TISSUE DISTRIBUTION AND BIOTRANSFORMATION OF ZOPOLRESTAT, AN ALDOSE REDUCTASE INHIBITOR, IN RATS

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ABSTRACT:

Zopolrestat (Alond) is a new drug that is being evaluated as an aldose reductase inhibitor for the treatment of diabetic complications. 14C-labeled zopolrestat was orally administered to rats for a tissue distribution study and a bile duct cannulation metabolism study. Tissue samples from the distribution study were analyzed by complete oxidation and liquid scintillation counting. Urine and bile samples from the bile duct cannulation study were analyzed by microbore HPLC, with simultaneous radioactivity monitoring and atmospheric pressure ionization tandem mass spectrometry. The mass balance in the distribution study demonstrated that the greatest exposure (AUC0–∞) occurred in the liver, followed by the ileum and large intestine. The time of maximal plasma concentration for nearly all tissues was 4 hr after the dose, and the half-life of radioactivity in most tissues (8–10 hr) was similar to the half-life in plasma. For the bile duct-cannulated rat study, most of the radioactivity was recovered in the bile, indicating that biliary excretion is a major route of elimination of zopolrestat and its metabolites in rats. Numerous oxidative metabolites, as well as phase II conjugates, were identified in the bile and urine samples. Acyl glucuronides of zopolrestat and unchanged drug accounted for >85% of biliary radioactivity, whereas unchanged drug and degradation products of glutathione conjugates were identified as the major urinary metabolites.

Methods for the treatment and prevention of the long-term complications of diabetes are medically needed. In the past 10 years, it has been shown that flux through the polyol pathway in diabetic animals and humans may be associated with complications such as neuropathy, nephropathy, cataractogenesis, and macrovascular disease (Kinoshita and Nishimura, 1988; Nathan, 1995; Raskin and Rosenstock, 1987; Sastry et al., 1995). The enzyme aldose reductase has been implicated in the pathogenesis of diabetic complications such as these (Lee et al., 1995; Sastry et al., 1995). The use of aldose reductase inhibitors in diabetic subjects has been shown to have a positive impact on motor nerve conduction velocity, sciatic nerve blood flow, tissue sorbitol levels, and sciatic nerve fructose and myo-inositol levels (Beyrer-Mears et al., 1984; Gonzalez et al., 1983; Hotta et al., 1995; Whiting and Ross, 1988). Several aldose reductase inhibitors have been shown to decrease erythrocyte sorbitol levels in diabetic subjects (Malone et al., 1984; Raskin et al., 1985).

Zopolrestat (CP-73,850) [3,4-dihydro-4-oxo-3-[5-(trifluoromethyl)-2-benzothiazolylmethyl]-1-phthalazineacetic acid] is a novel, carboxylic acid, aldose reductase inhibitor. It has been shown to normalize sorbitol, fructose, and myo-inositol levels in sciatic nerve, lens, retina, and kidney of diabetic rats (Beyer et al., 1990; Mylari et al., 1991) and to normalize renal plasma flow in galactosemic rats (Oates and Ellery, 1990). The pharmacokinetics of zopolrestat have been evaluated in normal and diabetic rats (Inskeep et al., 1991), healthy male volunteers (Inskeep et al., 1994), and non-insulin-dependent diabetic patients (Inskeep et al., 1995).

The pharmacological and pharmacodynamic effects of zopolrestat are briefly summarized above, but details of the metabolism and disposition of zopolrestat have not yet been reported. To assess the metabolism and disposition of zopolrestat in rats after a radioactive dose, we used simultaneous radioactivity detection and atmospheric pressure ionization MS with an electrospray ionization interface. Atmospheric pressure ionization MS has evolved into a common analytical tool that provides a soft ionization technique for many analytes. It has been extensively used for the quantitative analysis of drugs (Fouda and Schneider, 1995), the identification of neurotoxic metabolites (Subramanyam et al., 1991), the confirmatory identification of drug residues (Schneider et al., 1993), and the elucidation of the metabolic fate of drugs (Tomlinson et al., 1993). The studies described here made extensive use of these methods, and they summarize the metabolic profile of zopolrestat in rats after oral administration of radiolabeled drug.

Materials and Methods

Labeled Zopolrestat. Radiolabeled zopolrestat (fig. 1) was synthesized at Pfizer Central Research (Groton, CT) (Mylari and Zembrowski, 1991). The specific activity of [14C]zopolrestat (lot 22280–236-2, C-725) used in this study was 1.05 μCi/mg.

Reagents. All solvents and reagents were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Ecolite scintillation cocktail was purchased from ICN (Irvine, CA). Permafluor E+ scintillation fluid, Ultima Gold liquid scintillation fluid, and Carbo-Sorb trapping solvent were purchased from Packard Instrument Co. (Meriden, CT). Putative metabolites (CP-72,262, 5-trifluoromethyl-2-benzothiazol; CP-114,914, phthalazineacetic acid) were synthesized at Pfizer and used as standards for HPLC and MS reference.

