ABSTRACT:
The in vivo metabolism and excretion of zafirlukast [Accolate; 4,5-cyclopentoxycarbonylaminio-3-[(2-methoxy-4,2-methylphenylsulfonylaminocarbonyl)phenylmethyl]-1-methylindole], a selective peptide leukotriene receptor agonist, were investigated in mice, rats, and dogs. Leukotrienes are a class of compounds that have been identified as being responsible for the contraction of human airway and lung vascular smooth muscle. A chemical agent that is effective in blocking the induced constricting actions of leukotrienes could be used to treat inflammatory processes in the pulmonary system. Zafirlukast has been shown to be clinically efficacious and has been approved for the treatment of asthma in humans. To determine the metabolic fate of zafirlukast, the radiolabeled compound was administered orally to mice, rats, and dogs and iv to rats and dogs. Plasma, urine, and feces samples were collected, assayed for radioactivity, and profiled for metabolites. Nearly all of the [14C]zafirlukast-derived radioactivity was excreted in the feces of the test species, indicating biliary clearance as the major route of elimination from the systemic circulation. The primary routes of metabolism in all species studied involved hydrolysis of the amide linkage at the 5-aminoindole position and hydroxylation at one or more sites. Additional metabolites were formed by N-acetylation (not in dogs), demethylation of the indole nitrogen, and N-desmethylation. Accolate is a registered trademark, property of Zeneca Ltd.

The role LTs1 play in asthma was clarified by Samuelsson (1983). LTs are formed by the breakdown of arachidonic acid via the 5-lipoxygenase enzyme pathway, which produces the cysteinyl LTs LTD4, LTE4, and LTC4. Wenzel (1997) observed that these LTs produce effects on cell membranes that are characteristic of asthma, i.e. bronchoconstriction, increased endothelial membrane permeability leading to airway edema, and enhanced secretion of mucus. By providing patients with compounds that block the induced constricting action of LTD4, the symptoms of asthma could be reduced. Zafirlukast (Accolate; Zeneca Pharmaceuticals, Wilmington, DE) was developed as a potent, selective, oral, LT receptor antagonist for the treatment of asthma.

To determine the absorption, distribution, metabolism, and excretion of zafirlukast, the radiolabeled compound was administered to mice, rats, and dogs. This information was used to support nonclinical drug safety/toxicity trials, first-time-in-humans trials, and safety evaluations in humans.

Materials and Methods
Chemicals. Two versions of [14C]zafirlukast were used, i.e. methyl-labeled and carbonyl-labeled. Fig. 1 shows the chemical structure of zafirlukast and the locations of the labeling sites. One lot of methyl-labeled zafirlukast was used for the rat study with 1 mg/kg doses. It was synthesized by ICI Pharmaceuticals Group (Wilmington, DE) and had a specific activity of 16.2 μCi/mg and a radiochemical purity of >98%. Carbonyl-labeled zafirlukast was used for all other studies. Two lots were synthesized by the Radiochemistry Unit of the Safety of Medicines Group, Zeneca Pharmaceuticals (Alderly Park, Macclesfield, Cheshire, UK), and had specific activities of 13.6 and 29.2 μCi/mg. One lot of carbonyl-labeled zafirlukast was synthesized by Stuart Pharmaceuticals (Wilmington, DE) and had a specific activity of 45.4 μCi/mg. Radiochemical purity was >97% for all lots. Two batches of unlabeled zafirlukast were synthesized at Zeneca Pharmaceuticals, and each had a purity >98%. Appropriate amounts of radiolabeled and unlabeled zafirlukast were combined to adjust the specific activity for dosing.

Animals. All of the laboratory animals involved in these research studies were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86–23, 1985) and subsequent amendments.

