BIOTRANFORMATION OF DOXEPIN BY CUNNINGHAMELLA ELEGANS

JOANNA D. MOODY, JAMES P. FREEMAN, AND CARL E. CERNIGLIA

Division of Microbiology (J.D.M., C.E.C.) and Division of Chemistry (J.P.F.), National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas

(Received March 3, 1999; accepted June 10, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:
A filamentous fungus, Cunninghamella elegans ATCC 9245, was used as a microbial model of mammalian metabolism to biotransform doxepin, a tricyclic antidepressant drug. Doxepin is produced as an 85:15% mixture of the trans- (E) and cis- (Z) forms. After 96 h of incubation in Sabouraud dextrose broth, 28% of the drug was metabolized to 16 metabolites. No change in the trans- (E) and cis- (Z) ratio of doxepin was observed. Metabolites were isolated by reversed phase HPLC and identified by 1H NMR and mass spectroscopic analysis. The major metabolites were (E)-2-hydroxydoxepin, (E)-3-hydroxydoxepin, (Z)-8-hydroxydoxepin, (E)-2-hydroxy-N-desmethyldoxepin, (E)-3-hydroxy-N-desmethyldoxepin, (E)-4-hydroxy-N-desmethyldoxepin, (Z)- and (E)-8-hydroxy-N-desmethyldoxepin, (E)-N-acetyl-N-desmethyldoxepin, (E)-N-desmethyl-N-formyldoxepin, (E)-N-acytildidesmethyldoxepin, (E)- and (Z)-doxepin-N-oxide, and (E)- and (Z)-N-desmethyldoxepin. Six of the metabolites produced by C. elegans were essentially similar to those obtained in human metabolism studies, although nine novel metabolites were identified.

Doxepin, (3S/6H-dibenzo[c,j]oxepin-11-ylidene)dimethylamine (Fig. 1), is a tricyclic antidepressant drug with a structure similar to those of cyclobenzaprine, amitriptyline, imipramine, and protriptyline. Doxepin is marketed as an 85:15% mixture of the trans- (E) to cis- (Z) form with the cis form being more active pharmacologically (Pinder et al., 1977). It is marketed under the names Sinequan, Adapin, Aponal, Curatin, Quitaxon, or Zonalon cream (Budavari et al., 1997). Doxepin is used for treatment of depression and anxiety (Pinder et al., 1977), pruritus (Smith and Corelli, 1997), and fibromyalgia and chronic pain syndromes (Godfrey, 1996). Doxepin has selective action of guanethidine to the extent of some other tricyclic antidepressants. In animal studies, anticholinergic, antiserotonin, and moderate sympathomimetic effects have been demonstrated (Pinder et al., 1977).

The precise mechanism of action is not known. It is neither a central nervous system stimulant nor a monoamine oxidase inhibitor. The current hypothesis is that the clinical effects are due, at least in part, to influences on the adrenergic activity at the synapses so that deactivation of norepinephrine by reuptake into the nerve terminals is prevented (Physicians’ Desk Reference, 1997). Animal studies suggest that doxepin does not appreciably antagonize the antihypertensive action of guanethidine to the extent of some other tricyclic antidepressants. In animal studies, anticholinergic, antiserotonergic, and antihistamine effects have been demonstrated (Pinder et al., 1977).

