Residue Determination of 9α-Fluoroprednisolone Acetate and Its Metabolite 9α-Fluoroprednisolone in Bovine Tissues

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A method for the determination of 9α-fluoroprednisolone acetate (FΔFAc) and 9α-fluoroprednisolone (FΔF) in bovine milk, blood serum, fat, kidney, liver, and muscle has been developed which removes, through several partitions and a column chromatography step, most of the interfering materials before injection of the sample onto a high-pressure liquid chromatograph (lc) for FΔFAc and a gas–liquid chromatograph (glc) for FΔF. Observed recovery ± estimated standard deviation for FΔFAc was 97 ± 6% by lc and 99 ± 8% for FΔF by electron capture/gas–liquid chromatography in bovine tissue assayed over the 5–20-ppb range. Twenty-four ruminating beef heifer calves injected with 20 mg of FΔFAc (experimental formulation of S.A.S. Predef 2× (registered trademark of The Upjohn Co.)) for 3 consecutive days and slaughtered at 0, 1, 3, 5, 7, 10, and 13 days posttreatment showed that no residues were present 7 days after the last injection in muscle, liver, fat, kidney, and blood serum and 13 days after the last injection for the injection site. Milk from lactating cows injected with 20 mg of FΔFAc (experimental formulation of S.A.S. Predef 2×) at three consecutive 12-hr intervals showed that milk was free of detectable residues.

9α-Fluoroprednisolone acetate (1-dehydro-9α-fluorohydrocortisone acetate, or Predef, FΔFAc) is a potent corticosteroid with greater glucocorticoid activity than an equal quantity of prednisolone. Our preliminary work in rabbit muscle had shown that FΔFAc was partially hydrolyzed to the alcohol FΔF. Consequently, its use in bovine animals required a method that would detect both FΔFAc and FΔF in tissues with a minimum of interference from sample background. Our experience indicated that both steroids could be detected in tissue extracts by liquid chromatographic (lc) techniques, but that the retention time area for FΔF was masked by an unresolved contaminant. With electron capture–glc techniques, only FΔF could be detected; FΔFAc failed to appear on the chromatogram. Therefore, it seemed appropriate to analyze FΔF by glc and FΔFAc by lc. This paper describes the necessary cleanup steps for the assay of FΔFAc and FΔF in milk, blood serum, fat, muscle, liver, and kidney.

EXPERIMENTAL SECTION

Milk Residue Study. Five lactating Holstein cows were injected with a 1× dose of 20 mg of FΔFAc (10 ml of the experimental S.A.S. Predef 2×, lot No. 40,077) at 12-hr intervals. Each injection was given prior to the milking and designated −24, −12, and 0 hr posttreatment. Zero designated the start of the posttreatment sample collection after the third injection. Each cow was milked twice a day, the production noted, and three 50-g aliquots frozen in 1-pint polyethylene freezer cartons. All samples were stored in the deep freeze until assayed (less than 6 weeks).

Tissue Residue Study. Twenty-four ruminating beef heifer calves were weighed and housed three to a box stall. Feed and water were provided *ad libitum*. The diet was a coarse-ground complete feed with 50% roughage (Veterinary Research Experimental Diet D-78).

Seven groups (three calves/group) were randomly assigned for slaughter at 0, 1, 3, 5, 7, 10, and 13 days after final treatment. An eighth group of three calves served as the control. The calves were injected with a 1× daily dose of 20 mg of FΔFAc (10 ml of the experimental S.A.S. Predef 2×, lot No. 10973-TWY-36) for 3 days. The first two intramuscular injections were given in the left cervical area. The third injection was given in the left gluteal muscle after the injection site was shaved, cleansed, and marked with a deep suture which penetrated to subdermal muscle layer. The third injection was made adjacent to the suture knot.

Animals were slaughtered in an abattoir. Blood samples were collected in 250-ml centrifuge bottles from the severed superior vena cava. The injection site was removed as a core of muscle approximately 4 in. × 4 in. o.d. A similar sample was collected from the contralateral gluteal muscle (noninjection site) and designated for assay as the muscle tissue from a treated animal. Liver, kidney, fat, and muscle samples were chilled immediately after collection; blood was allowed to clot overnight at ambient temperature.

All samples were ground in a small hand grinder and hand mixed and two 50-g aliquots were weighed into pint freezer cartons. One pint of the surplus ground sample was held in the freezer as a reserve. All ground samples were frozen immediately. The blood was centrifuged for 20 min at 2000 rpm the morning after the collection, the serum was decanted, and two 50-g aliquots were weighed out into pint freezer cartons. The surplus serum was held in the deep freeze as a reserve.

Apparatus used included: Waring Blendor Model 702B, refitted with polyethylene bowl gaskets cut from polyethylene freezer containers; Lourdes homogenizer with 300-ml capacity stainless steel cups; A DuPont 820 liquid chromatograph, equipped with a precision spectrophotometer (sensitivity of 0.005 absorbance units, full scale deflection at 254 nm) and a 1 m × 2.1 mm i.d. hydrocarbon polymer column (HCP, DuPont); rotary evaporation unit or equivalent equipment (Gosline *et al.*, 1969); an F&M 402 gas chromatograph equipped with a 2-ft glass column packed with 1% OV-17 (100–120 mesh Gas Chrom Q), a Ni-63 detector, and a 1-mV recorder.

