METABOLISM AND EXCRETION OF ATORVASTATIN IN RATS AND DOGS

ANN E. BLACK, ROGER N. HAYES, BRUCE D. ROTH, PETER WOO, AND THOMAS F. WOOLF

Departments of Pharmacokinetics, Dynamics, and Metabolism (A.E.B., R.N.H., T.F.W.) and Chemistry (B.D.R., P.W.), Parke-Davis Pharmaceutical Research Co., Ann Arbor, Michigan

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ABSTRACT:

Atorvastatin (AT) is a second-generation potent inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, clinically approved for lowering plasma cholesterol. Using a mixture of [D_5/D_0] AT and/or [^{14}C]AT, the metabolic fate and excretion of AT were examined in rats and dogs following single and multiple oral doses. Limited biliary recycling was observed in one dog after a single dose of AT. AT-derived metabolites in bile samples were identified by metabolite screening of the [D_5/D_0] AT molecular clusters using tandem mass spectrometry. Bile was a major route of [^{14}C] drug-derived excretion, accounting for 73 and 33% of the oral dose in the rat and dog, respectively. The remaining radioactivity was recovered in the feces; only trace amounts were excreted in urine. Radioactive components identified in rat and dog bile were the para- and ortho-hydroxy metabolites, a glucuronide conjugate of ortho-hydroxy AT, and unchanged AT. Two minor radioactive components were identified as β-oxidation products of AT with one confirmed as a β-oxidized AT derivative. The reappearances of AT and major metabolites in bile from a dog administered a sample of its previously excreted bile indicated biliary recycling is an important component in AT metabolism. Multiple dose administration in rats did not alter biliary metabolic profiles. Rat and dog plasma profiles after multiple dose administration were similar and showed no additional metabolites not found in bile. Examination of rat and dog bile and plasma indicates that AT primarily undergoes oxidative metabolism.

Materials and Methods

Chemicals. AT and [^{14}C]AT with the carbon label at the 3 position of the pyrrole moiety were synthesized at Parke-Davis Pharmaceutical Research (Ann Arbor, MI) as the calcium salt (Baumann et al., 1992). Radiochemical purity was >98.8% and chemical purity was 99.1%. The [D_5/D_0] AT with the deuterium on the 3-phenyl group had a purity of >98.2%, as determined by HPLC and mass spectrometry (MS). All synthetic reference standards of AT and para- and ortho-hydroxy AT were synthesized shown in Fig. 1. (All boldface numbers in the following paragraph refer to Fig. 1.)

The synthesis of ring-labeled [^{14}C]AT ([9a] was accomplished by an adaptation of the methodology of Baumann et al. (1992). The label was introduced as [^{14}C]benzaldehyde (2). Sequential condensation of labeled benzaldehyde with isobutyrylacetanilide (3a and 4a) with para-fluorobenzaldehyde (5), in the presence of the thiazolium catalyst (6) and triethylamine, gave the key labeled intermediate diketone in 7. Reaction of 7 with the protected chiral dihydroxyaminohexanoic ester 8, synthesized separately (B. D. Roth, U.S. Patent 4,681,893, July 21, 1987 and Roth et al., 1991) gave a condensation product of AT in its protected form. Deprotection with sequential acid and base treatments followed by preparative HPLC purification and calcium salt formation yielded [^{14}C]AT ([9a] labeled at the C-3 position of the pyrrole ring. Using a similar reaction sequence, but with benzaldehyde (2) and the appropriately substituted isobutyrylacetanilide 3b or 3c, respectively, as the starting materials, 2-hydroxy AT as the sodium (9b) salt and 4-hydroxy AT as the sodium (9c) salt, were synthesized (S. M. Bjorge, A. E. Black, B. D. Roth, and T. F. Woolf, U.S. Patent 5,385,929, Jan. 31, 1995).

Other Chemicals. β-Glucuronidase-type H-1 was purchased from Sigma (St. Louis, MO). All other chemicals except organic solvents were commercial analytical grade reagents. The organic solvents were HPLC grade.