Animal Studies. Tissue Distribution Study. Eighteen male Long-Evans rats (body weight, 315 ± 9 g) were dosed with an aqueous solution of [14C]zopolrestat (5.10 mg/ml; specific activity, 0.92 μCi/mg) by gavage, for a total of
AUC 0–tissues.

the slope of the regression line of ln

rate constant was determined from the

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using blank oxidizer samples (processed through a burn cycle) fortified with

oxidizer (model 306). Burn efficiency standards were used throughout all

activity in all tissue samples was determined by sample oxidation in a Packard

vessels contained 0.5 ml of 1.0 M phosphoric acid for male rats and 0.5–1.0 ml

solution/kg of body weight, with a 8.02 mg/ml solution of [14 C]zopolrestat,

not survive surgery or did not provide bile for at least 48 hr were not included

additional animals that were dosed for use as substitutes for animals that did

Long-Evans rats were cannulated so that continuous collections of bile could

range of 80 –120 dpm) being defined as approximately 3 times background counts.

were counted for up to 30 min, with minimal quantifiable counts (generally in the

tissue available). The lower limit of quantification for tissue radioactivity levels

depended on the amount of tissue mass available for sample oxidation. Samples

urinary bladder (single determinations were made because of the small amount of

represented the mean of replicate determinations, except for adrenal glands and

i.e. adrenal glands and urinary bladder (contents removed) were analyzed as single

samples.

Tissue levels of radioactivity were expressed as microgram-equivalents per

gram (i.e. dpm per gram divided by the specific activity of the parent drug) and

represented the mean of replicate determinations, except for adrenal glands and

urinary bladder (single determinations were made because of the small amount of

tissue available). The lower limit of quantification for tissue radioactivity levels

depended on the amount of tissue mass available for sample oxidation. Samples

were counted for up to 30 min, with minimal quantifiable counts (generally in the

range of 80–120 dpm) being defined as approximately 3 times background counts.

The approximate lower limits of quantification (and approximate tissue masses)

were 0.4 µg-eq/g (0.1 g) for eyes, 0.8 µg-eq/g (0.05 g) for adrenal glands, 1.5

µg-eq/g (0.04 g) for sciatic nerve, 0.4 µg-eq/g (0.1 g) for whole blood, plasma, and

red blood cells, 0.3 µg-eq/g (0.15 g) for fat, and 0.15 µg-eq/g (0.3 g) for other

tissues.

Pharmacokinetic parameters were calculated from mean tissue levels.

AUCτ,∞, was calculated using a linear trapezoidal approximation. The elimina-

tion rate constant was determined from the slope of the regression line of ln

(tissue concentration) vs. time. AUCτ,∞ was calculated by adding to AUCτ,∞ the

mean terminal tissue concentration divided by the elimination rate constant.

Bile Duct-Cannulated Rat Study. The bile ducts of several male or female

Long-Evans rats were cannulated so that continuous collections of bile could be

obtained for at least 48 hr from four male and four female rats. Data from

additional animals that were dosed for use as substitutes for animals that did not

survive surgery or did not provide bile for at least 48 hr were not included in the

analysis. Male rats were dosed with approximately 12 ml of dosing solution/kg of body weight, with a 8.02 mg/ml solution of [13 C]zopolrestat, and female rats were dosed with approximately 20 ml of dosing solution/kg of body weight, with a 4.63 mg/ml solution. The total dose was approximately 100 mg/kg. The rats were housed in metabolism cages. Urine, bile, and feces samples were collected for 0–24 hr on days 1, 2, 3, and 4. Urine collection vessels contained 0.5 ml of 1.0 M phosphoric acid for male rats and 0.5–1.0 ml of 2 M phosphoric acid for female rats. All samples were stored frozen and were thawed just before assay.

Total Radioactivity Determination. Tissue Distribution Study. Radioac-

tivity in all tissue samples was determined by sample oxidation in a Packard

oxidizer (model 306). Burn efficiency standards were used throughout all

sample oxidizer runs. Samples were counted for up to 30 min in a Packard

scintillation counter (model 2000CA), and counting efficiency was determined

using blank oxidizer samples (processed through a burn cycle) fortified with

[14 C]toluene (18,330 dpm). For some samples, counting efficiency was deter-

mined by adding internal standard ([14 C]toluene) to the sample and recounting.

Bile Duct-Cannulated Rat Study. Radioactivity in bile and urine was deter-

mined by LSA after dilution in Packard Ultima Gold liquid scintillation fluid.

Radioactivity in feces was determined by LSA after sample oxidation in a

Packard sample oxidizer, using Packard Carbo-Sorb trapping solvent. The

oxidized samples were diluted with Packard Permafluor E+ scintillation fluid.

Internal standardization ([14 C]toluene) was used for all LSA determinations in

this study.