Metabolic studies of doxepin have been performed on animals and humans. Absorption of the drug is rapid and metabolism appears to take place mainly in the liver (Pinder et al., 1997). Phase I and phase II metabolites have been identified in plasma, urine, and cerebrospinal fluid. The urinary metabolites in humans are (E)-2-hydroxydoxepin, (E)-2-hydroxy-N-desmethyldoxepin, (Z)- and (E)-N-desmethyldoxepin, (Z)- and (E)-doxepin-N-oxide (Shu et al., 1990a), (E)-2-O-glucuronidodoxepin (Shu et al., 1990b), and a quaternary ammonium-linked glucuronide (Luo et al., 1991). (E)- and (Z)-N-Didesmethyldoxepin and N-desmethyldoxepin have been reported in the cerebrospinal fluid of humans (Deuschle et al., 1997). Rat bile metabolites include (E)-2-O-glucuronidodoxepin and (E)-3-O-glucuronidodoxepin (Shu et al., 1990b). The concept of using microorganisms, and in particular Cunninghamella elegans, as models of mammalian metabolism has been well documented (Zhang et al., 1995, 1996; Cerniglia, 1997). C. elegans can metabolize a wide variety of xenobiotics in a regio- and stereo-selective manner similar to mammalian enzyme systems (Rao and

![Fig. 1. Chemical structure of (E)- and (Z)-doxepin.](Image)
was 5 ml/min. Compounds with similar retention times were pooled, evaporated to dryness in vacuo at 34°C using a Buchi 011 rotary evaporator (Brinkmann Instruments, Westbury, NY). The residue was dissolved in 5 ml of methanol, transferred to a 13 × 100 mm test tube, and concentrated to approximately 100 µl in a model SS21 Savant Speed-vac system (Savant Instruments, Holbrook, NY) for analysis by HPLC.

Doxepin and its metabolites were resolved using reversed phase HPLC. The analyses were performed with a Hewlett-Packard series 1050 pump system (Hewlett-Packard, Palo Alto, CA) equipped with a Hewlett-Packard diode array model 1040A detector at 233 nm. The compounds were eluted using a linear gradient of 30 to 75% methanol-buffer (v/v) over 30 min at 1.0 ml/min and then 10 µg of doxepin hydrochloride (31.75 µmol) dissolved in 0.5 ml of sterile physiological saline solution was added. Control experiments consisted of cultures without doxepin and sterile flasks containing only media and doxepin.

Extraction, isolation, and identification of metabolites. After 96 h of incubation, the contents of each flask, including the controls, were filtered through glass wool into a separatory funnel and extracted with three equal volumes of ethyl acetate. The organic extracts were dried over sodium sulfate and evaporated to dryness in vacuo at 34°C using a Buchi 011 rotary evaporator. NMR measurements were carried out at 500.13 MHz on an AM500 spectrometer (Bruker Instruments, Billerica, MA). Chemical shifts are reported on the d6-sulfoxide (99.96 atom % 2H) for 1H NMR (NMR) analysis. The d6-sulfoxide was used as a solvent for NMR experiments, the d6-sulfoxide for 1H NMR (NMR) analysis.

Microbial culture and biotransformation conditions. Cultures of C. elegans ATCC 9245 were maintained on potato dextrose agar slants (Remel, Lenexa, KS) and stored at 4°C. The spores and/or mycelia were aseptically transferred to potato dextrose agar plates (Remel) and allowed to grow for at least 48 h at room temperature. The mycelia from five plates were then transferred to a sterile blender cup containing 90 ml of sterile physiological saline solution and homogenized for 5 min. Approximately 13.ml aliquots of the homogenate were used to inoculate 125 ml Erlemeyer flasks containing 30 ml of Sabouraud dextrose broth (Difco Laboratories, Detroit, MI). The cultures were incubated for 48 h at 26°C on a rotary shaker operating at 125 rpm and then 10 µg of doxepin hydrochloride (31.75 µmol) dissolved in 0.5 ml of sterile physiological saline solution was added. Control experiments consisted of cultures without doxepin and sterile flasks containing only media and doxepin.

Quantification of metabolites. In a separate study for quantitative analysis of doxepin metabolites, three flasks of 48-h cultures of C. elegans ATCC 9245 in 30-ml Sabouraud dextrose broth were dosed with approximately 10 mg of doxepin, incubated for 96 h, and extracted as above. HPLC with UV detection was used to separate the fungal metabolites produced by doxepin, as described previously. Each metabolite was dried completely and dissolved in methanol or dimethyl sulfoxide, respectively. Typical data acquisition parameters were: data size, 32,000; sweep width, 7042 Hz; filter width, 8900 Hz; acquisition time, 2.33 s; flip angle, 90°; relaxation delay, 0 s; temperature, 298.5 K. For spectra recorded under quantitative conditions, a 10- to 20-s relaxation delay was used. At 298.5 K, the resonances of the H6 methylene protons appear as a broad singlet because their exchange rate is slow...

