Metabolites of Caspofungin Acetate, a Potent Antifungal Agent, in Human Plasma and Urine

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ABSTRACT:

Caspofungin acetate (MK-0991) is a semisynthetic pneumocandin derivative being developed as a parenteral antifungal agent with broad-spectrum activity against systemic infections such as those caused by Candida and Aspergillus species. Following a 1-h i.v. infusion of 70 mg of [3H]MK-0991 to healthy subjects, excretion of drug-related material was very slow, such that 41 and 35% of the dose radioactivity was recovered in urine and feces, respectively, over 27 days. Plasma and urine samples collected around 24 h postdose contained predominantly unchanged MK-0991, together with trace amounts of a peptide hydrolysis product, M0, a linear peptide. However, at later sampling times, M0 proved to be the major circulating component, whereas corresponding urine specimens contained mainly the hydrolytic metabolites M1 and M2, together with M0 and unchanged MK-0991, whose cumulative urinary excretion over the first 16 days postdose represented 13, 71, 1, and 9%, respectively, of the urinary radioactivity. The major metabolite, M2, was highly polar and extremely unstable under acidic conditions when it was converted to a less polar product identified as N-acetyl-4(S)-hydroxy-4-(4-hydroxyphenyl)-l-threonine γ-lactone. Derivatization of M2 in aqueous media led to its identification as the corresponding γ-hydroxy acid, N-acetyl-4(S)-hydroxy-4-(4-hydroxyphenyl)-l-threonine. Metabolite M1, which was extremely polar, eluting from HPLC column just after the void volume, was identified by chemical derivatization as des-acetyl-M2. Thus, the major urinary and plasma metabolites of MK-0991 resulted from peptide hydrolysis and/or N-acetylation.

Systemic fungal infection often can be lethal in vulnerable patient populations, such as those immunocompromised with AIDS, or receiving cancer chemotherapy or immunosuppressive therapy following organ transplants. For such patients, new therapies are needed because of the ineffectiveness, resistance to, or toxicity of existing treatments (Horsburgh and Kirkpatrick, 1983; Rex et al., 1994, 1995). Newer echinocandins, such as LY30366 (Peritakis et al., 1998) and pneumocandins, e.g., caspofungin (MK-09911; Fig. 1), exhibit excellent activity against yeast and Pneumocystis carinii infection in animals and show potential as clinical antifungal candidates. Caspofungin (Adefarati et al., 1992; Graybill et al., 1997a,b; Powles et al., 1998) is a semisynthetic, water soluble, broad spectrum lipopeptide with potent fungicidal activity, e.g., against serious Candida and Aspergillus infections. MK-0991 is a complex, cyclic hexapeptide with 16 asymmetric centers, a molecular weight of 1093 Da and low lipophilicity (log \( \text{P} \)). As a result of this functional selectivity toward the fungal cell wall enzyme, the side effects due to MK-0991 in humans are expected to be low. In addition, MK-0991 has shown synergistic effects in the in vitro activities of amphotericin B and fluconazole against Cryptococcus neoformans, an infection often incurable in patients with AIDS (Franzot and Casadevall, 1997). This report describes the metabolism of MK-0991 in humans after i.v. administration to healthy volunteers.

Experimental Procedures

Materials. Specimens of plasma and urine were obtained from healthy adult volunteers given a single 70-mg [3H]MK-0991 i.v. dose (1-h infusion) (specific activity 3.0 \( \mu \text{Ci/mg} \)), with sample collection over 27 days. Urine samples were treated with albumin to a final concentration of approximately 0.5% to prevent binding of drug to containers. An authentic sample of metabolite M0 was prepared at Merck Research Laboratories, and its structure confirmed by MS and NMR analysis. All other chemicals were of analytical grade and solvents of HPLC grade. Samples were stored at −70°C until analysis.

