Identification of [^{14}C]Fluasterone Metabolites in Urine and Feces Collected from Dogs after Subcutaneous and Oral Administration of [^{14}C]Fluasterone

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Received July 30, 2008; accepted January 29, 2009

ABSTRACT:

The objective of this research was the identification of the metabolic profile of fluasterone, a synthetic derivative of dehydroepiandrosterone, in dogs treated orally or subcutaneously with [^{14}C]fluasterone. Separation and characterization techniques used to identify the principal metabolites of fluasterone in urine and feces included high-performance liquid chromatography (HPLC), liquid scintillation spectrometry, HPLC/tandem mass spectrometry, and NMR. In urine, the majority of the radioactivity was present as two components that had apparent molecular weights consistent with their tentative identification as monoglucuronide conjugates of 4 α -hydroxy-16 α -fluoro-5-androsten-17 β -ol and X(α or β)-4 α -dihydroxy-16 α -fluoro-5-androsten-17 β -ol. The identification of the monoglucuronide conjugate of 4 α -hy-

droxy-16 α -fluoro-5-androsten-17 β -ol was also supported by NMR data. In support of this identification, these metabolites were cleaved with glucuronidase enzyme treatment, which gave rise to components with molecular weights again consistent with the aglycones of a monohydroxylated, 17-keto reduced (dihydroxy) fluasterone metabolite and a dihydroxylated, 17-keto reduced (trihydroxy) fluasterone metabolite. In feces, nonconjugated material predominated. The primary metabolites eliminated in feces were the two hydroxy fluasterone metabolites arising from 17-reduction (16 α -fluoro-5-androsten-17 β -ol and 16 α -fluoro-5-androsten-17 α -ol) and 4 α -hydroxy-16 α -fluoro-5-androsten-17 β -ol that was present in urine in glucuronide form.

Fluasterone (Fig. 1) is a synthetic derivative of dehydroepiandrosterone (DHEA), an important intermediate in both testosterone and estrogen biosynthesis. In addition to its role as an intermediate in steroid hormone synthesis, DHEA and its sulfate conjugate (DHEAS) have been shown to have numerous direct physiological activities, including immunomodulatory and antiglucocorticoid effects, and are

believed to be important for the development and function of the central nervous system. In humans, DHEA is synthesized by the adrenal cortex, gonads, brain, and gastrointestinal tract. At certain times, DHEA and DHEAS constitute the most abundant steroid hormones in the circulation. Levels of DHEAS decrease rapidly after birth, increase to a peak at approximately 20 to 30 years of age, and then decrease again gradually over time (Rainey et al., 2002). Because of this, supplemental intake of DHEA and DHEAS is popular as an "aging remedy." In addition, DHEA and DHEAS are often used by athletes to improve performance and are also said to increase longevity and improve mood, cognition, and sexuality (Allolio and Arlt, 2002). Most interestingly, DHEA is an inhibitor of cancer induction in a wide range of in vivo experimental models for human cancer, including rat mammary gland (Li et al., 1994; McCormick et al., 1996), mouse mammary gland (Schwartz, 1979), mouse skin (Pashko et al., 1984), mouse colon (Nyce et al., 1984; Osawa et al., 2002), mouse lung (Schwartz and Tannen, 1981), mouse lymphatic system

This work was supported by the National Institutes of Health Division of Cancer Prevention and Control [Contract N01-CN-43306].

Parts of this work were previously presented as a poster as follows: Zhan Q, Hill JM, Chao A, Green JS, Lyubimov A, Kapetanovic IM, and Thomas BF (2007) Characterization of the metabolites of [^{14}C]fluasterone excreted in dog urine and feces using LC/MS/MS. *American Association of Pharmaceutical Scientists 2007 Annual Meeting*; 2007 Nov 10-16; San Diego, CA. American Association of Pharmaceutical Scientists, Arlington, VA.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.108.023614.

ABBREVIATIONS: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; 17 β -OH fluasterone, 16 α -fluoro-5-androsten-17 β -ol; 17 α -OH fluasterone, 16 α -fluoro-5-androsten-17 α -ol; HPLC/UV/LSS, high-performance liquid chromatography/UV/liquid scintillation spectrometry; LSS, liquid scintillation spectrometry; HPLC, high-performance liquid chromatography; HPLC/LSS, high-performance liquid chromatography/liquid scintillation spectrometry; HPLC/MS/MS, high-performance liquid chromatography/tandem mass spectrometry; 4 α -17 β -diOH fluasterone, 4 α -hydroxy-16 α -fluoro-5-androsten-17 β -ol; X(α or β)-4 α -17 β -triOH fluasterone, X(α or β)-4 α -dihydroxy-16 α -fluoro-5-androsten-17 β -ol; APCI, atmospheric pressure chemical ionization; G6PDH, glucose-6-phosphate dehydrogenase.

