IN VIVO METABOLISM AND FINAL DISPOSITION OF A NOVEL NONSTEROIDAL ANDROGEN IN RATS AND DOGS

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Running Title: Pharmacokinetics and Final Disposition of S-4 in Rats and Dogs.

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# of text pages: 36
# of tables: 3
# of figures: 5
# of references: 27
# of words in abstract: 234
# of words in introduction: 721
# of words in discussion: 1,372

Abbreviations: SARM, selective androgen receptor modulator; AR androgen Receptor: AUC, area under the plasma concentration-time curve; C_{max}, maximal drug plasma concentration; T_{max}, time to reach the maximal drug concentration (at the C_{max}); V_{ss}, steady state volume of distribution; V, volume of distribution; MRT Mean Residence Time; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry;
ABSTRACT

Compound S-4 (S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide) is a novel nonsteroidal androgen agonist that mimics many of the beneficial pharmacologic effects of testosterone with lesser effects on the prostate. S-4 demonstrated high androgen receptor (AR) binding affinity as well as anabolic specificity during in vivo pharmacologic studies in rats, identifying it as the first member of a new class of selective androgen receptor modulators (SARMs). The purpose of these studies was to determine the pharmacokinetics and metabolism of S-4 in dogs. S-4 showed linear pharmacokinetics after both intravenous (IV) and oral (PO) administrations at pharmacologically relevant doses, with a mean clearance of 4.6 ml/min/kg and a mean half-life of about 200 minutes. Interestingly, dose-dependent oral bioavailability was seen. However, at pharmacologically relevant doses, the oral bioavailability of S-4 was 91%. Species differences were observed in S-4 metabolism, the major metabolic pathway for S-4 in dogs was deacetylation of the B ring acetamide group and reduction of the A ring nitro group, while the major metabolic pathway for S-4 in rats was hydrolysis on the amide bond and reduction of the A ring nitro group. In addition, oxidative metabolites and phase II metabolites were identified in both rats and dogs. These studies demonstrate that S-4 maintains its promising pharmacokinetic properties in dogs (i.e., high oral bioavailability and linear kinetics) and is largely eliminated via hepatic metabolism by both phase I and phase II enzymes.
INTRODUCTION

Testosterone, an endogenous ligand for the androgen receptor (AR), plays important and diverse physiological roles in several different organ systems, including muscle and bone (George and Wilson, 1986; Hiort, 2002). Testosterone and related anabolic steroids are used clinically as replacement therapy for primary or hypogonadotrophic hypogonadism, delayed puberty, anemia, or muscle wasting conditions (Mooradian et al., 1987; Bagatell and Bremner, 1996). However, there are many disadvantages associated with testosterone and anabolic steroid therapy. (Basaria and Dobs, 1999). Firstly, current testosterone preparations can only be administered via intramuscular injections (Snyder and Lawrence, 1980). While 17α-alkylated androgens were developed for oral administration, they are associated with hepatic toxicity and are less efficacious; hence, they are not recommended for long-term androgen therapy (Swerdloff and Wang, 2002). Secondly, testosterone injections produce large fluctuations in testosterone plasma concentrations, which may lead to adverse effects such as hepatotoxicity, blood pressure effects, and gynecomastia (Heywood et al., 1977; Negro-Vilar, 1999). Thirdly, current steroidal preparations demonstrate equal anabolic and androgenic activity (i.e., they are not tissue selective). The non-selective nature of testosterone and its derivatives, poor pharmacokinetic properties, and toxicities have led to the search for androgens with improved properties.

A variety of selective androgen receptor modulators (SARMs) are being developed to address these deficiencies (Chen et al., 2005; Gao et al., 2005). The ideal SARM would have a minimum of side effects, good oral bioavailability, and a favorable pharmacokinetic profile (i.e., linear kinetics and a half-life conducive to once-a-day
dosing). Much like selective estrogen receptor modulators (SERMs), SARMs can be designed to be tissue specific. This would, for example, allow the drug to act as an androgen in muscle (anabolic) while having lesser or no effect on other androgenic organs like the prostate. Such a drug could be beneficial in disorders such as muscle, wasting, andropause, osteoporosis, or benign prostatic hyperplasia, and avoid the concerns related to the role of testosterone and androgens in prostate cancer.

The first SARM developed, acetothiolutamide (Figure 1, B), demonstrated high binding affinity (Ki = 2.1 nM) and full in vitro agonist activity (Yin et al., 2003b). However, pharmacologic studies showed that acetothiolutamide showed very low androgenic and anabolic activity in vivo (Yin et al., 2003a). Given that acetothiolutamide showed in vitro efficacy comparable to testosterone, these findings were unexpected. Pharmacokinetic studies showed that acetothiolutamide was completely bioavailable after subcutaneous administration. However, this compound showed rapid elimination from plasma with a clearance of 45 ml/min/kg and a plasma half life of less than 30 minutes. In addition, the metabolic profile of acetothiolutamide showed that the thioether linkage was oxidized to a sulfonyl linkage which led to the formation of metabolites with partial AR agonist or antagonist activity. These findings resolved the inconsistencies seen between in vitro and in vivo studies.

S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (hereafter referred to as S-4 as in (Yin et al., 2003a)) is a structural analog of bicalutamide and flutamide, nonsteroidal antiandrogens used for the treatment of prostate cancer. Metabolism studies with bicalutamide show that the majority of the drug is hydrolyzed at the amide bond along with oxidation of the aromatic rings (Boyle et
al., 1993). These reactions are species specific with hydrolysis occurring in the rat, but not in human or dog. Interestingly, the metabolite produced by hydrolysis was not seen with acetothiolutamide. However, extensive metabolism of the thioether linkage, which converted the compound to an antagonist, was seen; therefore modifications to a less labile ether linkage were made. In addition, the cyano group in the para-position or the A ring was changed to a nitro because of the increased binding affinity observed in prior studies. This novel SARM, S-4 (Figure 1, A), showed high binding affinity (Ki = 4 nM) and was a full androgen agonist in transcriptional activation studies with the androgen receptor (Yin et al., 2003a).