Quantitative Profiling of Radioactivity for Cannulated Rat Study Sam-

ples. Urine Profiling. Urinary metabolite profiles were determined for each

animal, using individual or pooled urine samples containing at least 70% of

total urinary radioactivity. When urine samples from one animal were com-

bined, aliquots from each interval were combined in the same proportion as the

total urine volumes for the intervals. In one case (male 20), profiles were
determined in two separate urine samples, and the results for the composite

sample were calculated with a similar volume-weighted summation.

Aliquots (0.5 ml) of thawed urine samples were centrifuged, and the

supernatant was transferred to another tube. The remaining pellet was quanti-

tatively extracted with 1 ml of ACN. The ACN extract was evaporated to

dryness, and the 0.5-ml supernatant layer was added to the residue. The

samples were flash-frozen and lyophilized. The residues of the urine samples

were reconstituted in 60 µl of 20 mM ammonium acetate plus 40 µl of ACN.

Aliquots (10 µl) were injected into a microbore HPLC system, and two-drop (approximately 30-sec) fractions were collected. Additional 10-µl aliquots of

reconstituted samples were removed to determine total radioactivity injected

onto the HPLC column. The amount of radioactivity in each fraction and in the

10–15 µl recovery aliquot was determined by LSA after addition of 6 ml of

Ultima Gold scintillation fluid. Metabolite peaks were quantified based on retention

times, relative to that of unchanged drug. Each metabolite was quantified by the

amount of radioactivity in the fractions collected at its HPLC retention
time.

Bile Profiling. To quantify biliary radioactivity, 5–15 µl injections of bile

(containing ~3000 dpm) were made. Bile samples that exhibited low activity

were concentrated ~2–3-fold by an ACN precipitation method. With this

procedure, mean recoveries of radioactivity were 89%.

A β-RAM (INUS Systems, Tampa, FL) was equipped with a specially
designed cell to accommodate low flow rates (i.e. 75 µl/min). The flow cell

minimized extracolumn effects during radioactivity monitoring, with reduced

tubing inner diameter and volume (cell tubing i.d., 0.028 inch; total cell

volume, 100 µl). A Harvard syringe pump with a 10- or 25-ml syringe was

used to deliver the cocktail to the liquid scintillation flow cell. The connecting

tubing between the MS ion-spray interface and the β-RAM was a combina-

tion of 50-µm i.d. fused silica (to the ion-spray interface) and 100-µm i.d. fused

silica (to the β-RAM). With this configuration, the LC flow rate of 75 µl/min

was split, such that the mass spectrometer received ~15 µl/min and the

β-RAM received ~60 µl/min.

After chromatographic resolution, the β-RAM was used to quantify the

radioactivity associated with each biliary metabolite. The β-RAM provided

peak representation, an integrated printout of dpm/peak, and the percentage of

radiolabeled material in each peak, relative to the total amount of activity

detected. The β-RAM was operated in the homogeneous liquid scintillation

counting mode, with the addition of scintillation cocktail to the LC effluent at

240 µl/min. Total flow to the β-RAM was ~300 µl/min, resulting in a cell

residence time of 20 sec.

Recovery of biliary radioactivity from the HPLC column was determined in

a separate experiment. After injection of 5 µl of bile, the HPLC effluent was

collected and counted by LSA. The recovery of radioactivity was approxi-

mately 96%.

HPLC. Urine Analysis. HPLC analysis used a Keystone BDS Hypersil C18
column (1 × 250 mm). An ABI 140A dual-syringe pump provided a constant

flow of 75 µl/min. The mobile phase and linear gradient conditions were as

follows: solvent A, 1.9 ACN/20 mM NH₄OAc (pH 5); solvent B, 9:1 ACN/20

mM NH₄OAc (pH 5); at 0 min, A = 100%; at 15 min, A = 90%; at 22 min,

A = 50%; at 30 min, A = 0%; at 33 min, A = 90% at 41 min, A = 100%.

1 Abbreviations used are: LSA, liquid scintillation analysis; CID, collision-

induced dissociation; ACN, acetonitrile; NH₄OAc, ammonium acetate; β-RAM,

β-radioactivity monitor; NAC, N-acetylcysteine; Cmax, maximal plasma concen-

tration; GSH, glutathione.
The column was equilibrated for an additional 15 min before the next injection. Under these conditions, zopolrestat eluted at approximately 35 min.

**Bile Analysis.** The analytical column and syringe pump were the same as described above. The mobile phase and linear gradient conditions were as follows: solvent A, 1:9 ACN/20 mM NH4 OAc (pH 4); solvent B, 9:1 ACN/20 mM NH4 OAc (pH 4); at 0 min, A = 90%; at 30 min, A = 70%; at 35 min, A = 35%; at 45 min, A = 35%; at 50 min, A = 90%. The column was equilibrated for an additional 15 min before the next injection. Under these conditions, zopolrestat eluted at approximately 35 min.

**Preparation of Biological Samples for MS Analysis.** For the characterization of urinary metabolites, aliquots of pooled male rat urine (1 ml; 0–24 hr after dosing) containing 392,000 dpm/ml were acidified with approximately 66.24% in feces (table 2). Exposure to radioactivity for all other tissues was 3–30-fold lower than for liver.