FIG. 1. The structure of fluasterone with atom numbering.

(Perkins et al., 1997), rat liver (Moore et al., 1986), rat thyroid (Moore et al., 1986), and rat prostate (McCormick et al., 2007).

Depending on the hormonal milieu, DHEA has either estrogen-like or androgen-like effects (Ebeling and Koivisto, 1994). For example, in premenopausal women DHEA is either an estrogen antagonist, possibly through the competitive binding of its metabolite 5-androstene-3 β , 17 β -diol, and estradiol to the estrogen receptor, or an androgen through its metabolism to androstenedione and testosterone. These estrogenic and androgenic properties can produce some significant adverse effects. Indeed, it has been shown that postmenopausal women with elevated serum androgens (including high DHEAS concentrations) are at an increased risk of breast cancer (Dorgan et al., 1996, 1997; Hankinson et al., 1998). In men with normal testosterone levels, DHEA produces predominantly estrogenic effects. Thus, treatment of men with DHEA can lead to gynecomastia and other unwanted side effects. These sex hormone-related side effects limit the therapeutic utility of DHEA in humans. The adverse effects seen with DHEA prompted the development of fluasterone, which in certain experimental paradigms seems to be devoid of the estrogenic and androgenic activity but retains many of DHEA's therapeutic properties (Schwartz and Pashko, 1995). Fluasterone is currently of sufficient interest that the National Cancer Institute has sponsored its preclinical development.

Biotransformation pathways for DHEA and steroid-based therapeutics can be extensive, involving both Phase I and Phase II processes, and can produce additional compounds that may retain the pharmacological or toxicological properties of the parent or possess new and unanticipated biological activities. Because the metabolism and disposition of fluasterone have not been previously thoroughly investigated, this investigation, of importance for any pharmaceutical, is particularly important for this close structural analog of such an important endogenous compound as DHEA. Indeed, the Food and Drug Administration mandates that such studies be performed and that reliable and accurate qualitative and quantitative methods for drugs and their metabolites in biological fluids be used to adequately assess the pharmacokinetic and pharmacodynamic processes of candidate therapeutics. Therefore, the main objectives of these studies were to 1) develop methods that allow for the separation of fluasterone and its metabolites in a variety of biological matrices; 2) obtain radiochromatographic profiles of biological fluids and matrices from dogs dosed with [14 C]fluasterone; 3) acquire spectrometric information on isolated metabolites using liquid chromatography/(tandem) mass spectrometry (LC/MS/MS or LC/MS, respectively) and NMR spectroscopy; and 4) elucidate the structure of urinary and fecal metabolites of [14 C]fluasterone in dogs.

The metabolism and elimination profiles determined in dogs receiving [14 C]fluasterone orally and subcutaneously are reported in this article. The pharmacokinetic and tissue distribution profiles of [14 C]fluasterone are to be presented elsewhere.

Materials and Methods

Chemicals. Radiolabeled [4- 14 C]fluasterone (lot 9676-29-07 from Midwest Research Institute, Kansas, MO) had a radiochemical purity of approximately 97% and specific activity of 58.3 mCi/mmol. Fluasterone (lot 99973-1/91) was supplied by Proquina (Orizaba, Mexico). Standards of 16 α -fluoro-5-androstene-17 β -ol (17 β -OH fluasterone) and 16 α -fluoro-5-androstene-17 α -ol (17 α -OH fluasterone) were supplied by Dr. Marvin L. Lewbart (Department of Medicine, Jefferson Medical College, Philadelphia, PA). Sulfatase-free β -glucuronidase, bacterial from *Escherichia coli*, was supplied with phosphate buffer Sigma G8396 (Sigma-Aldrich, St. Louis, MO). β -Glucuronidase-free sulfatase, type VI, from *Aerobacter aerogenes* was supplied with 0.01 M Tris, pH 7.5, Sigma S1629 (Sigma-Aldrich). Soluene-350 tissue solubilizer and Ultima Gold scintillation mixture were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Dosing and Collection of Biological Samples. Groups of male beagle dogs received either a single subcutaneous administration of [14 C]fluasterone in 30% hydroxypropyl- β -cyclodextrin at 1 mg/kg ($n = 4$) or a single oral administration of [14 C]fluasterone in corn oil at 15 mg/kg ($n = 4$) in a study performed by the Toxicology Research Laboratory at the University of Illinois at Chicago. Dosage formulations were analyzed by high-performance liquid chromatography/UV/liquid scintillation spectrometry (HPLC/UV/LSS) and proved to be within 10% of target before dosing.