As all of the prior studies were conducted in rats, we sought to examine the pharmacokinetics and metabolism of S-4 in dogs as a step towards continued development of this SARM. Our previous in vitro metabolism studies (Gao et al., 2006b) have identified species differences in the metabolism of S-4 between rats and dogs. Thus, we also further characterized and compared the metabolic profiles of S-4 in these two species. The studies reported herein provide compelling evidence of the promising pharmacokinetic and metabolic properties of this first nonsteroidal SARM.
MATERIAL AND METHODS

1. Chemicals

S-4 (S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl–N-(4-nitro-3-
trifluoromethyl-phenyl)-propionamide) was synthesized in our laboratories with purity
greater than 99%. Uniformly labeled $[^{14}\text{C}]$ S-4 (purity 95%, specific activity 5.4
mCi/mmol) was synthesized and purified in the Radiochemistry Laboratory at The Ohio
State University (OSU) Comprehensive Cancer Center. Polyethylene glycol 300 (PEG
300, reagent grade) was purchased from Sigma Chemical Company (St Louis, MO).
Ethyl alcohol USP was purchased from AAPER Alcohol and Chemical Company
(Shelbyville, KY). HPLC grade acetonitrile and ethyl acetate were purchased from Fisher
Scientific Company (Fair Lawn, NJ).

2. Animals

Beagle dogs were procured from approved vendors. The animals were between 2
and 6 years old and weighed 11.4 to 14.7 kg. Dogs were individually housed in an
animal facility accredited by the American Association for Laboratory Animal Care that
is directed by full-time veterinarians. Animals were acclimated in the facility for at least
one week prior to the study. Male Sprague Dawley rats weighing approximately 250 g
were purchased from Harlan (Indianapolis, IN). The animals were maintained on a 12-
hour cycle of light and dark, and had free access to food and water unless otherwise
stated. The animal protocols were reviewed and approved by the Institutional Laboratory
Animal Care and Use Committee of OSU.
3. Pharmacokinetic Study #1

A four treatment, four period crossover design was used to determine the pharmacokinetics of S-4 in six beagle dogs. Dogs were randomly assigned to a treatment group with a one-week washout period after each dose. Intravenous (IV) bolus dose of 10 or 3 mg/kg, an oral (PO) solution dose of 10 mg/kg, or an oral capsule dose of 10 mg/kg administered in a packed gelatin capsule (size 0) were administered during this study. Each animal was weighed prior to dosing. Animals were placed in metabolism cages 18 hours prior to dosing and for up to 72 hours after the dose was administered. Animals were fasted for 18 hours prior to dosing and for 8 hours after each dose was administered. Animals were allowed water *ad libitum*. IV and PO dosing solutions were freshly prepared by aseptic methods. An appropriate quantity of S-4 was weighed and dissolved in ethanol and diluted to a final volume of 3 mL with PEG 300. Ethyl alcohol content did not exceed 5% of the final volume. Solutions were stored at 4 °C and used within 24 hours. The gelatin capsule doses were prepared by weighing an appropriate amount of S-4 powder and loosely packing a size 0 gelatin capsule. Angiocatheters were placed in the right saphenous vein of the dog for blood collection. IV doses were administered as bolus doses through the left saphenous vein. PO doses were administered by gavage followed by a 10 mL water flush. Blood samples (5 mL) were collected into heparinized vacutainers prior to the dose and at 2 min, 5 min, 10 min, 15 min, 30 min, 1, 2, 4, 6, 8, 10, 24, 48, and 72 hours after IV doses and at 10 min, 30 min, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 24, 48, and 72 hours after PO doses. Blood samples were immediately centrifuged at 2000g for 10 minutes and the plasma collected and stored at –
20°C until analysis. In addition, feces and urine samples were collected and frozen at 8, 24 and 48 hours.

4. Pharmacokinetic Study #2:

The lack of bioavailability at high doses of S-4 prompted a second pharmacokinetic study. A three treatment, three period crossover design was used to determine the bioavailability of S-4 in four beagle dogs weighing approximately 12 kg. Dogs were randomly assigned to a treatment group with a one-week wash-out period after each dose of either an IV dose of 1 mg/kg or an PO dose of 1 or 0.1 mg/kg. Procedures for dosing and blood sampling were identical to the previous study.

5. Sample Preparation

An aliquot (0.5 mL) of plasma was spiked with an internal standard (S-3-(4-fluoro-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide) and mixed with two volumes of acetonitrile to precipitate plasma proteins. The samples were centrifuged at 2000g for 5 minutes, and the supernatants were removed and then mixed with 7 mL of ethyl acetate for 40 minutes on a shaker. The samples were then centrifuged at 2000g for 5 minutes to facilitate phase separation. The organic phase was collected and evaporated under a stream of nitrogen gas. The residue was reconstituted in 100 µL of mobile phase. An aliquot (20 µL) was injected and analyzed by high pressure liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS).
Urine samples were thawed, centrifuged at 1000g for 5 minutes, and spiked with the internal standard. The supernatant was filtered through a 0.22 µm filter and diluted 1:1 with HPLC grade water. An aliquot (20 µL) was injected in the LC/MS system for analysis.

Fecal samples were homogenized in 2 times the volume of HPLC grade water with a mechanical homogenizer. The samples were centrifuged at 3000g for 10 minutes, and spiked with the internal standard. The supernatant was collected and filtered through a 0.22 µm filter. The fecal pellet was extracted three times with a mixture of methanol/ethyl acetate [2:1 (v/v)]. The mixture was centrifuged at 3000g for 10 min and the supernatants of each extraction were pooled. The samples were evaporated under nitrogen. The resulting residue was reconstituted in 500 µL of mobile phase. The reconstituted sample was then filtered through a 0.22 µm filter and a 20 µL aliquot of each phase was injected for analysis by LC/MS.