**Tissue Distribution Study:** Maximal levels of radioactivity from [14C]zopolrestat were observed at 4 hr after dosing in all tissues except urinary bladder and large intestine, where maximal levels were observed at 8 hr after dosing (table 1). Radioactivity could be quantified in all tissues, except eyes, brain, and adrenal glands, for 48 hr after dosing. Only in testes and liver could radioactivity still be quantified 168 hr after dosing, and levels in these tissues were very close to the lower limits of quantification (approximately 0.15 μg·eq/g). Tissue exposure to radioactivity, as expressed by AUC_{0–t}, was greatest in liver, followed by the intestines, kidney, and plasma (table 1). Exposure to radioactivity for all other tissues was 3–30-fold lower than for liver.

**Bile Duct-Cannulated Rat Study.** Total Radioactivity Excreted in Urine, Bile, and Feces. Most of the radioactivity administered to the rats in this study was recovered in urine, bile, and feces within 2 days after dosing. The bile contained the most radioactivity (59.70–73.67% of the dose for individual males and 12.53–52.10% for individual females). Means ± SD of recoveries of administered radioactivity for the four male rats during the 4 days after dosing were 12.30 ± 4.59% in urine, 66.24 ± 6.45% in bile, and 14.83 ± 3.72% in feces (table 2). Means ± SD of recoveries of administered radioactivity for the four experiments included the following: precursor-ion scanning of m/z 204, indicative of a benzothiazole moiety; precursor-ion scanning of m/z 390, indicative of an oxidized m/z 374 product ion; precursor-ion scanning of m/z 452, indicative of a thiol addition; neutral-loss scanning of 129 amu, indicative of mercapturic acid conjugates.

The HPLC column effluent was split before the ion-spray needle. The β-RAM response was also recorded by the mass spectrometer, providing simultaneous detection of radioactivity and MS data. The delay in response between the two detectors was approximately 0.28 min, with the MS data being recorded first.

**Results**

**Tissue Distribution Study:** Maximal levels of radioactivity from [14C]zopolrestat were observed at 4 hr after dosing in all tissues except urinary bladder and large intestine, where maximal levels were observed at 8 hr after dosing (table 1). Radioactivity could be quantified in all tissues, except eyes, brain, and adrenal glands, for 48 hr after dosing. Only in testes and liver could radioactivity still be quantified 168 hr after dosing, and levels in these tissues were very close to the lower limits of quantification (approximately 0.15 μg·eq/g). Tissue exposure to radioactivity, as expressed by AUC_{0–t}, was greatest in liver, followed by the intestines, kidney, and plasma (table 1). Exposure to radioactivity for all other tissues was 3–30-fold lower than for liver.

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female rats were 19.11 ± 6.43% in urine, 32.25 ± 19.37% in bile, and 11.90 ± 7.07% in feces. Small amounts of radioactivity (<3% of the dose) were recovered in cage washes and in residual food. Mean total recovery of the administered dose was 94.20 ± 6.90% for male rats and 65.00 ± 14.22% for female rats. The recoveries of administered radioactivity in female rats might have been lower because the bile duct cannulations remained patent for 4 days for three of four males but for only one of four females. Patency for two of the females lasted for only 2 days. Total recovery of radioactivity in the two females with better patency was similar to total recovery in the four males.

MS Analysis of Zopolrestat and Identification of Metabolites. A CID spectrum of zopolrestat is shown in fig. 2. Structural assignments for the major product ions are noted. The structures of the proposed urinary and biliary metabolites are shown in fig. 3, and the structural descriptions are listed in table 3. Each metabolite was assigned a numerical notation (metabolite 1, 2, etc.) based on the order of HPLC elution.

Identification of Urinary Metabolites. Female rats excreted more radioactivity into urine than did male rats (table 2), and most of the difference was attributable to unchanged drug. Whereas males excreted 1.3 ± 1.3% of the dose in urine as unchanged drug in 24 hr, females excreted 6.9 ± 3.5% of the dose in 24 hr, plus an additional 9.5 ± 4.4% of the dose between 24 and 48 hr. Radioactivity from urine samples injected into the HPLC system was quantitatively recovered in the column fractions. In male rats, most of the radioactivity in urine was attributable to metabolites 8 and 9 (table 4 and fig. 4). Parent drug was the second most abundant form of radioactivity in the urine of males. All other identified metabolites (metabolites 6 and 7, 10 and 11, and 12) each accounted for approximately 10% of total urinary radioactivity. In female rat urine, the same metabolites were observed at approximately the same concentrations as in male urine. However, because of the high concentrations of parent drug in female urine, the relative proportion of metabolites in total urinary radioactivity was lower in female rats. For both genders, all metabolites accounting for >1% of total urinary radioactivity are listed in table 4. The mean sum of all unidentified metabolites was <1.8% for males and <0.5% for females.