Dosing and Sample Collection. Rats were administered AT as a suspension by gavage, whereas dogs were given AT as a suspension in a capsule. For plasma profile studies conducted in rats and dogs, unlabeled and labeled materials were prepared as suspensions in 0.5% methylcellulose. In rat and dog mass balance studies, labeled and unlabeled drug were first dissolved in the
minimal amount of dimethylacetamide followed by the addition of 0.5% methylcellulose to form a suspension. Dimethylacetamide constituted less than 10% of the final suspension.

**Rats.** Male and female Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) and were maintained on standard rodent chow with water provided ad libitum. For rat mass balance studies, six male (298–319 g) and six female (227–266 g) animals had their bile ducts cannulated, and the tubing was exteriorized at the back of the neck and attached to a 20-gauge swivel, allowing free movement about the cage. An additional cannula was also inserted into the duodenum. Each rat was fitted with a Velcro vest to protect the cannula. No antibiotics were necessary after this sterile surgical procedure. Approximately 16 h after surgery, animals were administered a single 10 mg/kg dose of a mixture of [D5/D0]AT and [14C]AT as a suspension (37–46 mCi/rat) and housed individually in stainless steel metabolism cages. During the initial 48 h, donor bile was infused at the rate of 0.8 ml/h to replace bile collected through the fistula. Bile samples were collected predose and at intervals of 0 to 2, 2 to 4, 4 to 8, and 8 to 24 h, and at 24-h intervals through 144 h. Urine samples were collected predose and at intervals of 0 to 8 and 8 to 24 h, and at 24-h intervals through 144 h. Fecal samples were collected predose and at 24-h intervals through 144 h. Samples were stored frozen at −20°C until analysis.

In the first multiple-dose study, 12 male rats (295–377 g) were divided into three groups of four animals each. A bile duct and a duodenal cannula were implanted 12 h before the last dose. Each group received a once-daily oral dose of 10 mg/kg [14C]AT (19.5 μCi/day) for either 1, 8, or 15 days with each animal housed individually in a stainless steel metabolism cage. Complete urine, bile, and feces were collected through 144 h and stored frozen at −20°C. As described for plasma profile studies, 15 male (204–245 g) and 15 female (175–193 g) rats were dosed by gavage with 20 mg/kg of AT suspension (0.5% methylcellulose) once daily for 20 days. On day 21, each fasted rat received an oral 20 mg/kg suspension dose of [14C]AT (100 μCi, males; 70 μCi, females.) At 3, 6, and 12 h postdose, 10 rats (5 males and 5 females) were anesthetized with diethyl ether and exsanguinated by cardiac puncture. Blood was collected in heparinized containers, and harvested plasma was stored frozen at −20°C until analysis.

**Dog.** Beagle dogs were obtained from Marshall Research Animals (North Rose, NY). Mass balance studies were conducted in two female bile-fistula dogs (9–10 kg) on separate occasions. Before each study, the dog was anesthetized and the common bile duct cannulated (gallbladder removed); the cannula was then directed into a ventral pocket and attached to a collector. A topical antibiotic was applied for 5 days after surgery because of irritation caused by the vest and was followed by another 5 to 9 days of recovery. Dogs were fed standard dog chow and water ad libitum, and each animal received a capsule of desiccated hog bile daily with food for the duration of the experiment. Dogs were fed 1 h before administration of a capsule dose. The first dog (dog 1) received a single 10 mg/kg dose given as mixture of [D5/D0]AT and [14C]AT (39 μCi); the second dog (dog 2) received 10 mg/kg dose of [14C]AT (74 μCi). Each animal was housed in a stainless steel metabolism cage. Bile samples were collected predose and at intervals of 0 to 2, 2 to 4, 4 to 8, and 8 to 24 h, and at 24-h intervals through 240 h. Urine and fecal samples were collected predose and at 24-h intervals although 240 h. Samples were stored frozen at −20°C until analysis. Biliary recycling was examined 8 days after the administration of [14C]AT to Dog 2 by giving the previously collected 4 to 8 h