Blood samples were collected in lithium heparin tubes 0.5 and 2 h after subcutaneous administration and 1 and 4 h after oral administration of [14 C]fluasterone. Blood was centrifuged at 3700 rpm for 15 min to separate red blood cells and plasma. Urine was collected into containers placed on dry ice through 48 h after dosing. Feces collected for metabolic profiling were removed from the cages at intervals of 0 to 8, 8 to 24, and 24 to 48 h postdosing. All of the collected samples were stored at approximately -80°C . The collected urine and feces and aliquots of plasma and red blood cells were shipped frozen by the Toxicology Research Laboratory at the University of Illinois at Chicago and stored at RTI International (Research Triangle Park, NC) at approximately -80°C until analyzed.

Determination of Radioactivity in Samples. Weighed aliquots of thawed urine and plasma were added directly to vials containing scintillation mixture (Ultima Gold). Each feces collection was thawed and homogenized with an approximately equal weight of deionized, distilled water. Weighed aliquots of feces homogenates and red blood cells (0.1–0.3 g) were digested in Soluene-350 (2 ml) and then neutralized and bleached by the addition of approximately 125 μl of 70% HClO_4 followed by the addition of approximately 0.3 ml of 30% H_2O_2 . After the samples were decolorized, scintillation mixture was added to the samples. Samples prepared as described above were analyzed for radioactivity content by liquid scintillation spectrometry (LSS) using a Packard Tri-Carb 2100TR Liquid Scintillation Spectrometer (PerkinElmer Life and Analytical Sciences). Eluent fractions (1 ml) from high-performance liquid chromatography (HPLC) analyses were collected directly into vials containing scintillation mixture and also analyzed by LSS.

Treatment of Urine and Plasma with Glucuronidase or Sulfatase Enzymes. Varying amounts of glucuronidase enzyme were reconstituted in urine and heated at 37°C for approximately 22 h. Likewise, aliquots of urine and plasma were treated with varying amounts of sulfatase and allowed to hydrolyze at 37°C for up to approximately 94 h.

Analysis by HPLC. Analysis by HPLC was accomplished using a Phenomenex (Torrance, CA) Luna 5- μm C18(2) column (150 $\times$ 4.6 mm) and two gradient systems, each varying the amount of acetonitrile and water in the mobile phase over the time course of linear gradient changes at a flow rate of 1.5 ml/min. For HPLC Method 1, the initial proportions of acetonitrile/water, 5:95 (v/v), were maintained for 5 min after sample injection; changed over 5 min to 10:90 and held constant for 6 min; changed over 6 min to 40:60 and held for 8 min; then changed over 5 min to 70:30 and held for 5 min; and finally changed over 2 min to 95:5 and held for 8 min. For HPLC Method 2, used to provide increased chromatographic resolution for the separation of fluasterone, 17 α -OH fluasterone, and 17 β -OH fluasterone, the initial proportions of acetonitrile/water, 70:30 (v/v), were maintained for 15 min after sample injection, changed to 100% acetonitrile over 2 min, and held for 7 min.

Metabolite Profiles. Aliquots of untreated urine and feces (after extraction with methanol) from all the collection samples that afforded analysis were

TABLE 1

Mean (S.D.) percentage cumulative recovery of total radioactivity postdose after subcutaneous and oral administration of [¹⁴C]fluasterone in male dogs

Collection Time	Urine	Feces	Total
Subcutaneous dose at 1 mg/kg (n = 4)			
0–24 h	4.4 (5.1)	5.8 (9.3)	10.0 (9.1)
24–48 h	7.7 (6.1)	22.0 (7.7)	30.0 (12)
Oral at 15 mg/kg (n = 4)			
0–24 h	3.8 (2.7)	36.0 (12)	39.0 (13)
24–48 h	5.1 (2.6)	45.0 (11)	50.0 (11)

profiled for fluasterone and its metabolites by high-performance liquid chromatography/liquid scintillation spectrometry (HPLC/LSS) (eluent collected at 1-min intervals) and high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) using an on-line radioactivity detector (β -RAM; IN/US Systems, Tampa, FL) using HPLC Methods 1 and 2. In addition, enzyme-treated aliquots of selected urine collections were profiled using the same procedures as those for untreated urine. Methanol extracts of plasma were also profiled by HPLC/LSS. Unless otherwise stated, results reported below are from analysis using HPLC Method 1.

For preliminary HPLC/MS/MS analyses, the mass spectrometer was operated in positive ionization mode using the atmospheric chemical ionization probe in series after the UV and β -RAM radioactivity detector (IN/US Systems). The time difference between a peak detected at each detector was approximately 0.15 min. All the data were recorded using Analyst software (MDS Sciex, Concord, ON, Canada).

When glucuronides were suspected, a Turbolon Spray ion source (Applied Biosystems, Foster City, CA) was operated in negative ion mode with the following operating conditions: curtain gas 40, nebulizer gas 75, vaporizer temperature 600°C, needle voltage 4500 V, collisionally activated dissociation 4, and collision energy 30 V. Information-dependent analysis was used during the data acquisition. An MS survey scan was conducted, followed by an information-dependent analysis experiment to select the two most intense ions for MS/MS analysis. The fragmentation patterns of these ions were collected in the MS/MS experiments and analyzed for qualitative identification using the Metabolite ID software (Thermo Fisher Scientific Inc., Waltham, MA).