Mouse Studies. Male and female C57BL/10J, Apo-Alpk mice were supplied by the Barrierm Animal Breeding Unit (Zeneca, Alderly Park, Macclesfield, Cheshire, UK). The animals weighed 19–31 g and were 4–6 weeks of age at the time of dosing. The mice were administered a single oral gavage dose of 10 or 300 mg/kg [14C]zafirlukast (approximately 50–200 μCi/kg). The mice were placed in glass metabolism cages (five mice/unit) and separated according to gender and dose. Food (powdered diet) and potable water were provided ad libitum throughout the study. Urine and feces were collected daily, for 3 days, from each metabolism cage. After the excretion phase, the dosing was repeated and plasma was collected for metabolite profiling and identification. A cage wash was made at each collection time and refrigerated.

Rat Studies. Four sets of rat studies are included in this report. The first involved three male Sprague-Dawley albino-Hsd:SDR rats, 8–9 weeks of age and weighing between 298 and 312 g, obtained from Hilltop Laboratory Animals (Scottsdale, PA). These rats received a single oral dose of 1 mg/kg (approximately 20 μCi/kg) with the methyl-labeled zafirlukast and were individually housed in glass metabolism cages. Respired air was pulled by vacuum through a mixture of 2-methoxyethanol and ethanolamine (2:1, v/v) to trap the carbon dioxide for intervals up to 24 hr. Urine and feces were collected over dry ice at the end of 12, 24, and 48 hr. A cage wash was obtained at each collection time and refrigerated.

The second set of rats included eight male and eight female Charles River...
Wistar CRL/W rats, 7–8 weeks of age, obtained from Charles River Laboratories (Wilmington, MA). At the time of dosing, the male rats weighed 272–294 g, whereas the females weighed 180–218 g. Four rats of each gender were administered a single oral dose of 40 mg/kg (approximately 40 μCi/kg) with the carbonyl-labeled zafirlukast. Four additional rats of each gender were administered a daily dose of 40 mg/kg (approximately 40 μCi/kg) for 14 days. The animals were individually housed in plastic metabolism cages for the daily collection of urine and feces for 7 or 21 days. The samples were collected over dry ice for the first 48 hr and at room temperature thereafter. A cage wash was collected at each collection time and refrigerated.

The third set involved male Sprague-Dawley albino:Hla(SD)BR rats obtained from Hilltop Laboratory Animals. The rats were 7–9 weeks of age and weighed 240–300 g at the time of dosing. These animals were anesthetized using Halothane, and bile duct cannulations were performed under aspetic conditions. After overnight recovery from surgery, the rats were dosed. Eight rats were administered an oral dose of 1 or 5 mg/kg (2–10 μCi/kg), whereas five rats were administered an iv dose of 1 mg/kg (2 μCi/kg). All bile duct-cannulated rats received carbonyl-labeled zafirlukast. The rats were housed as described above. In addition to urine and feces, bile was collected at intervals for 96 hr. A cage wash was obtained at each collection time and refrigerated.

A tissue distribution study was conducted with rats in the fourth set. The male Wistar rats were obtained from Charles River (Margate, Kent, UK) and were administered a single oral dose of 5 mg/kg (approximately 40 μCi/kg) with carbonyl-labeled zafirlukast. The rats weighed 180–200 g at the time of dosing. The rats were individually housed in polypropylene and stainless steel cages with raised wire-mesh floors or in metabolism cages for the collection of urine and feces (168-hr rats). After dosing, three rats were killed by CO2 narcosis at each time interval (1, 6, 12, 24, 48, and 168 hr). A total of 37 organs, tissues, and body fluids were removed and retained for analysis.

**Dog Studies.** Two sets of sexually mature male beagle dogs, weighing 9–14 kg, were obtained from Marshall Farms (Rose, NY). The dogs were fasted approximately 16 hr before dosing and for 4 hr after dosing. A certified laboratory diet of canned moist food and dry food was provided ad libitum. Drinking water was provided to all dogs throughout the studies. Dogs surgically cannulated for bile collection were allowed to recover from surgery for at least 14 hr before dosing. Gall bladder-cannulated dogs received additional electrolyte solutions by oral gavage, to help maintain homeostasis. Two dogs were given a single oral dose of 1 mg/kg (10 μCi/kg), whereas the remainder received an iv dose of 1 mg/kg. Three weeks later, the dosing was repeated using the opposite route. After another 3 weeks, all dogs received a single oral dose of 5 mg/kg (10 μCi/kg). Additional dogs were given a dose of 40 mg/kg (10 μCi/kg) to provide samples for metabolite profiling and identification. The carbonyl-labeled zafirlukast was used in all dog studies.

**Sample Analysis.** Lithium heparin was added to all blood samples to prevent clotting. Samples of blood were obtained from each species at selected time intervals. The plasma was separated from blood cells by centrifugation and was assayed for total radioactivity and for zafirlukast concentrations. All plasma samples were stored frozen at <15°C until assayed.

Urine from the dogs, rats, and mice was collected over dry ice. Feces from the dogs was collected at room temperature and then frozen until assayed. The feces samples from the rats and mice were collected over dry ice and stored frozen until assayed. Bile from the dogs was collected at room temperature and refrigerated and was assayed for total radioactivity and for zafirlukast concentrations. All bile samples were stored frozen at <15°C until assayed.

**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Time after final dose (hr)</th>
<th>Recovery (% of total radioactivity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>72</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Male</td>
<td>72</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Female</td>
<td>72</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Rat</td>
<td>92</td>
<td>1.46 ± 0.12</td>
</tr>
<tr>
<td>Male</td>
<td>92</td>
<td>1.46 ± 0.12</td>
</tr>
<tr>
<td>Female</td>
<td>92</td>
<td>1.46 ± 0.12</td>
</tr>
<tr>
<td>Dog</td>
<td>192</td>
<td>2.0 ± 0.10</td>
</tr>
<tr>
<td>Male</td>
<td>192</td>
<td>2.0 ± 0.10</td>
</tr>
<tr>
<td>Female</td>
<td>192</td>
<td>2.0 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE.

*Added to feces.*
then stored frozen until assayed. Bile from the rats was collected over wet ice (which was replenished after each collection) and then stored frozen.

**Sample Assay for Radioactivity.** Frozen samples were thawed and mixed before assays. Aliquots of plasma, urine, bile, and cage washes were assayed directly after the addition of Monofluor liquid scintillation fluid (National Diagnostics, Atlanta, GA). The samples were counted for total radioactivity using LSC in a Packard model B307 sample oxidizer using Carbo-Sorb carbon dioxide-trapping solution and Permafluor liquid scintillation fluid.

Metabolite Isolation and Quantitation. The samples were injected into a Shimadzu LC-10AD system (Shimadzu, Columbia, MD) equipped with a conventional electrospray ion source. The retention times of the column eluent and subsequent LSC. Metabolites were identified by retention times, compared with those of synthetic standards, and structures being returned to the starting concentration. Detection was by measurement of UV absorbance at 296 nm (Shimadzu SPD6A detector), by radioactivity detection with a flow-through detector with a 250-μm ID flow cell (Ramona 5LS; Raytest USA, Wilmington, DE), and by fraction collection (20 sec) of the column eluent and subsequent LSC. Metabolites were identified by retention times, compared with those of synthetic standards, and structures were confirmed by MS. Quantitation was based on the concentration of radioactivity associated with each identified peak.

**MS.** The column eluent from the HPLC separation was split, with approximately 100 μl/min being introduced into a Fisons Quattro quadrupole mass spectrometer equipped with a conventional electrospray ion source. The remaining 900 μl/min was directed to the Ramona 5LS radioactivity detector. Analysis was performed in two steps; first, zafirlukast-related molecular ions and corresponding retention times were identified in an HPLC/MS experiment; second, structural information for each metabolite was obtained by performing Mass Spectrometry of Zafirlukast.
**Fig. 2.** Biotransformation pathways for zafirlukast.

**Fig. 3.** Zafirlukast metabolites present in dog bile collected at various times after dosing.
sequential MS/MS product scans in the time window in which each metabolite eluted from the HPLC column. The mass spectrometer was operated in the positive-ion mode. Neutral-ion-loss experiments were performed with selected samples to assay for the presence of conjugates. Identifications were confirmed with synthetic standards and other methods, where possible.

**NMR Spectrometry.** NMR spectra for the synthetic standards were obtained for reference. Two hydroxylated metabolites that were isolated from dog bile by HPLC were subjected to NMR and MS analysis. The M5 and M6 metabolites (approximately 1 mg each) were dissolved in dimethylsulfoxide-$d_6$, and proton NMR spectra were obtained at 400 mHz. The sites of hydroxylation were unequivocally confirmed. The data were described in an internal technical report.

**Synthetic Standards.** Synthetic standards for zafirlukast and metabolites designated M1, M2, M3, M4, M9, and M13 were prepared by Zeneca Pharmaceuticals. The identities of the standards were confirmed by MS and NMR spectrometry.

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**TABLE 5**

**Identified zafirlukast metabolites and their structures**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Edited IUPAC Name Generated by Autonom (Only Required Punctuation Appears)</th>
<th>Name, Description</th>
<th>Mass m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure 1" /></td>
<td>[3-[2-Methoxy-4-(toluene-2-sulfonylaminocarbonyl)benzyl]-1-methyl-1H-indol-5-yl]carbamic acid cyclopentyl ester</td>
<td>ICI 204,219</td>
<td>575</td>
</tr>
<tr>
<td><img src="image2" alt="Structure 2" /></td>
<td>[3-[2-Methoxy-4-(toluene-2-sulfonylaminocarbonyl)benzyl]-1H-indol-5-yl]carbamic acid cyclopentyl ester</td>
<td>M8, N-desmethyl</td>
<td>561</td>
</tr>
<tr>
<td><img src="image3" alt="Structure 3" /></td>
<td>[3-[4-(Hydroxy-2-methyl-benzene-sulfonylaminocarbonyl)-2-methoxy-benzyl]-1-methyl-1H-indol-5-yl]carbamic acid cyclopentyl ester</td>
<td>M10, monohydroxy (toluyl)</td>
<td>591</td>
</tr>
<tr>
<td><img src="image4" alt="Structure 4" /></td>
<td>[1-Hydroxymethyl-3-[2-methoxy-4-(toluene-2-sulfonylaminocarbonyl)-benzyl]-1H-indol-5-yl]carbamic acid cyclopentyl ester</td>
<td>M5, monohydroxy 1 (N-methyl center ring)</td>
<td>591</td>
</tr>
<tr>
<td><img src="image5" alt="Structure 5" /></td>
<td>4-(5-Cyclopentyloxycarbonylamino-1-methyl-1H-indol-3-yl)methyl-3-methoxybenzoic acid</td>
<td>M9, des-sulfonamide acid</td>
<td>422</td>
</tr>
<tr>
<td><img src="image6" alt="Structure 6" /></td>
<td>[3-[2-Methoxy-4-(toluene-2-sulfonylaminocarbonyl)benzyl]-1-methyl-1H-indol-5-yl]carbamic acid 3-hydroxycyclopentyl ester</td>
<td>M11, monohydroxy (3-position of the CP ring)</td>
<td>591</td>
</tr>
<tr>
<td><img src="image7" alt="Structure 7" /></td>
<td>[3-[2-Methoxy-4-(toluene-2-sulfonylaminocarbonyl)benzyl]-1-methyl-1H-indol-5-yl]carbamic acid 2-hydroxycyclopentyl ester</td>
<td>M6, monohydroxy (2-position of the CP ring)</td>
<td>591</td>
</tr>
<tr>
<td><img src="image8" alt="Structure 8" /></td>
<td>Dihydroxy Dihydroxy-[3-[2-methoxy-4-(toluene-2-sulfonylaminocarbonyl)benzyl]-1-methyl-1H-indol-5-yl]carbamic acid cyclopentyl ester</td>
<td>M12, dihydroxy</td>
<td>607</td>
</tr>
</tbody>
</table>