Radioanalysis. Aliquots of HPLC fractions from plasma and urine samples were mixed with liquid scintillation cocktail and analyzed by scintillation spectrophotometry. Samples of individual or pooled (across time or subjects) plasma from humans (24 and 30 h; days 5/6, 11/12, and 19/20 pools) were deproteinized by the addition of a mixture of acetonitrile (ACN) and methanol containing 0.1% trifluoroacetic acid (TFA), followed by vortex mixing and centrifugation. The resulting supernatants then were evaporated on a Speed-Vac system, and the residues reconstituted in 0.1% TFA-H\(_2\)O. Aliquots of these samples were analyzed by HPLC using a DuPont Zorbax RX C8 column (4.6 x 250 mm, 5 \( \mu \text{m} \)) eluted isocratically at 1 ml/min with 5 mM pentane-sulfonate (S5) in 0.5% acetic acid-water for 10 min, followed by a 30-min linear gradient to 80% ACN. The effluent was mixed with Packard Ultima Flow scintillation cocktail flowing at 1.4 ml/min before being analyzed by a Packard 500/TR radiochemical detector (system A). A second system used

1 Abbreviations used are: MK-0991: L-743872, Caspofungin acetate. CANCIDAS, 1-[(4R,5S)-5-[(2-aminoethoxy)lamine]-N\(_2\)(10,12-dimethyl-1-oxotetradecyl)-4-hydroxy-L-ornithine]-5-[(3R)-3-hydroxy-L-ornithine] pneumocandin B\(_1\), diacetate (salt), CAS registry no. 179463-17-3; MS, mass spectrometry; LC, liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid.

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involved a Zorbax RX C8 column (4.6 × 250 mm, 5 μm) eluted for 4 min with 18% ACN in 0.1% TFA-H2O, followed by a 36-min linear increase to 50% ACN in 0.1% TFA-H2O. The LC effluent was fractionated (at 1-min intervals), mixed with scintillation cocktail, and analyzed by liquid scintillation spectrophotometry (system B).

Urine samples from two subjects (collected over 16 days) were concentrated on a Speed-Vac system, and analyzed individually on Luna C18 (4.6 × 250 mm, 5 μm, Phenomenex) and Zorbax SAX (4.6 × 250 mm, 5 μm, DuPont) columns, connected in series, and eluted for 15 min with 0.1% TFA-H2O, followed by a 25-min linear gradient to 80% acetonitrile in 0.1% TFA-H2O (system C). The LC effluent was mixed on-line with 1.4 volumes of Ultima-Flo M cocktail (Packard) before radiochemical detection.

**Metabolite Identification.** The major metabolite M0 in plasma was isolated using a Zorbax RX C8 column (4.6 × 250 mm, 5 μm) eluted with mixtures of aqueous 0.1% TFA and ACN, and subjected to MS analysis using a Finnigan LCQ mass spectrometer. Unchanged MK-0991 and M0 in urine specimens were identified by comparison of their LC and MS/MS properties with those of authentic standards. The major urinary metabolite, M2, was isolated using system C, although the compound proved to be unstable under acidic conditions and degraded to a less polar component termed M2D. This degradant was isolated and purified further on a Zorbax SB C18 column (4.6 × 250 mm, 5 μm) eluted with a 25-min linear gradient from 0 to 25% ACN in 0.1% TFA-H2O. The purified degradant was analyzed by NMR on a Varian Unity 400 MHz spectrometer.

Metabolite M2 was stabilized during HPLC isolation using system C by postcolumn infusion with 30% NH4OH delivered at ~60 μl/min. M2 obtained in this fashion was subjected to mass spectral analysis following treatment with an excess of ethyl chloroformate in Na2CO3 buffer, pH 9 (Hoffmann et al., 1985). After 4 h, the reaction mixture was extracted with methylene chloride, the organic and aqueous layers were evaporated, and the residues reconstituted in water and analyzed on a Zorbax RX C8 column (4.6 × 250 mm, 5 μm) eluted with 10% ACN in 0.1% TFA-H2O for 10 min, followed by a 30-min linear gradient to 40% ACN. Two derivatives were obtained by this procedure, which were analyzed by mass spectrometry and NMR spectroscopy.