Urinary metabolites were isolated by combining and concentrating fractions collected from multiple HPLC injections of dog urine. Proton NMR spectra of standards and the isolated urinary metabolites were obtained on a Bruker (Billerica, MA) AVANCE 300 NMR spectrometer in *d*₆-dimethyl sulfoxide at ambient temperature using a 5-mm QNP probe. Proton spectra, proton-proton correlation spectra, and proton-carbon correlation spectra of isolated urinary metabolites in D₂O were obtained on a Varian, Inc. (Palo Alto, CA) INOVA 500 NMR spectrometer at ambient temperature using a 5-mm broadband probe. Chemical shift calculations were performed using ACD Proton Predictor version 10.02 (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

Results

Total Radioactivity Content of Urine, Feces, Red Blood Cells, and Plasma. Excretion of radioactivity in urine and feces after dosing with [¹⁴C]fluasterone by oral and subcutaneous routes is shown in Table 1. After either subcutaneous or oral administration, a larger portion of the dose was eliminated in feces compared with urine. After a single subcutaneous dose of [¹⁴C]fluasterone, 8% of the administered radioactivity was eliminated in urine in 48 h compared with 22% eliminated in feces within 48 h. Likewise, after a single oral dose of [¹⁴C]fluasterone, approximately 5 and 45% of the administered radioactivity were eliminated in the urine and feces, respectively, after 48 h. There was large variability in both matrices among animals at all the time points.

Levels of radioactivity in red blood cells and plasma at all the collection time points were very low (0.004% or less of dose received/ml). Again there was large variability (coefficient of variation ranged

Fig. 2. HPLC/LSS profiles of nonenzyme- and enzyme-treated 8- to 24-h urine collected after subcutaneous administration of [¹⁴C]fluasterone at 1 mg/kg.

from 11–72%) in both plasma and red blood cells among animals at all the time points (data not shown).

HPLC/LSS and HPLC/MS Analysis of Urine and Feces. Representative profiles of radioactivity in urine from a dog administered fluasterone subcutaneously analyzed before and after treatment with glucuronidase or sulfatase are shown in Fig. 2. In the untreated urine sample, there are four primary regions where radioactive material elutes: a small peak eluting shortly after the column void volume (2–4 min), a slightly larger peak eluting at 7 to 10 min, a broad region between 13 and 20 min, and a large peak eluting at approximately 22 min. Treatment with sulfatase does not seem to alter the appearance of the chromatogram; however, glucuronidase treatment results in the disappearance of the aforementioned radioactivity regions and the appearance of two new peaks with retention time ranges of approximately 25 to 28 min and 31 to 35 min.

Representative profiles of radioactivity in 8- to 24-h urine collected from dogs administered fluasterone orally are shown in Fig. 3. The profiles are similar to those after subcutaneous administration with a broad peak eluting shortly after the column void volume (2–4 min), a peak at approximately 7 to 10 min, a broad region between 13 and 20 min, and a peak eluting after 22 min.

Recovery of radiolabel after the extraction of feces aliquots with methanol was greater than 90%. Representative profiles of radioactivity in feces of dogs administered fluasterone orally or subcutaneously are shown in Fig. 4, top. There are five primary regions of interest where radioactive material elutes: a broad peak eluting between 24 and 30 min, a peak at approximately 35 min, a small peak at approximately 38 min, a peak at approximately 43.0 min, and peaks eluting at approximately 47 min. The radioactive peaks seen in feces at approximately 27 and 35 min are at the same retention times as those that appear in urine after treatment with glucuronidase. The region at approximately 47 min is important because fluasterone elutes at approximately 47.5 min; 17 β -OH fluasterone and 17 α -OH fluasterone elute at 46.5 and 47.0 min, respectively. The results of further experiments conducted using HPLC Method 2 to verify the identity of the peaks at approximately 47 min are shown in Fig. 4, bottom. The radiolabeled peaks at 13, 15, and 17.5 min are identified as 17 β -OH fluasterone, 17 α -OH fluasterone, and nonmetabolized fluasterone, respectively, based on their similar retention time to authentic standards. In feces from the orally dosed dog the large peak that appears at the retention time of fluasterone in the 8- to 24-h

FIG. 3. HPLC/LSS profiles of 8- to 24-h urine collected from two dogs after oral administration of [^{14}C]fluasterone at 15 mg/kg.

collection decreases significantly in size in the 24- to 48-h feces collection, whereas a peak at the retention time of the 17β -OH fluasterone is observed in the feces obtained from the subcutaneously treated animal.

