

Metabolite Profiles of Degarelix, a New Gonadotropin-Releasing Hormone Receptor Antagonist, in Rat, Dog, and Monkey

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ABSTRACT:

Degarelix is a novel competitive gonadotropin-releasing hormone receptor blocker (antagonist). In this study, the nonclinical metabolism and excretion of degarelix was investigated in Sprague-Dawley rat, beagle dog, and cynomolgus monkey. Degarelix was found to be stable when incubated in microsomes and cryopreserved hepatocytes from animal liver tissue. Absorption, distribution, metabolism, and excretion studies in male rat, dog, and monkey showed that after a subcutaneous dose of tritium-labeled degarelix, the peptide was rapidly absorbed with C_{max} in plasma of 1 to 2 h. The predominant route of excretion was via the kidneys and the bile. In rat and dog, most of the degarelix dose was eliminated within 48 h via urine and feces in equal amounts (40–

50% in each matrix), whereas in monkey the major route of excretion was fecal (50%) and renal (22%). In plasma and urine samples from all three species, mainly intact degarelix was detected. In bile and feces samples from rats and dogs, the same truncated peptides of the parent decapeptide were detected. The major metabolites identified represented the N-terminal tetrapeptide, the pentapeptide, and the heptapeptide. From the animal studies, it could be concluded that degarelix is subject to common peptidic degradation in the liver and bile and is fully excreted via metabolic and biliary (as metabolites and parent compound) and urinary (mainly as parent compound) pathways. Systemic exposure to metabolic products seems to be low.

Introduction

Degarelix (Fig. 1) is a decapeptide that shows a high affinity/selectivity to human gonadotropin-releasing hormone receptors (Jiang et al., 2001). Gonadotropin-releasing hormone (GnRH) blockers bind to GnRH receptors in the pituitary gland without any subsequent receptor activation, which results in a prompt suppression of luteinizing hormone (and follicle-stimulating hormone) secretion from this gland, which in turn results in decreased production of testosterone from the Leydig cells of the testes (Ortmann and Diedrich, 1999; Chengalvala et al., 2003). The suppression of testosterone occurs almost immediately after administration of the peptide drug and continues as long as degarelix is available at the receptors (de Pinieux et al., 2001). The efficacy and duration of action of a single subcutaneous injection of degarelix at 2 mg/kg to the intact male rat was compared to that of surgical castration (Broqua et al., 2002; Princivalle et al., 2007). Degarelix suppresses testosterone to castration levels for a total of 42 days; thereafter, plasma testosterone levels began to increase gradually and returned to baseline levels at day 77 (Broqua et al., 2002). In nude mice and rats grafted with androgen-dependent human prostate tumor, degarelix subcutaneously injected at

2 mg/kg every 2 weeks produced complete suppression of tumor growth (de Pinieux et al., 2001). On the basis of the successful nonclinical findings, degarelix entered and underwent successful clinical development, which resulted in approval for the treatment of advanced prostate cancer by the U.S. Food and Drug Administration and European Medicines Agency. Degarelix can now be prescribed under the name Firmagon (Boccon-Gibod et al., 2009; Pommerville and de Boer, 2010).

The present study was aimed at elucidating the hepatic metabolism and excretion profile of this small peptide hormone analog with profound biologic activity on the pituitary gland and testicular function. Because of the presence of several non-natural amino acids in the molecule [D-2Nal, D-4Cpa, D-3Pal, 4Aph(L-Hor), D-4Aph(Cbm), ILys, and D-Ala; Fig. 1], degarelix was suspected not to follow the classical route of peptide hydrolysis, which made these explorations of particular interest. The metabolite profile of degarelix in plasma and excreta was compared with reported results of other luteinizing hormone-releasing hormone antagonists (Chan et al., 1991; Schwahn et al., 2000). Furthermore, it was also of interest to explore whether the compound is a substrate for the cytochrome P450 (P450) enzyme in hepatocytes with direct implications for the risk of drug-drug interactions.

This report summarizes studies conducted to investigate in vivo metabolism of degarelix in rat, dog, and monkey species as well as in vitro stability studies in liver microsomes and cryopreserved hepato-

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ABBREVIATIONS: GnRH, gonadotropin-releasing hormone; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; P450, cytochrome P450; RAD, radiochemical detection; SPE, solid-phase extraction; Ac, acetyl; 2Nal, 2-naphthylalanine; 4Cpa, 4-chloro-phenylalanine; 3Pal, 3-pyridylalanine; 4Aph, 4-aminophenylalanine; Hor, hydroorotyl; Cbm, carbamoyl; ILys, *N*(ϵ)-isopropyllysine; FE 200486, degarelix; ADME, absorption, distribution, metabolism, and excretion.

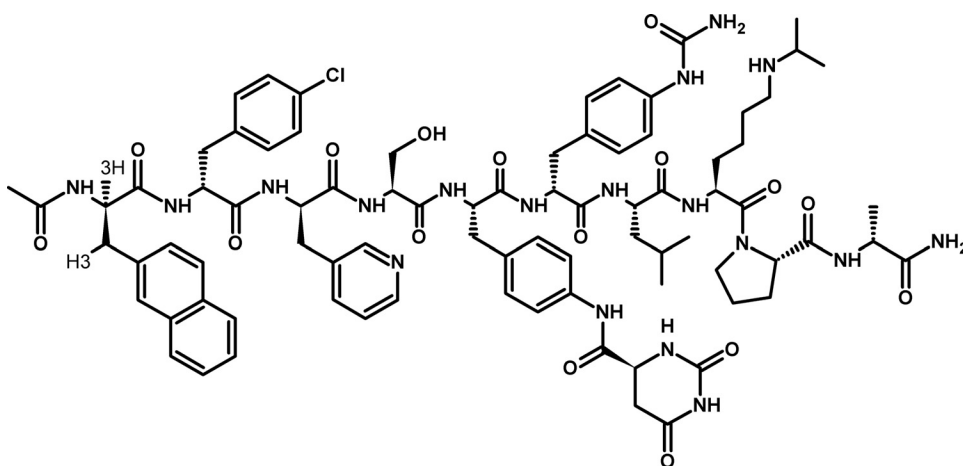


FIG. 1. Structure of degarelix (FE 200486) with ^3H indicating the positions of the tritium radiolabeled FE 200486. Peptide sequence: Ac-D-2NaI-D-4Cpa-D-3Pal-Ser-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys-Pro-D-Ala-NH₂.

cytes from the same animal species as a part of the preclinical development performed in compliance with regulatory demands.

