r_c = 0.7181, p < 0.0038) \).

The N-demethylation of the 2-dimethylaminoethyl group of almotriptan to M4 strongly correlated with dextromethorphan N-demethylation (3A4) \( (r_c = 0.8779, p < 0.0001) \) and testosterone 6β-hydroxylation (3A4/5) \( (r_c = 0.8899, p < 0.0001) \) and presented a weak correlation with CYP1A2 marker activity.

None of the P450 activities correlated with almotriptan N-oxidation to M5. The formation of the indole acetic acid metabolite M6 presented a weak correlation with CYP2E1 marker activity, whereas the formation of the indole ethyl alcohol derivative M7 presented only inverse correlations with CYP3A markers.

Metabolism by cDNA-Expressed Human Enzymes. Three recombinant P450 enzymes catalyzed the hydroxylation of 14C-almotriptan to M3, namely CYP1A1, CYP3A4, and CYP2D6. Six of ten P450s catalyzed the N-demethylation of the 2-dimethylaminoethyl group: CYP1A1, CYP1A2, CYP2C8, CYP2C19, CYP2D6, and CYP3A4. None of the cDNA-expressed P450s was able to generate metabolites M2 or M6, whereas M5 and M7 were only produced by CYP1A1. Table 3 shows the rates of formation of the different metabolites by recombinant P450 enzymes.

Incubation of almotriptan with cDNA-expressed human FMO-3 in the presence of NADPH produced a single metabolite at the same retention time of the N-oxide metabolite standard LAS-32195. The \( K_m \) value for almotriptan N-oxidation by recombinant FMO-3 was 6.3 mM, and the \( V_{max} \) was 1.83 nmol/min/mg of protein.

Metabolism by Human Liver Mitochondria. Human liver mitochondria converted almotriptan to two metabolites identified by co-chromatography with authentic reference standards as the indole acetic acid (M6) and the indole ethyl alcohol (M7) derivatives of almotriptan. These metabolites also present in human liver microsomal incubates, are formed by oxidative deamination of the 2-dimethylaminoethyl sidechain to an acetaldehyde intermediate, which is further oxidized to M6 and reduced to M7. The effect of substrate concentration on the metabolism of almotriptan by human liver mitochondria is shown in Table 1 and Fig. 3. The apparent \( K_m \) values for M6 and M7 were 62 and 363 μM, respectively. The \( V_{max} \) for the formation of these metabolites by human liver mitochondria were 41.7 pmol/min/mg of protein (M6) and 68.7 pmol/min/mg of protein (M7).

Dopamine, a reversible inhibitor of MAO-A and MAO-B inhibited the formation of M6 and M7 by liver mitochondria with \( K_i \) values of 0.8 and 3.2 μM, respectively. Clorgyline, a metabolism-dependent inhibitor of MAO-A completely inhibited the formation of mitochondrial metabolites within the concentration range of 0.1 to 5 μM (Fig. 4). Conversely, deprenyl, the metabolism-dependent inhibitor of MAO-B was a weaker inhibitor, and complete inhibition of almotriptan metabolism only occurred at the highest concentration tested (5 μM).

Role of Aldehyde Dehydrogenase in the Metabolism of Almotriptan. Preliminary experiments carried out using rat liver subcellular fractions showed that M3 was the major metabolite formed by liver microsomes, whereas M2 was the main metabolite in incubations with liver S9 fraction. This observation suggested the presence of a NAD(P)-dependent enzyme in rat liver cytosol that converts M3 to M2. In human liver microsomes, M3 was metabolized to M2 by a microsomal enzyme, although the presence of the same rat enzyme in human liver microsomes as a contaminant could not be discarded.

As a hypothesis the mechanism of enzymatic formation of M2 was attributed to aldehyde dehydrogenase that would oxidize the γ-aminobutyraldehyde derivative of almotriptan formed by tautomeration of the carbinolamine M3. Moreover, the initial aldehyde produced by MAO can be converted to the corresponding carboxylic acid M6 and alcohol M7 either by spontaneous oxidation/reduction, which likely explains why human liver microsomes can convert almotriptan to M6 and M7 in the absence of NADPH, but also by aldehyde dehydrogenase and alcohol dehydrogenase mediated metabolism.

To demonstrate the involvement of aldehyde dehydrogenase in the metabolism of almotriptan, inhibitors of this enzyme such as disulfiram and DDTC (Hu et al., 1997; Lipsky et al., 2001) were incubated with almotriptan and human liver subcellular fractions. Since aldehydes can also be oxidized by aldehyde oxidase (AO) or xanthine oxidase (XO), inhibitors of these enzymes were tested for its ability to...
interfere with the formation of M2 (Beedham, 1985; Clarke et al., 1995). PMSF, an inhibitor of serine proteases such as trypsin and chymotrypsin, and of mammalian acetylcholinesterase (Allid and Orrego, 1972), was also included in the study to rule out the presence of high concentrations of the AO substrate analog isovanillin do not affect the formation of M3 and M7. Allopurinol, the xanthine oxidase inhibitor, had no effect on almotriptan metabolism, while PMSF interfered with the formation of M2 by human liver microsomes. Similar results were obtained when human liver S9 fraction was used instead of microsomal preparations.