6. HPLC Analysis Method

An aliquot (100 µL) of each sample was injected onto a Nova-pak® C18 column (3.9 mm X 150 mm, 4 µm particle size, Waters Corp., Milford, MA), and eluted with a mobile phase containing acetonitrile:water (35:65, v/v) at a flow rate of 1.0 mL/min. The UV absorbance of the eluents was monitored at 270 nm. The HPLC system consisted of a Waters Model 510 solvent pump, a Waters Model 717 autosampler, and a Waters Model 486 absorbance detector. Calibration standards were prepared in untreated dog plasma with S-4 concentrations ranging from 0.08 to 200 µg/mL. Standard curve samples were run in triplicate along with each sample run. The coefficients of variation of the assay,
both intra-day and inter-day, were below 15% at all concentrations. The concentration of S-4 in plasma, urine and feces was determined from the area ratio of S-4 to internal standard in comparison to calibration standards.

7. Data Analysis

The plasma concentration-time profiles for each animal were analyzed by noncompartmental methods using WinNonlin (version 3.1, Pharsight, Mountain View, CA). The area under the concentration-time curve (AUC) was calculated using the trapezoidal rule with extrapolation to time infinity. The terminal elimination half-life ($T_{1/2}$) was calculated from $T_{1/2} = \frac{0.693}{\lambda}$, where $\lambda$ was the terminal elimination rate constant determined via linear regression of the terminal portion of the concentration versus time curve. The plasma clearance (CL) was calculated as $\text{CL} = \frac{\text{Dose}_{iv}}{\text{AUC}_{0-\infty, iv}}$, where $\text{Dose}_{iv}$ and $\text{AUC}_{0-\infty, iv}$ were the IV dose and the corresponding area under the curve from time 0 to infinity, respectively. The apparent volume of distribution at equilibrium ($V_{ss}$) was calculated by:

$$V_{ss} = \frac{\text{Dose} \times \text{AUMC}_{0-\infty}}{\left( \text{AUC}_{0-\infty} \right)^2}$$

where $\text{AUMC}_{0-\infty}$ was the area under the first moment of the plasma concentration-time curve extrapolated to infinity. The mean residence time (MRT) was calculated as:

$$\text{MRT} = \frac{\text{AUMC}_{0-\infty}}{\text{AUC}_{0-\infty}}$$

Oral bioavailability (F) was calculated as:

$$F = \frac{\left( \text{AUC}_{0-\infty, po} \times \text{Dose}_{iv} \right)}{\left( \text{AUC}_{0-\infty, iv} \times \text{Dose}_{po} \right)}$$

where $\text{Dose}_{po}$ and $\text{AUC}_{0-\infty, po}$ were the oral dose and the corresponding area under the curve from time 0 to infinity, respectively.
8. Statistical Analysis

Statistical analyses were performed using a Student’s t-test, with P-values less than 0.05 considered as statistically significant.

9. Radioactive Metabolite Determination

We also conducted a metabolism study using $^{14}$C-labeled S-4. Following cannulation of the jugular vein, Sprague Dawley rats were allowed to recover for 24 hours. Animals were then placed in Nalgene® metabolism cages. An appropriate amount of $[^{14}\text{C}]$ S-4 (uniformly labeled B-ring) was dissolved in ethanol and diluted in PEG 300. The final concentration of ethanol was less than 5% of the dosing solution. An IV bolus dose of $[^{14}\text{C}]$ S-4 (100 $\mu\text{Ci}$) was administered through the jugular catheter over a 5 minute period. The animals were sacrificed 24 after dosing and the following organs harvested: liver, spleen, heart, kidneys, intestines (small and large), levator ani muscle, pancreas, stomach wall, abdominal fat and prostate. Feces and urine samples were also collected prior to dosing and at 24 hours after the dose was administered. All samples were stored at -20° C until analysis. Fecal and urine samples were prepared as stated previously. Organ samples were weighed and minced with a scalpel. The organ samples were then placed in 1 mL of ScintiGest® tissue solubilizer (Fisher Scientific Company, Fair Lawn, NJ). The tissue samples were then homogenized in a Pro 200 homogenizer (Pro Scientific, Monroe, CT). The samples were incubated at 60°C until the tissue dissolved. An aliquot (200 $\mu$L) of 30% hydrogen peroxide was added to decolorize the samples and 70$\mu$L of glacial acetic acid was added to prevent
chemiluminescence within the samples. An aliquot (10 mL) of ScintiVerse® scintillation cocktail (Fisher Scientific Company, Fair Lawn, NJ) was added to each sample and the total radioactivity was determined in a Beckman LS6000 IC liquid scintillation counter (Beckman-Coulter, Fullerton, CA).

A new HPLC method was also developed to identify highly hydrophilic metabolites that eluted in the solvent front of the chromatogram. This method provided baseline separation of metabolites to facilitate quantitation using the internal standard as described previously. The fecal and urine samples were separated using a reverse-phase column (5 µm, 2mm x 250 mm, Ultrasphere ODS C$_{18}$ column). A gradient elution program with a mobile phase consisting of methanol and water at a flow rate of 0.2 mL/min was used. The effluent was introduced into a HPLC system consisting of a Hewlett Packard 1050 quaternary pump, Hewlett Packard 1050 autosampler, and Hewlett Packard 1050 diode array detector. Eluted fractions were collected at 2 minute intervals over each HPLC run. A 5 mL aliquot of EcoLite (+) scintillation cocktail was added to each collected fraction. Total radioactivity (dpm) in each sample was determined by a Beckman LS6000 IC liquid scintillation counter (Beckman-Coulter, Fullerton, CA). Data acquisition was controlled by ChemStation software.