A typical HPLC profile of urinary radioactivity is shown in fig. 4. Fifteen urinary metabolites were tentatively identified, and MS data were acquired for each metabolite. For the sake of brevity, we have chosen to present the MS evidence only for the major urinary metabolites. The data supporting the structure of each metabolite are given below.

Metabolites 6 and 7. The protonated molecular ion for these metabolites was m/z 557. Their respective HPLC retention times were 10.7 and 15.3 min. The structures were deduced from CID spectra, which were consistent with cysteine conjugation plus hydroxylation. These cysteine metabolites are proposed to be degradation products of GSH conjugates of hydroxylated zopolrestat. A product-ion spectrum for the ion at m/z 557 (fig. 5) provided evidence for the proposed structure. The two metabolites showed similar fragmentation spectra. The product-ion spectrum shows dehydration (557 − 18 = 539) and neutral loss of 121 amu (cysteine moiety; 557 − 121 = 436). In comparison with the product-ion spectrum of zopolrestat (fig. 2), the metabolite product ions of m/z 390 (374 + O), 248 (216 + S), and 232 (216 + O) indicate that the oxidation and conjugation of cysteine occur on the benzothiazole moiety. Two chromatographic bands were present in the extracted ion chromatogram and might be attributable to the existence of several positional isomers for the proposed structure.

The in vivo formation of the GSH conjugates is thought to be the result of cytochrome P450-mediated epoxidation, followed by nucleophilic addition of GSH (Armstrong et al., 1981; Baillie and Slatter, 1991; Buckpitt et al., 1987; Jones et al., 1993; Nicoll-Griffith et al., 1993). The sites of oxidation and conjugation on the benzothiazole ring may change, resulting in positional isomers. Because the C6-C7 bond is the least sterically hindered, it is suspected that epoxidation and GSH addition would occur at C6 and C7 of the benzothiazole moiety.

Metabolites 8 and 9. The protonated molecular ion of these metabolites was m/z 599. Their respective HPLC retention times were 16.5 and 16.7 min. The structures were deduced from CID spectra, which were consistent with NAC (or mercapturic acid) conjugation plus hydroxylation on the benzothiazole moiety. Evidence for this assignment can be found in the product-ion spectrum for the ion at m/z 599 (fig. 6). The product ion at m/z 232 suggests oxidation of the benzothiazole moiety, and the product ion at m/z 162 represents the molecular ion of NAC. Corroborating product ions are m/z 248 (216 + S), 452 (420 + S), and 581 (599 − H2O). Two chromatographic bands were present in the extracted ion chromatogram and may be attributable to the existence of several positional isomers for the proposed structure.

These metabolites were most likely formed by the enzymatic activity of N-acetyltransferase on metabolites 6 and 7. Isoforms of N-acetyltransferase catalyze the transfer of an acetyl group from acetyl-CoA to the amino termini of most eukaryotic proteins (Kulkarni and Sherman, 1994). Acetylation of the S-substituted cysteine conjugate is the final step in the biosynthesis of mercapturic acid (Duffel and Jakoby, 1982; Tate, 1980).

Metabolite 10. The protonated molecular ion of this metabolite was m/z 581. The HPLC retention time was 19.4 min. Its structure was deduced from the CID spectrum, which was consistent with NAC.

### TABLE 2

Recoveries of radioactivity from Long-Evans rats after oral administration of [14C]zopolrestat at 100 mg/kg.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Gender</th>
<th>Collection Days</th>
<th>Recovery</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>1–4</td>
<td>5.83</td>
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<tr>
<td>14</td>
<td>M</td>
<td>1–4</td>
<td>14.39</td>
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<td>16</td>
<td>M</td>
<td>1–3</td>
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<tr>
<td>20</td>
<td>M</td>
<td>1–4</td>
<td>12.59</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>12.30 ± 4.59</td>
<td>66.24 ± 6.45</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>1–3</td>
<td>22.82</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>1–2</td>
<td>9.58</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>1–3</td>
<td>23.13</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>1–4</td>
<td>20.90</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>19.11 ± 6.43</td>
<td>32.25 ± 19.37</td>
</tr>
</tbody>
</table>

Values are percentages of total administered radioactivity.

* Other sources of radioactivity include animal cage washes and intestinal contents.
conjugation at the benzothiazole moiety. The product-ion spectrum of the protonated molecular ion at m/z 581 is shown in fig. 7. Unlike metabolites 8 and 9, the mass spectrum shows no product ion at m/z 162 representing the NAC moiety. However, the immediate loss of an acetyl group (539 = 581 - 42) and the presence of an ion at m/z 452 (420 + S) are indicative of the NAC conjugate. It is notable that the ion at m/z 232 is absent from the spectrum but an ion at m/z 248 (216 + S) is present. These data indicate that the sulfur atom is attached to the benzothiazole ring (216 + 32 = 248 and 374 + 32 = 406), but no oxidation occurs (216 + 16 = 232) on that moiety. The chromatographic peak shape reveals a very narrow bandwidth for this metabolite.