Materials and Methods

Radiolabeled Test Material. Tritium-labeled degarelix (^3H degarelix; Fig. 1) was prepared by a modification of the method used for synthesis of the tritiated version of the structurally related GnRH antagonist in Parnes and Shelton (1986). In brief, 2-*N*-acetylamino-3-(2-naphthyl)acrylic acid was stereospecifically tritiated to *N*-acetyl-D-[2,3- ^3H]2-naphthylalanine with the homogeneous catalyst (2*S*,4*S*)-*N*-tert-butyloxycarbonyl-4-diphenylphosphino-2-diphenylphosphinomethylpyrrolidine-Rhodium in methanol. After evaporation of the solvent, the crude product was coupled to the unprotected (2–10) degarelix fragment D-4Cpa-D-3Pal-Ser-4Aph(L-Hor)-D-4Aph(Cbm)-Leu-ILys-Pro-D-Ala-NH₂ with benzotriazol-1-yl-oxytripyrrolidinophosphonium/diisopropylethylamine in dimethylformamide in 2 h at room temperature. During this coupling step, approximately 20% racemization of the *N*-acetyl-D-naphthylalanine occurred, but the resulting [Ac-2NaI]degarelix epimer could easily be separated during the final purification. No byproduct from coupling to the secondary nitrogen of isopropyllysine⁸ was found, possibly because of steric hindrance. Tritiated degarelix was obtained by reversed phase liquid chromatography (LC) (C18) purification using triethylamine phosphate (pH 2.3)/acetonitrile buffer followed by counterion exchange for acetate using the same column. The radiosynthesis was performed at Moravex Biochemicals (Brea, CA) to afford [Ac-D-[2,3- ^3H]2NaI]degarelix with specific radioactivity of 40 Ci/mmol and radiopurity of >95%. The radiolabeled peptide was stored in solution in ethanol/water (1:1) at -40°C , conditions under which degarelix was found to be chemically stable for at least 4 months.

Chemicals and Reagents. The development compound code of degarelix was FE 200486; all metabolites are named according to the usual conventions for modified peptides (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1983) using FE 200486 as basis. Degarelix (FE 200486) was synthesized by Polypeptide Laboratories (Torrance, CA). The unlabeled reference compounds FE 200486(1–4)-OH, FE 200486(1–6)-OH, FE 200486(1–7)-OH, FE 200486(1–9)-OH, FE 200486(1–10)-OH, FE 200486(4–10), and FE 200486(7–10) were synthesized by Polypeptide Laboratories (Wolfenbüttel, Germany). Internal standard [^3H]barusiban was prepared at Ferring Research Institute, Inc. (San Diego, CA). Reagents and solvents were of analytical grade and were obtained from commercial sources.

Liver Tissue. Pooled liver microsomes from three male species: beagle dogs (pool of 4), cynomolgus monkey (pool of 6), and Sprague-Dawley rats (pool of 200) were purchased from XenoTech, LLC (Lenexa, KS) through Tebu-Bio (Le Perray-en-Yvelines, France). The liver microsome preparations were stored at -80°C until use.

Cryopreserved preparations of male rat Sprague-Dawley hepatocytes (M00005/MSE) and male beagle dog (M00205/VWL) hepatocytes were purchased from In Vitro Technologies (Baltimore, MD) and stored in liquid nitrogen until use.

In Vitro Studies. Microsomal incubation conditions. Degarelix (10 μM) was incubated in a 1.5-ml solution containing magnesium chloride (3 mM) and

EDTA (1 mM) in potassium phosphate buffer (0.1 M, pH 7.4). The liver microsome protein concentration was 3 mg/ml. The reaction was started by adding NADPH (2 mM). Control samples containing no NADPH were also included in the study. All incubations were performed at 37°C in a shaking water bath. Samples, aliquots of 100 μl , were removed after 0, 5, 10, 20, 40, and 60 min and added to polypropylene tubes containing 100 μl acetonitrile/glacial acetic acid [96:4 (v/v)]. The precipitated microsomal solutions were vortexed and put in ice for at least 30 min. Internal standard, [^3H]barusiban, was added to a concentration of 952 nM. The test tubes were centrifuged, and the supernatant was diluted with an equal volume of water in an LC autosampler vial. The samples were analyzed for degarelix substance stability and screening of metabolites using LC-MS.

Stability studies with cryopreserved hepatocytes. The stability of degarelix in hepatocytes was studied using cryopreserved preparations from male Sprague-Dawley rats and male beagle dog origin. Cryopreserved hepatocytes were thawed and prepared according to a protocol from In Vitro Technologies. The thawed cell suspensions were counted and diluted to 2 million viable cells per milliliter in a Krebs Henseleit buffer (pH 7.4) containing 2.2 g/l sodium hydrogen carbonate, 22.6 mM HEPES, and 1 mM calcium chloride. The metabolic activity of the hepatocyte suspensions were investigated by degradation studies of test substrates naloxone (glucuronic acid activity) (Wahlström et al., 1989), propranolol (CYP2D6 activity) (Masubuchi et al., 1994), and verapamil (CYP3A4 activity) (Kroemer et al., 1993). These activity control samples were analyzed by LC-MS/MS.

Substance stability studies were performed at concentrations of 40 μM [^3H]degarelix. Hepatocyte suspensions were tempered at 37°C for 5 min before adding the test substrate. Samples were removed after 0, 10, 30, 60, and 120 min of incubation. Aliquots of 100 μl were withdrawn and terminated in 100 μl acetonitrile/glacial acetic acid [96:4 (v/v)] in Eppendorf polypropylene tubes. After centrifugation, 100 μl of supernatant was diluted with 100 μl Milli-Q water (Millipore Corporation, Billerica, MA) in an LC autosampler vial. The samples were stored at 8°C until analysis by LC-RAD and LC-MS.

In Vivo Samples. Animals and treatments. All animal studies were performed at contract laboratory facilities. The animal experiments were performed according to ethical guidelines of animal experimentation, and all animal work was performed in compliance with United Kingdom or Dutch legislation and was approved by a local ethical review process as appropriate. Sprague-Dawley rats, beagle dogs, and cynomolgus monkeys were used. All samples collected were frozen within 1 h after collection and stored at -80°C until the time of analysis. For metabolite screening analysis, the biologic samples were shipped on dry ice to the Ferring Pharmaceuticals laboratory facility and stored at -80°C until analysis, except for the samples from rat study R1 (see *Rats*), in which the sample analyses were performed at the contract research organization. All mass balance determinations were performed at the contract research organizations.

Rats. Three studies in rats were performed. The first study (R1) was an absorption, distribution, metabolism, and excretion (ADME) study and comprised one group (A) dosed intravenously and two groups (B and C) dosed subcutaneously, respectively. Male and female rats were used. All rats (200–

400 g b.wt.) were dosed with 30 μg [^3H]degarelix free base peptide/kg (~ 300 $\mu\text{Ci/kg}$). Feces, heparin plasma, and urine samples from groups A and B were collected up to 240 h after dosing. Bile sampling from cannulated rats (group C) as well as urine and feces were collected for up to 48 h. All samples in this study were analyzed by LC-RAD at the contract research organization.

The second rat study (R2) was a biliary excretion study. Three male rats under anesthesia had a flexible plastic cannula inserted in the common bile duct to allow for complete collection of bile. A second cannula was inserted into the stomach to be able to infuse sodium taurocholate in saline solution during the study as a replacement for lost bile salts. The animals were administered a single subcutaneous dose of [^3H]degarelix (26 $\mu\text{g/kg}$ b.wt. with nonradiolabeled degarelix added to a total dose of 30 $\mu\text{g/kg}$ b.wt.) and placed in metabolism cages. Bile, urine, and feces samples were collected for 48 h after dosing. The collected samples were analyzed by LC-RAD and LC-MS.

The third rat study (R3) was performed to study the enterohepatic circulation of degarelix and its metabolites. Three pairs of male rats were surgically prepared under anesthesia. Bile duct cannulation was surgically established so that bile was directly and quantitatively transferred from the bile duct of the first ("donor") rat to the duodenum of the second ("recipient") rat. A further cannulation of the bile duct of the recipient rat allowed for the completion of collection of bile produced in the recipient animal. A single subcutaneous dose of [^3H]degarelix (26 $\mu\text{g/kg}$ b.wt. with nonradiolabeled degarelix added to a total dose of 30 $\mu\text{g/kg}$ b.wt., ~ 300 $\mu\text{Ci/kg}$) was administered to the donor rats, and during the following 48 h, urine and feces were collected from donor and recipient rats, and bile was collected from the recipient rats.