Menadione, the aldehyde oxidase inhibitor, inhibited the P450- and MAO-mediated metabolism of almotriptan by human liver microsomes and hindered the study on the possible role of AO in this metabolic pathway. However, the presence in the incubation medium of high concentrations of the AO substrate analog isovanillin do not affect the formation of M3 and M7. Allopurinol, the xanthine oxidase inhibitor, had no effect on almotriptan metabolism, while PMSF produced only a slight inhibition on the formation of M2 and M6.

**Discussion**

In human liver microsomes, 14C-almotriptan undergoes NADPH-dependent metabolism to four metabolites (M2, M3, M4 and M5) and by human liver microsomes. Similar results were obtained when human liver S9 fraction was used instead of microsomal preparations.

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**Discussion**

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**TABLE 1**

Apparent kinetic parameters for almotriptan biotransformation by human liver microsomes and mitochondria calculated by non-linear regression analysis of the untransformed data.

<table>
<thead>
<tr>
<th>Liver Fraction</th>
<th>Metabolite</th>
<th>Apparent K_m (µM)</th>
<th>Apparent V_max (pmol/min/mg)</th>
<th>CL_int (µl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>M2</td>
<td>102 (47)^a</td>
<td>401 (5)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>106 (35)</td>
<td>951 (8)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>434 (45)</td>
<td>1996 (12)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>387 (86)</td>
<td>56.0 (4)</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>387 (39)</td>
<td>93.8 (6)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>1245 (148)</td>
<td>304.3 (19)</td>
<td>0.24</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>M6</td>
<td>62 (5)</td>
<td>41.7 (1)</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>363 (123)</td>
<td>68.7 (19)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

^a Values in parenthesis represent the standard error of measurement.

**TABLE 2**

Correlation of various P450-selective monoxygenase activities with the formation of almotriptan metabolites in a panel of human liver microsomes. The Spearman correlation coefficients were determined using liver tissue from 14 different organ donor subjects.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>P450 Form</th>
<th>Spearman Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD</td>
<td>CYP1A2</td>
<td>M2 0.15, M3 0.38, M4 0.59^a, M5 0.36, M6 0.22, M7 0.25</td>
</tr>
<tr>
<td>COH</td>
<td>CYP2A6</td>
<td>M2 0.23, M3 0.35, M4 0.44, M5 0.20, M6 0.37, M7 0.17</td>
</tr>
<tr>
<td>TOLH</td>
<td>CYP2C9</td>
<td>M2 -0.13, M3 0.11, M4 0.21, M5 -0.49, M6 0.11, M7 0.31</td>
</tr>
<tr>
<td>MepH</td>
<td>CYP2C19</td>
<td>M2 -0.13, M3 0.19, M4 0.28, M5 -0.06, M6 0.11, M7 0.15</td>
</tr>
<tr>
<td>DEXOD</td>
<td>CYP2D6</td>
<td>M2 0.72**, M3 0.64*, M4 -0.47, M5 0.28, M6 0.58*</td>
</tr>
<tr>
<td>CHLH</td>
<td>CYP2E1</td>
<td>M2 -0.15, M3 0.10, M4 0.21, M5 -0.28, M6 0.00, M7 -0.58</td>
</tr>
<tr>
<td>DEXON</td>
<td>CYP3A4</td>
<td>M2 0.47, M3 0.69**, M4 0.88***, M5 0.54, M6 0.00, M7 0.00</td>
</tr>
<tr>
<td>STSH</td>
<td>CYP3A4/5</td>
<td>M2 0.49, M3 0.74**, M4 0.89***, M5 0.29, M6 0.01, M7 0.00</td>
</tr>
<tr>
<td>LAH</td>
<td>CYP4A9/11</td>
<td>M2 -0.24, M3 -0.02, M4 0.11, M5 -0.15, M6 0.24, M7 0.15</td>
</tr>
</tbody>
</table>

EROD, 7-ethoxyresorufin O-deethylation; COH, coumarin 7-hydroxylation; TOLH, tolbutamide methyl-hydroxylation; MepH, S-mephenytoin 4′-hydroxylation; DEXOD, dextromethorphan-O-demethylation; CHLH, chloroxazone 6-hydroxylation; DEXON, dextromethorphan N-demethylation; STSH, testosterone 6β-hydroxylation; LAH, lauric acid 12-hydroxylation.

^a Values in parenthesis represent the standard error of measurement.