*CP, cyclopentyl.
Results

Total Recoveries in Excreta. The total recovery of [14C]zafirlukast-derived radioactivity in urine, feces, and cage wash fractions during the mouse, rat, and dog studies is presented in table 1. Recovery was good, ranging from 79.5 to 100%. The 79.5% recovery was determined in a single 48-hr test that did not measure residual amounts of radioactivity in the carcasses. With exclusion of that result, the total recoveries ranged from 89.5% to 100%, with an average of 94.7%.

Excretion Patterns in Animal Studies. The data in table 1 also indicate that there were no qualitative differences in the excretion patterns attributable to the gender of the animal (for rats and mice), the dose level, the dosing route, or the administration of single or multiple doses. The majority of the doses were recovered in the feces, with <2.7% being recovered in the urine of all tested species.

Excretion during Repetitive Dosing. Mean recoveries of [14C]zafirlukast-derived radioactivity in feces during repetitive dosing studies are presented in table 2. After single oral doses, [14C]Zafirlukast was well absorbed after oral doses, i.e. 69.6% after a
1 mg/kg dose and 52.1% after 5 mg/kg. The majority of the absorbed radioactive material was excreted in the bile.

**Biliary Excretion in Dogs.** Excretion of [14C]zafluralkast-derived radioactivity by bile duct-cannulated dogs after iv or oral doses is shown in table 3. Dogs administered a 1 mg/kg iv dose eliminated almost all [14C]zafluralkast-derived radioactivity in the bile. Dogs that received a 1 mg/kg oral dose excreted a majority of the [14C]zafluralkast-derived radioactivity in the feces. Absorption of zafluralkast was increased to 59.2% by predosing one dog with control bile before a 1 mg/kg dose of zafluralkast. A total of 34.7% of the [14C]zafluralkast-derived radioactivity was excreted in the feces, with <3% being excreted in the urine.

**Tissue Distribution in Rats.** The tissues with the highest concentrations of [14C]zafluralkast-derived radioactivity are listed for each sampling time interval in table 4. Total radioactivity generally peaked at 6 hr after dosing, with highest concentrations being found in the liver, gastrointestinal tract, plasma, whole blood, mesenteric lymph nodes, lungs, and trachea. Tissues consistently having the lowest levels of radioactivity were brain, spinal cord, and eyes. The distribution of radioactivity at other sample times was similar to that observed at 6 hr. By 168 hr, levels of radioactivity remaining were generally at or near background concentrations.

**Biotransformation Pathways of Zafirlukast.** The metabolites of zafirlukast were produced by five major reactions, as follows: (a) cleavage of the carbamate linkage, (b) hydroxylations at various sites, (c) N-demethylation (at the indole nitrogen), (d) N-acetylation (at the 5-aminoindole position), and (e) cleavage of the sulfonamide linkage. The biotransformation pathways for zafirlukast, as first reported by Spreen (1994), are presented in fig. 2. Metabolites derived from these routes were found to be capable of undergoing subsequent metabolism by various combinations of the primary routes. The identified metabolites of zafirlukast and their structures are presented in table 5.

**Metabolite Profiles for Plasma.** In mouse, rat and dog plasma samples, most (>90%) of the compound-related material in circulation during the first 8 hr was associated with zafirlukast. No single metabolite accounted for >4% of the radioactivity in any animal plasma sample. The M1 metabolite accounted for approximately 1% of the total radioactivity in dog plasma. The M2 metabolite was present in rat plasma (1–4%) at peak concentrations (252 ng equivalents/ml).

**Metabolite Profiles for Urine.** The total percentage of administered radioactivity excreted in the urine by mice, rats, and dogs was generally <1% of the dose. The M9 metabolite (des-sulfonamide acid) was the major metabolite in urine from male mice, whereas the M3 metabolite was the most abundant metabolite in urine from female mice. The M13 metabolite (N-acetyl-des-sulfonamide) accounted for almost 50% of the radiolabeled material in rat urine. The major metabolite in the urine from dogs was M6 (2-hydroxycyclopentyl). Additional metabolites were identified in urine samples from one or more animal species and included M5 (N-hydroxymethyl), M7 (hydroxycyclopentyl-N-desmethyl), and M14 (hydroxycyclopentyl des-sulfonamide). Some zafirlukast was detected in rat and mouse urine; however, this was thought to be the result of a small amount of unavoidable fecal contamination during the collection. A few other minor metabolites were present in urine but were unidentifiable because of the sensitivity of the assay method.

**Metabolite Profiles for Feces.** In dog and rat feces samples that contained the largest amounts of excreted radioactivity (24–48 hr),
zafirlukast accounted for 47 and 29%, respectively, of the total radioactivity, whereas the percentage in mouse samples ranged between 54 and 69% (0–24 hr). The M5 and M9 metabolites were identified in all of the species tested. The M10 metabolite (hydroxytoluyl) was found in rat and mouse feces. Dog feces contained the M11 (3-hydroxycyclopentyl) and M12 (dihydroxy) metabolites.

**Metabolite Profiles for Bile.** The main radiolabeled component in dog bile was zafirlukast, accounting for 52, 43, and 33% in bile samples collected at 0–3, 3–6, and 6–12 hr, respectively (fig. 3). Other metabolites identified in dog bile included M5, M6 (both major components), M10, and M11. A representative profile of radioactivity in dog bile is presented in fig. 4. The mass spectrum for M5 and M6 displayed a molecular peak at 592 amu (an increase of 16 amu, compared with that of zafirlukast). MS and MS/MS experiments indicated that hydroxylation occurred on the N-methyl group for M5 and that the cyclopentyl group had been hydroxylated and subsequently reduced to a cyclopentadiene group. The structures were confirmed by NMR analysis. Rat bile contained the same metabolites as dog bile plus M2, M3, M4, M8, M9, and M12.

**Discussion**

The primary route of excretion for [14C]zafirlukast-derived radioactivity is in bile and, subsequently, feces, with <3% excreted in urine. Absorption of zafirlukast was >52% in rats at a dose of 5 mg/kg. In dogs, absorption was nearly doubled when the bile-cannulated animal was pretreated with bile. The patterns of excretion in rats were similar after single and multiple administrations.

Biotransformation reactions that are common to mice, rats, and dogs include cleavage of the amide linkage at the 5-aminoindole position, hydroxylation at one or more sites, and demethylation at the indole nitrogen. N-Acetylation was observed in rats and mice. Hydroxylation at the toluene ring and hydrolysis of the sulfonamide linkage were biotransformation reactions found only in animals. Hydroxylation at the 3-position cyclopentyl ring was found only in dogs. Only the hydroxylated metabolites showed any activity as LT antagonists, and only at a low fraction of zafirlukast activity. Several minor components were detected in bile, feces, and urine, but they were present in insufficient quantities for identification.

**Acknowledgments.** We thank the following people from Zeneca Pharmaceuticals for their help with this work: Dr. John Harding and Robert Dedinas for radiosyntheses, James Hall for NMR analyses, Dr. Peter Bernstein and Dr. Chris Veale for help with metabolite identification, Sandra Merritt for fraction collections and LSC, Virginia Crego for conducting the mouse balance study, and Deborah Raybuck for conducting the rat quantitative tissue distribution study.

**References**