ADME Study in Dogs. Male and female dogs (10–15 kg b.wt.) were treated with either a single subcutaneous (group A) or intravenous dose (group B) of [^3H]degarelix at 3 $\mu\text{g/kg}$ b.wt. Feces, heparin plasma, and urine samples from groups A and B were collected up to 240 h after dosing.

In addition, two male dogs had a surgically inserted catheter placed in the gall bladder via the bile duct for bile collection. Before surgery, the dogs fasted for at least 24 h and were allowed a recovery period of 3 days before dosing. The dogs were administered a subcutaneous injection of 1 mg of nonradiolabeled degarelix per kilogram body weight (injection volume 0.2 ml/kg), and heparin plasma and bile samples were collected up to 24 h postdose for metabolite screening analysis.

ADME Study in Monkeys. Four male cynomolgus monkeys (4.2–7.5 kg) were dosed for a disposition of radioactivity study. The animals were administered a single subcutaneous dose of 8.2 $\mu\text{g/kg}$ (200 $\mu\text{Ci/kg}$) [^3H]degarelix. Urine and feces samples were collected quantitatively from each animal after dosing until the time of sacrifice. Blood samples were collected at the time of the sacrifice of the individual animal (6, 24, 48, and 240 h) into tubes containing EDTA as an anticoagulant.

Analysis of Total Radioactivity. Plasma, blood, urine, cage washings, homogenates of feces and tissue, and carcass digests were combusted in oxygen using a Packard model 307 automatic sample oxidizer (PerkinElmer Life and Analytical Sciences, Waltham, MA). The products of combustion were absorbed into 15 ml Monophase (Canberra Industries, Meriden, CT) for radioactivity measurement. Radioactivity was measured by liquid scintillation spectrometry using a Wallac 1409 automatic scintillation counter (PerkinElmer Life and Analytical Sciences). Radioactivity in hepatocyte uptake studies was measured using a Tricarb model 1600TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences).

Analysis of Plasma Samples for Metabolites. Plasma samples were thawed and then centrifuged at 3000g for 20 min at 8°C. Plasma (0.5–1.0 ml) was transferred to a 6-ml glass vial and precipitated by adding two equivalents of acetonitrile/glacial acetic acid [96:4 (v/v)]. Internal standard [50 μl of 4 $\mu\text{g/ml}$ [$\text{Ile}^3\text{H}_{10}$]barusiban] was added to each tube. The tubes were vortexed and thereafter centrifuged at 3000g for 20 min at 8°C. The supernatants were transferred to new 6-ml glass vials and evaporated to dryness under vacuum in a SpeedVac (Thermo Fisher Scientific, Waltham, MA) at 40°C. The sample residues were redissolved in 0.2 ml water/acetonitrile [64:36 (v/v)] containing 0.05% (v/v) trifluoroacetic acid, and the supernatants were transferred to LC autosampler vials. The samples were analyzed by LC-RAD and LC-MS.

Analysis of Urine Samples for Metabolites. Urine samples were purified and concentrated by solid-phase extraction (SPE) using Isolute (Hengood, Mid-Glamorgan, UK) C18 columns. The SPE column was first conditioned with one column volume of methanol followed by an equal volume of aceto-

nitrile and finally with 5 mM ammonium acetate buffer (pH 4.6). The urine sample, with 20 ng/ml internal standard ([$\text{Ile}^3\text{H}_{10}$]barusiban) added, was applied, and the container and resin were washed with a column volume of 5 mM ammonium acetate buffer (pH 4.6). Retained material on the SPE C18 column was eluted using 3 ml acetonitrile/water [9:1 (v/v)] containing 5 mM ammonium acetate (pH 4.6). The acetonitrile/water 9:1 eluate was evaporated to dryness under reduced pressure using a SpeedVac. Each sample residue was redissolved in 0.2 ml water/acetonitrile [64:36 (v/v)] containing 0.05% (v/v) trifluoroacetic acid and transferred to a total recovery vial (Waters, Milford, MA). The samples were analyzed by LC-RAD and LC-MS. Recoveries of degarelix and synthesized standards FE 200486(1–4)-OH, (1–6)-OH, (1–7)-OH, (1–9)-OH, and (1–10)-OH; FE 200486(4–10) and (7–10); and ([$\text{Ile}^3\text{H}_{10}$]barusiban) in the SPE method for spiked urine samples at concentrations of 10 and 100 ng/ml were $>80\%$ for all peptides tested.

Analysis of Bile Samples for Metabolites. Bile samples were thawed at room temperature. For direct analysis of the bile, 100 μl of bile from each sample was transferred to autosampler vials for analysis by LC-RAD and LC-MS. Larger volumes of bile were also purified and concentrated by SPE before analysis according to the SPE procedure described for urine above. Recoveries in the SPE method for spiked bile samples at concentrations of 10 and 100 ng/ml were $>80\%$ for all peptides tested.

Analysis of Feces Samples for Metabolites. Thawed feces samples, approximately 0.9 to 2.3 g material, were transferred to preweighed Kimax tubes (Fisher Scientific, Loughborough, UK) (100 \times 16 mm). The feces material was homogenized in 1 ml of 1.5% (v/v) aqueous formic acid using a Covaris (Woburn, MA) acoustic mixing system. Internal standard [50 μl of 4 $\mu\text{g/ml}$ [$\text{Ile}^3\text{H}_{10}$]barusiban in 5 mM ammonium acetate (pH 4.6)] was added, and 5 ml of water/acetonitrile [6:4 (v/v)], was added to each sample tube. The tubes were extracted for 30 min using a Heidolph (Schwabach, Germany) multi Reax at 90% of the maximal setting. The Kimax tubes were centrifuged at 3000g for 30 min at 8°C. The supernatant from each tube was evaporated under vacuum in a SpeedVac at 40°C. The sample residue in the first test tube was redissolved in 0.5 ml water/acetonitrile [64:36 (v/v)] containing 0.05% (v/v) trifluoroacetic acid and transferred to a LC autosampler vial. The feces extract samples were analyzed by LC-RAD and LC-MS. Recoveries in the extraction method after spiking homogenate of blank rat feces at concentrations of 50 and 500 ng/ml were approximately 50% for all peptides tested. The test peptides used were the same as those used for investigating the SPE recovery for the bile and urine samples.

Metabolite Profiling Using Radiochemical Detection. Rat bile, feces, and urine from rat study R1 were analyzed using a LC-RAD system consisting of a Spectra Series P200 binary gradient pump (Thermo Fisher Scientific) and a Canberra Packard A515 radiometer. The LC analysis was achieved using a Hypersil (Thermo Fisher Scientific) base deactivated phenyl column (5 μm , 250 \times 4.6 mm) eluted at 1 ml/min with 70% eluent A (20 mM aqueous phosphate buffer, pH 6.9 with 0.3% triethylamine) and 30% eluent B (20 mM phosphate buffer in acetonitrile with 0.3% triethylamine) for 10 min followed by a linear gradient to 34% B in 1 min and an isocratic elution (66% A/34% B) for an additional 34 min. The column temperature was 40°C. The RAD had a liquid scintillation flow cell with a volume of 0.5 ml, and the scintillation flow was 3 ml/min of FloScint A (PerkinElmer Life and Analytical Sciences).