FIG. 4. Top, HPLC/LSS profiles using HPLC Method 1 of methanol extracts of 8- to 24- and 24- to 48-h feces collected from a single dog after oral administration of [^{14}C]fluasterone at 15 mg/kg and 24- to 48-h feces collected from a dog after subcutaneous administration of [^{14}C]fluasterone at 1 mg/kg. Bottom, HPLC/LSS profiles using HPLC Method 2 of the same extracts shown in A.

FIG. 5. HPLC radiochromatographic profile recorded during LC/MS analysis of nonenzyme-treated urine collected from a single dog between 8 and 24 h after a subcutaneous dose of [^{14}C]fluasterone at 1 mg/kg.

Recovery of radiolabel after the treatment of plasma aliquots with methanol was greater than 90%. The lower level of radioactivity in plasma results in a radiochromatogram with significant noise; however, three primary regions of eluting radioactivity were observed (data not shown). Based on retention times and comparison with the results of the analysis of methanol extraction of feces, these regions are tentatively identified as those containing nonconjugated 4α -hydroxy- 16α -fluoro-5-androsten- 17β -ol (4α - 17β -diOH fluasterone), $X(\alpha$ or β)- 4α -dihydroxy- 16α -fluoro-5-androsten- 17β -ol [$X(\alpha$ or β)- 4α - 17β -triOH fluasterone], and the one containing 17β -OH fluasterone, and fluasterone.

Because urine samples can be directly analyzed by HPLC/MS without rigorous sample purification, this method was used to characterize the [^{14}C]fluasterone-derived radioactive metabolites eliminated during the first 24 h postdose administration in representative urine samples collected from an orally dosed dog and a subcutaneously dosed dog. Figures 5 and 6 described below are from the analysis of the urine from a subcutaneously treated dog. Analysis results from the urine from an orally treated dog (data not shown) support the results described. A radiochromatogram observed in non-

FIG. 6. HPLC radiochromatographic profile recorded during LC/MS analysis of glucuronidase-treated urine collected from a single dog between 8 and 24 h after a subcutaneous dose of [^{14}C]fluasterone at 1 mg/kg.

hydrolyzed urine collected from a subcutaneously dosed dog during in-line radiometric detection is shown in Fig. 5. The radiolabeled peak eluting at approximately 8 min had a negative electrospray ionization mass spectrum with a prominent ion at m/z 499, an ion that was not present with any significant intensity at this retention time in nondosed dog urine. An ion at this mass is consistent with the presence of a fluasterone metabolite resulting from hydrogenation at the 17 position (turning the ketone into a hydroxyl) and hydroxylation at two sites on the molecule, with one of these hydroxyl groups also glucuronidated. The more retained (potentially less polar) radiolabeled peak at approximately 22 min was seen to have an intense ion at m/z 483, which is also consistent with the presence of a glucuronidated metabolite. The lower mass results from the metabolite having only two hydroxyl groups, one formed from hydrogenation and the other from oxidation. Again, this ion was not present with any significant intensity at this retention time in nondosed dog urine. The identification of the two predominant peaks in urine as glucuronides is consistent with the results obtained in the enzyme hydrolysis experiments described previously (Fig. 2).

The radiochromatogram obtained during LC/MS of urine after glucuronidase treatment is shown in Fig. 6. Incomplete cleavage of the material results in some radioactivity eluting at approximately 22 min; however, new peaks are observed at 24.5 and 32.7 min. If the identifications described in the nonenzyme-treated material are correct, then the metabolites liberated after glucuronidase treatment would be expected to be a hydrogenated + monohydroxylated metabolite of m/z 308 and a hydrogenated + dihydroxylated metabolite of m/z 324. Furthermore, if these metabolites behaved as one might anticipate, then one might expect that the more polar peak at 7 min, when cleaved, would elute first in the glucuronidase-treated chromatogram (i.e., at 25 min) and that the less polar peak at 21 min would elute later in the glucuronidase-treated chromatogram (i.e., the peak at 35 min). This was observed when the glucuronidase-treated urine samples were analyzed using the atmospheric pressure chemical ionization (APCI) source, with the mass spectrum of the more polar eluting material (25 min) showing a modestly intense ion at m/z 325 (the $M + 1$ ion expected in positive ion mode APCI mode for the hydrogenated + dihydroxylated metabolite). The intense ion at 307 can be attributed to the loss of one hydroxyl group, the ion at 289 to the loss of the second hydroxyl group, the ion at 271 to the loss of the third hydroxyl group, and the ion at 269 to the loss of two hydroxyl groups and HF. Also as predicted, the data obtained at approximately 33 min are consistent with the elution of a hydrogenated, monohydroxylated compound. In this instance, a molecular ion is not observed, but the ion at 291 represents the loss of the first hydroxyl group, the ion at 273 the loss of both hydroxyls, the ion at 271 the loss of one hydroxyl and HF, and the ion at 253 the loss of both hydroxyls and HF. These characteristic fragmentation patterns, where the molecular ion may be weak or not observed, are seen with hydroxylated fluasterone analogs in APCI mode.