This metabolite may be a product of direct GSH conjugation without epoxidation. It is known that hepatic GSH S-alkenetransferase catalyzes an efficient reaction of the xenobiotic material with GSH (Hutson, 1970). Substitution typically occurs at an activated carbon atom, β to the electronegative functionality. It has been reported that benzothiazoles preferentially undergo electrophilic substitution on the carbocyclic ring, principally at C4 and C6 (Rance, 1989) (position 4 of this zopolrestat moiety is expected to be the most electrophilic site). The location of the CF₃ group on the zopolrestat molecule fulfills all of the aforementioned criteria to accommodate GSH conjugation to the carbocyclic ring of the benzothiazole moiety. Another possible origin for this metabolite could involve the in vivo dehydration of metabolite 8 and/or metabolite 9. Dehydration may also have occurred in vitro, after the addition of acid to the urine during sample collection and extraction.

Metabolite 12. The protonated molecular ion of this metabolite was m/z 248; its HPLC retention time was approximately 23 min. Weak responses were observed in the positive-ion mode, and the structure of this metabolite was deduced from a parent-ion scanning experiment for the ion at m/z 204. Additional evidence was obtained from a Q1 experiment and product-ion scanning of the ion at m/z 265 (the ammoniated molecular ion). Because the positive-ion conditions yielded poor sensitivity, negative ion-spray conditions were used. With negative ion-spray ionization, an intense response was observed at the retention time of the radioactive metabolite, and a molecular ion of m/z 306 was deduced to be the acetate adduct molecular ion. Its product-ion spectrum is shown in fig. 8 and supports the proposed structure.
**Unchanged Drug.** Nonmetabolized zopolrestat eluted at a retention time (approximately 24.5 min) identical to that of an authentic standard, showed the expected molecular ion of \( m/z \) 420 at the time of radioactivity elution, and demonstrated a matching CID product-ion spectrum.

**Identification of Biliary Metabolites.** Bile samples from eight rats (four of each gender) were analyzed by simultaneous MS and \( \beta \)-RAM detection. The amount of radioactivity associated with each metabolite or drug peak was quantified. A typical profile of biliary radioactivity is shown in fig. 9, and table 5 provides a summary profile of radioactivity percentages for each bile sample. In male rats, unchanged zopolrestat was the major radioactive component (73.1%), and metabolite 18 was the next most abundant component (12.2%). In female rats, metabolite 18 was the major radioactive component...

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**TABLE 3**

<table>
<thead>
<tr>
<th>Metabolite No.</th>
<th>Elimination Route</th>
<th>Molecular Ion</th>
<th>Significant CID Product Ions</th>
<th>Description of Metabolite Structure</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>( m/z )</td>
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<td></td>
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<tr>
<td>1</td>
<td>U</td>
<td>205</td>
<td>159, 149, 103</td>
<td>Phthalazineacetic acid</td>
</tr>
<tr>
<td>2</td>
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<td>743</td>
<td>449, 420, 406, 406, 177, 130</td>
<td>Benzothiazole glutathione conjugate and hydroxylation product</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>686</td>
<td>539, 495, 449, 406, 249, 130</td>
<td>Benzothiazole glutamyl cysteine conjugate and hydroxylation product</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>B</td>
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<td>493, 449, 406, 146</td>
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</tr>
<tr>
<td>6, 7</td>
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<td>557</td>
<td>539, 436, 390, 248, 232</td>
<td>Benzothiazole cysteine conjugate and hydroxylation product</td>
</tr>
<tr>
<td>8, 9</td>
<td>B, U</td>
<td>599</td>
<td>581, 539, 452, 420, 406, 162</td>
<td>Benzothiazole mercapturic acid conjugate and hydroxylation product</td>
</tr>
<tr>
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<td>B, U</td>
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<td>539, 452, 406, 248</td>
<td>Benzothiazole mercapturic acid conjugate</td>
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<tr>
<td>11</td>
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<td>612</td>
<td>436, 390, 216</td>
<td>Phthalazion oxidation product and glucuronic acid conjugate</td>
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<tr>
<td>12</td>
<td>U</td>
<td>306</td>
<td>246, 202</td>
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<tr>
<td>13, 14</td>
<td>U</td>
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<td>390, 232, 171, 146</td>
<td>Single oxidation of benzothiazole moiety product</td>
</tr>
<tr>
<td>15, 7</td>
<td>U</td>
<td>436</td>
<td>390, 216, 187, 162</td>
<td>Single oxidation of phthalazione moiety product</td>
</tr>
<tr>
<td>18</td>
<td>B, U</td>
<td>596</td>
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<td>Acyl glucuronide conjugate</td>
</tr>
<tr>
<td>19</td>
<td>U</td>
<td>204</td>
<td>184, 157, 108</td>
<td>Benzothiazole</td>
</tr>
</tbody>
</table>

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*Information for all of the metabolites identified in rat urine and bile is given here. The text addresses only the major urinary and biliary metabolites, as determined by the percentage of radioactivity in each matrix.