LC-RAD of bile, urine, and feces samples from rat studies R2 and R3 were performed using an Agilent Technologies (Santa Clara, CA) 1100 LC system connected to a Packard (PerkinElmer Life and Analytical Sciences) flow scintillation analyzer. The column used was an Agilent HP Zorbax SB-C18, 5 μm (150 \times 2.1 mm) with guard column SB-C8, 5 μm (12.5 \times 2.1 mm). The LC flow rate was 0.25 ml/min. The samples were analyzed using a gradient system starting at 9% (v/v) acetonitrile and 91% aqueous 5 mM ammonium acetate (pH 4.6) buffer to 56% acetonitrile over 50 min. The RAD flow-cell volume was 0.5 ml, and the scintillation flow was 1 ml/min Ultima Flo Gold scintillation cocktail (PerkinElmer Life and Analytical Sciences).

LC-RAD of monkey plasma and urine samples were performed by using a Waters 2695 Alliance system connected to an IN/US Systems (Tampa, FL) β -RAM model 3. FlowLogic 1:1 scintillation cocktail (AIM Research, Hockessin, DE) was delivered by an ARC (AIM Research) StopFlow Model B unit. The column used was an YMC (Kyoto, Japan) basic C18, 5 μm (150 \times 2.1 mm) lot 3096 with guard column C18, 5 μm (10 \times 2.1 mm). The LC flow rate was 0.22 ml/min. The gradient used was as for the Agilent system. The

agent/eluate ratio was 2.0; thus, 0.44 ml/min of scintillation cocktail was mixed with the LC column eluant. Samples were analyzed in nonStopFlow mode.

Metabolite Profiling and Metabolite Identification by Mass Spectrometry Detection. Plasma, bile, urine, and feces samples and liver microsome and hepatocyte samples were analyzed for metabolite identification using LC-MS. The LC instrument was a Waters 2690 Alliance system, and the MS system was a Micromass (Manchester, UK) Q-time of flight 1.0 upgraded to a 3.6-GHz time-to-digital converter. The column used was an YMC basic C18, 5 μm (150 \times 2.1 mm) with guard column C18, 5 μm (10 \times 2.1 mm). The LC flow rate was 0.22 ml/min. The samples were analyzed using a gradient starting at 5% acetonitrile isocratic for 5 min, and then a linear increase to 60% acetonitrile for 50 min. The mobile phase was acidified with 0.05% (v/v) trifluoroacetic acid. The MS interface used was an electrospray ionization interface operating in positive ionization mode.

The LC retention times of the synthesized standard truncated peptides were known, as were their MS and product ion spectra. In addition, all MS data files were screened for m/z values of possible truncated peptide ions as FE 200486(1–3)-OH, FE 200486(1–5)-OH, FE 200486(1–8)-OH, and FE 200486(6–10) as well as oxidation products of degarelix and oxidation products of truncated metabolites of degarelix. The possible presence of a glucuronyl derivative of degarelix was also investigated. Also, total ion current data from postdose samples were compared with the total ion current data from predose samples for any unique ions present in the postdose samples. ACD/Labs (Toronto, Ontario, Canada) software MS Manager was used for comparing data sets to detect unique differences. Product ion spectra of detected metabolite ions were recorded. The collision energy used was optimized for each metabolite.

Results

Radiolabeling of Degarelix. Tritium (two atoms per peptide molecule) was chosen as a radiolabel because of higher specific radioactivity than the alternative ^{14}C . The label was placed on the alpha and beta carbon atoms of D-2Nal in position 1 of the peptide (^3H degarelix; Fig. 1), where tritium would not be expected to exchange with hydrogen under physiologic conditions. The choice of D-2Nal as the tritiation site was dictated by ease of synthesis. At the same time, loss of label via enzymatic cleavage in this part of the molecule is highly unlikely because of the D-configuration of the amino acid bearing the label and two consecutive amino acids following it.

Stability in Liver Microsomes. No degradation of degarelix was detected when the substrate was incubated in liver microsomes from dog and monkey. A tendency for a minor decrease in degarelix peak area was seen when incubating the peptide in liver microsomes from rats. However, no metabolites of degarelix could be detected in the samples representing 60-min incubation in buffered rat liver microsome mixture.

Stability in Hepatocytes. No degradation of degarelix was detected when ^3H degarelix was incubated in cryopreserved hepa-

tocytes from male rat and male dog for up to 120 min at 37°C. The metabolic activity of the cryopreserved hepatocytes as evaluated using three test substrates demonstrated that all of the hepatocyte batches used expressed metabolic activities such as CYP3A4- and CYP2D6-mediated metabolism and glucuronic acid conjugation activity.

Recovery of Radioactivity in Rat. After a subcutaneous dose of 30 $\mu\text{g}/\text{kg}$ (study R1), ^3H degarelix radioactivity appeared to be rapidly absorbed with a remainder of 10 to 14% of the radioactivity at the injection site after 10 days. Maximal plasma concentration was achieved within 1 to 2 h. The predominant routes of excretion were via the kidneys (50%) and the bile (40–50%). Most radioactivity was excreted within 48 h.

Metabolite Profiling in Rats. In the first study (R1), the samples were only analyzed by LC-RAD. The identity of the peaks detected was assigned by retention time comparison with standards, but the identities were not confirmed by LC-MS analysis. In plasma, most radioactivity detected was the parent compound ^3H degarelix, representing >94% of total radioactivity measured in female rats and >87% in male rats, respectively. One metabolite assumed to represent FE 200486(1–9)-OH that was detected in the plasma samples represented approximately 6% of the peak integrated radioactivity in female rats whereas in male rats approximately 13% was detected (Table 1). In urine, the major part of excreted radioactivity was the parent compound for both genders. Low levels of six metabolites were also detected. In bile and feces, a clear difference in metabolic profile was detected depending on the gender. In bile, the same five metabolites were detected in both genders, but in female rats most of the excreted radioactivity was the parent compound and the two metabolites were assumed to represent FE 200486(1–4)-OH and FE 200486(1–6)-OH, which were present in almost equal amounts. In bile collected from male rats, the amount of metabolite FE 200486(1–6)-OH was almost twice as large as the parent compound. In feces, the same six metabolites were detected in samples from female and male rats. The major part of excreted radioactivity in feces from male rats was metabolite FE 200486(1–4)-OH, whereas in female rats equal amounts of FE 200486(1–4)-OH and FE 200486(1–10)-OH were the largest metabolites detected.

In the second rat study (R2), bile, feces, and urine samples were analyzed by LC-RAD and LC-MS. In urine, most radioactivity (94%) detected was the parent compound ^3H degarelix (see Fig. 2). In addition, seven smaller metabolites were detected, representing <5% of total radioactivity detected. In bile, four metabolites were identified, and the metabolites were identified by LC-MS/MS to represent the truncated peptides of degarelix: FE 200486(1–4)-OH, FE

TABLE 1

Metabolite pattern in plasma, urine, bile, and feces after subcutaneous administration of 30 μg ^3H -degarelix/kg in rats (rat study R1)

Gender	Sample	Sampling Period	Percentage of Component in Sample ^a						U	U	FE 200486 (1–10)-OH
			^3H -Degarelix	FE 200486 (1–4)-OH	FE 200486 (1–6)-OH	FE 200486 (1–5)-OH	FE 200486 (1–9)-OH	FE 200486 (1–7)-OH			
		<i>h</i>									
Male	Plasma	0–24	87.4					12.6			
	Urine ^b	0–48	96.4	0.44					0.17	0.32	
	Bile ^b	0–48	17.2	25.9	30.5	1.17	9.98	13.3			
Female	Feces ^b	0–48	5.84	25.7	19.2	12.9	15.5	3.90		14.4	
	Plasma	0–24	94.3				5.75				
	Urine ^b	0–48	92.4	2.97	2.97			0.07	0.17	0.12	
	Bile	0–48	28.3	24.9	23.4	1.36	7.91	14.1			
	Feces ^b	0–48	5.95	26.6	12.0	9.95	15.2	3.15		24.7	

U, unknown.