1 Abbreviations used are: NOE, nuclear Overhauser effect; DEP, direct exposure probe; EI, electron ionization; PICI, positive ion chemical ionization.
(E)-N-desmethyldoxepin. To verify this, several NOE experiments were conducted. At 255 K, the H6 methylenes appeared as two distinct doublets rather than a broad singlet, as they did at 298.5 K. Irradiation of each doublet at 255 K produced an NOE at 7.42 ppm, H7. The decoupling experiments already completed led to the subsequent assignment of the other protons on the B ring. When the α-resonance was irradiated, an NOE was produced at 7.31 ppm. Because that doublet was not part of the B ring, it was assigned as H1 and so the larger component of the metabolite was proven to be in the (E)-isomeric form. A rigorous proof of structure was not performed on the smaller component due to resonance overlap. The smaller component was assumed to be (Z)-N-desmethyldoxepin because of its similarity to the one present in authentic doxepin and its 15.85% ratio with the major component. Based on this evidence, peak IX was identified as a mixture of (E)- and (Z)-N-desmethyldoxepin.

The EI mass spectra of peaks V through VII each contained an intense base peak ion at \( m/z \) 58 \( \left[ \text{CH}_2 = \text{NH} - (\text{CH}_3)_2 \right]^+ \) and the base peak ion at \( m/z \) 295 \([M^+]\). The corresponding PICI mass spectra contained a base peak ion at \( m/z \) 296, the \([M + H]^+\) ion and a significant fragment ion at \( m/z \) 58 (Table 1). The UV-visible spectra of peaks V through VIII were similar so that NMR became the key analytical technique for unambiguously identifying the metabolites.

The NMR analyses of metabolite peaks V through VII were similar (Table 2). All exhibited seven aromatic resonances, two of them shifted upfield from those of doxepin and having the ABX pattern characteristic of a single substitution on one of the 6-membered rings. NOE experiments provided the (E)- or (Z)- designation, as described above. Homonuclear decoupling experiments were used to provide information about the connectivity between the resonances on a particular ring and ultimately led to the determination of the sites of substitution for these metabolites. The three metabolites were identified as (E)-3-hydroxydoxepin [peak V], (E)-2-hydroxydoxepin [peak VI], and (Z)-8-hydroxydoxepin [peak VII].

Peaks I through IV produced similar EI and PICI mass spectra (Table 1). The EI base peak ion was at \( m/z \) 44, probably \([\text{CH}_2 = \text{NH} - \text{CH}_3]^+\), and the molecular ion at \( m/z \) 281 \([M^+]\); the PICI base peak ion was at \( m/z \) 282 \([M + H]^+\), with a significant fragment ion at \( m/z \) 44 (Fig. 3, C and D). Although peak III appeared to be a single component in the HPLC chromatogram, the NMR spectrum (Fig. 4C) revealed two similar components. One had a NMR spectrum almost identical with that of metabolite VII; the other had a NMR spectrum similar to that of metabolite VI. The only differences were that the methyl resonance of each metabolite integrated as three instead of six, indicating N-demethylation. The (E)- or (Z)- designation as well as the site of substitution was determined by NOE experiments, as described above. The metabolites were identified as (Z)-8-hydroxy-N-desmethyldoxepin and (E)-2-hydroxy-N-desmethyldoxepin.

