METABOLISM AND EXCRETION OF THE NOVEL ANTIPSYCHOTIC DRUG ZIPRASIDONE IN RATS AFTER ORAL ADMINISTRATION OF A MIXTURE OF $^{14}$C- AND $^{3}$H-LABELED ZIPRASIDONE

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ABSTRACT:

The metabolism and excretion of ziprasidone (5-[2-{4-(1,2-benzisothiazol-3-yl)piperazin-1-yl}ethyl]-6-chloro-1,3-dihydroindol-2-one hydrochloride hydrate) were studied in Long Evans rats after oral administration of a single dose of a mixture of $^{14}$C- and $^{3}$H-labeled ziprasidone. The radioactive dose was quantitatively recovered over 7 days in both male and female rats. The percentage of the dose excreted in urine, bile, and feces of rats was 21.6, 19.2, and 55.6%, respectively. The total excretion in urine and bile suggested that at least 41% of the drug was absorbed. Absorption of ziprasidone was rapid, and the mean plasma concentrations of the unchanged drug and metabolites were slightly higher in the female rats than in the males. The maximal plasma concentrations for ziprasidone and metabolites were reached at 1 hr in both male and female rats. Based on AUC (0–12 hr) values, approximately 59 and 52% of the circulating radioactivity (average of $^{14}$C and $^{3}$H) was attributable to metabolites in male and female rats, respectively. Ziprasidone was extensively metabolized in rats, and only a small amount of ziprasidone was excreted as unchanged drug. Twelve metabolites were identified by ion spray LC/MS, using a combination of parent ion and product ion scan techniques. The structures of eight metabolites were unambiguously confirmed by co-elution on HPLC with synthetic standards, and four additional metabolites were partially identified. There was a gender-related difference in the excretion of urinary metabolites in Long Evans rats. The major route of metabolism in male rats involved N-dealkylation. In female rats the major metabolites were due to oxidation at the benzisothiazole ring. Based on the structures of these metabolites, four major and two minor routes of metabolism of ziprasidone were identified. The major routes included 1) N-dealkylation of the ethyl side chain attached to the piperazinyl nitrogen, 2) oxidation at the sulfur, resulting in the formation of sulfoxide and sulfone, 3) oxidation on the benzisothiazole moiety (other than sulfur), and 4) hydration of the C–N bond and subsequent oxidation at the sulfur of the benzisothiazole moiety. The minor routes involved N-oxidation on the piperazine ring and hydrolysis of the oxindole moiety.

Classical antipsychotic drugs of the phenothiazine and butyrophenone classes have been established as effective agents for the treatment of schizophrenia, a mental disorder estimated to afflict 1% of the world population. However, these drugs are not effective in all patients or against all symptoms (1, 2). Secondly, an unacceptably high incidence of extrapyramidal symptoms limits the usefulness of these drugs (3). Laboratory and clinical findings have suggested that antagonism of serotonin 5-HT$_2$ receptors in the brain limits the undesirable side effects associated with dopamine receptor blockade and improves efficacy against the negative symptoms of schizophrenia (4–9).

ZIP is the hydrochloride salt of a benzothiazolylpiperazine analog structurally related to the atypical antipsychotic drug tiospirone (10, 11). It was developed during a structure-activity investigation to find a compound that potently blocks dopamine D2 receptors while binding with even greater affinity to cerebral serotonin 5-HT$_2$ receptors (12–14). ZIP has extremely potent central serotonin 5-HT$_2$ ($K_i = 0.42$ nM) and potent dopamine D2 ($K_i = 4.8$ nM) receptor antagonistic properties. The ratio between these two affinities (5-HT$_2$/D2 = 11.4) is greater than observed for other antipsychotic drugs (12–14). In phase II clinical studies, ZIP has demonstrated good tolerability, particularly with regard to extrapyramidal symptoms, at doses that are associated with efficacy in patients with schizophrenia (15). The oral bioavailability of ZIP in humans is 59% and its elimination half-life is 4 hr (16). ZIP is well tolerated in animals at doses producing effective blockade of dopaminergic behaviors and has a favorable separation between activities predictive of efficacy and side effect liability. ZIP is partially absorbed in rats and dogs, with absolute oral bioavailability of 39–60%. The elimination half-life is about 1 hr in rats and 2.3 hr in male dogs (17).

As with all new drugs, it is necessary that the metabolic fate of ZIP be evaluated in vivo. Metabolic studies conducted in living animals provide the ultimate information regarding metabolic pathways and disposition of the molecule that can be used to correlate or interpret efficacy and toxicity data. In addition, the identified metabolites can be evaluated for pharmacological activity. The objective of the present study was to investigate the metabolism and excretion of ZIP in LE...
acetyl chloride, followed by triethylsilane reduction of the aryl carbonyl and HPLC. [3H]ZIP, specifically labeled at the C7-position of the benzisothiazole and are uncorrected. FAB mass spectra of synthetic standards were obtained on a VG-70/70 mass spectrometer, using glycerol as a matrix.