^a Numbers represent mean values of four individual animals.

^b Radioactivity detected in void peak adds up to 100%.

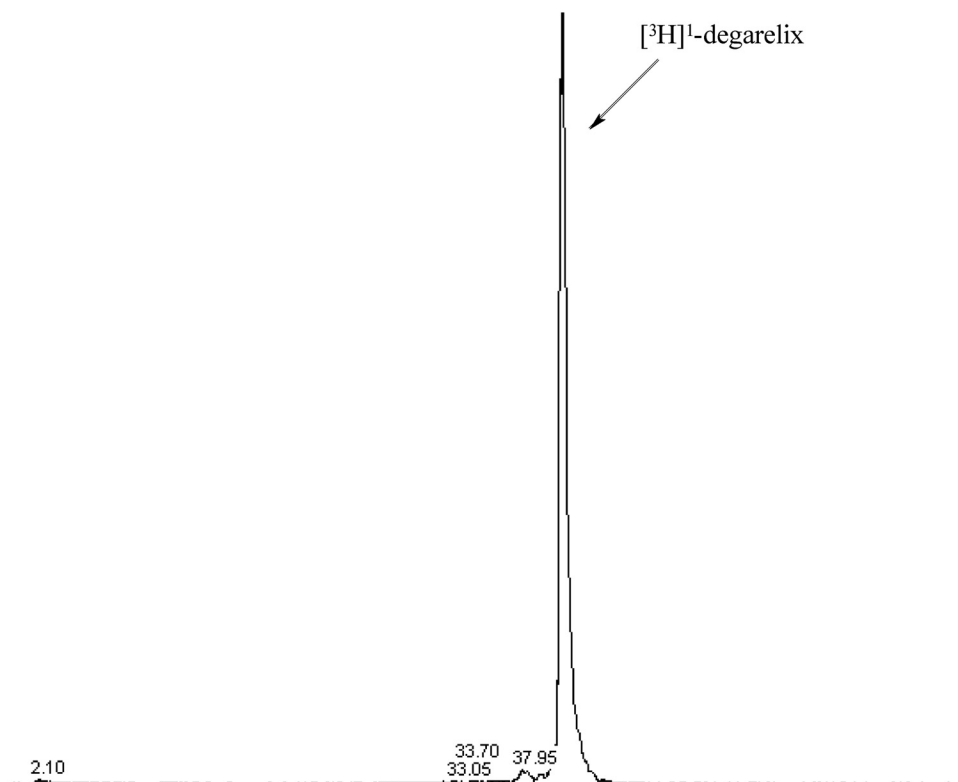


FIG. 2. Radiochromatogram from LC-RAD analysis of urine collected between 0 and 6 h postdose from a male rat administered a subcutaneous dose of 30 $\mu\text{g}/\text{kg}$ [^3H]degarelix.

200486(1–6)-OH), FE 200486(1–7)-OH, and FE 200486(1–9)-OH. The product ion spectra data of metabolites detected as well as the product ion spectrum of degarelix (FE 200486) are summarized in Table 2. In addition, the product ion spectrum of degarelix is shown in Fig. 3. In addition to the b-ions and yⁿ-ions present in the product ion spectrum of degarelix, the product ions *m/z* 121.1, 154.1, and 170.1 represent immonium ions of amino acids 3Pal³, 4Cpa², and Nal¹, respectively. The origin of product ion *m/z* 171.2 is related to amino acid Ilys⁸.

A triethylamine phosphate buffer containing mobile phases was used for LC-RAD analysis in rat study R1, which enabled baseline separation of the metabolites. However, to use a mass spectrometer as a detector, ammonium acetate was the buffer salt used in the mobile phases for LC-RAD and LC-MS analysis of the samples from rat study R2. Using this mobile-phase mixture, the metabolites FE 200486(1–4)-OH and FE 200486(1–6)-OH unfortunately coeluted, as

did the metabolites FE 200486(1–7)-OH and FE 200486(1–9)-OH (see Fig. 4). The peak representing the tetrapeptide and the hexapeptide was 46%, the peak representing the heptapeptide and the nonapeptide was 31%, and the peak representing [^3H]degarelix was 22% of the total radioactivity detected, respectively, in the 0- to 6-h bile samples, which are close to the results found in study R1 for male rats (Table 1). Feces samples were investigated to confirm the completeness of the surgical procedure of bile-duct cannulation. No metabolites of [^3H]degarelix or the parent compound were detected by LC-RAD or LC-MS in the feces extracts.

Enterohepatic Circulation of Degarelix in Rat (Study R3). After a single subcutaneous dose of 30 $\mu\text{g}/\text{kg}$, [^3H]degarelix radioactivity was almost entirely absorbed from the dose site, and approximately one half of the dose was excreted in bile. Only approximately 1% of this biliary excreted fraction was reabsorbed from the gastrointestinal tract; thus, enterohepatic recirculation of [^3H]degarelix and/or its

TABLE 2

Identifications of degarelix and metabolites of degarelix using product ion mass spectrometry data