10. Sample Preparation and Identification of Metabolite

The samples were separated using a reverse-phase column (Ultrasphere ODS C$_{18}$ column, 2mm x 250 mm, column length, 5µm particle size,) and eluted with a gradient mobile phase consisting of methanol and water at a flow rate of 0.200 mL/min. After separation by the column, the effluent was introduced into a ThermoFinnigan LCQ Deca
Ion Trap System, which consisted of a Surveyor Pump, Surveyor Autosampler, and Surveyor PDA coupled to a LCQ Deca Ion Trap mass spectrometer through an electrospray ionization (ESI) interface set at the following conditions: sheath gas (nitrogen) 1185 mL/min, auxiliary gas (nitrogen) 60mL/min, Spray voltage 3.5 kV, capillary temperature 180°C, capillary voltage –6V, tube lens offset –55V, multipole 1 offset 6.75V, lens voltage 22V, multipole 2 offset 8.50V and entrance lens voltage 58V or an atmospheric pressure chemical ionization (APCI) interface set at: vaporization temperature 450°C, sheath gas (nitrogen) flow 660 mL/min, auxiliary gas (nitrogen) flow 60 mL/min, discharge current 10 µA, capillary temperature 150°C, capillary voltage –19.00V, tube lens offset 35.00V, multipole 1 offset 2.25V, lens voltage 88 V, multipole 2 offset 16.5 V, multipole RF amplitude 510.0 V p-p, entrance lens voltage 30 V, and electron multiplier voltage –900.0 V. All analyses were conducted in negative ion mode. The scan range for full-scan spectrometry analysis was 100- 2000 m/z. For LC/MS² analysis, precursor ions were isolated with a width of 4 m/z and fragmented with a collision voltage of 26 V. Data acquisition was controlled by Xcaliber software.
RESULTS

1. Pharmacokinetic Results

Plasma concentrations after IV doses declined in a bi-exponential manner and the drug concentration remained detectable for up to 600 minutes after the dose. Mean (± S.D.) concentration-time profiles for S-4 after IV administration are shown in Figure 2. Pharmacokinetic parameters determined after the IV doses are presented in Table 1. No statistically significant differences were found between clearance, volume of distribution or T1/2 between all doses, indicating no dose-dependent pharmacokinetics for S-4. The mean values for Cl, Vss, and T1/2 over the dosing range were 4.6 mL/min/kg, 1.4 L/kg and 200 min, respectively.

S-4 appeared rapidly in plasma after PO administration. Figure 3 shows the plasma concentration-time profile for S-4 following 0.1, 1, and 10 mg/kg oral solution doses of S-4. Table 2 summarizes the pharmacokinetic parameters of S-4 PO doses in beagle dogs. Interestingly, the Tmax for the 1 and 0.1 mg/kg PO doses were not significantly different, with values of 53.5 and 60 minutes, respectively, while the Tmax for the 10-mg/kg PO dose was significantly longer, 110 minutes, suggesting decreased solubility and/or delayed absorption at the highest oral dose. The AUC did not increase proportionally with dose, due to either increased clearance or decreased oral absorption over the PO dosing range. As such, oral clearance values were significantly different between all doses and the calculation of oral bioavailability showed a dose-dependent pattern. Plasma concentrations of S-4 after administration of the capsule preparation (data...
not shown) were erratic, precluding determination of pharmacokinetic parameters for this data.

2. S-4 Metabolites in Rat and Dog

We also determined the in vivo metabolites of S-4 in rats and dogs using LC/MS and radiochromatographic methods. Five major metabolic pathways were seen: 1) reduction of the nitro group on the A ring to either an amine or a nitroso group, 2) deacetylation of the B ring acetamido substituent, 3) oxidation of the compound and subsequent phase II metabolic conjugation, 4) hydrolysis of the amide bond, and 5) methylation of the parent compound. The proposed in vivo metabolic pathway of S-4 is shown in Figure 4. Figure 5, A-D, H and I shows the LC/MS spectra and proposed structure of these primary metabolites along with the parent compound, S-4.

A. Urinary Metabolites in Rats

Post-dose urine samples collected at 0 to 8 hours and 8 to 24 hours showed similar metabolic profiles. Urine samples showed molecular ions ([M-H] deprotonated anion) corresponding to the parent drug (m/z 440) as well as a methylated metabolite (S4-CH3), a reduced metabolite (M4), an oxidized metabolite (M4-B-OH) and a sulfated metabolite (M4-A-OH-S).

Parent compound (m/z 440)

The theoretical mass of the parent compound is 441. Figure 5, A shows the LC/MS spectra for the parent compound. The predominant product ions of S-4 are m/z 150, 261, and 289. Metabolite identification was determined by comparison of the metabolite product ions to that of the parent compound.
Metabolite S4-CH$_3$ ($m/z$ 454)

This [M-H]$^-$ ion corresponds to the addition of a methyl group (14 Da.) to the parent compound, S-4. LC/MS fragmentation produced product ions at $m/z$ 150 and 303. The product ion at $m/z$ 303 also gained 14 Da. relative to the product ion at $m/z$ 289 produced from the fragmentation of the parent drug. There are two potential methylation sites: i) N-methylation of the amide nitrogen para- to the A-ring nitro substituents, or ii) O-methylation of the hydroxyl group of the chiral carbon. Differentiation between these sites was not possible from further fragmentation because of the relatively low abundance of the daughter ions. Although N-methylation of amides is rare, the presence of the amide carbonyl and para-nitro substituent on the adjoining aromatic ring make this an acidic proton and increase the likelihood of N-methylation. Previous in vitro and in vivo metabolism studies with a close structural relative of S-4 demonstrated that N-methylation occurs in the aryl propionamide SARMs (Wu et al., 2006). Further, MS/MS spectra of the S4-CH$_3$ metabolite were similar to those of synthetic standards of N-methylated aryl propionamide SARMs previously reported by our laboratories (cite Nair et al).