For the isolation of metabolite M1, urine was filtered through Centricon plus-80 centrifugal filter devices fitted with Biomax-8 membranes, with a molecular weight cut-off of 8 kDa, to remove added bovine serum albumin and high molecular weight endogenous urinary components. Filtrates were concentrated using a Speed-Vac system, three volumes of acetone were added, and the resulting mixtures centrifuged to remove precipitated salts. Acetone extracts were evaporated to dryness, reconstituted and the metabolite M1 isolated by HPLC using system C. Fractions containing M1 were pooled and concentrated on a Speed-Vac. Precipitated salts were removed either directly by centrifugation or after two successive treatments with equal volumes of ace-
trole and ACN. The M1 isolate was derivatized in the aqueous phase by adding 10% potassium tetraborate in the presence of an excess of acetic anhydride. After overnight reaction at room temperature, the solvents were evaporated. The residue was dissolved in water and subjected to solid phase extraction using C18 cartridges. Elution with ACN yielded a fraction enriched in acetylated M1, which was concentrated further and analyzed by LC-MS/MS, using a Phenomenex Luna C18 column (4.6 × 250 mm, 5 μm) eluted with a 30-min linear gradient from 0 to 30% ACN in 0.1% formic acid-water. The effluent was split to admit 20% of the effluent to an LCQ mass spectrometer, while the remaining 80% was mixed with liquid scintillation cocktail and analyzed by an on-line radiochemical detector. The acetylated derivatives of M1 were compared by LC-MS/MS and/or NMR with those obtained from acetylation of metabolite M2, and of 4(S)-hydroxy-4-(4-hydroxyphenyl)-L-threonine, which was obtained by treatment of MK-0991 with IN-HCl at −95°C, for 2 h.

Results and Discussion

[^3H]MK-0991 is cleared slowly after i.v. administration to man, with a plasma clearance of 0.15 ml/min/kg (Stone et al., 1998). Consequently, the excretion of radioactivity into urine and feces was slow, with 40.7 and 34.4% of the dose, respectively, excreted over 27 days (Merck Research Laboratories, unpublished data). This slow excretion, in turn, resulted in low concentrations of MK-0991 metabolites in the urine that, coupled with the relatively low specific activity and lower efficiency of detection of tritium (compared to 14C), made identification of metabolites difficult. Nevertheless, tritium was the most suitable and convenient isotope for labeling due to the semisynthetic nature of MK-0991 (Bouffard et al., 1994). Analysis of urine samples was further confounded by the presence of albumin (~5 g/l), which was added extraneously during sample collection to prevent the drug from binding to sampling containers. Attempts to identify metabolites directly in concentrated urine by LC-MS and LC-NMR were not successful. Furthermore, the major metabolites of MK-0991 were extremely polar, and one was highly unstable under the acidic conditions of isolation. Methods were developed to partially purify the urine before isolation and stabilization of metabolites, followed by chemical derivatization to facilitate identification.

HPLC analysis of the early time point (24- or 30-h) plasma specimen showed that MK-0991 was the major circulating species, whereas M0 was a minor component. M0 is formed from the hydrolysis of the aminol in the ornithine residue and recyclization to form a five-membered cyclic hemiaminal, with the loss of ethylenediamine. At later times, the proportion of MK-0991 in plasma decreased and that of M0 increased, such that ≥day 5 plasma samples contained M0 as the major component (Fig. 2), while MK-0991 was barely detectable beyond that time point.

In early urine collections (~24 h) MK-0991 was the major component, with a trace of M0. A representative urinary profile of radioactivity from later time points is shown in Fig. 2. Quantitation of metabolites in urine showed that the cumulative excretion of unchanged MK-0991 accounted for 9% of the urinary radioactivity over the first 16 days postdose, during which time approximately 33% of the administered radioactivity was recovered in urine. The major metabolite in urine, M2 (accounting for 71% of the radioactivity), was accompanied by minor metabolites M1, M4, and M0, which represented 13, 2, and <1% of the urinary radioactivity, respectively.