Analyte	Precursor Ion	Characteristic Product Ions	
		<i>m/z</i>	<i>m/z</i>
Degarelix	816.4	121.1, 154.1, 170.1, 171.2, 186.1 (y ₂ ⁿ), 240.1 (b ₁), 356.3 (y ₃ ⁿ), 421.2 (b ₂), 469.4 (y ₄ ⁿ), 569.2 (b ₃), 606.3 (y ₈ ⁿ), 656.1 (b ₄), 674.4 (y ₅ ⁿ), 696.9 (y ₉ ⁿ), 794.9 (y ₁₀ ⁿ), 807.9 (b ₁₀), 958.4 (b ₅), 976.6 1 (y ₆ ⁿ), 1063.6 1 (y ₇ ⁿ), 1163.6 (b ₆), 1211.7 (y ₈ ⁿ), 1276.5 (b ₇), 1393.0 (y ₉ ⁿ)	
FE 200486(1–10)-OH	816.9	121.1, 154.1, 170.1, 171.2, 187.1 (y ₂ ⁿ), 240.1 (b ₁), 357.3 (y ₃ ⁿ), 421.2 (b ₂), 470.4 (y ₄ ⁿ), 569.2 (b ₃), 606.8 (y ₈ ⁿ), 656.1 (b ₄), 675.4 (y ₅ ⁿ), 697.4 (y ₉ ⁿ), 795.4 (y ₁₀ ⁿ), 807.9 (b ₁₀), 958.4 (b ₅), 977.6 1 (y ₆ ⁿ), 1064.6 1 (y ₇ ⁿ), 1163.6 (b ₆), 1212.7 (y ₈ ⁿ), 1276.5 (b ₇)	
FE 200486(1–9)-OH	781.4	121.1, 154.1, 170.1, 171.2, 240.1 (b ₁), 286.3 (y ₂ ⁿ), 399.4 (y ₃ ⁿ), 421.2 (b ₂), 569.2 (b ₃), 604.6 (y ₄ ⁿ), 656.1 (b ₄), 662.0 (y ₈ ⁿ), 760.1 (y ₉ ⁿ), 773.6 (b ₉), 906.8 (y ₅ ⁿ), 958.4 (b ₅), 993.8 (y ₆ ⁿ), 1141.9 (y ₇ ⁿ), 1163.6 (b ₆), 1323.2 (y ₈ ⁿ), 1276.5 (b ₇)	
FE 200486(1–7)-OH	1294.5	121.1, 154.1, 240.1 (b ₁), 337.2 (y ₂ ⁿ), 421.2 (b ₂), 569.2 (b ₃), 639.3 (y ₃ ⁿ), 656.1 (b ₄), 726.3 (y ₄ ⁿ), 874.4 (y ₅ ⁿ), 958.4 (b ₅), 1055.4 (y ₆ ⁿ), 1163.6 (b ₆), 1251.5 (y ₇ ⁿ), 1276.5 (b ₇)	
FE 200486(1–6)-OH	1181.4	121.1, 154.1, 240.1 (b ₁), 421.2 (b ₂), 526.3 (y ₂ ⁿ), 569.2 (b ₃), 613.2 (y ₃ ⁿ), 656.1 (b ₄), 761.3 (y ₄ ⁿ), 942.3 (y ₅ ⁿ), 958.4 (b ₅), 1139.4 (y ₆ ⁿ), 1163.6 (b ₆)	
FE 200486(1–5)-OH	976.4	121.1, 154.1, 240.1 (b ₁), 321.2 (y ₁ ⁿ), 408.1 (y ₂ ⁿ), 421.2 (b ₂), 556.2 (y ₃ ⁿ), 569.2 (b ₃), 656.1 (b ₄), 737.4 (y ₄ ⁿ), 934.3 (y ₅ ⁿ), 958.4 (b ₅)	
FE 200486(1–4)-OH	674.3	121.1, 154.1, 240.1 (b ₁), 254.1 (y ₂ ⁿ), 421.2 (b ₂), 435.1 (y ₃ ⁿ), 569.2 (b ₃), 632.2 (y ₄ ⁿ), 656.1 (b ₄)	

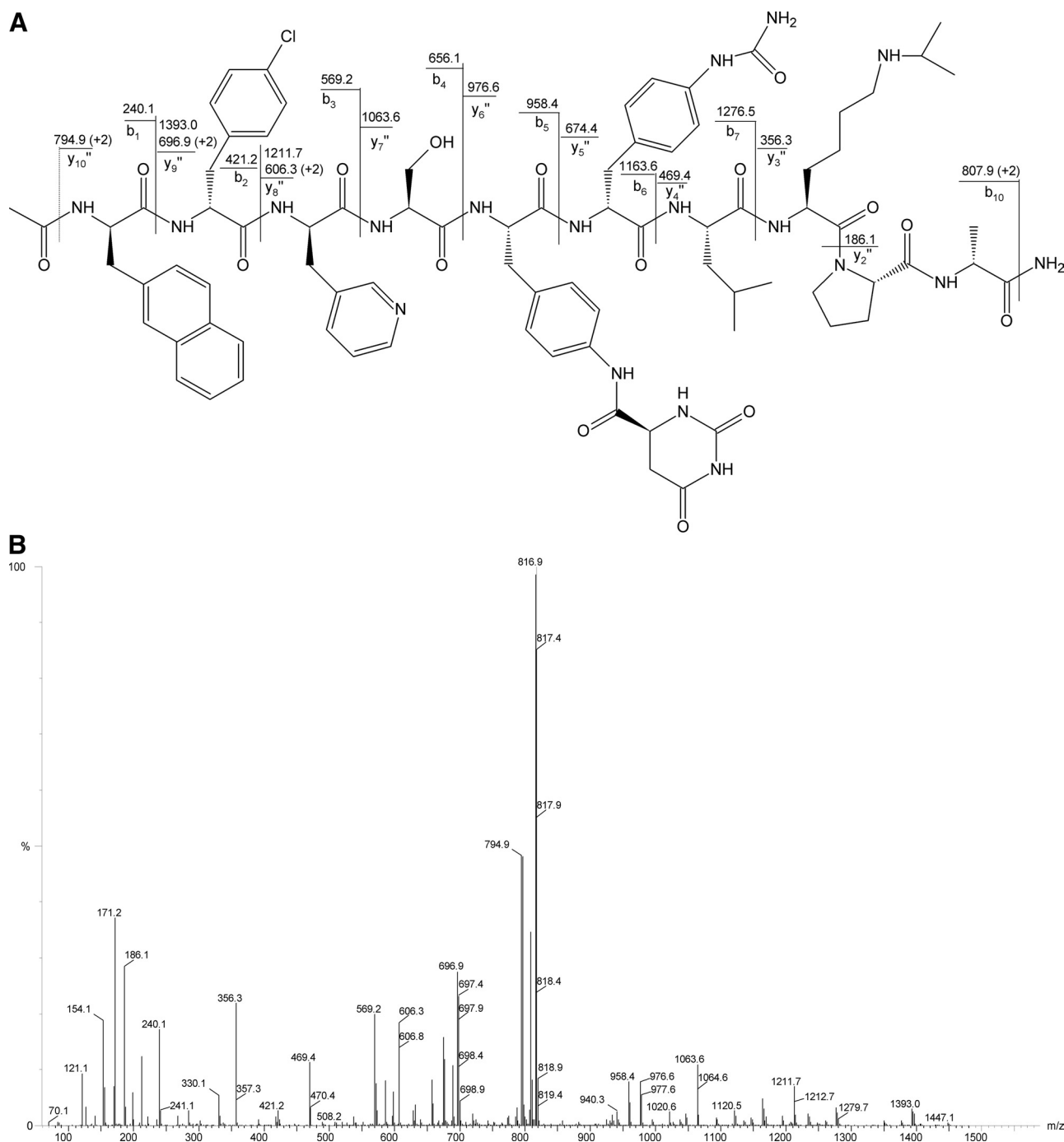


FIG. 3. Product ion spectrum of degarelix (below) with fragmentation pattern indicated (above).

metabolites plays virtually no part in the disposition of the drug in the rat.

Recovery of Radioactivity in Dogs. Beagle dogs were dosed with a single subcutaneous dose of 3 $\mu\text{g}/\text{kg}$ [^3H]degarelix. The radiolabeled compound reached a maximum in plasma after 2 h. The radioactivity was excreted rapidly, with most excreted within 48 h. The routes of excretion were via the kidneys (40–42%) and the bile (50–52%).

Metabolite Profiling in Dogs. In plasma, only the parent compound degarelix was detected in postdose plasma samples; no metabolites of degarelix were detected. In bile samples, intact degarelix and five metabolites of degarelix were detected in postdose bile samples.

The identity of the four metabolites FE 200486(1–4)-OH, FE 200486(1–6)-OH, FE 200486(1–7)-OH, and FE 200486(1–9)-OH were confirmed by retention time comparison and MS/MS product ion spectra data with synthesized compounds. The fifth metabolite was identified to be FE 200486(1–5)-OH on the basis of the product ion spectrum recorded (Table 2). The heptapeptide, the largest metabolite detected, constituted 47% of total integrated peak area, whereas the other peaks detected were smaller: the pentapeptide (26%), the hexapeptide (10%), the nonapeptide (6%), the tetrapeptide (4%), and degarelix (7%).