Metabolite M4 ($m/z$ 410)

Metabolite M4 corresponds to a decrease of 30 Da. from the parent compound, corresponding to reduction of the nitro group on the A ring to an amine. Figure 5, C shows the LC/MS spectra and proposed structure of M4. This metabolite was previously identified as a metabolite during in vitro metabolism studies (Gao et al., 2006b). As in the previous study, the same LC/MS fragmentation pattern was seen. Fragmentation of the $m/z$ 259 ion produced product ions at $m/z$ 209, 201, and 175 ($410 \rightarrow 259 \rightarrow 175$), the
last of which was also 30 Da. lower than the equivalent product ion at m/z 205 (440 → 289 → 205) produced from fragmentation of the parent drug, further confirming the proposed structure of M4.

**Metabolite M4-B-OH (m/z 426)**

Metabolite M4-B-OH corresponded to an addition of 16 Da. to metabolite M4, suggesting that it represented an oxidized metabolite of M4. There are four possible sites of oxidation: i) oxidation of the aromatic para-amino group of M4, ii) hydroxylation of the aromatic A ring, iii) hydroxylation of the methylene carbon, or iv) hydroxylation of the aromatic B ring. The LC/MS spectra shows a 16 Da. increase in the product ion at m/z 166 relative to the corresponding product ion at m/z 150 produced by metabolite M4, suggesting an oxidation of the B ring of metabolite M4. The presence of the m/z 259 ion further corroborated that there was no oxidation occurring at the other three sites mentioned above, confirming that M4-B-OH is a B-ring hydroxylated metabolite of M4 (Figure 5, D).

**Metabolite M4-A-OH-S (m/z 506)**

Metabolite M4-A-OH-S corresponds to an addition of 96 Da. to metabolite M4, suggesting oxidation of M4 followed by sulfation. However, the site of oxidation observed in M4-A-OH-S differed from that observed with metabolite M4-B-OH. LC/MS fragmentation of the [M-H]⁻ ion at m/z 506 produced product ions of m/z 426 and 275. The ion at m/z 426 was 80 Da. lower than the [M-H]⁻ ion at 506, consistent with the loss of a SO₃ moiety. Further LC/MS² fragmentation of the m/z 426 ion produced at m/z 275, 255, 406, and 366 (listed in order of abundance, data not shown). The presence of the m/z 275 ion upon fragmentation of the m/z 506 and 426 ions indicated that the site of oxidation...
oxidation and subsequent sulfation was located within the A-ring of M4 as shown in Figure 5, E.

B. Fecal Metabolites in Rats

Fecal samples collected at 8 and 24 hours after dosing showed similar metabolic profiles. In addition, fecal samples (both aqueous and extracted fecal pellet) showed molecular ion peaks similar to those found in urine samples; specifically peaks corresponding to the parent drug and metabolites M4, M4-B-OH, and M4-A-OH-S. However, the S4-CH₃ metabolite was not observed. These peaks produced fragmentation patterns that were similar to those found in rat urine, indicating that these molecular ions were structurally the same as those found in the urine samples. Two additional glucuronidated metabolites (m/z 602) were found in the feces (M4-A-OH-G and M4-B-OH-G). Figure 5, F and G shows the LC/MS spectra and proposed structure of these metabolites.

*Metabolite M4-A-OH-G and M4-B-OH-G (m/z 602)*

Metabolites M4-A-OH-G and M4-B-OH-G corresponded to an addition of 192 Da. to metabolite M4, suggesting oxidation of M4 followed by glucuronidation. LC/MS fragmentation of both [M-H]⁻ ions at m/z 602 produced product ions at m/z 426, which were the result of the loss of the glucuronide moiety (176 Da). Further fragmentation of the base peak at m/z 426 of M4-B-OH-G produced product ions at 166, 337, and 396, similar to those produced by metabolite M4-B-OH in rats (Figure 5, G). The presence of the fragment at m/z 166 suggested that the M4-B-OH-G metabolite was hydroxylated and then conjugated on the B ring. Fragmentation of the second product ion of M4-A-OH-G at m/z 426 produced product ions at m/z 275, 406, and 255 (Figure 5, F). These product
ions were similar to those produced by metabolite M4-A-OH-S, the sulfated conjugate of M4. The presence of the fragment ion at m/z 275 suggested that the metabolite was hydroxylated and then conjugated with the glucuronide moiety on the A ring, similar to M4-A-OH-S. As such, M4-A-OH-G and M4-B-OH-G represented the A-ring and B-ring glucuronide conjugates, respectively, of M4. The A-ring oxidized metabolite of M4 was not detected in rat urine or feces, likely indicative of oxidation followed by rapid sulfate and glucuronide conjugation of M4.

C. Final Disposition of S-4 in Rats

To determine the overall disposition of S-4 and its metabolites, a 100 µCi dose of [C\textsuperscript{14}] S-4 was administered via a jugular catheter to Sprague Dawley rats. The rats were sacrificed at 24 hours and radioactivity was measured in urine, feces and selected organs. The percentage of the injected dose recovered after 24 hours was 94%. The majority of radiolabeled S-4 and metabolites was found in the feces (36%) and urine (41%). An additional 17% of total radioactivity was found in the organs, with the highest percentages in liver (3.3%) and intestines (8.9%).

The 24-hour urine and feces (both aqueous and extracted fecal pellet) samples were fractionated to determine the relative percentages of S-4 and metabolites. Given the lower specific activity, small percentage of some metabolites, and sensitivity of the radiochromatographic technique, we were only able to quantify the major metabolites of S-4. While LC/MS analysis of a peak that eluted at 49 minutes confirmed the presence of M4 in the urine (11% of injected dose), an additional hydphilic urinary metaboite that eluted at approximately 5 minutes and represented 28% of the injected radiolabeled dose was also identified. Separate LC/MS analysis of this fraction corresponding to the 5
minute peak showed a metabolite (M3. m/z 252) that was produced from hydrolysis of the amide bond in the parent compound (Figure 5, H). This metabolite was highly hydrophillic due to the presence of a carboxylic acid. This metabolite was therefore missed during LC/MS determinations due to its elution in the solvent front, and was only detected upon radiochromatographic determination. Table 3 shows the percentage of radioactive dose of the parent compound and metabolites found in urine and feces.