The typical radiochromatogram obtained during LC/MS of a methanol extract of feces is shown in Fig. 7. Peaks derived from [^{14}C]fluasterone were observed at approximately 32.6, 47.5, 49.3, and 52.3 min. An overlay of the radiochromatogram and total ion chromatogram obtained with this feces sample, along with the total ion chromatogram obtained with a feces sample obtained before dosing, shows that the separation achieved during the HPLC analyses was sufficient to minimize coelution of endogenous substances at the elution time of the radiolabeled peaks (data not shown). The radiolabeled peak at 32.6 min, based on its similar retention time to the peak observed in urine, can tentatively be identified as hydroxylated and reduced (17-position) fluasterone. The radiolabeled peaks at 47.5, 49.3, and 52.3 min, based on their similar retention time to authentic standards, can

FIG. 7. HPLC radiochromatographic profile recorded during LC/MS analysis of a methanol extract of feces collected between 8 and 24 h after oral dosing with [^{14}C]fluasterone at 15 mg/kg.

tentatively be assigned the structural identification as 17 β -OH fluasterone, 17 α -OH fluasterone, and nonmetabolized fluasterone, respectively. This assignment is supported by the spectra recorded using APCI in positive ion mode. The mass spectrum recorded at 32.6 min is similar to the peak eluting at the same time in urine with an $M + 1$ ion observed at 309. As described before, the ion at m/z 291 could represent the loss of the first hydroxyl group, the ion at m/z 273 the loss of both hydroxyls, the ion at m/z 271 the loss of one hydroxyl and HF, and the ion at m/z 253 the loss of both hydroxyls and HF. This fragmentation and the retention time again lead to the identification of this material as a 17-hydrogenated, monohydroxylated metabolite of fluasterone. Although $M + 1$ ions at m/z 293 were not observed for the putative 17-hydrogenated metabolites, the ions observed at m/z 275, 273, and 255 can be explained as resulting from loss of H_2O (18), HF (20), and $\text{H}_2\text{O} + \text{HF}$ (38), respectively. The retention times are also consistent with their structural assignments. The mass spectrum recorded at the retention time of fluasterone also agrees with this identification. An $M + 1$ ion at m/z 291 and an $M + \text{Na}$ ion at m/z 313 are apparent with the fragment ions consistent with the loss of HF and water from the $M + 1$ ion at m/z 291. Furthermore, the spectra recorded for the radioactive peaks at 47.5, 49.3, and 52.3 min in the feces extracts appear similar to the spectra recorded for authentic, nonradiolabeled standards dissolved in organic solvents.

Together, the radiochemical profiling and metabolite identification from representative dogs reveal that after oral dosing approximately 30% of the radioactivity in the 8- to 24-h feces is fluasterone, 8% 17 α -OH fluasterone, 6% 17 β -OH fluasterone, and the remainder monohydroxylated and dihydroxylated metabolites of 17-hydrogenated (presumably 17 β -OH) fluasterone, with the majority being the monohydroxylated, 17-hydrogenated metabolite (i.e., 4 α -17 β -diOH fluasterone). During the 24- to 48-h period after oral dosing, the relative amount of fluasterone present in feces was reduced to less than 1%, the amount of 17 β -OH fluasterone to approximately 4%, and the amount of 17 α -OH fluasterone to approximately 3% with the remainder of the radioactivity eluting at 35 min as 4 α -17 β -diOH fluasterone and at 27 min as X(α or β)-4 α -17 β -triOH fluasterone. In the animals dosed subcutaneously, levels of radioactivity eliminated in feces during the 8- to 24-h period were not sufficient for quantitative analysis. In the 24- to 48-h period after subcutaneous dosing for the representative dog, approximately 24% of the radioactivity in feces is 17 β -OH fluasterone, approximately 2% 17 α -OH fluasterone,

FIG. 8. Proton NMR spectra of isolated urinary metabolite eluting at approximately 21.6 min in nonenzyme-treated urine collected between 8 and 24 h after oral dosing with [^{14}C]fluasterone at 15 mg/kg (A), 7- α -hydroxyfluasterone (B), 7- β -hydroxyfluasterone (C), 7- β -hydroxy-17- α -dihydrofluasterone (D), and 7- β -hydroxy-17- β -dihydrofluasterone (E).

less than 1% fluasterone with the remaining material eluting at 35 min as 4 α -17 β -diOH fluasterone.

In urine, radiochemical profiling and metabolite identification from the representative dog reveal that in the first 24 h after oral dosing the primary urinary metabolites, tentatively identified as glucuronides of X(α or β)-4 α -17 β -triOH fluasterone and di-OH fluasterone, account for approximately 5 and 25%, respectively, of the radioactivity excreted. After subcutaneous dosing in the representative dog, these same glucuronides account for approximately 20 and 35%, respectively, of the radioactivity excreted in the first 24 h after dosing. For both dosing regimens, profiling by HPLC/LSS revealed that most of the remaining radiolabel excreted in the 0- to 24-h period after dosing eluted shortly after the column void volume and in the broad region between 13 and 20 min.