**TABLE 1**

<table>
<thead>
<tr>
<th>Excreta</th>
<th>Rats Male</th>
<th>Female</th>
<th>Dogs Female (dog 1)</th>
<th>Female (dog 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>20.0</td>
<td>17.8</td>
<td>9.29</td>
<td>0.45</td>
</tr>
<tr>
<td>Bile</td>
<td>77.7</td>
<td>69.7</td>
<td>32.8</td>
<td>35.4</td>
</tr>
<tr>
<td>Feces</td>
<td>21.8</td>
<td>24.2</td>
<td>55.5</td>
<td>55.0</td>
</tr>
<tr>
<td>Total</td>
<td>99.7</td>
<td>95.7</td>
<td>97.6</td>
<td>90.9</td>
</tr>
</tbody>
</table>

**Fig. 1.** Synthetic schemes for ring-labeled [14C]AT (9a), ortho-hydroxy AT (9b), and para-hydroxy-AT (9c).
bile sample (10 μCi). This sample, which contained the largest amount of radioactivity in a reasonable volume, was given by gavage. Urine, bile, and feces were collected as described in the initial experiment. The study was stopped at 24 h when the collector became detached from the dog. For plasma profiling, six beagle dogs, three males (11.0 – 15.5 kg) and three females (8.8 – 10.0 kg), were administered an AT suspension placed in gelatin capsules (7 mg/kg) three times daily for 10 days. On day 11, each fasted animal received a dose of AT followed 6 h later by a 7 mg/kg dose of [14C]AT (300 m Ci).

Heparinized blood was collected 3 and 6 h postdose, and the harvested plasma was stored frozen at −20°C until analysis.

**Enzyme Inhibition Assay.** Inhibitory activities of AT and AT metabolites in the in vitro HMG-CoA reductase activity assay were determined as described (Shum et al., 1993).

**Determination of Radioactivity.** Radioactivity was determined by liquid scintillation counting using a model 2500 TR TriCarb System (Packard, Downers Grove, IL) with quench correction by external standardization. Aliquots of plasma, bile, and urine were counted directly in Ready Safe (Beckman, Fullerton, CA). Fecal samples were homogenized in distilled water (10% homogenate), and duplicate 0.5-ml samples were air dried. Samples were combusted by a Packard Tri Carb Sample Oxidizer model 306l, and the resulting CO2 was trapped in Carbosorb and counted in Permafluor. Disintegration/min values for urine, bile, and feces were converted to percentage of administered dose.

**Extraction of Plasma.** Rat (1–1.5 ml) and dog (3–6 ml) plasma samples were prepared for chromatography by precipitating proteins with 5 ml of ice-cold absolute ethanol for each ml of plasma. After centrifugation, the

<table>
<thead>
<tr>
<th>Species</th>
<th>Approximate Retention Time and Component Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>5.9 (1) 10 (2) 35 (3) 62 (4) 64 (5) 66 (6) 86 (7) 89 (8) Total</td>
</tr>
<tr>
<td>Dog</td>
<td>0.24 3.75 16.0 3.98 ND 7.37 ND 0.41 22.6</td>
</tr>
</tbody>
</table>

ND, not detected. Retention time given in minutes; values in parentheses are component numbers.

Glucuronide conjugate of ortho-hydroxy AT.

para-hydroxy AT.

ortho-hydroxy AT.

AT.

β-Oxidized AT.

**TABLE 2**

Major radioactive components in 0- to 24-h rat and dog bile (percentage of dose) after a single 10 mg/kg oral suspension dose of a mixture of [D5/D0] and/or [14C]AT

<table>
<thead>
<tr>
<th>Sample</th>
<th>Approximate Retention Time and Component Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, Day</td>
<td>8 (1) 12 (2) 40 (3) 68 (4) Total</td>
</tr>
<tr>
<td>1</td>
<td>5.81 11.5 28.6 11.1 18.8 1.05 1.05 78</td>
</tr>
<tr>
<td>8</td>
<td>2.01 8.23 22.6 8.51 23.6 1.57 1.99 69</td>
</tr>
<tr>
<td>15</td>
<td>2.97 9.37 28.6 11.8 15.1 1.24 1.39 71</td>
</tr>
</tbody>
</table>

ND, not detected. Retention time given in minutes. Values in parentheses are component numbers.

Glucuronide conjugate of ortho-hydroxy AT.

para-hydroxy AT.

ortho-hydroxy AT.

AT.

β-Oxidized AT.