**Metabolite M3 (m/z 252)**

Fragmentation of the [M-H]⁻ ion at m/z 252 produced product ions at m/z 193, 208, 254, and 155, as shown in Figure 5, H. This metabolite was also observed during *in vitro* metabolism studies with S-4, with structural confirmation by LC/MS and biotin modification (Gao et al., 2006b). The origin of the metabolite (i.e., from parent of M4) could not be determined given that the A-ring portion of the metabolite could not be detected. In the *in vitro* studies, this metabolite was produced directly from the parent compound. Other previously identified metabolites were not seen, however, this may be due to the relative insensitivity of the radiochromatographic technique as compared to the LC/MS in detection of minor metabolites.

Fecal samples from the radioactive study confirmed the presence of M4, M4-A-OH-S, M4-A-OH-G and M4-B-OH-G, which were seen in the LC/MS study. M4 found in feces accounted for 4% of the injected radioactive dose with each of the phase II metabolites accounting for less than 1% of the injected radioactive dose in rats.

**D. Urinary Metabolites in Beagle Dogs**

Like our findings in rats, urine samples collected at 8 and 24 hours after administration of S-4 to dogs showed similar metabolic profiles. Urine samples showed
the presence of the parent compound and M4. Characterization of parent compound and M4 by LC/MS and LC/MS² analysis produced fragmentation patterns and retention times that were similar to those found in rat urine. These similarities suggest that these molecular ions were structurally the same as those found in rat urine. Canine specific metabolites included a reduced metabolite (M6), a deacetylated metabolite (M5), and two oxidized metabolites (M4-A-OH (1) and M4-A-OH (2)) that were not detected in rats.

Metabolite M6 (m/z 424)

Metabolite M6 corresponds to the loss of 16 Da. from the parent compound. LC/MS fragmentation of the [M-H]⁻ ion at m/z 424 produced product ions at m/z 245, 273, 150, and 189 (Figure 5, I). Data supporting the proposed structure included the 16 Da. decrease in the product ions at m/z 245 and 189, relative to the corresponding product ions at m/z 261 and 205 produced upon fragmentation of the parent compound. As a whole, these data suggest the loss of an oxygen atom from the nitro group on the para-position of the A-ring. The presence of the product ion at m/z 273 further supports this structural identification, showing a loss of 16 Da from the product ion at m/z 289 produced by the parent.

Metabolite M5 (m/z 368)

Metabolite M5 corresponded to the loss of 42 Da. from metabolite M4. LC/MS fragmentation of the [M-H]⁻ ion at m/z 368 produced product ions at m/z 259, 201, 231, 175, and 108 (Figure 5, J). This metabolite and an identical fragmentation pattern were also observed during in vitro metabolism studies of S-4 (Gao et al., 2006b). As further confirmation, LC/MS² fragmentation of the base peak at m/z 259 produced a
fragmentation spectrum similar to that seen with M4 metabolite, confirming M5 as a B-ring deacetylated metabolite of M4.

**Metabolite M4-A-OH (1) and M4-A-OH (2) (m/z 426)**

Two m/z 426 metabolites were also identified in dog urine. Although both of these [M-H]- ions corresponded to oxidized metabolites of M4, they demonstrated different fragmentation patterns. As stated previously, there are four possible sites for oxidation on the M4 metabolite. Fragmentation of M4-A-OH (1) resulted in predominant product ions at m/z 245 and 275, suggesting that oxidation occurred in the A-ring. Likewise, fragmentation of M4-A-OH (2) resulted in ions at m/z 216, 275, and 245, also suggestive of oxidation in the A-ring. However, important differences in ion intensity at m/z 216, 245, and 275 were noted, suggesting that M4-A-OH (1) and M4-A-OH (2) represented separate metabolites with differing sites of oxidation. We could not unequivocally determine the sites of oxidation of these metabolites based on their fragmentation patterns.

E. Fecal Metabolites in Beagle Dogs

Post-dose extracted fecal samples collected at 0 to 8 hours and 8 to 24 hours showed similar metabolic profiles. Fecal samples (both aqueous and extracted fecal pellet) showed molecular ions similar to those found in urine samples corresponding to the parent drug and M4.
DISCUSSION

Although S-4 is a structural relative of bicalutamide, its pharmacokinetic behavior was quite different. Bicalutamide has a plasma T\(_{1/2}\) of 7 days in dogs after IV administration (Cockshott et al., 1991). The T\(_{1/2}\) of S-4 is shorter, at 3.5 to 4 hours after IV administration. This difference is due to more rapid clearance of S-4 from plasma. S-4 showed a mean plasma clearance and T\(_{1/2}\) of 4.6 mL/min/kg; while R-bicalutamide has a clearance of 0.8 mL/min/kg. The contribution of higher clearance to the shorter T\(_{1/2}\) of S-4 is corroborated by the relatively small difference in volume of distribution between S-4 and bicalutamide (1.4 L/kg for 3 mg/kg dose of S-4 and 0.8 L/kg for a 2.5 mg/kg dose of bicalutamide).

The low oral bioavailability seen at the 10 mg/kg dose of S-4 in dogs was unexpected considering the mean plasma clearance of S-4 after an IV dose was 4.2 mL/hr/kg. Assuming that all clearance of the drug is hepatic, the hepatic extraction ratio of S-4 would be approximately 0.25 (i.e., assuming a hepatic blood flow of 30.9 mL/min/kg in the dog and hematocrit of 45% (Davies and Morris, 1993)). This would suggest that the oral bioavailability of S-4 should be approximately 75%. The decreased bioavailability seen after oral doses of S-4 may be attributed to several reasons: 1) low aqueous solubility of the compound in the gastrointestinal tract, 2) active transporter mediated efflux in the GI membrane, and/or 3) pre-systemic degradation or metabolism of the compound in the intestinal lumen prior to absorption.