The NMR spectra of peaks I (Fig. 4D) and IV were similar to those of metabolites V and VII with a single substitution on one of the rings and demethylation. The techniques described above identified them as (E)-3-hydroxy-N-desmethyldoxepin [peak I] and (E)-8-hydroxy-N-desmethyldoxepin [peak IV]. Metabolite II was also demethylated, but the splitting pattern of the proton resonances on the substituted

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>HPLC Retention Time</th>
<th>UV ( \lambda_{max} )</th>
<th>Mass Spectral Diagnostic Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-3-hydroxy-N-desmethyldoxepin (I)</td>
<td>281</td>
<td>17.3</td>
<td>207, 269, 299</td>
<td>44(100), 238(22), 281(1.9)</td>
</tr>
<tr>
<td>(E)-4-hydroxy-N-desmethyldoxepin (II)</td>
<td>281</td>
<td>18.2</td>
<td>206, 262, 300</td>
<td>44(100), 281(4)</td>
</tr>
<tr>
<td>(Z)-8-hydroxy-N-desmethyldoxepin, (E)-2-hydroxy-N-desmethyldoxepin (III)</td>
<td>281</td>
<td>19.2</td>
<td>205, 256, 286</td>
<td>44(100), 281(1.0)</td>
</tr>
<tr>
<td>(E)-8-hydroxy-N-desmethyldoxepin (IV)</td>
<td>281</td>
<td>21.7</td>
<td>204, 262, 285</td>
<td>44(100), 281(4)</td>
</tr>
<tr>
<td>(E)-3-hydroxydoxepin (V)</td>
<td>295</td>
<td>22.7</td>
<td>208, 267, 298</td>
<td>58(100), 295(0.8)</td>
</tr>
<tr>
<td>(E)-2-hydroxydoxepin (VI)</td>
<td>295</td>
<td>23.6</td>
<td>211, 318</td>
<td>58(100), 295(0.8)</td>
</tr>
<tr>
<td>(Z)-8-hydroxydoxepin (VII)</td>
<td>295</td>
<td>24.1</td>
<td>205, 261, 284</td>
<td>58(100), 295(1.2)</td>
</tr>
<tr>
<td>(E)-N-desmethyldoxepin (IX)</td>
<td>265</td>
<td>26.6</td>
<td>214, 254, 297</td>
<td>44(100), 265(1.2)</td>
</tr>
<tr>
<td>(E)-doxepin-N-oxide (X)</td>
<td>295</td>
<td>30.2</td>
<td>204, 260, 298</td>
<td>58(100), 295(0.8)</td>
</tr>
<tr>
<td>(E,Z)-doxepin (XI)</td>
<td>295</td>
<td>34.1</td>
<td>207, 264, 298</td>
<td>58(100), 279(0.4)</td>
</tr>
<tr>
<td>(E)-N-formyl-N-desmethyldoxepin (XII)</td>
<td>293</td>
<td>34.8</td>
<td>206, 256, 298</td>
<td>58(100), 294(100), 311(15)</td>
</tr>
<tr>
<td>(E)-N-acetyldidesmethyldoxepin (XIII)</td>
<td>293</td>
<td>35.6</td>
<td>207, 255, 297</td>
<td>219(50), 221(12), 234(100), 293(21)</td>
</tr>
<tr>
<td>(E)-N-acetyl-N-desmethyldoxepin (XIV)</td>
<td>307</td>
<td>37.9</td>
<td>207, 256, 297</td>
<td>44(67), 219(37), 221(26), 234(100), 307(14)</td>
</tr>
</tbody>
</table>