**TABLE 3**

Major radioactive components in (0–24 h) rat bile (percentage of chromatogram) after single and multiple oral dose administration of 10 mg/kg [14C]AT

![HPLC-radioactivity profile of rat bile before (A) and after (B) treatment with β-glucuronidase/sulfatase.](image)

**Fig. 2.** HPLC-radioactivity profile of rat bile before (A) and after (B) treatment with β-glucuronidase/sulfatase.
ethanol was transferred to a clean tube. The pellet was resuspended with 2 ml of acetonitrile by vortexing for 1 min. After centrifugation, the acetonitrile was added to the ethanol and the organic mixture was taken to near dryness by a stream of nitrogen at room temperature. Samples were reconstituted in 200 µl of 50% acetonitrile: 0.1 M ammonium acetate buffer (pH 4.0) and were transferred to amber vials for injection (150 µl) and HPLC analysis. Recovery of radioactivity averaged 87 and 66% for rat and dog plasma samples, respectively.

Gradient HPLC-Radioactivity Profiles. Separation of radioactive components in biological extracts and bile was performed on a Waters HPLC system (Milford, MA) with radioactivity detection (Radiomatic, Model CR, Packard). Profiling was performed using a BioSil ODS-5S column (5 µm particle size, 4.6 × 150 mm) in series with a Brownlee RP-18 Speri-5 guard cartridge. The mobile phase consisted of buffers A (0.1 M ammonium acetate) and B (acetonitrile). The gradient had an initial solvent composition of 75% buffer A and 25% buffer B, which was held for 5 min. Over the next 40 min, buffer B increased to 32%, followed by increase of buffer B to 40% for another 20 min. This mixture was held until 75 min into the run, when buffer B increased to 80% over the next 15 min, and this mixture was held to 110 min. Flow rate of the mobile phase was 1 ml/min with UV detection at 270 nm. Radioactivity was detected in a 1-ml flow cell using Flo-Scint III at 3 ml/min. Recovery of 14C activity applied to the column was approximately 100%. Aliquots of bile (50–150 µl) were analyzed after direct injection. Fractions were collected as selected radioactive peaks eluted from the column by a fraction collector.

MS. Identification of collected fractions was done with the aid of MS and comparison to standards. MS was done with a Fisons AutoSpec Ultima-Q hydrid instrument (VG Analytical Ltd., Manchester, UK). Continuous-flow liquid secondary ion mass spectrometry was achieved by using a Harvard Apparatus model 11 syringe pump (South Natick, MA) and a Valco C14W micro-bore LC injector (Valco Instrument Co.) with a 1-µl sample loop.

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>Male rata</td>
<td>17,192 (8.6)</td>
</tr>
<tr>
<td>Female rata</td>
<td>18,798 (57)</td>
</tr>
<tr>
<td>Male dogb</td>
<td>2,598 (45)</td>
</tr>
<tr>
<td>Female dogb</td>
<td>3,967 (26)</td>
</tr>
</tbody>
</table>

ND, not determined (radioactivity insufficient for analysis).

* a n = 5.

b n = 2–3.

**Fig. 3.** HPLC-radioactivity profile of dog (A) and rat (B) plasma after multiple-dose administration. Peak 1 is para-hydroxyAT, peak 2 is ortho-hydroxyAT, peak 3 is AT, and peaks 4 and 5 are β-oxidation products.
Table 5
Cumulative urinary, biliary, and fecal recovery of radioactivity in a female dog after oral administration of previously collected 4- to 8-h bile (10 μCi) sample

<table>
<thead>
<tr>
<th>Collection Intervals (h)</th>
<th>Urine</th>
<th>Bile</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2</td>
<td>NS</td>
<td>2.52</td>
<td>NS</td>
</tr>
<tr>
<td>2–4</td>
<td>NS</td>
<td>4.10</td>
<td>NS</td>
</tr>
<tr>
<td>4–8</td>
<td>0.24</td>
<td>2.93</td>
<td>NS</td>
</tr>
<tr>
<td>8–12</td>
<td>0.77</td>
<td>NS</td>
<td>46.8</td>
</tr>
<tr>
<td>12–24</td>
<td>0.24</td>
<td>NS</td>
<td>46.8</td>
</tr>
<tr>
<td>Total</td>
<td>2.42</td>
<td>9.55</td>
<td>46.8 = 57.6 total</td>
</tr>
</tbody>
</table>

NS, no sample (dog destroyed collection apparatus).