Materials and Methods

General Chemicals. Commercially obtained chemicals and solvents were of HPLC or analytical grade. β-Glucuronidase (from Helix pomatia, type I-H, with sulfatase activity) was obtained from Sigma Chemical Co (St. Louis, MO). YMC basic columns were obtained from YMC (Wilmington, NC). Ecolite (+) scintillation cocktail was obtained from ICN (Irvine, CA). Carbosorb and Permafluor scintillation cocktails were purchased from Packard Instrument Co. (Downers Grove, IL). Diamethane was generated just before use from N-methyl-N-nitroso-p-toluene sulfonamide obtained from Aldrich (Milwaukee, WI). Silica gel was obtained from Fisher Scientific (Springfield, NJ).

General Instrumentation. The structures of all synthetic compounds were supported by their 1H-NMR (Bruker AM-300) and FAB mass spectra. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. FAB mass spectra of synthetic standards were obtained on a VG-70/70 mass spectrometer, using glycerol as a matrix.

Radiolabeled Drug and Reference Compounds. [14C]ZIP, specifically labeled at C2 of the ethyl group attached to the piperazinyl nitrogen, was synthesized by Friedel-Crafts acylation of 6-chloroindole with [14C]chloracetyl chloride, followed by triethylsilane reduction of the aryl carbonyl and coupling with BITP (20). [14C]ZIP showed a specific activity of 9.0 μCi/mmol (21.8 μCi/mg) and a radiochemical purity of ≥98%, as determined by radio-HPLC. [3H]ZIP, specifically labeled at the C7-position of the benzisothiazole ring, was synthesized by reduction of 7-bromo-ZIP with trimethylborane (20). [3H]ZIP showed a specific activity of 20.66 Ci/mmol (50.0 μCi/mg) and a radiochemical purity of ≥99% (by radio-HPLC). BITP, BITP-SO, BITP-SO₂, BITP-SO₃-lactam, OXCl, and OH-ZIP were prepared as described previously (20, 21). ZIP-N-oxide was prepared by reaction of ZIP with m-chloroperbenzoic acid.

ZIP-SO. A mixture of BITP-SO (75 mg, 0.32 mmol), OX-Cl (73 mg, 0.32 mmol), Na₂CO₃ (100 mg, 0.96 mmol), and KI (5 mg, 0.03 mmol) in 1.0 ml of acetonitrile was stirred and heated to reflux for 32 hr. After cooling, the solvent was removed in vacuo and the residue was redissolved in 5 ml of anhydrous CH₂Cl₂, filtered through a pad of diatomaceous earth, and concentrated in vacuo to a brown oil. Chromatography on silica gel (40 mm) eluted with CH₃Cl/CH₂Cl₂/CH₃OH (96:4) gave the desired product as a pale yellow solid (20 mg; m.p. 215–218°C; FAB mass spectrum: m/z 447 [14C], 445 (MH+) + [14C]) NMR (dimethylsulfoxide-d₆); δ 10.45 (s, 1H, NH), 8.23 (d, 1H, BIT C7-H), 8.05 (d, 1H, BIT C4-H), 5.75 (m, 2H, BIT C5,6-H), 7.25 (s, 1H, indole C7-H), 6.82 (s, 1H, indole C4-H), 3.95 (m, 4H, NCH₂), 3.45 (s, 2H, indole C3-H), 2.80 (m, 2H, ArCH), 2.70–2.50 (m, 6H, NCH₆).

ZIP-SO₂. A mixture of BITP-SO₂ (81 mg, 0.32 mmol), OX-Cl (73 mg, 0.32 mmol), Na₂CO₃ (100 mg, 0.96 mmol), and KI (5 mg, 0.03 mmol) in 1.0 ml of acetonitrile was stirred and heated to reflux for 32 hr. Work-up of the reaction mixture as described for ZIP-SO gave the desired product as a pale yellow solid (20 mg). It was converted to the hydrochloride salt by stirring with 3 N HCl in vacuo and was purified by recrystallization from CH₂Cl₂/CH₃OH (96:4) gave the desired product as a pale yellow solid (20 mg; m.p. 215–218°C; FAB mass spectrum: m/z 447 [14C], 445 (MH+) + [14C]). NMR (dimethylsulfoxide-d₆); δ 10.45 (s, 1H, NH), 8.22 (dd, 1H, BIT C7-H), 8.04 (dd, 1H, BIT C4-H), 7.74 (m, 2H, BIT C5,6-H), 7.23 (s, 1H, indole C7-H), 6.78 (s, 1H, indole C4-H), 3.95 (m, 4H, NCH₂), 3.45 (s, 2H, indole C3-H), 2.84 (m, 2H), 2.70–2.50 (m, 6H, NCH₆).

Animals. LE rats were purchased from Charles River Laboratories (Stonewall, NY). Animals were quarantined for a minimum of 7 days before treatment and were maintained on a 12-hr light/dark cycle. Animals were fed food and water ad libitum and maintained with United States Department of Agriculture guidelines for the care and use of laboratory animals.