**TABLE 1** Chromatographic data and mass spectral diagnostic ions of doxepin and its metabolites produced by C. elegans

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Shifts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acetyl&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-3-hydroxy-N-desmethyldoxepin (I)</td>
<td>7.13 6.35 6.16 5.47 7.41</td>
<td>7.33 7.38 7.23</td>
</tr>
<tr>
<td>(E)-4-hydroxy-N-desmethyldoxepin (II)</td>
<td>6.79 6.72 6.70 5.52 7.44</td>
<td>7.36 7.40 7.25</td>
</tr>
<tr>
<td>(Z)-8-hydroxy-N-desmethyldoxepin (III)</td>
<td>7.13 6.93 7.17 6.86 6.10</td>
<td>6.73 7.13 5.61</td>
</tr>
<tr>
<td>(E)-2-hydroxy-N-desmethyldoxepin (III)</td>
<td>6.74 6.60 6.56 7.40 6.34</td>
<td>7.38 7.24 5.97</td>
</tr>
<tr>
<td>(E)-8-hydroxy-N-desmethyldoxepin (IV)</td>
<td>7.29 6.85 7.10 6.71 6.84</td>
<td>7.07 5.94 5.26</td>
</tr>
<tr>
<td>(E)-3-hydroxydoxepin (V)</td>
<td>7.11 6.35 6.16 5.43 7.40</td>
<td>7.33 7.38 7.23</td>
</tr>
<tr>
<td>(E)-2-hydroxydoxepin (VI)</td>
<td>7.61 6.57 6.54 7.38 7.33</td>
<td>7.36 7.34 5.98</td>
</tr>
<tr>
<td>(Z)-8-hydroxydoxepin (VII)</td>
<td>7.14 6.91 7.15 6.84 6.09</td>
<td>6.87 7.12 6.11</td>
</tr>
<tr>
<td>(E)-N-desmethyldoxepin (IX)</td>
<td>7.31 6.86 7.11 6.70 5.33</td>
<td>7.39 7.26 6.01</td>
</tr>
<tr>
<td>(E)-doxepin-N-oxide (X)</td>
<td>7.28 6.86 7.10 6.70 5.00</td>
<td>7.41 7.36 5.99</td>
</tr>
<tr>
<td>(E)-doxepin (XI)</td>
<td>7.26 6.85 7.07 6.69 5.20</td>
<td>7.41 7.36 6.00</td>
</tr>
<tr>
<td>(E)-N-formyl-N-desmethyldoxepin (XII)</td>
<td>7.25 6.84 7.08 6.68 7.39</td>
<td>7.36 7.23 6.00</td>
</tr>
<tr>
<td>(E)-N-acetyldidesmethyldoxepin (XIII)</td>
<td>7.27 6.84 7.08 6.68 5.48</td>
<td>7.31 7.36 6.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dissolved in methanol-d<sub>4</sub>.

<sup>b</sup>Blank spaces indicate the proton was not visible due to overlap with the H<sub>2</sub>O resonance or exchange.

<sup>c</sup>The (Z)-form was also present. Data not shown.

<sup>d</sup>Only first order coupling constants are reported.
(E)-3-hydroxy-N-desmethyldoxepin (I)
1.25 3.93 1.67 ± 0.34 5.03 ± 1.46
(E)-4-hydroxy-N-desmethyldoxepin (II)
0.14 0.44 0.16 ± 0.04 0.47 ± 0.15
(Z)-8-hydroxy-N-desmethyldoxepin, (E)-2-hydroxy-N-desmethyldoxepin (III)
1.02 3.20 0.83 ± 0.05 2.48 ± 0.38
(E)-8-hydroxy-N-desmethyldoxepin (IV)
0.11 0.33 0.10 ± 0.01 0.29 ± 0.04
(E)-3-hydroxydoxepin (V)
0.49 1.54 0.42 ± 0.01 1.16 ± 0.16
(E)-2-hydroxydoxepin (VI)
0.24 0.75 0.33 ± 0.03 1.03 ± 0.86
(Z)-8-hydroxydoxepin (VII)
0.55 1.74 0.42 ± 0.03 1.26 ± 0.16
(E)-N-desmethyldoxepin (IX)
2.87 9.05 4.16 ± 0.49 12.40 ± 1.67
(E)-doxepin-N-oxide (X)
0.34 1.08 0.46 ± 0.04 1.34 ± 0.06
(E)-doxepin (XI)
0.44 1.40 0.70 ± 0.18 1.60 ± 0.35
(E)-N-formyl-N-desmethyldoxepin (XII)
0.13 0.40 0.12 ± 0.05 0.36 ± 0.18
(E)-N-acetyldidesmethyldoxepin (XIII)
0.59 1.86 0.57 ± 0.01 1.71 ± 0.21
(E)-N-acetyl-N-desmethyldoxepin (XIV)
0.40 1.26 0.43 ± 0.01 1.30 ± 0.17