Combined β-Glucuronidase/Sulfatase Enzyme Hydrolysis. Bile samples (approximately 500 μl) were incubated at 37°C overnight with 2 mg of material (300,000–400,000 U/g β-glucuronidase containing 15,000–40,000 U/g sulfatase) after adjustment of the pH to 5.0 with acetate buffer.

Results

Single-Dose Mass Balance Studies in Bile Fistula Rats. Mean recoveries of radioactivity in male and female rats are presented in Table 1 after a 10 mg/kg oral suspension dose of a mixture of [D5/D0] AT and/or [14C] AT. Data were combined because the excretion pattern in male and female animals was similar. The majority of the radioactivity was recovered within the first 48 h of dosing, with bile being the primary route of excretion. Peak biliary excretion occurred in the 4- to 8-h collection interval. Gradient HPLC-radioactivity analysis of directly injected bile revealed several unchanged radioactive components, including unchanged drug (Table 2). Within the first 24 h, four identified metabolites (Fig. 2, components 2, 3, 4, and 8 based on HPLC retention characteristics) and AT (Fig. 1, component 3) accounted for 39% of the administered dose, whereas approximately 62% of dose was excreted in bile. The presence of a major biliary conjugate was revealed after treatment with β-glucuronidase, as shown in Fig. 2. After treatment, the percentage of dose represented by component 2 (Fig. 2) disappeared and a peak with similar retention time as the ortho-hydroxy metabolite of AT (Fig. 2, component 4) increased by a similar percentage. An average of 75% of the administered dose was absorbed in rats based on the combined biliary and urinary recoveries.

Multiple-Dose Studies in Rats. In these studies, animals were given a single daily 10 mg/kg dose of [14C]AT for 1, 8, or 15 days. Mean recoveries of radioactivity after a single dose were 0.23% in urine, 60.3% in bile, and 34.7% in feces (total 95.2%). Recovery of the radiolabel was not determined on days 8 or 15 because of radioactivity carryover. Radioactivity profiles of 0 to 24 h bile after [14C] AT administration on days 1, 8, or 15 were qualitatively similar (Table 3). Radioactivity profiles of 0 to 24 h bile after [14C]AT administration on days 1, 8, or 15 were qualitatively similar (Table 3). The presence of a glucuronide conjugate after treatment with β-glucuronidase also was confirmed.

HPLC-Radioactivity Profiles of Rat Plasma Extracts. Mean concentrations of radioactivity in rat plasma after an oral dose of [14C] AT on the last day of the multiple-dose regimens are shown in Table 4. Whereas peak plasma concentrations of radioactivity occurred later in female rats, no difference in metabolic pattern was noted between gender. Identification of para-hydroxy AT (peak 1), ortho-hydroxy AT (peak 2), AT (peak 3), and β-oxidized metabolites (peaks 4 and 5) was based on retention time comparisons with injected standards (Fig. 3).

Single-Dose Mass Balance Studies in Bile Fistula Dogs. Mean recovery data in female dogs after a 10 mg/kg oral suspension dose of a mixture of [D5/D0] AT and/or [14C] AT are shown in Table 1. The majority of the radioactivity was recovered by 48 h postdose, with bile and feces being the primary routes of excretion (Table 1). Dogs absorbed an average of 39% of the dose, based on biliary and urinary recovery. Gradient HPLC-radioactivity analysis of directly injected dog bile shows the presence of several radioactive components that are summarized in Table 2. Within the first 24 h postdose, four metabolites (components 2, 3, 4, and 8) and AT (component 6) accounted for 22.4% of the dose (Table 2). An average of 32.6% of the dose was excreted in the bile during this time. The profiles of dog bile had fewer smaller drug-derived peaks than were noted in rat bile; however, the metabolite profiles were qualitatively similar. After treatment with β-glucuronidase, the percentage of dose represented by a conjugate (component 2) disappeared, and the ortho-hydroxy metabolite of AT (component 4) increased by a similar percentage.