Urinary, Biliary, and Excrement Studies. A group of three male and three female LE rats (240–260 g) were implanted with a PE-10 cannula into the common bile duct under anesthesia (sodium phenobarbital via ip injection). The animals were housed individually in stainless steel metabolic cages and were allowed to recover overnight before drug administration. The animals were orally administered 10 mg/kg radiolabeled ZIP. The dose was composed of a mixture of [3H]- and [14C]-labeled ZIP diluted with unlabeled drug to specific activities of 3.72 nCi/mg and 2.82 nCi/mg, respectively, and was administered as a suspension in 0.5% methylcellulose at a concentration of 1.5 mg/ml. Each animal received 23 μCi of [3H]-labeled and 17 μCi of [14C]-labeled material. All animals were fed at 2.5 hr after the dose and received electrolyte Krebs-Ringer solution throughout the study. Urine, bile, and feces from each animal were quantitatively collected for 7 days (168 hr after the dose) at 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144, and 144–168 hr after the dose; the first feces sample was collected at 0–24 hr after the dose. The volumes of urine and bile samples were recorded, and all of the biological samples were stored at −20°C until analysis.

Plasma Time Course Study. Another group of LE rats (N = 14/gender, 270–320 g) were dosed by gavage with a 10 mg/kg dose of a mixture of [3H]- and [14C]-labeled ZIP. Blood was collected in heparinized tubes, by decapitation of two male and two female rats, at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 hr after the dose. The blood samples were centrifuged at 1000 g for 10 min to obtain the plasma. Samples were transferred to clean tubes and stored at −20°C until analysis.

Determination of Radioactivity. Total radioactivity in urine, bile, and plasma was quantitated by counting sample aliquots (20–50 μl) using a [14C] "dual-label" program with a Packard 2500 TR liquid scintillation counter. Ecolite (+) scintillation cocktail (5 ml; ICN) was used for determination of the radioactivity in the samples. Quench curves were prepared separately for [14C]ZIP and [3H]ZIP. Fecal samples were lyophilized overnight and homogenized on a paint can shaker (Red Devil, model 5410), using chrome-plated 3/8 and 7/16 ball bearings. A small aliquot (20–60 mg) was combusted using a Packard Tricarb oxidizer (Irvine, CA). The liberated 14CO₂ and H₂O were trapped, and the radioactivity in the trapped samples was determined by counting in the liquid scintillation counter. Combustion efficiencies were determined by combusting 14C and 3H standards in an identical manner. The samples obtained at 0 hr after the dose were used as controls and counted to obtain a background count rate.

Pharmacokinetic Analysis. Plasma concentrations of the unchanged ZIP were determined by a validated HPLC/MS/MS assay (22). Pharmacokinetic parameters were determined by standard methods. The AUCs were calculated from plasma concentrations of ZIP and total radioactivity, using a trapezoidal approximation of area and using 0 as the time 0 concentration. The t½ value was the time of the first occurrence of the maximal plasma concentration.

Materials and Methods

BIOTRANSFORMATION OF A BENZISOTHIAZOLYP Piperazine 207

Fig. 1. Structures of [14C] and [3H]-labeled ZIP.
Quantitative Assessment of Metabolite Excretion. Quantification of the metabolites was carried out by measuring radioactivity in the individual peaks that were separated on HPLC, using a β-RAM. The β-RAM provided an integrated printout in dpm and percentage of the radiolabeled material, as well as peak representation. The β-RAM was operated in the homogeneous liquid scintillation counting mode, with addition of 4 ml/min Ecolite scintillation cocktail to the eluent after UV detection. For simultaneous monitoring of 3H and 14C-labeled compounds, efficiencies of 37% for 3H and 55% for 14C were used, with a compensation for 14C spill-over into the 3H window of 31%. These parameters were determined through separate injections of singly labeled standards.

Enzyme Hydrolysis. Pooled rat bile and urine samples (0–24 hr, 0.5 ml each) were adjusted to pH 5 with sodium acetate buffer (0.1 M) and treated with 2500 units of β-glucuronidase/sulfatase. The mixture was incubated in a shaking water bath at 37°C for 12 hr and diluted with acetonitrile. The precipitated protein was removed by centrifugation. The pellet was washed with an additional 2 ml of acetonitrile, and the two supernatants were combined. The supernatant was concentrated and dissolved in 0.5 ml of mobile phase, and an aliquot (50 μl) was injected into the HPLC system. Incubation of bile and urine samples without the enzyme served as a control.

Derivatization. One polar metabolite, M4, was isolated by HPLC and methylated with diazomethane. The purified metabolite M4 (100–200 ng) was dissolved in methanol (100 μl), and freshly prepared ethereal diazomethane (200 μl) was added. After standing for 15 min at room temperature, the solvent was removed with a stream of nitrogen and the residue was dissolved in the HPLC mobile phase.

MS. Analysis of the metabolites was performed on a Perkin-Elmer (Norwalk, CT) SCIEX API III HPLC/MS/MS system using ion spray. The effluent from the HPLC column was split and about 50 μl/min was introduced into the atmospheric ionization source via an ion spray interface. The remaining effluent was directed into the flow cell of the β-RAM. The β-RAM response was recorded in real time by the mass spectrometer data system, which provided simultaneous detection of radioactivity and MS data. The delay in response between the two detectors was about 0.2 min, with the mass spectrometric response being recorded first. The ion spray interface was operated at 6000 V, and the mass spectrometer was operated in the positive mode. CID studies were performed using argon gas at a collision energy of 25–28 eV and a collision gas thickness of 3.5 × 10^14 molecules/cm^2.

Results

Excretion. Preliminary studies showed that, at 24 hr after po administration of 10 mg/kg [3H]ZIP to rats, excretion of tritiated water amounted to only 0.2% of the dose (data not shown). The plasma sample obtained 24 hr after the dose had only 0.2% of the radioactive dose excreted in the recovered water. These findings suggest that the tritium label was on a metabolically stable position.

As shown in table 1, the radioactive dose was quantitatively recovered in urine, bile, and feces of LE rats after oral administration of a single dose of a mixture of [14C]ZIP and [3H]ZIP over a period of 0–168 hr. After a po dose of a mixture of [3H]ZIP and [14C]ZIP to rats, the fraction of the dose excreted in the urine of female rats tended to be slightly smaller than that in the males, and the fraction of the dose excreted in urine, bile, and feces as 14C label was somewhat smaller than the 3H label. The cumulative recovery in urine, bile, and feces from male and female rats is graphically depicted in fig. 2. Of all the radioactivity recovered in urine and bile of rats, 77 and 79% was excreted in the first 24 hr, respectively.

Circulation. The mean plasma concentration-time curves for ZIP and total radioactivity for male and female rats are shown in fig. 3. Absorption of ZIP was rapid in both male and female rats, as indicated by the early appearance of radioactivity in plasma after oral administration. The plasma concentration of ZIP and metabolites reached a peak of 1.92 μg equivalents/ml and 2.42 μg equivalents/ml for male and female rats, respectively, at 1 hr after the dose. Based on AUC (0–12 hr) values, 41 and 48% of circulating radioactivity, respectively, was attributable to parent drug (table 2).

Identification of the Major Metabolites. Metabolite Profile in Urine. An HPLC method was developed for the separation of ZIP and its metabolites. Baseline separation of all metabolites was obtained using this system. Representative profiles of the metabolites in urine samples (0–24 hr) from a male rat after oral administration of [3H]ZIP and [14C]ZIP, with on-line radioactivity monitoring of 14C and 3H, are given in fig. 4. Treatment of the urine samples with β-glucuronidase/arylsulfatase did not result in any changes in the HPLC profile of the sample, indicating that few, if any, of the metabolites were present as conjugates. A total of 11 radioactive peaks were detected in the chromatograms from both male and female rats. The radio-HPLC data are presented with the peak height normalized to that of the largest peak. Metabolites that did not undergo cleavage at the piperazinyl nitrogen are expected to be dual-labeled and are represented by HPLC peaks with similar heights and retention times in the two traces. Metabolites that underwent cleavage are expected to be singly labeled. Each of the cleaved metabolites is represented by a HPLC peak in one of the traces and a smaller peak representing spillover in the other trace. The metabolites were quantified with on-line integration of the radiochromatographic peaks. The percentages of the metabolites excreted in relation to the total radioactivity in urine are presented in table 3. Judging from the quantities of cleaved metabolites, there was a gender-related difference in the amounts of cleaved metabolites excreted. Approximately 45% of the total radioactivity in male rat urine and 26.9% of the total radioactivity in female rat urine was attributable to the cleaved products (table 3).

The structures of metabolites were elucidated by ion spray LC/MS/MS using a positive-ion mode. A strong pseudomolecular ion (M+H)^+ signal for ZIP at m/z 413 was observed; the minimum amount required to obtain a full-scan spectrum (Q1 scan) with a good signal-to-noise ratio was ~10 ng. The product ion mass spectrum of m/z 413 showed signals at m/z 220, 194, 177, 166, and 159 (fig. 5, top). The fragment ions at m/z 220 and 194 resulted from cleavage of the C—N (piperazine) bond and corresponded to BTP and...
(OX-CH₂CH₂)⁺, respectively. The fragment ions at m/z 177 and 159 were due to losses of 43 (C₅H₅N) and 35 Da (Cl) from the ions at m/z 220 and 194, respectively. The assignment of these ions was verified by a parallel CID spectrum of m/z 415 (MH⁺, 37Cl), which gave the fragment ions at m/z 220, 196, 177, 166, and 159 (fig. 5, bottom).

Combined liquid chromatography/ion spray MS (full scan) and tandem MS techniques such as precursor ion monitoring, product ion monitoring, selected ion monitoring, and multiple reaction monitoring scanning were used for the identification of metabolites (23–25). Where possible, the identities were confirmed by HPLC coelution with synthetic standards. The identified metabolites accounted for 88% of the total radioactivity present in urine. The remaining radioactive components were present only in very small amounts and could not be characterized.