The high oral bioavailability is a key issue in the development of this compound as a novel nonsteroidal SARM, given that oral bioavailability is a disadvantage of testosterone therapy. Studies by Cockshott et al. (Cockshott et al., 1991) showed dose-
dependent oral bioavailability of bicalutamide in both rats and dogs. This led to the investigation of oral bioavailability of S-4 at lower oral doses. The increase in oral bioavailability of S-4 (i.e., 59% and 91% at doses of 1 and 0.1 mg/kg, respectively) as the dose of S-4 decreases suggests that aqueous solubility in the GI tract is responsible for the low oral bioavailability seen at higher doses. These findings are supported by the erratic absorption seen after the capsule dosing as well as the increase in $T_{\text{max}}$ at the higher PO doses of S-4, assuming that placement of un-formulated S-4 in a capsule causes larger particle size within the gastrointestinal tract and therefore lower solubility. The pharmacologic activity study showed that S-4 showed anabolic activity at doses below 1 mg/day (Yin et al., 2003a; Gao et al., 2004). At these lower doses, S-4 would show high bioavailability of 91%.

Prior studies determined that acetothiolutamide was metabolically converted from an agonist to an antagonist by the oxidation of the thioether linkage to a sulfonyl linkage. Therefore, an ether linkage was employed in S-4 to prevent oxidation at this labile site. We investigated the metabolism of S-4, and the structures of these metabolites were determined by LC/MS and LC/MS$^2$ analysis. The fragmentation patterns of each of the metabolite(s) were compared to those of the parent compound to determine the site of metabolism.

Three of the metabolites seen in this study were also identified in the paper by Gao et al. that investigated the \textit{in vitro} metabolism of S-4 in human, rat and dog liver preparation (Gao et al., 2006b). Many of the metabolites found during \textit{in vitro} study were derived directly from the parent compound, while many in this \textit{in vivo} study were generated from the M4 metabolite, suggesting that the reduction reaction occurs more
rapidly in a living system, and explaining the differences that we observed between the metabolites observed during \textit{in vitro} and \textit{in vivo} metabolism studies of S-4.

Similar to the \textit{in vitro} observations, the presence of the ether linkage prevented oxidation at the linkage site, but there was an increase in hydrolysis of the amide bond. The hydrolysis product (M3) accounted for 28% of S-4 metabolites in rats. These findings are also similar to the metabolic pathways seen with bicalutamide. Rats and mice cleave approximately 20 to 30\% of the bicalutamide dose to the carboxylic acid metabolite (Boyle et al., 1993). However, this metabolite is absent in humans after bicalutamide dosing. The A-ring product of amide hydrolysis of S-4 was not identified in either urine or feces due to its low molecular weight, the conditions used for LC/MS analyses, and use of S-4 radiolabeled in the B-ring.

Radiographic analysis showed the presence of the parent drug, S-4, in the feces, which was interesting given that the dose of radioactive S-4 was given intravenously. This may suggest biliary excretion of S-4. One of the most important factors in biliary excretion is the molecular weight of the compound. A minimum molecular weight of between 200 and 325 is necessary for biliary excretion in rats (Wright and Line, 1980), while the human molecular weight threshold is around 400 to 500 (Hirom et al., 1972). Therefore, S-4 and many of its metabolites would meet this requirement. Bile is an aqueous solution, hence drugs that are commonly excreted in the bile are relatively hydrophilic or have been conjugated by phase I or phase II processes (Roberts et al., 2002). In addition, intestinal flora expressing \(\beta\)-glucuronidase enzymes can hydrolyze the drug conjugates to liberate the parent compound within the intestines, which can then be reabsorbed into the plasma (Hazenberg et al., 1988). Boyle et al. which showed that
30% of the dose of bicalutamide given to rats was recovered in the bile as glucuronide conjugates that were then cleaved within the intestines (Boyle et al., 1993). Slight evidence of enterohepatic recirculation of S-4 is also observed in our pharmacokinetic data. The concentration time profile of S-4 after IV administration showed an increase in the plasma concentration at around 30 to 60 minutes after the 0.1 mg/kg dose was given (Figure 2). Additional bile studies are needed to determine if S-4 is eliminated unchanged in the feces, or if it is cleaved within the intestines to the parent compound.

In comparison, the M3 metabolite was not detected in dogs. Although the hydrolysis pathway was observed during in vitro experiments using dog liver S9 fractions, the non-reversible deacetylation of the B-ring acetamido group (Gao et al., 2006a) makes the metabolite more difficult to separate and detect in in vivo biological samples. The lack of N-acetyltransferase activity in dogs (King and Glowinski, 1983) was also responsible for our observation that the deacetylated metabolite M5 was observed in the dog, but not in the rat.

Although replacement of the cyano group with a nitro substitucent improves the androgen receptor binding affinity of the aryl propionamide SARMs, it introduced an additional metabolically labile site that is less suitable for in vivo use. Although we (Kim et al., 2005) recently demonstrated that replacement of the nitro substituents with a cyano substituent has minimal to no effect on the in vivo clearance and half-life of the aryl propionamide SARMs in rats, due to the more rapid metabolism via other pathways in this species, this is unlikely to be the case in higher species (Gao et al., 2006a). As such, inclusion of a cyano substituent in the para-position of the A-ring of aryl propionamide...
SARMs is likely to be beneficial on the basis of pharmacokinetic and toxicologic considerations (Hornsten et al., 1990).