Recirculation of Bile. After nearly complete recovery of administered [14C]AT dose in the second bile-fistula dog (Dog 2), the 4- to 8-h bile sample was administered by gavage (approximately 10 μCi) to the same dog. By 24 h, at least 58% of the radioactivity was recovered in urine, bile, and feces, with bile contributing 10% to the total (Table 5). The biliary recycling study was halted at this time because the bile collection system no longer functioned. Most of the metabolites, as well as AT, were excreted again in the bile, and no new peaks were noted (Table 6). Thus, AT-derived radioactivity can be reabsorbed from bile. The extent of absorption, however, is not known.

HPLC-Radioactivity Profiles of Dog Plasma Extracts. Mean plasma concentrations of radioactivity after multiple-dose administration of [14C]AT are shown in Table 4. Dog radioactivity concentrations were considerably lower at all time points than rat plasma radioactivity concentrations. The radioactivity in the 12-hour plasma

Table 6
Major radioactive components (percentage of dose) in female dog bile after administration of 4- to 8-h bile sample (10 μCi) collected after a single 10 mg/kg dose of [14C]AT

<table>
<thead>
<tr>
<th>Interval (h)</th>
<th>Approximate Retention Time and Component Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2</td>
<td>8 (1) 12° (2) 40° (3) 68° (4) 70° (6) 87° (7) 89° (8)</td>
</tr>
<tr>
<td>2–4</td>
<td>ND 0.12 0.28 0.23 0.23 0.23 0.23 0.23</td>
</tr>
<tr>
<td>4–8</td>
<td>ND 0.28 0.28 0.28 0.28 0.28 0.28 0.28</td>
</tr>
<tr>
<td>Total</td>
<td>0.63 3.98 1.85 2.23 ND ND 8.69</td>
</tr>
</tbody>
</table>

ND, not determined. Retention times given in minutes. Values in parentheses are component numbers.

1 Glucuronide of ortho-hydroxy AT.
2 para-hydroxy AT.
3 ortho-hydroxy AT.
4 AT.
5 β-Oxidized hydroxy AT.
6 β-Oxidized AT.
samples was too low to provide any information. Up to five radioactive components were detected in plasma profiles, but not all profiles (Fig. 3) contained all components all the time. No qualitative differences in these profiles were observed between male and female dogs. Identification of the para-hydroxy AT (component 1), ortho-hydroxy AT (component 2), AT (component 3), and β-oxidized metabolites (components 4 and 5) was based on comparison of retention times with injected standards.

Metabolite Identification. Metabolite-profiling studies were conducted with the inclusion of stable isotope labeled AT that served to detect metabolites and facilitate structural identification. A pentadeutero analog of AT was used in this study. The ratio of stably labeled AT to AT was measured from the ion ratio (i.e., M + 5/M) of the dose solution (Fig. 4) to be 38/62 for [1H5]AT/AT. Collected HPLC fractions from rat and dog bile (single dose) were analyzed by MS using the method of continuous-flow fast atom bombardment ionization. The mass spectra of HPLC-collected fractions showed prominent protonated molecular ion clusters at m/z 756/751 (retention time of 10 min), m/z 580/575 (35 and 62 min, see Fig. 5), and m/z 564/559 (66 min), corresponding to expected molecular ions of an ether glucuronide of a monohydroxylated AT, two monohydroxylated AT metabolites, and unchanged AT, respectively. Two additional AT metabolites were detected at m/z 520/515 (87 min) and m/z 504/499 (89 min), which correspond to the anticipated molecular ions of a hydroxylated β-oxidized AT and β-oxidized AT, respectively.