**Urinary Peak II (M1).** Peak II had a retention time of 7.30 min on HPLC and was detected only in the ³H chromatogram, indicating that it was a cleaved product. It showed a protonated molecular ion at m/z 252, 32 Da higher than BITP, and its CID product ion spectrum showed fragment ions at m/z 235 and 209. M1 coeluted with authentic BITP-SO₂ on HPLC and had an identical CID product ion spectrum. Based on these data, M1 was identified as BITP-SO₂.

**Urinary Peak III (M2).** Peak III had a retention time of 8.02 min and was detected only in the ³H chromatogram, indicating that it was a cleaved product. It showed a protonated molecular ion at m/z 252, 32 Da higher than BITP, and its CID product ion spectrum showed fragment ions at m/z 235 and 209. M1 coeluted with authentic BITP-SO₂ on HPLC and had an identical CID product ion spectrum. Based on these data, M1 was identified as BITP-SO₂.

**TABLE 2**

<table>
<thead>
<tr>
<th>Animals</th>
<th>ZIP and Total Radioactivity</th>
<th>Pharmacokinetic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cₘₐₓ ng equivalents/ml</td>
</tr>
<tr>
<td>Male rats</td>
<td>¹⁴C radioactivity</td>
<td>1897.5</td>
</tr>
<tr>
<td></td>
<td>³H radioactivity</td>
<td>1932.0</td>
</tr>
<tr>
<td></td>
<td>ZIP</td>
<td>981.6</td>
</tr>
<tr>
<td></td>
<td>% of ZIPᵇ</td>
<td>51.3</td>
</tr>
<tr>
<td>Female rats</td>
<td>¹⁴C radioactivity</td>
<td>2374.5</td>
</tr>
<tr>
<td></td>
<td>³H radioactivity</td>
<td>2459.0</td>
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<tr>
<td></td>
<td>ZIP</td>
<td>1435.1</td>
</tr>
<tr>
<td></td>
<td>% of ZIPᵇ</td>
<td>59.4</td>
</tr>
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</table>

ᵃ N = 2.
ᵇ Average of ¹⁴C and ³H.
also a cleaved product. It showed a protonated molecular ion at \( m/z \) 236, 16 Da higher than BITP, suggesting that an oxygen atom had been added to the BITP moiety. CID product ions of \( m/z \) 236 showed intense fragment ions at \( m/z \) 177, 150, 134, and 85. M2 coeluted with authentic BITP-SO on HPLC and had an identical CID product ion spectrum. Based on these data, M2 was identified as BITP-SO.

**Urinary Peak IV (M3).** Peak IV showed a protonated molecular ion at \( m/z \) 266, 46 Da higher than BITP, suggesting the addition of three oxygen atoms with subsequent loss of two hydrogen atoms. The presence of only \(^3\)H label in the chromatogram further suggested that it was a cleaved product. The CID product ion spectrum of \( m/z \) 266 at \( m/z \) 266, 46 Da higher than BITP, suggesting the addition of three oxygen atoms with subsequent loss of two hydrogen atoms. The presence of only \(^3\)H label in the chromatogram further suggested that it was a cleaved product. The CID product ion spectrum of \( m/z \) 266

![FIG. 4. HPLC radiochromatograms of ZIP metabolites in male rat urine.](image)

![FIG. 5. CID product ion spectra of ZIP \( m/z \) 413 (\(^{35}\)Cl) (top) and \( m/z \) 415 (\(^{37}\)Cl) (bottom).](image)

**TABLE 3**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Identification of Metabolite</th>
<th>Relative Amounts$^a$</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>M1</td>
<td>BITP-SO$_2$</td>
<td>5.1</td>
</tr>
<tr>
<td>M2</td>
<td>BITP-SO$_2$</td>
<td>22.9</td>
</tr>
<tr>
<td>M3</td>
<td>BITP-SO$_2$-lactam$^b$</td>
<td>9.3</td>
</tr>
<tr>
<td>M4</td>
<td>OX-COOH$^c$</td>
<td>44.6</td>
</tr>
<tr>
<td>M5</td>
<td>BITP$_2$</td>
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</tr>
<tr>
<td>M6</td>
<td>ZIP-O$_2$</td>
<td>24.4</td>
</tr>
<tr>
<td>M7</td>
<td>Dihydro-ZIP-O$_2$</td>
<td>4.7</td>
</tr>
<tr>
<td>M8</td>
<td>ZIP-SO$_2$</td>
<td>1.2</td>
</tr>
<tr>
<td>M9</td>
<td>ZIP-O</td>
<td>6.9</td>
</tr>
<tr>
<td>M10</td>
<td>ZIP-SO</td>
<td>3.4</td>
</tr>
<tr>
<td>M11</td>
<td>ZIP-NO</td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>ZIP-O-COOH</td>
<td></td>
</tr>
<tr>
<td>M13</td>
<td>ZIP</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

$a$ Relative amounts are expressed as mean percentage of \(^{14}\)C and \(^3\)H excreted in excreta.