The acidic HPLC conditions for metabolite isolation led to degradation of M2 to the species quoted as M2D. LC-MS analysis of M2D demonstrated a molecular weight of 251; this information, coupled with the presence in M2D of tritium, favored attention on the 4-(4-hydroxyphenyl)-L-threonine moiety as the likely source of the metabolite M2. A reference sample of M2D was generated by i.v. infusion of MK-0991 to rats at 45 mg/kg over 8 h. The NMR spectrum of this material (Table 1) showed a broad signal at 7.10 ppm (1H) and a methyl resonance at 1.96 ppm. A double irradiation experiment determined that these protons were coupled, suggestive of an –NHCOCH3 group. This view was supported by a double irradiation experiment that established coupling of the 7.10 ppm proton to an α-methylene proton at 4.38 ppm (>CH–NHCOCH3). A long-range correlation spectroscopy spectrum established that the doublet at 4.94 ppm was coupled weakly to ortho aromatic protons, thus identifying it as a benzylic methine. Relative to its chemical shift in NMR spectrum of MK-0991, the 0.7 ppm downfield shift of this CH proton suggested that the 4-hydroxylethreanine moiety had undergone cyclization to form a lactone. Spectra acquired in dimethyl sulfoxide revealed two additional exchangeable hydrogens, one at 9.57 ppm (s) assigned to the phenolic OH, and one at 5.89 ppm (d) assigned to the β-OH group. The M2D from human urine was identical with that from rat urine on analysis by NMR, which demonstrated its structure to be N-acetyl-L-4(S)-hydroxy-4-(4-hydroxyphenyl)-L-threonine γ-lactone (Fig. 1). The MS/MS spectrum of the degradant [m/z 252 (M + H)+, 175 (loss of NH2COCH3 and H2O), 147 (further loss of CO), 119 (a second loss of CO), 107 (tropanyl hydroxide)+, 72 (CH3N–NH–COCH3)+] was consistent with the NMR structure.

In subsequent experiments, M2 was stabilized during isolation from urine by postcolumn alkalination of the LC-effluent. The isolate, upon derivatization with ethyl chloroformate under aqueous basic conditions, yielded two products, which also were produced in large quantities from the urine of rats. The NMR spectra of the products (Table 1) collectively showed that M2 was N-acetyl-L-4(S)-hydroxy-4-(4-hydroxyphenyl)-L-threonine.

The M1 isolate from human urine was highly impure due to its poor retention on various HPLC columns. Added albumin and other high molecular weight endogenous contaminants could be removed easily.
**TABLE 1**  
$^1$H NMR chemical shifts (ppm) of derivatives of the human urinary metabolites M1 and M2, and the degradant M2D