Table 3: Production of doxepin metabolites by cultures of C. elegans

*Injection volume was 20 μl of a 150 μl dilution. Sum includes (E)- and (Z)- forms for metabolites IX, X, and XI.

The aromatic region of the 'H NMR spectrum of peak X (Table 2) was similar to that of authentic doxepin; the only difference was a slight (0.01–0.05 ppm) downfield shift of those resonances. However, the methyl and β-methylene resonances were shifted downfield considerably more, 0.63 and 0.24 ppm, respectively. The γ-methylene resonance was visible only as a broad hump in the 298.5 K spectrum. At 255 K, the spectrum revealed two separate γ-methylene resonances, each integrating as one. The methyl resonances were also consistent with that substitution being at either the H1 or H4 position. Selective saturation of the α-proton and H6 produced NOEs that proved the site of substitution. The metabolite was identified as (E)-4-hydroxy-N,N-desmethyldoxepin.

The NMR spectrum of peak VIII showed eight aromatic protons, indicating that the ring system was intact. However, the usually prominent α-proton resonance was missing. The β- and γ-methylene resonances were not obvious due to impurities and sample quantity limitations. Therefore, this metabolite has not been identified.

The aromatic region of the 'H NMR spectrum of peak X (Table 2) was similar to that of authentic doxepin; the only difference was a slight (0.01–0.05 ppm) downfield shift of those resonances. However, the methyl and β-methylene resonances were shifted downfield considerably more, 0.63 and 0.24 ppm, respectively. The γ-methylene resonance was visible only as a broad hump in the 298.5 K spectrum. At 255 K, the spectrum revealed two separate γ-methylene resonances, each integrating as one. The methyl resonances were also moved apart enough for the two of them to be resolved at that temperature. These observations are consistent with N-oxide compounds. Selective saturation of the α-methylene resonance produced an NOE at H1 consistent with the (E)-isomeric form. However, a subspectrum was observed in a 15:85% ratio with the major spectrum, indicating that this metabolite was in the same isomeric ratio as doxepin. The EI mass spectral data were inconclusive due to the lack of certain characteristic ions, but the PICI data showed the presence of an additional oxygen atom (Table 1). The major component of peak X was identified as (E)-doxepin N-oxide and the minor as (Z)-doxepin-N-oxide.

The NMR spectrum of authentic doxepin (Fig. 4E) appeared to show two compounds in an 85:15% ratio, as did the gas chromatography mass spectra (data not shown). NOE experiments proved that the larger of the two was in the (E)-conformation. Rigorous proof that the smaller component was present in the (Z)-form was not conducted, because doxepin is known to be marketed as an 85:15% ratio of (E)- to (Z)- isomers. The NMR spectrum (Fig. 4E), mass spectral data (molecular ion at m/z 280, Fig. 3E), and HPLC retention time of peak XI were the same as those of authentic doxepin, thus confirming that it was unmetabolized (E)- and (Z)-doxepin.

The EI mass spectra of peaks XII and XIII were similar, with molecular ions at m/z 293 and base peak ions at m/z 234. The intense ion at m/z 234 is presumed to be formed from the loss of the substituted amine (C₃H₄NO) from the molecular ion. The PICI mass spectra (Fig. 3A) were also virtually identical, with ions at m/z 311, 294, and 234. The ion at m/z 311 is presumed to be the ammoniated molecule, [M + NH₄]⁺ and the ion at m/z 294 is presumed to be the protonated molecule, [M + H]⁺.