Recovery of Radioactivity in Monkey. Four male monkeys were dosed with a single subcutaneous dose of 8.2 $\mu\text{g}/\text{kg}$ [^3H]degarelix.

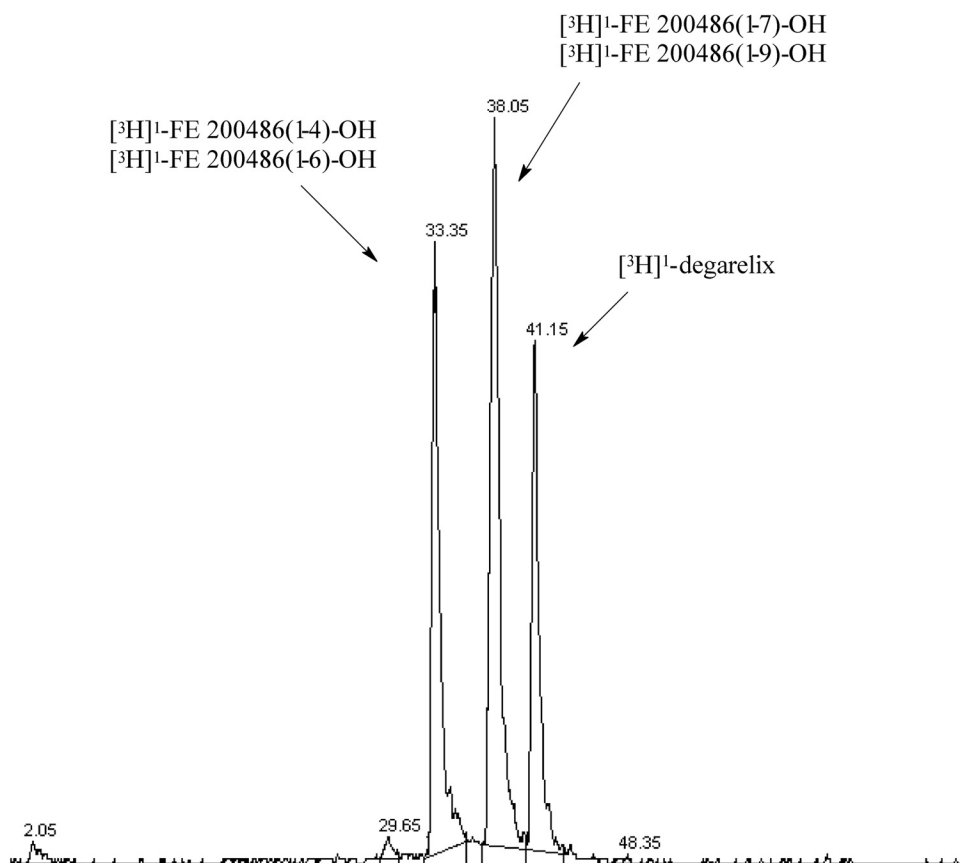


FIG. 4. Radiochromatogram from LC-RAD analysis of bile sampled between 0 and 1 h postdose from a male rat administered a subcutaneous dose of 30 $\mu\text{g}/\text{kg}$ [^3H]degarelix.

The major route of excretion was fecal, with 50% of the radioactivity recovered at 240 h. Excretion by the fecal route was slow, with only 1% excreted at 6 h postdose. This rose to 30% at 48 h postdose and 50% at 240 h postdose. Urinary excretion was lower but more rapid. At 6 h postdose, 10% of the radioactivity was excreted, rising to 20% at 240 h postdose. The mean overall recovery of radioactivity from the time of dosing up to the time of sacrifice, including excreta, tissues, and remaining carcass, was $83.3 \pm 5.9\%$ of dose.

Metabolite Profiling in Monkeys. In plasma samples, only the parent compound was detected. No metabolites of [^3H]degarelix were detected in the postdose plasma samples. Also, most of the radioactivity detected (92%) in the urine samples collected during the first 24 h after dosing was the parent compound. At least five low-abundant metabolites of [^3H]degarelix were detected in the urine samples. The major metabolite peak detected (5% of total radioactivity detected) was identified to be a coelution of the metabolites FE 200486(1-9)-OH and the FE 200486(1-10)-OH by retention time comparison with synthesized standards.

Discussion

This is the first detailed report on the nonclinical testing of degarelix that investigates the metabolic and excretion profile of the compound in numerous animal species using *in vitro* and *in vivo* approaches. On the basis of previous experiences with peptide drug candidates containing less than 10 amino acids and that the degarelix molecule contains 7 non-natural amino acids, the peptide was subjected to stability studies in liver microsomes and hepatocytes as performed for a small molecule drug. However, degarelix was found to be a very poor substrate to any degrading enzymes, e.g., P450 enzymes (Mabic et al., 1999) present in the buffered liver microsome suspensions or in hepatocyte suspensions. The minor loss of degarelix

in the rat microsomes could not be explained and was not further pursued. Similar results have been reported for cetorelix (ganirelix), a luteinizing hormone-releasing hormone receptor antagonist developed by ASTA Medica AG that has several structural similarities to degarelix. In incubations with liver fractions from rat and dog, no degradation of cetorelix was detected (Schwahn et al., 2000).

The ADME studies of degarelix performed in rat, dog, and monkey showed that degarelix and its metabolites were fully excreted via the biliary and urinary pathways in almost equal amounts. The total recovery of administered radioactivity was $>90\%$ in rat and dog. The overall recovery of radioactivity was somewhat lower than expected in the monkey; however, it was considered likely that incomplete collection of excreta from the primates accounted for most of the radioactivity not recovered.

Other peptide-based GnRH blockers have been reported to be mainly excreted by the biliary route in nonclinical studies. An ADME study of cetorelix in rats and dog (Schwahn et al., 2000) showed that rats excreted 70% of radiolabeled drug via feces and 24% into urine, whereas in dogs equal amounts were excreted into feces and urine. In bile collected from bile-duct-cannulated female rats, 70% of the radioactive dose of cetorelix administered was recovered. A disposition study of RS-26306 (ganirelix), a luteinizing hormone-releasing hormone antagonist developed by Syntex Research, showed that 16% and 55 to 69% of the radioactive dose was recovered in urine and feces, respectively, in rat and monkey (Chan et al., 1991).

Enterohepatic circulation was found to play no part in the disposition of degarelix or metabolites of degarelix in the rat. The same result has been reported for cetorelix in the rat (Schwahn et al., 2000).

The *in vivo* metabolism of degarelix was similar in all species investigated. In plasma, mainly intact degarelix was detected. Low levels of circulating metabolites were detected in plasma from rats,

whereas no circulating metabolites could be detected in plasma from dogs and monkey. Also in urine, mainly intact degarelix (>90%) was detected. Only low levels of truncated peptide metabolites were detected in the urine samples from animals. In bile and feces samples, mainly degradation products of degarelix (i.e., truncated peptides) were detected. In rat and dog, the tetrapeptide, the pentapeptide, and the heptapeptide metabolites of degarelix were found to represent the major part of the radioactivity excreted in feces.