$b$ Only \(^3\)H label.

$c$ Only \(^{14}\)C label.
showed an intense fragment ion at \( m/z \) 209. It coeluted with authentic BITP-SO\(_2\) lactam on HPLC and had an identical CID product ion spectrum. Based on these data, M3 was identified as BITP-SO\(_2\) lactam.

**Urinary Peak V (M4).** Peak V had a retention time of 22.1 min on HPLC and it had only \(^{14}\)C label, indicating that this metabolite was a cleaved product and contained an oxindole moiety. The metabolite did not show any distinct pseudomolecular ion, probably due to a low molecular weight. However, the reconstructed selected ion profile of peak V indicated an ammoniated adduct ion at \( m/z \) 243. The metabolite was, therefore, isolated by HPLC and was reacted with diazomethane. Treatment of M4 with diazomethane resulted in the disappearance of the peak coinciding with M4 and appearance of a new peak that had the retention time of 36.4 min on HPLC (fig. 6). The derivatized metabolite M4 showed an intense ion at \( m/z \) 257 (\( M+\text{NH}_4 \))\(^+\), 14 Da higher than that of the parent metabolite, indicating that the COOH functionality was present in the molecule. This was further supported by the fact that the CID product ion spectrum of the derivatized metabolite (\( m/z \) 257) showed intense ions at \( m/z \) 240 (\( M+\text{H}^+ \)) and 180, showing loss of a \( \text{CH}_3\text{COOH} \) molecule from the molecular ion (fig. 6). Based on these data, M4 was identified as 6-chloro-2-oxo-(2,3-dihydro-1H-indol-5-yl)acetic acid.

**Urinary Peak VI (M5).** Peak VI had a retention time of 32.1 min and was found to be a mixture of two metabolites (M5 and M6). One of the metabolites, M5, showed a protonated molecular ion at \( m/z \) 220, and its CID mass spectrum exhibited the fragment ion at \( m/z \) 177 (loss of 43), the same as BITP. M5 coeluted with synthetic BITP and had an identical CID product ion spectrum. Based on these data, M5 was identified as BITP.

**Urinary Peak VI (M6).** The second metabolite in peak VI showed a protonated molecular ion at \( m/z \) 445, 32 Da higher than the parent drug, suggesting that the molecule had undergone two oxidations. Its CID product ion spectrum (\( m/z \) 445) showed a fragment ion at \( m/z \) 166, suggesting the presence of two oxygen atoms on the benzisothiazole moiety. The presence of fragment ions at \( m/z \) 194, 263, and 280 in its CID spectrum further suggested that the oxidations had occurred.
Fig. 7. CID product ion spectrum of m/z 447 for ZIP metabolite M7.

Fig. 8. CID product ion spectra of m/z 445 for ZIP metabolite M8 and synthetic standard.
at positions remote from the oxindolylpiperazine part of the molecule. M6 did not coelute with synthetic ZIP-SO₂ standard on HPLC. Based on these data, M6 was identified as the intact molecule with the addition of two oxygen atoms at the benzisothiazole moiety. The exact sites for oxidation were, however, not established by the mass spectral data.

Urinary Peak VII (M7). M7 showed a protonated molecular ion at m/z 447, 34 Da higher than the parent drug, suggesting that the molecule had undergone monooxidation and addition of a molecule of water. Its CID product ion spectrum (m/z 447) showed a fragment ion at m/z 168, indicating the presence of an oxygen atom and a water molecule on the benzisothiazole moiety (fig. 7). This was further supported by the presence of other characteristic fragment ions at m/z 194 and 280 in its CID product ion spectrum. Based on these data, M7 was tentatively identified as 2-\{4-[2-(6-chloro-2-oxo-2,3-dihydro-1H-indol-5-yl)ethyl]piperazine-1-carbonyl\}benzenesulfonic acid amide.

Urinary Peak VIII (M8). M8 showed a protonated molecular ion at m/z 445, 32 Da higher than the parent drug, suggesting that the molecule had undergone two oxidations. Its CID product ion spectrum (m/z 445) showed an intense fragment ion at m/z 194, indicating that the oxidations had occurred at positions remote from the oxindole part of the molecule. M8 coeluted with authentic ZIP-SO₂ on HPLC and had an identical CID product ion spectrum (fig. 8). Based on these data, M8 was identified as ZIP-SO₂.

Urinary Peak IX (M9). M9 exhibited a protonated molecular ion at m/z 429, 16 Da higher than the parent drug, suggesting that it was a monooxidation product of ZIP. Its CID product ion spectrum (m/z 429) showed fragment ions at m/z 194, 280, and 150, suggesting the presence of an oxygen atom on the benzisothiazole moiety. The retention time of M9 was different from those of the synthetic ZIP-SO and OH-ZIP standards. Based on these data, M9 was identified as the intact molecule with the addition of one oxygen atom at the benzisothiazole moiety, other than the sulfur. These data could not establish the exact sites for the oxidation.