In general, levels of radiolabel in plasma were too low for successful radiolabel profiling. The HPLC/LSS profile of plasma collected 4 h after oral dosing, although containing significant baseline noise, revealed that approximately 28, 19, and 16% of the radiolabel in the sample eluted at retention times consistent with those for nonconjugated di-OH fluasterone, tri-OH fluasterone, and the region containing 17 β -OH fluasterone and fluasterone.

NMR Spectroscopy of Purified Urinary Metabolite. Efforts to further characterize the glucuronides present in urine by NMR were

only partially successful. The primary difficulty was purification of sufficient quantities of material using HPLC separation-based strategies. The material isolated from the radiolabel peak eluting at approximately 8 min was of insufficient quantity and purity for characterization of this peak by NMR. The proton NMR of the isolated radiolabeled peak eluting at approximately 22 min in nonhydrolyzed urine is shown in Fig. 8, along with the NMR spectra obtained with structural analogs of fluasterone. The proton NMR spectrum is consistent with a glucuronide conjugate of a 4 α -17X(α or β)-diOH fluasterone metabolite. The chemical shifts of key groups do not unequivocally indicate the position of the glucuronide. Characteristic resonances were determined by examination of the experimental spectra of a series of aglycone standards and from the calculated spectrum of a series of aglycone and glucuronide conjugates. From these standards the following characteristics were observed and used to partially determine the structure of the isolated material:

1. The methyl group H18 undergoes a characteristic upfield shift in the di-OH-fluasterone derivatives (see H18 in Fig. 8, D and E). This same upfield shift is observed in the isolated urinary metabolite, indicating that the metabolite is 17-hydroxylated.

Fig9

Fig. 10. Proton-proton correlation spectrum of isolated urinary metabolite eluting at approximately 21.6 min in non-enzyme-treated urine collected between 8 and 24 h after oral dosing with [^{14}C]fluasterone at 15 mg/kg. The correlations for H3 to H4 are labeled.

- The vinyl proton H6 is sensitive to functionalization near the C5 to C6 olefin. Allylic hydroxylation would be anticipated to produce a downfield shift of the H6 proton in both the 7-OH and 4-OH compound. This downfield shift, predicted by calculation for both the 7- and 4-hydroxy isomers at approximately 5.28 ± 0.32 and 5.60 ± 0.12 ppm, respectively, is observed in the experimental spectra at approximately 5.1 ppm for the 7 β -OH standards (see Fig. 8, C-E) and at 5.4 ppm for the 7 α -OH standard (Fig. 8B). The calculated values for the H6 proton in the 7- and 4-position *O*-glucuronides are 5.28 ± 0.47 and 5.64 ± 0.13 ppm. In the isolated fraction, H6 can be identified by the characteristic chemical shift of the proton (at 5.33 ppm in Fig. 8A) and its correlation to a carbon with the chemical shift of an olefin, observed at 119.0 ppm in the proton-carbon correlation spectrum (Fig. 9). The calculated chemical shift is 124.37 ± 3.46 for the olefin carbon in 7 β -hydroxy-16 α -fluoro-5-androsten-17-one, 123.44 ± 3.22 for the olefin carbon in 7 α -hydroxy-16 α -fluoro-5-androsten-17-one, and 115.51 ± 4.49 for the olefin carbon in either isomer of the 4-hydroxy-16 α -fluoro-5-androsten-17-one. In the *O*-glucuronide series, the calculated chemical shift is 119.30 ± 2.71 for the olefin carbon in 7 α -*O*-glucuronide, 119.08 ± 2.83 for the olefin carbon in 7 β -*O*-glucuronide, and 116.67 ± 4.38 for the olefin carbon in either isomer of the 4-*O*-glucuronide. Thus, it is difficult to distinguish the structure solely on the basis of the H6 vinyl proton.
- A broad triplet is observable at 3.70 ppm, which is similar to the 3.63-ppm shift observed for H7 in 7 β -hydroxy-16 α -fluoro-5-androsten-17-one and is similar to the expected chemical shift of 4.41 ± 0.44 ppm for H4 in a 4-hydroxy analog. This proton is on a carbon with a chemical shift of 67.5 ppm as observed in the proton-carbon correlation spectrum (Fig. 9). This chemical shift is consistent with a proton on a hydroxylated carbon, which is possible for any oxygenated fluasterone analog or any glucuronide carbon. However, analysis of the proton-proton correlation spectrum (Fig. 10) indicates that the putative H4 or H7 only correlates to upfield aliphatic protons, therefore eliminating the possibility that this resonance is caused by a glucuronide proton. The aliphatic proton correlations have chemical shifts of 2.11 and 1.15 ppm. These chemical shifts are consistent with the chemical shift of H3 in the calculated spectra of 4-hydroxy analogs. Furthermore, the strong coupling ($J = 14$ Hz) between H4 and H3 suggests a 1,2 diaxial relationship between H4 and H3. Thus, H4 must be axial and the OH group equatorial. That results in a 4 α -hydroxyl or 4 α -*O*-glucuronide conjugate. These data also eliminate the possibility of the proton being the H7 proton of a 7-hydroxy analog because H7 should correlate to the olefinic proton H6 in either the 7 α -OH or the 7 β -OH analog (full characterization of the synthetic standards of the 7-OH compounds is described in Burgess et al., 2006). Figure 11 summarizes the proposed metabolic scheme developed from results of the HPLC/LSS, HPLC/MS/MS, and NMR analyses of the study samples.