**TABLE 3**

Metabolism of almotriptan in the presence of human B lymphoblastoid microsomes containing cDNA-expressed P450 proteins. The Spearman correlation coefficients were determined using liver tissue from 14 different organ donor subjects.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Activity</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mmol P450</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>N.D.*</td>
<td>124.3</td>
<td>178.9</td>
<td>40.0</td>
<td>N.D.</td>
<td>87.2</td>
<td></td>
</tr>
<tr>
<td>2A2</td>
<td>N.D.</td>
<td>34.6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2A6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>N.D.</td>
<td>23.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2C8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2C9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2C19</td>
<td>N.D.</td>
<td>27.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2D6</td>
<td>88.4</td>
<td>32.1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>2E1</td>
<td>N.D.</td>
<td>36.7</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>3A4</td>
<td>48.8</td>
<td>36.7</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

^a N.D., no activity detected.

---
non-NADPH-dependent metabolism to two metabolites, namely M6 and M7. The reactions leading to the formation of these metabolites occur at the pyrrolidine group by hydroxylation of the \( -\)-carbon to form M3, a carbinolamine that is stable and is further oxidized to the open ring \( \alpha \)-aminobutyric acid metabolite M2. The 2-dimethylaminoethyl moiety undergoes different metabolic reactions that include N-oxidation (M5), N-demethylation (M4) and oxidative deamination to form the indole acetic acid (M6) and indole ethyl alcohol (M7) metabolites. The formation of the six metabolites was best described by monophasic Michaelis-Menten kinetics. The apparent \( K_m \) values and intrinsic clearances (CL_{int}) for the different metabolites in liver microsomes suggest that at high substrate concentrations, the oxidation of the pyrrolidine ring is the predominant route of metabolism. This hypothesis is not in agreement with excretion data from volunteers treated with \(^{14}\)C-almotriptan, demonstrating that M2 and also M6 are the major metabolites of almotriptan in vivo (unpublished data). As discussed later on, this inconsistency is related to the fact that MAO activity in liver microsomes is present presumably as a result of contamination with mitochondrial enzymes, and the capacity of human liver to catalyze the oxidative deamination of almotriptan is under-estimated.

Hydroxylation of \(^{14}\)C-almotriptan at the pyrrolidine moiety to form M3 was catalyzed by recombinant human CYP1A1, CYP2D6, and CYP3A4. CYP1A1 probably contributes nothing to the formation of this metabolite because in humans it appears to be expressed only in extrahepatic tissues (Parkinson, 1996). The sample-to-sample variation in the rate of formation of M3 by a bank of human liver microsomes was strongly correlated with CYP2D6 and CYP3A4 activities, indicating that both isoforms contribute to the formation of M3. In support to this interpretation, quinidine inhibited pyrrolidine \( \alpha \)-hydroxylation by 23\%, which suggests that this fraction of M3 is attributable to CYP2D6, whereas ketoconazole inhibited M3 formation by 80\%, being this fraction of M3 formation attributable to CYP3A4. However, in this study the substrate concentration (100 \( \mu \)M) was extremely high compared with pharmacological concentrations of almotriptan in humans at the highest oral dose administered to humans (1.7 \( \mu \)M) (Cabarrocas and Salva`, 1997). Consequently, the relative contribution of CYP2D6 and CYP3A4 to the formation of M3 may be different at pharmacologically relevant concentrations of almotriptan.

The generation of aldehyde intermediates from carbinolamines, which are recognized as common intermediates in the metabolism of
activity is the carbinolamine M3, which is an intermediate in the metabolism of P450 oxidase. The pyrrolidine ring of almotriptan by liver subcellular fractions under-oxidases, which usually prevents build-up of this reactive intermediate (Sladek et al., 1989; Beedham, 1997; Vasiliou et al., 2000). Therefore and similarly to what occurs with cyclophosphamide, the conversion of M3 to the γ-aminobutyric acid metabolite M2 was attributed to the formation of the aldehyde acyclic tautomer of M3 which would be then oxidized by aldehyde dehydrogenase.

Aldehyde dehydrogenase inhibitors disulfiram and DDTC turned out to be potent inhibitors of the oxidation of M3 to M2, as well as the formation of the indole acetic acid metabolite M6 in human liver microsomes, with IC_{50} values in the low micromolar range (IC_{50} = 10–16 μM). By contrast, inhibitors of the molybdenum-containing oxidases had little or no effect upon the biotransformation pathways that yield M2 and M6, indicating that neither AO nor XO are involved. The slight inhibition produced by PMSF was considered not relevant, whereas the complete inhibition of P450 and MAO activities produced by menadione remains unexplained.