Urinary Peak X (M10). Peak X also indicated a protonated molecular ion at m/z 429, 16 Da higher than the parent drug, suggesting that a single atom of oxygen had been added to the molecule and it was an isomer of M9. Its CID product ion spectrum showed the fragment ions at m/z 194 and 232, indicating that the oxindole moiety was unchanged. The characteristic fragment ion at m/z 99 (piperazine-CH₂⁺)
further suggested that the oxidation had occurred at the benzisothiazole moiety. M10 coeluted with synthetic ZIP-SO on HPLC and had an identical CID product ion spectrum (fig. 9). Based on these data, peak X was identified as ZIP-SO.

Urinary Peak XI (M13, Unchanged Drug). Peak XI showed the pseudomolecular ion at \( m/z \) 413, similar to ZIP. Its CID product ion spectrum showed fragment ions at \( m/z \) 194 and 159, indicating that the oxindole ring was unchanged. The fragment ions at \( m/z \) 220 and 177 suggested that there was no substitution on the BITP moiety. M13 coeluted with synthetic ZIP on HPLC and had an identical CID product ion spectrum. Based on these data, peak XI was identified as unchanged drug.

Metabolites in Bile. Metabolite Profile in Bile. A major portion of the radioactivity in bile was recovered from 0–8 and 8–24 hr. Therefore, bile samples from 0–8 and 8–24 hr were pooled, and the pooled sample was used for the identification of metabolites. Pooled bile samples were diluted with 4 volumes of acetonitrile, and the precipitated proteins were removed by centrifugation. Approximately 89% of the total radioactivity was recovered in the supernatant and 9% remained in the pellet. Representative profiles of the metabolites in bile (0–24 hr) from a male rat, with on-line radioactivity monitoring of \( ^{14}C \) and \( ^{3}H \), are given in fig. 10. The relative amounts of metabolites excreted in the bile, in relation to the total radioactivity, are presented in table 3. There was no substantial gender-related difference in the profiles of the biliary metabolites. Only the unchanged drug was excreted 3 times more in the bile of female rats than male rats. ZIP and five radioactive peaks, accounting for 66% of the total radioactivity, were identified in bile. Five of these metabolites were identical to those identified from the urine, i.e., peaks I (M6), II (M7), III (M8), IV (M9), and VI (M13). Only one additional metabolite was identified from the bile.

Biliary Peak V (M12). Biliary peak V showed a protonated molecular ion at \( m/z \) 447, 34 Da higher than the parent drug, suggesting that the molecule had undergone monooxidation and addition of a water molecule. The CID product ion spectrum of peak V showed three characteristic fragment ions, at \( m/z \) 212, 298, and 150 (fig. 11). The fragment ions at \( m/z \) 212 and 298 suggested that a molecule of water had been added to the oxindole moiety. The fragment ion at \( m/z \) 150 indicated that the monooxidation had occurred on the benzisothiazole
in their identification. ZIP was extensively metabolized before excretion in bile and urine. Only a small percentage of the unchanged drug was found in the bile. Approximately 41% of the radioactivity was excreted in the urine and bile of rats after administration of \(^{14}\text{C}\) and \(^{3}\text{H}\)-labeled ZIP. The major portion of the unabsorbed dose recovered in feces was attributable to the parent drug.

The profiles of metabolites before and after treatment with \(\beta\)-glucuronidase were similar, suggesting that the elimination of the drug was due to only phase I metabolism and that phase II metabolic pathways did not play a significant role in the metabolism of ZIP. In addition to the minor amounts of drug, a total of 12 metabolites were identified in rats. The structures of eight metabolites were unambiguously confirmed by comparison of their chromatographic and mass spectral fragmentation properties with those of synthetic standards, and four metabolites were tentatively identified based on their fragmentation patterns. The major metabolites found in bile and feces were also found in urine, although there were significant quantitative differences (table 3). Metabolites were identified by ion spray LC/MS/MS, using parent ion and product ion scanning techniques, with simultaneous radioactivity monitoring. These techniques have proved to be very useful for rapid screening and identification of drug metabolites (23–25). ZIP and its synthetic metabolites gave very intense protonated molecular ions. CID product ion spectra of protonated molecular ions provided structurally significant fragment ions.

In an attempt to establish the exact sites of oxidation, synthesis of some potential metabolites (ZIP-SO, ZIP-SO\(_2\), and ZIP-NO) was undertaken. Preliminary attempts to oxidize ZIP directly at the sulfur atom, using published procedures (21), gave a mixture of several products. This may be the result of instability of the oxindole moiety under acidic conditions. The metabolites ZIP-SO and ZIP-SO\(_2\) were, therefore, synthesized by oxidation of BITP and then condensation of the intermediates with OX-Cl.

The metabolism of ZIP occurred by four major and two minor routes (fig. 14). The major routes included N-dealkylation of the ethyl side chain attached to the piperazinyl nitrogen, oxidation at sulfur (resulting in the formation of sulfoxide and sulfone), oxidation on the benzisothiazole moiety (other than sulfur), and hydration of the C==N bond and subsequent oxidation at the sulfur of the benzisothiazole moiety. The minor routes involved N-oxidation at the piperazine ring and hydrolysis of the oxindole moiety. The metabolite patterns in male and female rats were qualitatively very similar. However, there were some gender-related quantitative differences in the excretion of urinary metabolites. N-Dealkylation was the major route of metabolism in male rats. In female rats, the major metabolites were due to oxidation at the sulfur of the benzisothiazole ring.