The aromatic area of the NMR spectra of XII and XIII indicated demethylation and no ring substitution (Fig. 4A). The mass spectral data indicated that an exchangeable proton might be present on each metabolite, so XII and XIII were dissolved in dimethyl-d₅-sulfoxide.

The NMR spectrum of peak XII revealed a singlet at 8.53 ppm, the formyl proton resonance (Table 2). The NMR spectrum of peak XIII showed a broadened triplet resonance at 7.91 ppm (the N-H proton) that was coupled to the γ-methylene resonance at 3.13 ppm. The other proton resonances were assigned by techniques already described. These metabolites were identified as (E)-N- and (Z)-formylididesmethyldoxepin [peak XII] and (E)- and (Z)-acetyldidesmethyldoxepin [peak XIII].

The EI mass spectrum of peak XIV had a base peak at m/z 44, again presumed to be [CH₂ = NH – CH₃]⁺, and the molecular ion was at m/z 307 [M⁺]. The corresponding PICI mass spectrum contained a base peak ion at m/z 308 [M + H]⁺ (Table 1). The 'H NMR spectrum of peak XIV shows all the resonances doubled with an extra set of singlets at 2.64 and 2.89 ppm (Table 2). Thus the data are consistent with N-acetylation (Zhang et al., 1996). The metabolite was identified as (E)-N-acetyl-N-desmethyldoxepin.

Table 3 shows the percentages of metabolites formed from cultures dosed with 10 mg doxepin per flask using NMR and HPLC peak area analysis for quantitation. Approximately 28% of the doxepin was metabolized to N-demethylated, ring-hydroxylated, N-oxide, N-acetylated, or N-formylated derivatives. The residual doxepin and/or metabolites that adhered to cellular material were not extracted. The mono-N-demethylated metabolites, including (E)- and (Z)-N-desmethyldoxepin, were the major metabolites.

Discussion

The present investigation has shown that C. elegans transformed doxepin to the following major metabolites: (E)-2-hydroxydoxepin, (E)-3-hydroxydoxepin, (Z)-8-hydroxydoxepin, (E)-2-hydroxy-N-desmethyldoxepin, (E)-3-hydroxy-N-desmethyldoxepin, (E)-4-hydroxy-N-desmethyldoxepin, (Z)- and (E)-8-hydroxy-N-desmethyldoxepin, (E)-N-acetyl-N-desmethyldoxepin, (E)-N-desmethyl-N-formylididesmethyldoxepin, (E)-N-acetyldidesmethyldoxepin, (E)- and (Z)-doxepin-N-oxide, and (E)- and (Z)-N-desmethyldoxepin. The structures of these compounds and the proposed biotransformation pathways are presented in Fig. 5.
FIG. 3. PICI mass spectra of selected doxepin metabolites.
A, (E)-N-acetylidesmethyldoxepin; B, (E)-, (Z)-N-desmethyldoxepin; C, (Z)-8-hydroxy-N-desmethyldoxepin and (E)-2-hydroxy-N-desmethyldoxepin; D, (E)-3-hydroxy-N-desmethyldoxepin; and E, doxepin.