Taken together these experiments suggest that the oxidation of the pyrrolidine ring of almotriptan by liver subcellular fractions undergoes two metabolic fates. The initial metabolite of P450 oxidase activity is the carbinolamine M3, which is an intermediate in the conversion of almotriptan to M2. The second metabolic reaction requires in rat the presence of a cytosolic enzyme, aldehyde dehydrogenase, which oxidizes the acyclic tautomer of M3 to the γ-aminobutyric acid metabolite M2. In humans, this reaction occurs also in liver microsomes, which could be explained by either the involvement of microsomal ALDH3A2 and/or the presence in human liver microsomes as contaminants of cytosolic ALDH1A1/ALDH9A1 and/or mitochondrial ALDH1B1/ALDH2 activities (Yoshida et al., 1998; Vasiliou et al., 1999). No further experiments were conducted to identify the human aldehyde dehydrogenase isoform that catalyzes the conversion of M3 to M2.

The N-demethylation of 14C-almotriptan to M4 was strongly correlated with CYP3A4 activity. This assignment was supported by the observation that ketoconazole and troleandomycin inhibited this reaction by 69 and 59%, respectively. However, the formation of M4 was catalyzed by several recombinant human P450s (namely 1A2, 2C8, 2C19, 2D6, 2E1, and 3A4). Therefore, it is possible that at pharmacologically relevant concentrations of almotriptan, the formation of M4 will be dominated by one or several of the isoenzymes listed above other than CYP3A4.

The N-oxidation of the 2-dimethylaminoethyl group of almotriptan was catalyzed by recombinant human FMO-3, a result consistent with the lack of correlation of almotriptan N-oxidation with P450 activities from the hepatic panel and the fact that none of the recombinant P450s converted almotriptan to M5.

The oxidative deamination of the 2-dimethylaminoethyl side chain is the major metabolic pathway of sumatriptan in animals and humans, to form the indole acetic acid metabolite (Dixon et al., 1993). The importance of this reaction is related to the low systemic bioavailability of sumatriptan in humans (14%) because of a first-pass effect (Fowler et al., 1991; Dechant and Clissold, 1992; Scott, 1994). Studies undertaken to investigate the enzymes responsible for the metabolism of sumatriptan in man, showed that monoamine oxidase A (MAO-A) was the major enzyme responsible for the oxidative deamination reaction (Dixon et al., 1994). Almotriptan was also metabolized in human liver microsomes and mitochondria by oxidative deamination to the indole acetic acid metabolite M6 and the indole ethyl alcohol M7. This reaction was not dependent on the presence of NADPH in the incubation medium, a cofactor required by P450 but not by MAO. The formation of M6 and M7 were inhibited by the mechanism-based inhibitors of monoamine oxidases clorgyline (MAO-A) and deprenyl (MAO-B), clorgyline being more effective than deprenyl.

Oxidative deamination of xenobiotics by amine oxidases affords the corresponding aldehydes, which are further metabolized to alcohols either by aldehyde reductases or alcohol dehydrogenases, or to acids by aldehyde dehydrogenases or acid oxidases (Strolin-Benedetti and Dostert, 1994; Beedham, 1997). The inhibition of M6 formation by disulfiram and DDTC in human liver microsomes, strongly suggests that aldehyde dehydrogenase is the enzyme that catalyze the conversion of the indole ethyl aldehyde intermediate formed by MAO to M6. Since MAO is a mitochondrial enzyme, it seems reasonable to assume that in vivo the aldehyde formed by MAO is further oxidized by a mitochondrial aldehyde dehydrogenase to the indole acetic acid metabolite M6.

In summary, almotriptan major metabolites are formed by oxidative metabolism of the pyrrolidine group and the dimethylaminoethyl moiety. The pyrrolidine ring is hydroxylated by CYP3A4 and CYP2D6 to a carbinolamine metabolite, which is further oxidized by aldehyde dehydrogenase to the open ring γ-butyric acid metabolite. The dimethylaminoethyl group is oxidized by MAO-A to form the inactive indole acetic acid metabolite that had been identified in vivo as the major metabolite of almotriptan. The N-demethylation and...
N-oxidation of the dimethylaminoethyl group are minor metabolic reactions both in vitro and in vivo. Different clinical trials conducted to study the effect of CYP3A4, CYP2D6, and MAO-A on the pharmacokinetics of almotriptan showed that verapamil and fluoxetine modestly inhibited almotriptan clearance, a result consistent with the assignment of CYP3A4 and CYP2D6 as the enzymes responsible for the oxidation of the pyrrolidine moiety (Fleishaker et al., 2000, 2001a). Moreover, moclobemide increased plasma concentrations of almotriptan by 37%, thus confirming that oxidative deamination of almotriptan by MAO-A was the major route of metabolism and that the degree of interaction was much less than that seen previously for sumatriptan, rizatriptan, or zolmitriptan given with moclobemide (Fleishaker et al., 2001b).

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References