The first major route, N-dealkylation, was analogous to those observed for the structurally related drugs (18, 26, 27). The N-dealkylation products of ZIP were identified as BITP-SO\(_2\) (M1), BITP-SO\(_2\)-lactam (M3), 6-chloro-2-oxo-(2,3-dihydro-1H-indol-5-yl)acetic acid (M4), and BITP (M5). The metabolites M1, M2, M3, M4, and M5, resulting from N-dealkylation, accounted for 42–45% of the radioactivity in male rats and only 22–27% of the radioactivity in female rats. On the other hand, the metabolites resulting from oxidation on the benzisothiazole ring represented 40 and 60% of the total radioactivity present in urine of male and female rats, respectively (table 3). It is not clear whether metabolites M1 and M2 were derived by N-dealkylation of ZIP-SO and ZIP-SO\(_2\), respectively, or by oxidation of BITP. However, the formation of M3 could be speculated to be by oxidation of BITP-SO\(_2\). Based on these data, it could be concluded that the N-dealkylation of ZIP formed BITP, which was further oxidized to form metabolites M1, M2, and M3.
The formation of sulfoxide and sulfone is common with sulfur-containing drugs (18, 19, 28), and this suggests that the benzisothiazole moiety is more susceptible to oxidation at sulfur than to aromatic hydroxylation. Oxidation at the α-carbon to the nitrogen, resulting in the formation of a lactam, has been observed previously for nicotine (29), encainide (30), flecainide (31), and several other drugs (32). Oxidation on the benzisothiazole moiety other than sulfur was, however, not observed for tiospirone and similar drugs (18, 19, 28). The novel metabolite M7, formed by the addition of water and subsequent oxidation of the benzisothiazole moiety, was tentatively identified as 2-{4-[2-(6-chloro-2-oxo-2,3-dihydro-1H-indol-5-yl)ethyl]piperazine-1-carbonyl}benzenesulfinic acid amide. The proposed structure of M7 did not immediately suggest the routes for its formation. Obviously there may be some intermediate compounds that were not detected as the precursors of this metabolite. A possible reaction sequence is given in fig. 15. Hydration of the C==N double bond of ZIP could afford the carbinoldiamine, which could be hydrolytically rearranged by a reaction identical to the second step of N-dealkylation (33, 34). Either of the two C==N bonds could be cleaved to yield [(2-piperazine-1-yl)ethyl]-6-chloro-1,3-dihydroindol-2-one and hydrated ZIP. Because only metabolite M7 was detected, it could be speculated that the hydrolytic cleavage was selective. The results of this study do not indicate whether hydration of the benzisothiazole moiety occurs before or after oxidation at the sulfur. Because ZIP-SO was detected as a metabolite, the only conclusion that can be drawn is that sulfur oxidation is a prerequisite for hydration to occur.

Two of the oxygenated metabolites (M6 and M9) detected in the urine and bile appear to be modified on the benzisothiazole moiety other than at the sulfur atom. There are two possible sites for such oxidation, i.e. oxidation at the nitrogen of the benzisothiazole ring to form ZIP-thiazole-N-oxide and aromatic hydroxylation. Metabolites derived from these oxidations could then undergo subsequent oxidation at sulfur to form the dioxygenated metabolites ZIP-N-oxide-S-oxide and ZIP-OH-S-oxide. Monooxygenated metabolite M9 did not coelute with synthetic OH-ZIP, suggesting that hydroxylation had not occurred at the 5-position of the benzisothiazole ring. However, the formation of other regiosomeric hydroxy metabolites could not be ruled out. The N-oxide of BIT has been prepared by the reaction of 1,2-benzisothiazolium chloride with hydroxylamine (35). In addition, the oxidation of two heterocyclic atoms in an aromatic ring was reported earlier by Offen et al. (36). They identified 4,5-dimethylthiazol-N-oxide-S-oxide, a metabolite of chloromethiazole, in humans (36). Attempts to synthesize a thiazole-N-oxide derivative of ZIP and to methyleate metabolites M6 and M9 were unsuccessful. Therefore, metabolites M6 and M9 were only partially identified. Complete structural characterization of partially identified metabolites requires further work to establish the exact sites of oxidation.

In summary, the clearance of ZIP in rats was mainly by phase I
oxidative metabolism, followed by elimination. Phase II metabolism did not play any significant role in the elimination of ZIP in rats. The use of two labels was very helpful in identifying the cleaved metabolites. The pharmacological properties of the major identified metabolites are currently under investigation. The present study will contribute to our understanding of the metabolism of other benzisothiazolylpiperazine drugs. In addition, the study of ZIP metabolism in human subjects will be facilitated by these data.

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References