FIG. 4. 1H NMR spectra of selected doxepin metabolites.
A, (E)-N-acetylidesmethyldoxepin; B, (E)-, (Z)-N-desmethyldoxepin; C, (Z)-8-hydroxy-N-desmethyldoxepin and (E)-2-hydroxy-N-desmethyldoxepin (denoted by assignments in quotation marks); D, (E)-3-hydroxy-N-desmethyldoxepin; and E, doxepin. The large resonance at approximately 2 ppm is an impurity.
The formation of (E)-2-hydroxydoxepin, (E)-3-hydroxydoxepin, and (Z)-8-hydroxydoxepin by C. elegans can be explained by a National Institutes of Health shift mechanism through doxepin 2,3- and doxepin 8,9-epoxide intermediates by the action of a cytochrome P-450 monooxygenase (Duhart et al., 1999). (E)-4-Hydroxy-N-desmethylidoxepin could have been formed from a doxepin 3,4-epoxide intermediate producing (E)-4-hydroxydoxepin that was subsequently demethylated. Several N-demethylated products were formed. This was not unexpected, because N-desmethyldoxepin was the most abundant metabolite. The hydroxy-N-desmethyl metabolites were presumably produced from a 2,3-, 8,9-, or 3,4-epoxide intermediate with concurrent demethylation. It is not clear which occurred first, the ring oxidation or the N-demethylation. Demethylation was probably required before acetylation or formylation, but ring oxidation never occurred in the N-acetyl or N-formyl products. It is interesting that neither 7-hydroxy-N-desmethylidoxepin nor 9-hydroxy-N-desmethylidoxepin was formed, although (E)-4-hydroxy-N-desmethylidoxepin and 2-hydroxy-N-desmethylidoxepin were formed on the A ring. This could be due to steric hindrance by the H6-methylene and the β-methylene protons, respectively.

The (E)-isomer of doxepin gave predominantly hydroxylation on the A ring and (Z)-isomer primarily on the B ring. This could be due to the steric hindrance of the β- and γ-carbons on the ring cis to them causing the ring trans to them being more open to enzymatic attack. (E)-8-Hydroxy-N-desmethylidoxepin does not conform to this explanation, but it is possible that if demethylation occurred first, then the ring cis to the β- and γ-carbons might be more vulnerable to enzymatic attack.

(E)- and (Z)-Desmethyldoxepin, (E)-2-hydroxy-N-desmethyldoxepin, (E)-2-hydroxydoxepin, (Z)- and (E)-doxepin-N-oxide, a hydroxy doxepin glucuronide and a hydroxy-N-desmethyldoxepin glucuronide have been reported as human and animal metabolites (Shu et al., 1990a; Hobbs, 1969). However, (E)-3-hydroxydoxepin, (Z)-8-hydroxydoxepin, (E)- and (Z)-8-hydroxy-N-desmethylidoxepin, (E)-3-hydroxy-N-desmethylidoxepin, (E)-4-hydroxy-N-desmethylidoxepin, (E)-N-desmethyl-N-formyldoxepin, (E)-N-acetylidesmethyldoxepin, and (E)-N-acetyl-N-desmethylidoxepin formed by C. elegans are novel metabolites not previously reported in animals or humans.

The phase I metabolism and stereoselectivity of the fungal metabolism of doxepin correspond to that reported in humans (Shu et al., 1990a). C. elegans produced (E)- and (Z)-doxepin N-oxide and (E)- and (Z)-N-desmethylidoxepin. These metabolites, present in both isomeric forms, retained the same 15:85% (Z)- to (E)-ratio as in human metabolism. Yan et al. (1997) state that more rapid metabolism of (E)-N-desmethylidoxepin accounts for distortion of the (Z)/E ratio over time. The present investigation shows twelve metabolites in the (E)-form but only three in the (Z)-form. Perhaps the microbial system also metabolizes the (E)-form of the drug faster, but instead of making a larger amount of a particular metabolite, it makes a larger variety of them.

Previous studies using C. elegans as a model for mammalian metabolism have indicated that the fungus is highly efficient in its production of metabolites from tricyclic antidepressants and related drugs. C. elegans produced six metabolites from doxepin that were similar to those obtained in human metabolism studies, as well as nine previously unreported metabolites. The ability of C. elegans to mimic mammalian metabolism and to perform novel biotransformations clearly demonstrates that microbial systems represent an attractive alternative to the use of actual mammalian systems or chemical synthesis of metabolites. The quantities of doxepin metabolites produced were sufficient not only for complete structural characterization, but also for future use in neurotoxicology studies at NCTR.
Acknowledgments. We thank Bruce D. Erickson, Thomas M. Heinze, and John B. Sutherland for critical reading of this manuscript, and Kim Cooney for illustrations.

References