*Signifies that the metabolite was detected in bile (B) or urine (U).
(56.3%), and unchanged zopolrestat was the next most abundant form of radioactivity (34.0%). The contribution of all other metabolites was 15% of total drug-related material in the analyzed samples.

As with the urinary metabolites, we have chosen to present the MS evidence only for the major biliary metabolites. The data supporting the structure of each metabolite are given below.

**Metabolite 2.** The protonated molecular ion of this metabolite was \( m/z \) 743, and its HPLC retention time was approximately 3 min. Its structure was deduced from the CID spectrum of the ion at \( m/z \) 743, which was consistent with GSH conjugation plus hydration of the benzothiazole moiety of zopolrestat. MS evidence was found in neutral-loss scanning of 129 amu and in the product-ion spectrum for the ion at \( m/z \) 743 (fig. 10). The product ion at \( m/z \) 306 represents the GSH adduct, and the product ions at \( m/z \) 130 and 177 represent the secondary fragmentation products of the GSH adduct. The molecular ion of zopolrestat \( (m/z \ 420) \) is present in conjunction with other commonly seen product ions that indicate conjugation with the sulfur atom of GSH \( (406 = 374 + S; \text{for } m/z \ 449, \text{see proposed structure in fig. 10}). \)

**Metabolite 3.** The protonated molecular ion of this metabolite was \( m/z \) 686, and its HPLC retention time was approximately 4 min. Its structure was deduced from the CID spectrum of the ion at \( m/z \) 686, which was consistent with formation of a glutamyl-cysteine conjugate plus hydroxylation of the benzothiazole moiety. The product-ion spectrum for the ion at \( m/z \) 686 is shown in fig. 11. Supporting evidence can be found in the product ion at \( m/z \) 449, representing the glutamyl-cysteine moiety. The product ions at \( m/z \) 406/408, 449, 495, and 539 represent different stages of fragmentation. Because of the similar elution volumes of metabolites 2 and 3, the radioactivity associated with these metabolites was combined.

**Metabolite 10.** This metabolite had been previously identified in rat

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Radioactivity Attributable to Each Metabolite % of total</th>
<th>Male</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td>Metabolite 6</td>
<td>Metabolite 7</td>
<td>Metabolites 8 &amp; 9</td>
<td>Metabolites 10 &amp; 11</td>
</tr>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>&lt;1.0</td>
<td>12.4</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>&lt;1.0</td>
<td>9.9</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>15.0</td>
<td>1.9</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>4.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>4.9 ± 7.1</td>
<td>6.8 ± 5.1</td>
<td>42.2 ± 7.0</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>28</td>
<td>F</td>
<td>&lt;1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>&lt;1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>&lt;1.0</td>
<td>0.8 ± 1.0</td>
<td>7.0 ± 4.9</td>
</tr>
</tbody>
</table>

For the calculations of mean and SD, the values shown as <1.0 were treated as equal to 0.
urine. The molecular ion of this metabolite was m/z 581, and its HPLC retention time was approximately 19.5 min. MS evidence was gathered from neutral-loss scanning of 129 amu and parent-ion scanning of m/z 452. The CID spectrum for the ion at m/z 581 for the biliary metabolite matches the CID spectrum for the ion at m/z 581 for the urinary metabolite (fig. 7).

Metabolite 18. The protonated molecular ion of this metabolite was m/z 596. Several partially resolved chromatographic peaks exhibited HPLC retention times between 23 and 26 min (fig. 9). Its structure was deduced from the CID spectrum for the ion at m/z 596, which was consistent with an acyl glucuronide of zopolrestat. The product-ion spectra of the chromatographic peaks, although not identical in ion ratios, were nearly identical in ion masses. A common product-ion spectrum is shown in fig. 12. The initial loss of 176 amu indicates the presence of a glucuronic acid conjugate. All product ions of less than m/z 420 were identical to those in the unchanged zopolrestat spectrum. The presence of several chromatographic peaks for this mass may be explained by the formation of different acyl glucuronide isomers (i.e. 2'-, 3'-, or 4'-O-acyl). Rearrangement of the acyl glucuronide conjugate by intramolecular transesterification at the hydroxyl groups of the glucuronic acid moiety has been reported (Akira et al., 1997; Benet and Spahn, 1988; Faed, 1984; Lenz et al., 1996). The stabilities of these conjugates are reportedly pH dependent, and acid stabilization has been recommended (Benet and Spahn, 1988). It was shown in previous studies that the acyl glucuronides of zopolrestat could be hydrolyzed under alkaline conditions, so that concentrations of the aglycone could be determined (Inskeep et al., 1991). The product-ion spectra from these biliary chromatographic peaks were similar, and the product ions of less than m/z 420 were the same as those of unchanged zopolrestat. These data suggest that the drug molecule is unchanged, with the exception of the acyl glucuronidation.

Unchanged Drug. Nonmetabolized zopolrestat eluted at a retention time identical to that of an authentic standard, exhibited the expected molecular ion of m/z 420 at the time of radioactivity elution, and yielded a matching CID product-ion spectrum.