The elucidation of metabolite structure was performed by acquiring collision-induced dissociation mass spectra by using linked sector scans (i.e., MS/MS). The position of stable label aided in the elucidation of structure by producing differentially labeled fragment ions on collisional activation. The MS/MS spectrum of the protonated molecular ion of m/z 575 detected in the 35-min collected fraction (Fig. 6) is identical with that of reference para-hydroxy AT. Fragmentation of the amide bond gives rise to ions of m/z 466 and 440. Similarly, the MS/MS spectrum of the protonated molecular ion of m/z 580 shows complimentary fragment ions of m/z 471 and 445 consistent with the expected 5 amu mass increase relative to unlabeled metabolite. In a similar manner, the compounds detected in the 62- and 66-min HPLC fractions were identified as ortho-hydroxy AT and AT based on reference compounds. Elucidation of the structure of the glucuronide conjugate eluting at 10 min was possible because in the positive mode, a protonated molecular ion cluster was detected at m/z 751/756; in the negative mode, at m/z of 749/754. In addition to parent, similar metabolites were identified in rat and dog bile that include a glucuronide conjugate of hydroxylated AT and ortho- and para-hydroxylated AT.

Enzyme-Inhibitory Activity of Metabolites. Standards of major metabolites identified in bile were assayed for bioactivity against AT as reference in the enzyme inhibition assay. Nanomolar concentrations were used to determine the percent inhibition. These values were used to assess IC50, using a computer program, DOSE (Biosoft, Cambridge, UK). Only the hydroxylated forms were as potent as AT, whereas β-oxidation of the heptanoic side chain caused loss of enzyme inhibition activity, as shown in Table 7.

Discussion
In these rat and dog studies, AT metabolism and excretion patterns were elucidated by using a mixture of stable and radioactively labeled materials and combined chromatography and MS. After an oral dose of [14C]AT, rats and dogs excreted most of the radioactivity in the bile and feces, with urinary excretion being only a relatively minor pathway. The mixture of stably labeled drug and radioactive AT allowed rapid identification of AT and major metabolites in the bile. At the time these studies were performed, limited in vitro data were available to assist in metabolite detection and identification. Incorporation of
the stable isotope of AT allowed rapid detection and structural elucidation of metabolites. By looking for molecular ion clusters of M/M + 5, the fragmentation behavior of AT and metabolites as well as structural confirmation were readily available. In addition, because ortho- and para-hydroxylated metabolites of AT were well separated by chromatography, the identification of glucuronide conjugate of ortho-hydroxy AT was readily apparent, because the ortho-hydroxy AT peak increased after treatment with β-glucuronidase/sulfatase. Mass spectral analysis provided a molecular ion consistent with a glucuronide conjugate. The β-oxidation pathway was a relatively minor route (based on biliary recovery) of AT metabolism with β-oxidation products barely detectable in dog bile. The low amounts of β-oxidation products excreted are similar to other HMG-CoA reductase inhibitors (Vickers et al., 1990; Komai et al., 1992; Halpin et al., 1993). Mouse was the only species to use the β-oxidation pathway extensively in the metabolism of AT (Black et al., 1998). Based on biliary recovery, the extent of AT absorbed was greater in the rat than in the dog. Despite this difference, approximately 66% of the radioactivity in the initial 24-h rat and dog bile was identified. The remaining radioactivity was contributed by many small components that individually accounted for less than 1% of the dose. Like other HMG-CoA inhibitors, enterohepatic circulation is probably responsible for the persistence of radioactivity in rat liver long after other organs were cleared (Duggan and Vickers, 1990; Bocan et al., 1992). The recirculation of radioactive components in bile was studied in a dog after nearly complete recovery of the initial radioactive dose. The reappearance of AT and major metabolites in dog bile after administration of a previously collected bile sample indicates that biliary recycling is an important component in AT metabolism and excretion profile. Multiple-dose administration of AT in the rat did not produce any new metabolites or change the pattern of metabolites in bile and plasma, consistent with other data indicating low induction potential.

Plasma concentrations of the reductase inhibitors and/or their metabolites are usually low due to extensive first pass metabolism (Desager and Horsmans, 1996). Plasma profiling was conducted after multiple dose administration because plasma concentrations in rats and dogs were very low after a single dose administration. Plasma profiles were similar to those observed for bile. Considerably less para-hydroxy AT was found in plasma, and no conjugates were detected. Dog absorbed approximately 50% less of the dose than rat and contributed to the lower plasma concentrations of radioactivity in the dog than the rat. The mouse is the only animal model that, when administered AT, displays different metabolism and presents plasma

![Figure 6](https://example.com/fig6.png)