In summary we determined the pharmacokinetic parameters and metabolic profile of the novel SARM S-4 in rats and dogs. The metabolism of S-4 in dogs shows that this compound has linear pharmacokinetics and is highly bioavailable at pharmacologically meaningful doses. The major metabolic products of S-4 in rats are the result of hydrolysis of the amide bond and reduction of the nitro group on the aromatic A ring. In comparison, reduction of A-ring nitro group and deacetylation of B ring acetamido moiety were the predominant metabolism pathways in dogs. These findings show that even though S-4 has similar half life in rats and dogs, there are significant differences in S-4 metabolism between species. These *in vivo* findings corroborate our *in vitro* metabolism studies and suggest that S-4 may demonstrate suitable pharmacokinetics, oral bioavailability, and metabolism for use in humans.
REFERENCES


FOOTNOTES:

These studies were supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (R01 DK59800-06).

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Legends for Figures

Figure 1. Structures of S-4 and acetothiolutamide.

Figure 2. Mean (± S.D.) plasma concentration-time profiles of S-4 in beagle dogs after IV administration of 10, 3 and 0.1 mg/kg doses. Plasma S-4 levels were analyzed using the LC/MS method detailed in the Materials and Methods section. Each point represents the mean and standard deviation of 5 to 6 animals.

Figure 3. Mean (± S.D.) plasma concentration-time profiles of S-4 in beagle dogs after PO administration of 10, 1 and 0.1 mg/kg doses. Plasma S-4 levels were analyzed using the LC/MS method detailed in the Materials and Methods section. Each point represents the mean and standard deviation of 3 to 4 animals.

Figure 4: Proposed Major In Vivo Metabolism Pathways of S-4 in Rats and Dogs. The urinary and fecal metabolites of S-4 were determined in rats and dogs using LC/MS^n and/or ¹⁴C-radiolabeled drug. Canine specific metabolites were noted.

Figure 5: Proposed Metabolites of S-4 and Their Mass Spectrometric Spectra. The urinary and fecal metabolites of S-4 were determined in rats and dogs using LC/MS^n and/or ¹⁴C-radiolabeled drug. S-4 and metabolites were separated using gradient reversed-phase chromatography prior to structural elucidation using a ThermoFinnigan LCQ Deca Ion Trap System with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) interface. All analyses were conducted in negative ion mode.
Table 1. Mean (± S.D.) Pharmacokinetic Parameters of S-4 after IV Administration (n=5-6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dose (mg/kg)</th>
<th>10</th>
<th>3</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µg*min/mL)</td>
<td></td>
<td>2497 ± 544</td>
<td>664 ± 123</td>
<td>21.1 ± 7.2</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td></td>
<td>242 ± 42</td>
<td>213 ± 34</td>
<td>143 ± 97</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td></td>
<td>4.2 ± 0.9</td>
<td>4.6 ± 0.9</td>
<td>5.2 ± 1.9</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td></td>
<td>1.4 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>
Table 2. Mean (± S.D.) Pharmacokinetic Parameters of S-4 after PO Administration (n=3-4).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dose (mg/kg)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µg*min/mL)</td>
<td>940 ± 357&lt;sup&gt;a&lt;/sup&gt;</td>
<td>131.5 ± 31.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.2 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>234 ± 49</td>
<td>161 ± 27</td>
<td>335 ± 254</td>
</tr>
<tr>
<td>CL/F (mL/min/kg)</td>
<td>12.1 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V&lt;sub&gt;f&lt;/sub&gt;/F (L/kg)</td>
<td>3.9 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>2.5 ± 0.62</td>
<td>0.460 ± 0.03</td>
<td>0.083 ± 0.06</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>110&lt;sup&gt;c&lt;/sup&gt;</td>
<td>53.5</td>
<td>60</td>
</tr>
<tr>
<td>F</td>
<td>0.38</td>
<td>0.59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.91</td>
</tr>
</tbody>
</table>

- a) AUC did not change proportionally to dose.
- b) Oral CL between doses was significantly different (p = 0.05).
- c) T<sub>max</sub> was significantly longer in the 10 mg/kg dose (p = 0.05).
- d) Calculated using 3 mg/kg IV dose.
Table 3. Final Disposition of Radiolabeled S-4 in Urine and Feces of Rats.

<table>
<thead>
<tr>
<th>Compounds found in</th>
<th>Percentage Injected Dose</th>
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<tbody>
<tr>
<td>urine</td>
<td></td>
</tr>
<tr>
<td>M-2</td>
<td>11%</td>
</tr>
<tr>
<td>M-7</td>
<td>28%</td>
</tr>
<tr>
<td>Parent Compound</td>
<td>31%</td>
</tr>
<tr>
<td>S-4</td>
<td></td>
</tr>
<tr>
<td>M-2</td>
<td>4%</td>
</tr>
<tr>
<td>M-4</td>
<td>1%</td>
</tr>
<tr>
<td>M-5</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>M-6</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Compounds found in</td>
<td></td>
</tr>
<tr>
<td>feces</td>
<td></td>
</tr>
</tbody>
</table>

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Figure 1

A.

B.
Figure 2

- **10 mg/kg i.v.**
- **3 mg/kg i.v.**
- **0.1 mg/kg i.v.**

**Concentration (µg/ml)**

**Time (min)**
Figure 3

![Graph showing concentration over time for different doses of medication.](image-url)
Figure 4

Deacetylation

M-4-B-OH

M-4-B-OH-G

M-4-A-OH (dog)

M-4-A-OH-G

M-4-A-OH-S

S-4-CH₃

M-3

M-5 (dog)

M-6 (dog)

Reduction

Hydrolysis

Methylation
Figure 5:

M-4-
B-OH
m/z = 426
Figure 5:

M-4-A-OH-G
m/z = 602
Figure 5:

G

100303-19 #1929-1963 RT: 52.82-54.21 AV: 19 NL: 1.72E4
F: - ESI Full ms3 602.00@30.00-426.00@60.00 [115.00-2000.00]

166.0

124.1

161.3 167.2 220.1 250.2 258.9 309.2 319.1 324.2 337.2 344.1 366.1 367.2 396.1 408.2

m/z

0 150 200 250 300 350 400

M-4-
B-OH-G
m/z = 602
Figure 5:

M-4-A-OH (1)

m/z = 426
Figure 5: