Determination of the Tissue Distribution and Excretion of ^{14}C-Fertirelin Acetate in Lactating Goats and Cows

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Fertirelin acetate is a synthetic luteinizing hormone-releasing hormone (LH-RH) analogue that is used in the treatment of cystic ovaries and for enhanced conception rates in lactating dairy cows. To satisfy human food safety issues, the concentration of the compound and its metabolites in the edible tissues of the cow must be determined. Because the study required the use of a radiolabeled compound, the release of $^{14}CO_2$ was a possibility. The goat was used as a model to determine the extent of the release of $^{14}CO_2$. In the dairy cow, intramuscularly administered ^{14}C-fertirelin acetate was rapidly absorbed, with a plasma t_{max} of 0.5 h, and was rapidly eliminated, dropping below 0.14 ng/mL within 4 h posttreatment. Analysis of the tissues revealed concentrations of ^{14}C-fertirelin acetate residue equivalents in the injection site at 12 h posttreatment ranging from <0.5 to 4 ng/g. The concentrations in the kidney were ≤0.6 ng/g. All other tissues were below the sensitivity of the method of 0.5 ng/g. The residue in the milk at 12 h varied between 1.2 and 1.7 ng/mL, and none of the residue in the milk was found to be parent compound at a concentration greater than the limit of detection (0.2 ng/mL) of the HPLC method used.

Keywords: *Tissue distribution; excretion; fertirelin acetate; ^{14}C-fertirelin acetate; goats; cows*

INTRODUCTION

Fertirelin acetate (Figure 1) is a synthetic analogue of luteinizing hormone-releasing hormone (LH-RH) that has been shown to have 5 times the biological activity of the natural hormone (Rippel et al., 1973; Fujino et al., 1973). It is being used for the treatment of reproduction disorders in cattle such as cystic ovaries in lactating dairy cows (Mori et al., 1974; Yamauchi et al., 1976). Studies conducted in the rat using the natural hormone, LH-RH, radiolabeled with ^{14}C show a rapid metabolism of the carbon skeleton to $^{14}CO_2$ (Takahashi et al., 1974). Prior to a residue depletion study being conducted in the cow, a preliminary ^{14}C-fertirelin acetate distribution study was conducted in the goat at an elevated dose to determine the rate and extent of $^{14}CO_2$ release in expired air in a ruminant animal and to establish the validity of the procedures to be used in the cow study.

To satisfy human food safety issues (Food and Drug Administration, 1994), a residue depletion study was completed and the concentration of the compound and its major metabolites in the edible tissues and milk of the cow were determined. The ^{14}C-fertirelin acetate cow residue depletion study was designed on the basis of the results of the preliminary goat study described above. The results of both studies are reported here.

EXPERIMENTAL PROCEDURES

Apparatus. The goats were housed in a subchronic inhalation chamber made of stainless steel and glass (Hornish et al., 1987). The 1 cm^3 chamber has two doors with access ports in them and is connected to a similar smaller chamber. Access between the chambers was allowed by additional port holes. Airtight seals secured the chambers. The floor of the chamber was fitted with a rubber-coated metal grating held off the floor

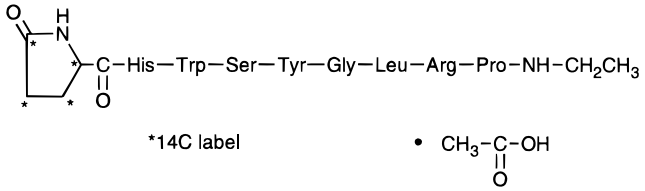

Figure 1. Chemical structure of ^{14}C-fertirelin acetate.

by rubber stoppers. The chamber air flow was maintained at 25−35 L/min, ∼4−6 times the resting respiratory rate of goats (National Academy of Sciences, 1958). The air supply to the chamber was from an in-house air compressor and was passed through a silica gel dryer. The chamber air was exhausted through a series of CO_2 traps, air filters, and valve systems via a rotary exhaust pump. The air supply and exhaust were regulated so that the chamber atmospheric pressure was slightly negative (−1 cm water) relative to ambient atmospheric pressure. A schematic representation of the chamber is provided in Figure 2. The CO_2 traps consisted of two 9.5 L Pyrex bottles modified to accept a No. 15 rubber stopper. The rubber stopper was fitted with a gas dispersion device made from five coarse frits cut from 30 mL fritted disk Büchner funnels and connected to a 480 mm × 7 mm i.d. heavy wall glass tube. The two bottles were filled with 7.0 L of 1.5 N NaOH trapping solution and were connected in series. A 6-in. 13-gauge needle was inserted through the rubber stopper and a $^1/_8$-in. diameter Teflon tube was connected to reach the bottom of the trap. A three-way Teflon valve was placed on the hub of the needle and was used for sampling of the trapping solution.

Materials. The ^{14}C-fertirelin acetate (MW = 1213.36) with a specific activity of 274.0 mCi/mmol (8.35 Bq/ng) was prepared by NEN-DuPont, Boston, MA, by the addition of *N*-hydroxy-5-norbornene-2,3-dicarboximide and *N*,*N*′-dicyclohexylcarbodimide to uniformly ring labeled (^{14}C) pyroglutamic acid followed by the addition of despyroglutamyl fertirelin acetate. The structure of the final product is shown in Figure 1.

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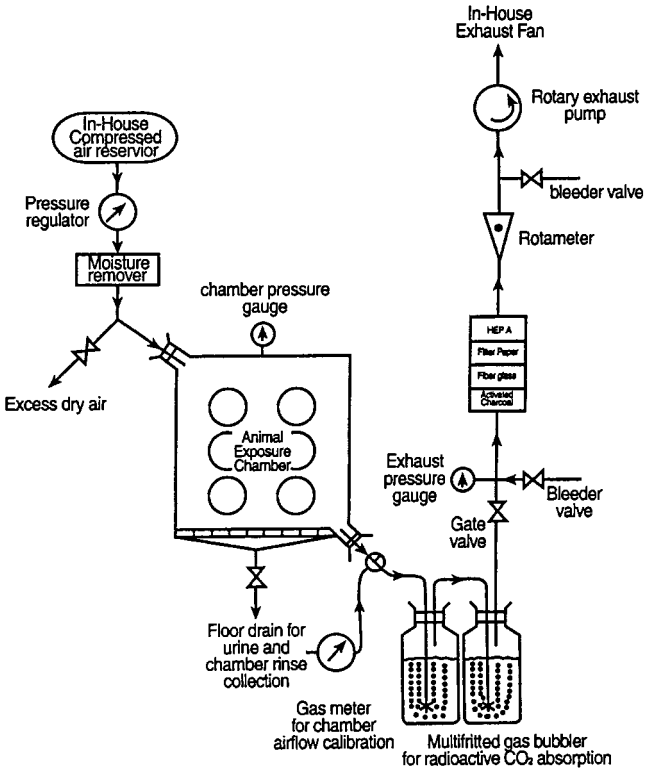

Figure 2. Schematic diagram of the system for trapping expiratory $^{14}CO_2$ from large animals.

Animal Husbandry—Goat. Two lactating female La Mancha goats ~2 years of age weighing between 30 and 45 kg were housed individually in the metabolism chamber shown in Figure 2. The first goat was not acclimated in the chamber before treatment; the second goat was acclimated for 24 h in the chamber before treatment. The goats were hand-milked every 12 h and were provided both standard pelleted sheep feed and water ad libitum.

Animal Husbandry—Cow. Six lactating Holstein dairy cows ~3 years of age and in their second or third lactation weighing between 470 and 650 kg were acclimated in metabolism stalls 3 days prior to dosing. The cows were milked using automatic milkers every 12 h during acclimation and while on treatment. The cows were provided corn silage, grain, and water ad libitum.

Animal Dosing—Goat. The goats were placed in the metabolism chamber (the first goat immediately prior to dose administration and the second goat 24 h prior to dose administration). Each goat was dosed by intramuscular (IM) injection with 2 mL of a 75 μg/mL aqueous solution of ^{14}C-fertirelin acetate (3−5 μg/kg of body weight—15× the expected use dose) previously sterilized by passage through a 0.2 μm filter. The dose was administered in the shaved gluteal region of the goat. The chamber was immediately sealed to prevent loss of expired $^{14}CO_2$.

Animal Dosing—Cow. The cows were dosed by IM injection with 3 mL of a 50 μg/mL aqueous solution of ^{14}C-fertirelin acetate (0.2−0.3 μg/kg of bodyweight—1.5× the expected use dose) previously sterilized by passage though a 0.2 μm filter. The dose was administered in the shaved gluteal region of the cow, and the location of the injection site was marked with spray paint. Immediately after injection, all personnel entering the area in which the animals were housed wore self-contained breathing apparatus to prevent the inhalation of expired $^{14}CO_2$.

Sample Collection and Analysis—Goat. During all sample collections, after dosing, and prior to euthanasia of the animals, the animal handlers wore self-contained breathing apparatus to protect against contamination from $^{14}CO_2$. The goats were euthanatized by captive bolt stunning followed by exsanguination according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Urine, feces, and cage wash were collected as a composite sample from both animals at 24 h intervals until euthanasia at 24 h in goat 001 and at 48 h in goat 002. Subsamples were weighed into Combusto cones, dried, combusted to $^{14}CO_2$, and trapped using a Packard B-306 sample oxidizer. Milk samples were collected by hand every 12 h until the animals were euthanatized. The samples were analyzed directly by standard scintillation counting techniques (1 mL of sample to 15 mL of Packard Instagel emulsifier). The CO_2 traps were sampled at 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 24 h for goats 001 and 002 and additionally at 32, 40, and 48 h for goat 002. The samples were analyzed directly by scintillation counting techniques. At euthanasia of the animals, representative samples of muscle and fat, the entire kidney, the entire liver, and the injection site were ground to a uniform consistency using a meat grinder. A representative sample of each tissue was combusted as described above for the excreta.

Sample Collection and Analysis—Cow. During all sample collections, after dosing, and prior to euthanasia of the animals, the animal handlers wore self-contained breathing apparatus to protect against contamination from $^{14}CO_2$. The cows were euthanatized by captive bolt stunning followed by exsanguination according to the AAALAC guidelines. Feces were collected from a tray inserted in the floor of the metabolism stall. The feces and urine were collected at 0 h (pretreatment) and at 12 h posttreatment (just prior to euthanasia). The feces samples were combusted as described above for the goat. Urine was collected as a runoff from the trays in the metabolism stall. Milk was collected by an automatic milking machine at 0 h (pretreatment) and 12 h posttreatment. The urine and milk samples were analyzed by standard scintillation counting techniques. Blood was collected from the jugular vein of each cow at 0 (pretreatment), 0.25, 0.5, 1, 2, 4, 8, and 12 h posttreatment. Representative samples of muscle and fat, the entire liver, the entire kidney, and the injection site (500 g) were collected from each cow. The tissues were ground uniformly using a meat grinder. Representative samples of each tissue and blood were analyzed by standard combustion techniques as described above.

Milk HPLC—Radioactive Monitor (RAM) Analysis. In addition to the analysis of the milk by direct scintillation counting, the milk from both the goats and cows was analyzed for parent ^{14}C-fertirelin by HPLC equipped with a RAM. A brief description of the procedure used follows: A 50 mL volume of milk was treated with 50 mL of an aqueous solution of acid (FTSH) consisting of 10% formic acid, 30% trifluoroacetic acid (TFA), 20 g/L NaCl, and 2 M HCl. The mixture was centrifuged (1000*g*), and the aqueous layer between the fat and precipitate was removed and centrifuged a second time. The supernatant was placed on a solid-phase extraction (SPE) cartridge (Bond Elut, C18, 6 cm^3, Analytichem International), and the cartridge was washed with 5 mL of a 50% FTSH solution and then with 5 mL of 10% acetonitrile in water. The compound was eluted with 30 mL of 40% acetonitrile in water. The eluant was evaporated to dryness and taken up in 2 mL of 0.1% TFA and loaded on a 20 cm × 1 cm desalting gel filtration column (Excellulose, 5000 Da exclusion, 40−100 μm GF-5 desalting gel, Pierce Chemical Co.). The column was eluted with 150 mL of 0.1% TFA. The first 20 mL of eluant was discarded, and the next 40 mL fraction was collected. The pH of the solution was adjusted to between 7 and 9.5 with 1.0 N NH_4OH and then evaporated to dryness. The residues were redissolved in 2 mL of 0.1% TFA, and 0.5 mL was injected on an HPLC equipped with a Bakerbond widepore octyl (C-8) 5 μm, 10 cm × 4.6 mm column. A gradient consisting of 0.1% TFA in water (mobile phase A) versus 0.1% TFA in acetonitrile (mobile phase B) was run at 1 mL/min. The gradient was as follows: 85% A and 15% B at time 0, 80% A and 20% B at 20 min (0.25%/min), and 40% A and 60% B at 30 min (4%/min). The column was connected to a Radiomatic model HP radioactive monitor (Radiomatic, Inc). The limit of detection (LOD) of the system for the radiolabeled compound was 0.2 ng of fertirelin acetate equivalents/mL of milk.

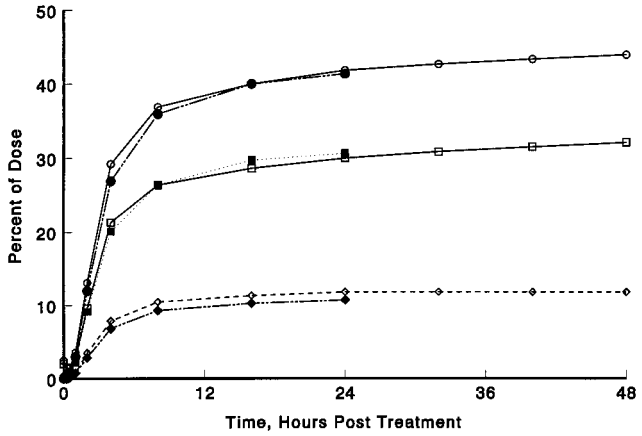

Figure 3. $^{14}CO_2$ expired from goats treated with ^{14}C-fertirelin acetate: (■) goat 1, first trap; (◆) goat 1, second trap; (●) goat 1, total; (□) goat 2, first trap; (◇) goat 2, second trap; (○) goat 2, total.

Table 1. Accountability for ^{14}C-Fertirelin Acetate in IM-Treated Animals (Percent of Dose)

animal	$^{14}CO_2$	milk	urine	feces	excreta[a]	total
goat						
001	41.4	9.5	*a*	*a*	0.3[b]	51.2
002	44.0	11.1	*a*	*a*	27.5	82.6
cow						
66	nc[c]	7.9	nc	0.9	0.9	46.8[d]
67	nc	8.2	35.0	2.9	37.9	84.1[d]
73	nc	8.4	6.5	18.6	25.1	71.5[d]
74	nc	5.7	7.8	17.4	25.2	68.9[d]
75	nc	8.1	18.5	5.2	23.7	69.8[d]
76	nc	6.8	27.0	6.2	33.9	78.7[d]

[a] Urine, feces, and cage wash collected as a composite and considered excreta. [b] Limited urine and feces excreted. [c] nc, not collected. [d] Assuming the amount of $^{14}CO_2$ expired in the cow is equivalent to 38% (estimate of the goat at 12 h posttreatment).

RESULTS AND DISCUSSION

Appropriateness of Radiolabel. Fertirelin acetate was uniformly labeled with ^{14}C in the pyroglutamyl ring. The LH-RH labeled in that position is known to undergo metabolism with subsequent release of $^{14}CO_2$ in the rat (Takahashi et al., 1974). Loss of the pyroglutamyl ring has been shown to cause the loss of the peptide's hormonal activity (Schally et al., 1972) and, therefore, the radiolabel in this position is appropriate for metabolism studies.

Accountability. In the goat study, the cumulative amount of expired $^{14}CO_2$ is shown graphically in Figure 3. Greater than 40% of the dose for both goats was recovered in the trapping solutions. In the cow study, the expired $^{14}CO_2$ was not collected. The estimate of $^{14}CO_2$ expired in the cow can be based upon the percent expired in the goat at 12 h (38%), but caution should be used because the dose in the goat was 10 times that in the cow on a milligram per kilogram basis. Using the estimate of 38% of the dose expired as $^{14}CO_2$ for the cow provided similar accountabilities for both studies as shown in Table 1. The overall accountability was reasonable (70−80%) for large animals studies but somewhat lower than expected. Tissue residue concentrations were not taken into account in estimating accountability primarily because these concentrations were extremely low and in most instances were below the LOD of the method used. Because of the low dose concentration and the large mass of the animal, small (unmeasurable) concentrations of the residue could have remained in the carcass, producing lower than expected recovery.

Table 2. ^{14}C-Fertirelin Acetate Milk and Tissue Concentrations (Nanograms per Gram)[a]

animal	milk (12 h)	kidney	liver	muscle	fat	injection site
goat						
001[b]	17.1	4.27	0.98	<0.5	<0.5	1.09
002[c]	17.0	3.96	0.83	<0.5	<0.5	<0.5
cow[d]						
66	1.23	0.50	<0.50	<0.50	< 0.50	<0.50
67	1.34	0.51	<0.50	<0.50	<0.50	<0.50
73	1.26	0.61	<0.50	<0.50	<0.50	4.01
74	1.23	<0.50	<0.50	<0.50	<0.50	0.67
75	1.66	0.50	<0.50	<0.50	<0.50	<0.50
76	1.15	0.58	<0.50	<0.50	<0.50	2.03

[a] LOD = 0.5 ng/g for tissue and 0.1 ng/mL for milk. [b] Goat euthanatized at 24 h. [c] Goat euthanatized at 48 h. [d] Cows euthanatized at 12 h.

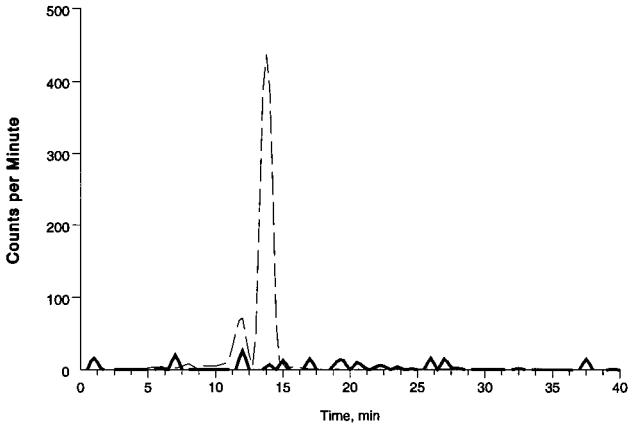

Figure 4. HPLC milk extract profile from a 0 h ^{14}C-fertirelin acetate fortified pretreatment milk sample and a 12 h posttreatment milk sample from a ^{14}C-fertirelin acetate treated goat: (- - -) ^{14}C-fertirelin acetate fortified 0 h pretreatment milk sample; (heavy solid line) 12 h posttreatment milk sample from treated goat.

Milk and Tissue Distribution. The concentrations of total residues in the edible tissues are presented in Table 2. The concentrations of total residue found in the kidney of the goat relative to the cow are approximately proportional to the 10-fold higher dose rate of fertirelin acetate administered to the goat. The same may be true for the liver as well; however, in the cow study, the liver residue concentrations dropped below the LOD of the assay. The concentrations in the injection sites were similar and reflect a dependency on dose amount rather than animal size and dose rate. No differences were observed for muscle and fat, which suggests that the distribution of fertirelin into the tissues is low.

As in the kidney, the concentration of total residue found in the milk of the goat relative to the cow (Table 2) is approximately proportional to the 10-fold higher dose rate of fertirelin acetate. The milk from both the cow and goat was analyzed for parent fertirelin acetate by an HPLC-RAM procedure. Figure 4 shows the results of the analysis of a pretreatment milk sample fortified with ^{14}C-fertirelin acetate along with the 12 h posttreatment milk sample from the goat. Figure 5 shows the results of the analysis of a pretreatment milk sample fortified with ^{14}C-fertirelin acetate along with the 12 h posttreatment milk sample from the cow. In both cases, the fortified samples showed a peak consis-

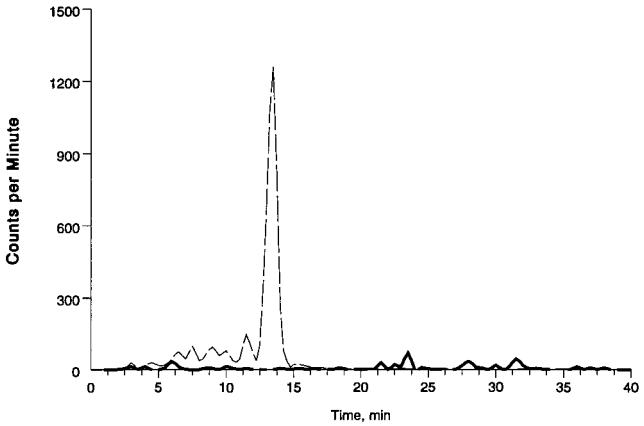

Figure 5. HPLC milk extract profile from a 0 h ^{14}C-fertirelin acetate fortified pretreatment milk sample and a 12 h posttreatment milk sample from a ^{14}C-fertirelin acetate treated cow: (- - -) ^{14}C-fertirelin acetate fortified 0 h pretreatment milk sample; (heavy solid line) 12 h posttreatment milk sample from treated cow.