Discussion

Tissue Distribution Study. The values for \( C_{\text{max}} \) and the time of \( C_{\text{max}} \) reported for zopolrestat in plasma and tissues in this study should be regarded as approximations, because of the long intervals between samplings. However, in a previous pharmacokinetic study of zopolrestat in rats dosed orally at 50 mg/kg (Inskeep et al., 1991), plasma and tissue (nerve, kidney, and lens) samples were collected at more frequent intervals in the early phase after dosing (0.5, 1, 2, 4, 8, and 12 hr) than in the current study, and these results were used to guide sampling time selection in the current study. In general, the times of \( C_{\text{max}} \) were similar for the two studies, although \( C_{\text{max}} \) values were slightly higher in the previous study.

The half-life of radioactivity in most tissues (8–10 hr) was similar to the half-life in blood. The longest half-life, 46 hr, was for testes, whereas liver, skin, and sciatic nerve had intermediate half-lives of approximately 20 hr. Although the half-life in testes was relatively long, total exposure to radioactivity, as expressed by AUC\(_{0-\infty}\), was low, compared with exposure in most of the other tissues (table 1).

Tissue levels of radioactivity were below the lower limit of quantification (generally representing <2% of maximal tissue levels of radioactivity) for all tissues except testes, skin, sciatic nerve, liver, and intestines by 96 hr after dosing. Only testes (1.4% of the maximal level) and liver (1.6% of the maximal level) had quantifiable levels of radioactivity remaining at 168 hr after dosing. No radioactivity could be quantified at 48 hr after dosing (<12% of maximal levels) in eye, brain, and adrenal glands. By 168 hr after dosing, radioactivity rep-
resented <2% of maximal tissue levels for all tissues except eye, brain, sciatic nerve, adrenal glands, and fat (for which the amount of tissue available for assay was limiting). For these latter tissues, the limit of quantification at 96 hr after dosing represented 4–20% of its maximal level by the end of the study at 168 hr after the dose.

Bile Duct-Cannulated Rat Study. The greater recovery of administered radioactivity in male rats than in female rats was most likely the result of better bile duct cannula patency over the 4-day period in males. Most of the radioactive dose was recovered in the bile, indicating that biliary elimination is the major route of elimination for zopolrestat and/or its metabolites. In both genders, approximately 15% of the dose was recovered in feces, indicating that ~85% of the dose of zopolrestat was absorbed. Urinary elimination of radioactivity was slightly greater in females. The greater urinary elimination of radioactivity in females represents a significant gender effect on the disposition of zopolrestat in rats. In multiple-oral dose studies in Sprague-Dawley, Fischer, and Long-Evans rats, using doses of 50 or 100 mg/kg/day, systemic exposure (AUC and approximate Cₘₐₓ) was consistently 2-fold higher in males than in females (data not shown).

The proposed metabolic scheme for zopolrestat in rat urine and bile is depicted in fig. 3. A major portion of orally administered zopolrestat was excreted as unchanged drug in urine (females) and bile (males and females).

Fifteen metabolites of zopolrestat were identified in a male rat urine sample. For male rats, most of the radioactivity in urine was attributable to the degradation products of GSH conjugates of hydroxylated zopolrestat (metabolites 6–9). Parent drug was the second most abundant form of radioactivity in urine. Benzothiazole carboxylic acid...
metabolite 12) accounted for approximately 10% of total urinary radioactivity. In addition, approximately 10% of urinary radioactivity was present as the mercapturic acid and/or ether glucuronide conjugates (metabolites 10 and 11, respectively), which were not chromatographically resolved. In female rat urine, the same metabolites were observed in approximately the same amounts, but the relative proportions in urine were lower because of the excess of unchanged drug in females. For both genders, only metabolites representing 1% of total urinary radioactivity are shown in table 4. All GSH-related metabolites accounted for only 7.8 and 2.1% of the administered dose in male and female rats, respectively. Female rats excreted more radioactivity in urine than did male rats, and most of the increased material was attributed to unchanged drug. The amounts and proportions of the urinary metabolites were similar for males and females. It is noteworthy that, among the minor metabolites whose radioactive contributions were not quantifiable, five single-oxidation products were identified. The metabolic characteristics of all of the minor metabolites have been summarized in table 3.

Eight metabolites of zopolrestat were identified in rat bile samples, of which four were also identified in rat urine. Parent drug and acyl glucuronidation products accounted for 85% of the identified radioactivity.
activity in male and female bile samples. Other minor metabolites included products of GSH conjugation and subsequent degradation of the GSH moiety (<9.5% of the administered dose).

In conclusion, the pharmacokinetics of zopolrestat in rats primarily involve biliary and urinary excretion of parent drug and/or its acyl glucuronide. Several oxidative pathways exist but are quantitatively much less important in the overall disposition of zopolrestat. Gender-related differences in renal elimination have been associated with higher systemic exposure in male rats than in female rats.

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References