<table>
<thead>
<tr>
<th>Proton</th>
<th>M1 Lactone R = Acetyl</th>
<th>M1 Acid R = Acetyl R’ = H</th>
<th>M2 Lactone R = Ethoxycarbonyl</th>
<th>M2 Acid R,R’ = Ethoxycarbonyl</th>
<th>M2D Lactone R = H</th>
<th>M2D Lactone R = H</th>
</tr>
</thead>
<tbody>
<tr>
<td>H α</td>
<td>4.40 (m*, 1H)</td>
<td>4.84 (bs, 1H)</td>
<td>4.74 (dd, J = 2.2, 9.0 Hz)</td>
<td>4.16 (m, 1H)</td>
<td>4.38 (dd, 1H, J = 7.8, 15.0 Hz)</td>
<td>4.35 (m, 1H)</td>
</tr>
<tr>
<td>H β</td>
<td>4.40 (m, 1H)</td>
<td>4.06 (d, 1H, J = 9.2 Hz)</td>
<td>4.54 (dd, 1H, J = 2.2, 7.5 Hz)</td>
<td>4.53 (dd, 1H, J = 6.5, 12.2 Hz)</td>
<td>4.40 (dd, 1H, J = 7.8, 15.0 Hz)</td>
<td>4.35 (m, 1H)</td>
</tr>
<tr>
<td>H γ</td>
<td>5.07 (d, 1H, J = 7.5 Hz)</td>
<td>4.26 (d, 1H, J = 8.9 Hz)</td>
<td>5.02 (d, 1H, J = 7.5 Hz)</td>
<td>5.71 (d, 1H, J = 6.5 Hz)</td>
<td>4.94 (d, 1H, J = 7.8 Hz)</td>
<td>4.92 (bd, 1H, J = 7.4 Hz)</td>
</tr>
<tr>
<td>OH β</td>
<td></td>
<td></td>
<td>4.74 (d, 1H, J = 5.4 Hz)</td>
<td>5.89 (bd, 1H, J = 6.0 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.57 (s, 1H)</td>
<td></td>
</tr>
<tr>
<td>NHCOCH₃</td>
<td>1.97 (s, 3H)</td>
<td>1.85 (s, 3H)</td>
<td>1.97 (s, 3H)</td>
<td>1.89 (s, 3H)</td>
<td>1.96 (s, 3H)</td>
<td>1.89 (s, 3H)</td>
</tr>
<tr>
<td>NHCOCH₃</td>
<td>7.07 (bs, 1H)</td>
<td>6.97 (bs, 1H, J = 7.3 Hz)</td>
<td>7.02 (bd, 1H, J = 8.4 Hz)</td>
<td>8.63 (d, 1H, J = 7.7 Hz)</td>
<td>7.10 (bs, 1H)</td>
<td>8.52 (bd, 1H, J = 6.2 Hz)</td>
</tr>
<tr>
<td>H3</td>
<td>7.16 (d, 2H, J = 8.6 Hz)</td>
<td>7.05 (d, 2H, J = 8.5 Hz)</td>
<td>7.23 (d, 2H, J = 8.6 Hz)</td>
<td>7.23 (d, 2H, J = 8.5 Hz)</td>
<td>6.84 (d, 2H, J = 8.6 Hz)</td>
<td>6.79 (d, 2H, J = 8.3 Hz)</td>
</tr>
<tr>
<td>H2</td>
<td>7.49 (d, 2H, J = 8.6 Hz)</td>
<td>7.41 (d, 2H, J = 8.5 Hz)</td>
<td>7.44 (d, 2H, J = 8.6 Hz)</td>
<td>7.30 (d, 2H, J = 8.5 Hz)</td>
<td>7.28 (d, 2H, J = 8.6 Hz)</td>
<td>7.21 (d, 2H, J = 8.3 Hz)</td>
</tr>
<tr>
<td>COOCH₂CH₃</td>
<td>4.27 (q, 2H, J = 7.0 Hz)</td>
<td>4.24 (q, 2H, J = 7.0 Hz)</td>
<td>4.24 (q, 2H, J = 7.0 Hz)</td>
<td>4.01 (q, 2H, J = 7.0 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COOCH₂CH₃</td>
<td>2.26 (s, 3H)</td>
<td>2.06 (s, 3H)</td>
<td>1.33 (t, 3H, J = 7.0 Hz)</td>
<td>1.28 (t, 3H, J = 7.0 Hz)</td>
<td>1.17 (t, 3H, J = 7.0 Hz)</td>
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</table>


*b* The resonance was shifted downfield by 0.7 ppm relative to that in MK-0991.
by membrane filtration. However, only after extensive desalting and derivatization with acetic anhydride to a mixture of three diacetylated products (retention times: 18, 20, and 25 min) could M1 be studied effectively. The acetylated derivatives of M1 were identical by LC-MS/MS to those formed from 4(S)-hydroxy-4-(4-hydroxyphenyl)-l-threonine derived from acid hydrolysis of MK-0991. NMR data (Table 1) on the acetylated product (20 min) from the acid degradant indicated the structure to be N-acetyl-4(S)-hydroxy-4-(4-acetoxyphenyl)-l-threonine. From these results, M1 was deduced to be 4(S)-hydroxy-4-(4-hydroxyphenyl)-l-threonine. The remaining minor metabolite, M4, was not identified due to insufficient quantity and purity of the isolate.

Overall, the results of the present investigation indicated that, following i.v. infusion of [3H]MK-0991 to healthy men, plasma specimens contain unchanged drug and the ring-opened degradant, M0. In contrast, urine was found to contain small amounts of MK-0991, whereas low molecular weight products of peptide hydrolysis dominated the radiochemical profile. In view of the location of the tritium label in [3H]MK-0991, only fragments retaining the 4-(4-hydroxyphenyl)-l-threonine moiety were detected in this study, and therefore the fate of the remaining elements of the native cyclic hexapeptide await future evaluation with the aid of appropriately labeled tracer of this drug. Nevertheless, the fact that the majority of the drug-related material excreted into urine could be accounted for by derivatives of 4-(4-hydroxyphenyl)-l-threonine suggests that hydrolytic pathways, as opposed to oxidative reactions, dominate the biotransformation of MK-0991 in man.

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References