Table 3. Blood Concentration of ^{14}C-Fertirelin Acetate Residues in the Lactating Dairy Cow (Nanograms per Milliliter)[a]

	0 h	0.25 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h	12.0 h
mean	<0.14	0.31	0.40	0.30	0.21	<0.14	<0.14	<0.14
SD[b]		0.14	0.08	0.06	0.07			

[a] LOD = 0.14 ng/mL. [b] SD, standard deviation.

tent with fertirelin acetate at ~14 min, the retention time for the fertirelin acetate standard, whereas in the 12 h posttreatment sample only minor peaks (near background) were seen and no discernible peak was observed at 14 min. All of the remaining posttreatment milk samples analyzed showed no significant radioactive peak at the retention time of fertirelin acetate (13−14 min). Recovery of the fortified samples was between 45 and 55%. At this recovery rate, the method would be able to detect 0.2 ng/mL of parent fertirelin acetate in the treated milk had it been present.

Very little of the radioactivity that was present in the milk was observed in the chromatogram. Most of what was present was eliminated in the sample workup during the protein precipitation step. This would suggest that the residue which was present in the milk was not intact parent fertirelin acetate and therefore would have no biological activity.

Blood Concentration. Blood samples were not taken in the goat study. In the cow study, analysis of the blood samples revealed (Table 3) that measurable concentrations of ^{14}C-fertirelin acetate equivalents occurred out to only 2 h. After that time point (4 h and beyond), the concentration of ^{14}C-fertirelin acetate equivalents was below the LOD (0.14 ng/mL) of the combustion technique used.

With only four data points per cow, it was not possible to accurately calculate pharmacokinetic parameters for the cow. However, the results of the blood analysis do indicate that ^{14}C-fertirelin acetate is rapidly absorbed, with a t_{max} of ~0.5 h. The compound is also rapidly eliminated, with concentrations in the blood reaching levels below the LOD of the assay (0.14 ng/mL) within 4 h posttreatment.

Conclusions. In the goats treated with 150 μg of ^{14}C-fertirelin acetate by IM injection, >40% of the dose was excreted as $^{14}CO_2$ and represented the primary route of excretion. The total residue concentration in the milk was 17 ng/mL at 12 h posttreatment, and none of the residue in the milk was found to be parent compound at a concentration greater than the LOD (0.2 ng/mL) of the HPLC-RAM method used. The highest tissue concentration was in the kidney (4 ng/g), followed by the liver (0.8−1 ng/g) and the injection site (0.5−1 ng/g). The remaining tissue concentrations (muscle and fat) were <0.5 ng/g.

In the dairy cow treated with 150 μg of ^{14}C-fertirelin acetate by IM injection, the data suggest that the drug is rapidly absorbed, with a t_{max} of 0.5 h, and is rapidly eliminated, with blood concentration reaching the LOD of the assay (0.14 ng/mL) within 4 h posttreatment. The concentration of ^{14}C-fertirelin acetate residue equivalents in the injection site at 12 h ranged from <0.5 to 4 ng/g, whereas the concentrations in the kidney were ≤0.6 ng/g. All other tissues were below the LOD of the method of 0.5 ng/g.

The residue in the milk of the cow at 12 h varied between 1.2 and 1.7 ng/mL, and none of the residue in the milk was found to be parent compound at a concentration >0.2 ng/mL.

Fertirelin acetate has been approved for use by the Working Party on the Safety of Residues of the Committee for Veterinary Medicinal Products (CVMP) under annex II and does not require a tolerance level or maximum residue limit (MRL) to be set. It is a natural product consisting of amino acids and is subject to degradation by the proteases present in the human gut. Because of this degradation, fertirelin acetate is 20 000 times less active when administered orally to the rat as compared to intravenously or intramuscularly. The low milk and tissue residues observed in this study coupled with its low oral activity and low toxicity demonstrate that fertirelin acetate when properly used in the cow will not present human food safety concerns.

ABBREVIATIONS USED

IM, intramuscular; i.d., inside diameter; RAM, radioactive monitor; TFA, trifluoroacetic acid; FTSH, 10% formic acid, 30% trifluoroacetic acid, 20 g/L NaCl, and 2 M HCl; LOD, limit of detection; AAALAC, Association for the Assessment and Accreditation of Laboratory Animal Care.

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