Discussion

Investigation of the metabolism and disposition of fluasterone is of importance for the development of this compound as a pharmaceutical. Because of fluasterone's close structural similarity to DHEA, one might suspect that the biotransformation pathways for fluasterone could be extensive involving both Phase I and Phase II processes and could produce compounds that retain pharmacological activity. Because of the need to understand the biological fate of fluasterone, this study in dogs to characterize the absorption, distribution, metabolism, and elimination profiles of fluasterone was conducted. The pharmacological properties of fluasterone and its metabolites might also be anticipated to be dependent on the hormonal milieu within which they are interacting (Ebeling and Koivisto, 1994). It is well recognized that the variation in hormone concentrations can be dramatic over time and

FIG. 11. Proposed metabolic scheme of fluasterone.

across sex. For example, in humans endogenous plasma concentrations of both DHEA and DHEAS are age- and sex-dependent, and concentrations of both decrease from the 3rd decade onward. Furthermore, DHEA concentrations have often been reported to be higher in women, whereas DHEAS concentrations have been consistently reported as higher in men (Frye et al., 2000).

Elimination in feces in the 48 h after administration accounts for approximately 22 and 45% of the dose after subcutaneous dosing and oral dosing, respectively. The mean cumulative percentage of dose eliminated in urine is significantly lower than in feces with less than 10% eliminated in urine over 48 h after either oral or subcutaneous dosing. Because less than 50% of the radiolabel received is excreted in the first 48 h, it appears that there is considerable distribution of fluasterone and its metabolites into tissues up to 48 h after either subcutaneous or oral administration.

The two primary metabolites of fluasterone excreted in dog urine are tentatively identified as monoglucuronides of 4 α -17 β -diOH fluasterone and X(α or β)-4 α -17 β -triOH fluasterone. The orientation of the 17-OH group is assumed to be β because of the stereospecificity of the enzyme 17-ketosteroid reductase. The α -orientation of the 4-OH group was inferred from NMR data. The position and orienta-

tion of one of the sites of hydroxylation in the trihydroxylated metabolite remain to be characterized, but it is possible it is a 7-hydroxylated metabolite because this position is allylic, as is the 4-position. The tentative identification of the metabolites in urine and the products produced by glucuronidase treatment of urine was applied to the determination of the metabolites detected in feces and plasma. The identifications in dog feces were confirmed by LC/MS analysis of feces extracts. Based on similar HPLC retention time, LC/MS data, and the NMR identification described above, it seems that the radioactive peak eluting at 27 min in extracts of feces is caused by the presence of the nonconjugated, keto-reduced, and dihydroxylated metabolite X(α or β)-4 α -17 β -triOH fluasterone, which is seen in its monoglucuronidated form in urine. Likewise, the radioactive material eluting at 34 min in fecal extracts seems to be the nonconjugated, keto-reduced, and monohydroxylated analog, most likely 4 α -17 β -diOH fluasterone, which also occurs as the monoglucuronidated form in urine. Finally, based on retention time and mass spectral data, the later eluting radioactive material in feces seems to be 17 α -OH fluasterone, 17 β -OH fluasterone, and nonmetabolized fluasterone. A similar logic based on both retention time and LC/MS data can be applied to allow for tentative assignment of metabolites in plasma.

The data reported in the article show that the primary fluasterone metabolites in the dog have the 17-oxo group reduced to 17-hydroxy. This is an important finding because it suggests that the active drug in the dog is fluasterone and not a metabolite. That is, the two primary biological effects of fluasterone are inhibition of inflammation and concomitant hyperplasia, which is apparently mediated by glucose-6-phosphate dehydrogenase (G6PDH) inhibition, and the antiglucocorticoid action, which is almost certainly not mediated by G6PDH inhibition. The compound 17 β -OH fluasterone has greatly reduced G6PDH inhibitory activity, as well as greatly reduced antiglucocorticoid action, indicating the importance of the 17-oxo group for activity.

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