Similar metabolite profiles have been reported for rats and dogs receiving cetorelix or RS-26306 (Chan et al., 1991; Schwahn et al., 2000). For both compounds, plasma and urine samples were found to mainly contain intact parent, and in bile and feces samples the four main metabolites were truncated peptides of the parent decapeptide. The metabolites detected were (1–4), (1–6), (1–7), and (1–9) for cetorelix (Schwahn et al., 2000) and the (1–4), (1–6), and (1–7) form of RS-26306 (Chan et al., 1991), i.e., the same size truncated peptides of degarelix found in bile from *in vivo* rat and dog studies in this study.

The *in vivo* excreta results reported in this study show that degarelix is mainly excreted unchanged via the urine and is subject to extensive sequential peptidic degradation during its elimination via the hepatobiliary pathway. The results are in line with data for other compounds of similar size and structure as degarelix (Chan et al., 1991; Schwahn et al., 2000) and in line with the fact that compounds of this size (molecular weight 1632) are more likely to be eliminated by the hepatobiliary pathway (Sahi, 2005). Peptidases that play an important role in the metabolism of several other peptide drugs (Lin,

2009; Malm-Erjefält et al., 2010) do not degrade degarelix in the blood circulation.

Seven of the 10 amino acids in degarelix are unnatural; despite this, proteolytic fragments from cleavage at most positions were found with two notable exceptions. Proteolysis of the very unnatural all *D* and lipophilic (1–3) partial sequence does not appear to have occurred. In addition, no products of oxidative metabolism of the (1–4) fragment were observed, suggesting that it may not be a substrate of the P450 enzymes. The fragment by cleavage between Ile⁸ and Pro⁹ was not observed either, suggesting that none of the proteases that came into contact with degarelix were able to productively bind the partial sequence around these amino acids. It is noteworthy that the (1–5) and (1–6) fragments were found in high proportion in the bile, urine, or feces of several species, which would require proteases capable of accommodating in their S₁ pocket a bulky side chain (Aph(Hydroxyrotyl)⁵) or a *D*-amino acid (*D*-Aph(Cbm)⁶). Because all mass spectrometry data collected were screened for the ions representing all theoretical cleavages of the peptide backbone, we most likely can rule out that other proteolytic cleavages should have occurred to a great extent because they were not detected by LC-MS. No other metabolites of degarelix than the truncated peptide metabolites reported above were detected when the LC-MS data were evaluated using the ACD/Labs compare MS data set software function.

The metabolite FE 200486(1–10)-OH was only detected in feces. FE 200486(1–10)-OH was not detected in the bile of rats. The metabolite might have been formed by two possible mechanisms:

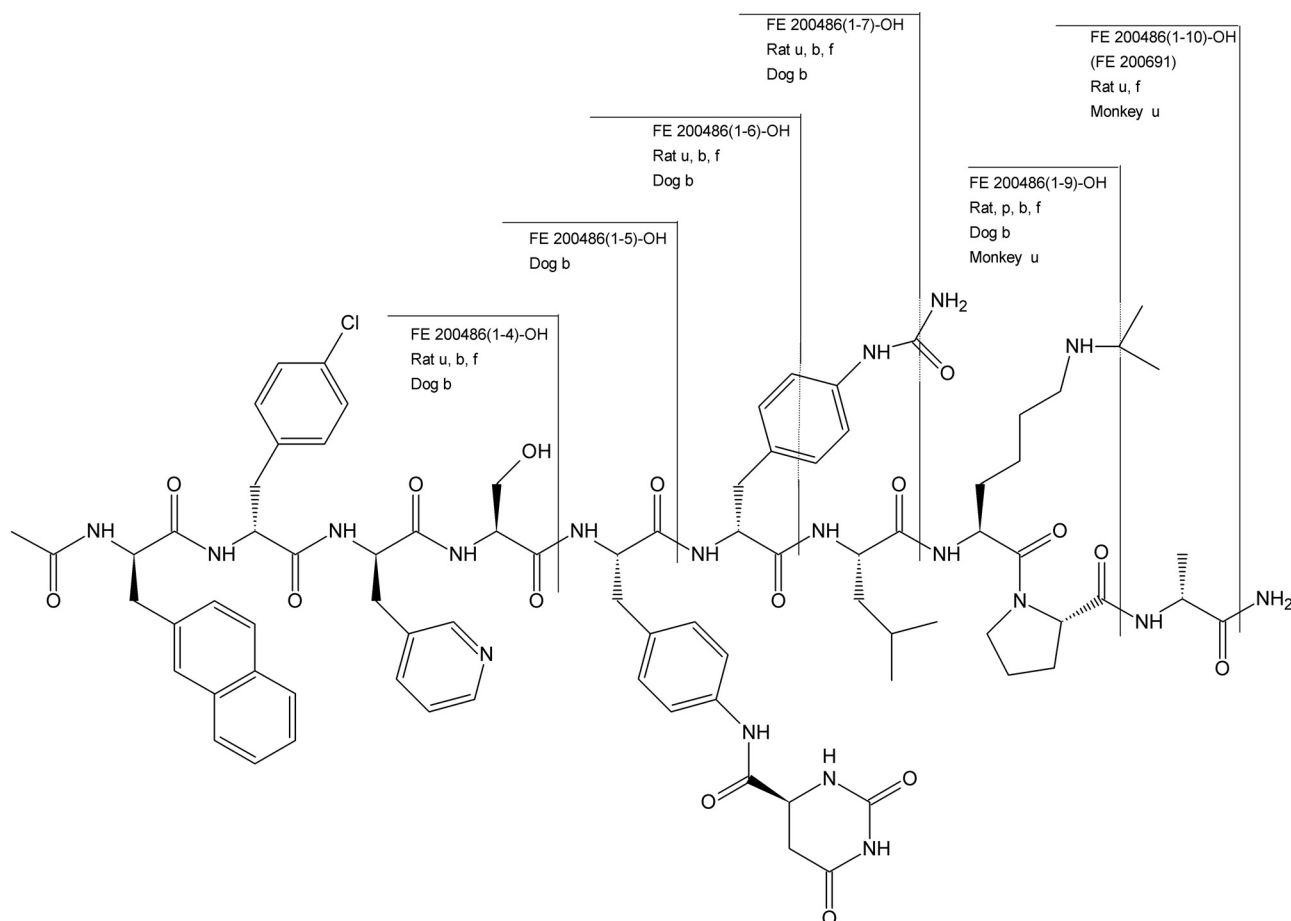


FIG. 5. Outline of the *in vivo* metabolism of degarelix based on animal data. b, bile; f, feces; p, plasma; u, urine, matrix.

proteases present in the duodenum and jejunum of the rat, or bacteria in the large intestine (cecum/colon) of the rats (Langguth et al., 1994). However, incubations of degarelix with rat jejunal juices or with colonic contents/feces have not been performed to confirm if any of these mechanisms is responsible for the formation of FE 200486(1–10)-OH.

As outlined in the summary figure (Fig. 5), degarelix is subject to proteolysis by endopeptidases, and unchanged degarelix and metabolites are fully excreted via the hepatic and urinary pathway. Systemic exposure to any metabolic products seems to be low.

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Authorship Contributions

Participated in research design: Sonesson, Koechling, and Buur Rasmussen.

Conducted experiments: Sonesson.

Contributed new reagents or analytic tools: Stalewski.

Performed data analysis: Sonesson and Buur Rasmussen.

Wrote or contributed to the writing of the manuscript: Sonesson, Koechling, Stalewski, Tankó, and Buur Rasmussen.

Other: Tankó.

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