**Fig. 6.** Continuous-flow fast atom bombardment MS/MS positive ion spectrum of the component eluting at ca. 35 min.

<table>
<thead>
<tr>
<th>Oxidized AT</th>
<th>AT</th>
<th>para-hydroxy AT</th>
<th>meta-hydroxy AT</th>
<th>ortho-hydroxy AT</th>
<th>β-Oxidized AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>3.71</td>
<td>3.62</td>
<td>3.52</td>
<td>3.62</td>
<td>0</td>
</tr>
<tr>
<td>Metabolites</td>
<td>3.21</td>
<td>4.22</td>
<td>5.41</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Normalized*</td>
<td>3.71</td>
<td>3.29</td>
<td>4.45</td>
<td>5.54</td>
<td>0</td>
</tr>
</tbody>
</table>

*Normalized to AT.

**TABLE 7**

In vitro HMG-CoA reductase inhibitory activity (IC₅₀) value of AT and metabolites in human plasma

![Chemical Structure](https://example.com/struct.png)
concentrations affected by multiple dose administration (Black et al., 1998).

HMG-CoA reductase inhibitors have similar potencies; however, difference in efficacy in vivo can often be related to delivery and residence time of the active drug and metabolites to the target, the liver. Animal mass balance studies with HMG-CoA reductase inhibitors have shown that these drugs, including AT and its metabolites, are usually excreted in feces (Duggan et al., 1989; Vickers et al., 1990; Komai et al., 1992; Tse et al., 1995). This is usually not because of a lack of absorption of the drug; a major portion of absorbed inhibitor and metabolites are excreted preferentially in the bile. Each of the current reductase inhibitors on the market varies in the amount of unchanged drug and active metabolites excreted in the bile. First-pass metabolism and biliary excretion can actually enhance the efficacy of the drug. Keeping active moieties in the liver and returning them through enterohepatic recirculation could prolong the action of the drug. In these bile fistula rat and dog studies, over 50% of the radioactivity in the bile is associated with AT and its active metabolites. After an oral dose of lovastatin or simvastatin, the metabolism of these compounds is based on the dynamic and reversible conversion of the lactone form to the active hydroxy acid form. The biliary products were composed of a small amount of the active acid form and metabolites that were 20 to 50% as active as the parent acid form (Duggan et al., 1989; Vickers et al., 1990). Disposition and metabolism studies of pravastatin in rats and dogs after an oral dose indicates that biliary excretion occurs to a lesser extent in dog than in rats. Most of the biological activity is due to unchanged drug, and the minor amounts of metabolites possess little activity. Examination of rat bile indicated that pravastatin is the prominent excretory product in bile and undergoes substantial enterohepatic circulation (Komai et al., 1992). Dogs administered fluvastatin excrete approximately 56% of the radioactive dose in the bile, with fluvastatin contributing about 12% (Tse et al., 1995) of this total. The contribution from metabolites as active inhibitor was not discussed. Compared with monkeys given fluvastatin, dogs metabolized this compound to a lesser extent. Thus, AT, like other HMG-CoA reductase inhibitors, is relatively well absorbed with minimal systemic exposure. Radioactivity is primarily excreted via the feces in rats and dogs, and biliary excretion contributes to a large portion of fecal radioactivity. Oxidative metabolism plays the largest role in AT metabolism in rats and dogs. In addition, our studies with a bile fistula dog show that biliary recycling occurs. AT is a very potent inhibitor of HMG-CoA and has an additional advantage of biliary metabolites that are also potent inhibitors as AT itself. Because AT undergoes a large first-pass effect that produces active metabolites that are in turn recirculated via the bile to the liver, the target organ, efficacy is enhanced, and the duration of action is prolonged. A single-dose study of [14C]AT in humans with a T tube found that biliary excretion was the major route of elimination (Le Couteur et al., 1996). AT was extensively metabolized to ortho- and para-hydroxylated glucuronides, with trace amounts of AT present in the bile, and these metabolites are very likely to undergo enterohepatic recycling as active moieties. Thus, many HMG-CoA reductase inhibitors such as AT can provide a long and effective duration of action because of their metabolism to active metabolites, and these active metabolites and drug undergo biliary recycling.

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References