Reagents and Solutions. Experimental S.A.S. Predef 2× (9α-fluoroprednisolone acetate) aqueous suspension (2 mg/ml) and 9α-fluoroprednisolone acetate and 9α-fluoroprednisolone standards (99.5% purity, The Upjohn Co.) were used.

Solvents used were: ethylene glycol monoethyl ether (Fisher Scientific Co., redistilled in the laboratory prior to use for glc); Florisil 60–100 mesh, 1225–1250° activated (Floridin Co.); dried at 130° for at least 2 days prior to use. Longer drying is not detrimental to its properties and storage of the Florisil in the oven is recommended.

Extraction Procedure for Milk and Blood Serum (Figure 1). A 50-g frozen sample was placed into a Waring Blendor with 50 ml of deionized water and 200 ml of ethyl acetate and allowed to blend for 1 min at low speed

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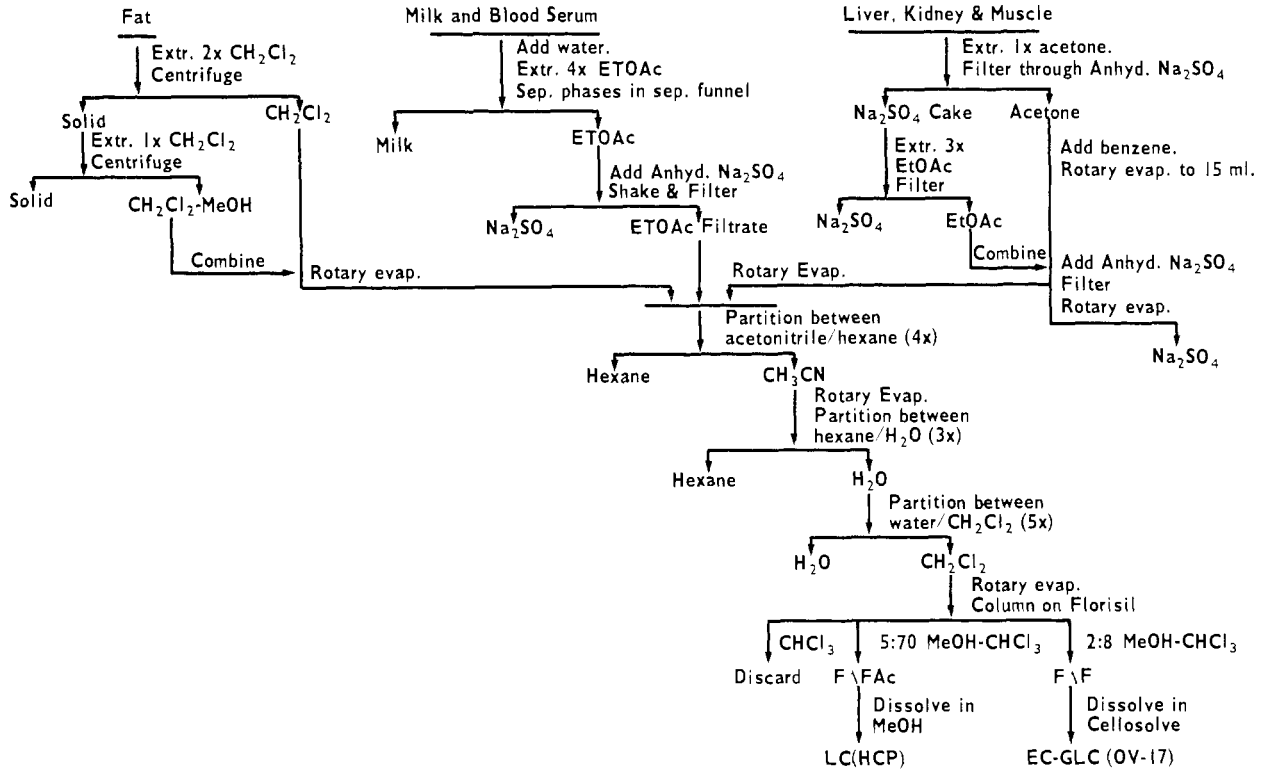

Figure 1. Flow diagram for cleanup steps.

until slushy. Then 200 ml of additional ethyl acetate was added and blended for 5 min at low speed. The homogenate was poured into a 1-l. separatory funnel. The sample was drained into the Waring Blendor bowl and the ethyl acetate was drained into a 1-l. round-bottomed flask. The sample was reextracted three times with 250 ml of ethyl acetate. The ethyl acetate extractions were combined, 100 g of anhydrous sodium sulfate was added, and the mixture was vigorously shaken for 1 min and filtered through a medium porosity sintered glass funnel. The cake was washed with 75 ml of ethyl acetate and the solvent rotary evaporated.

The residue was partitioned between 100 ml of acetonitrile and 200 ml of hexane in a 500-ml separatory funnel. *All partitions here and throughout the method were shaken vigorously for 1 min and allowed to stand for 15–30 min.* The acetonitrile layer was drained into a 1-l. round-bottomed flask. The hexane was extracted three times with 100 ml of acetonitrile and the combined acetonitrile phases were rotary evaporated. The hexane–water partition steps were followed next.

Extraction Procedure for Liver, Kidney, and Muscle (Figure 1). Frozen tissue (50 g) was placed into the Lourdes cup with 150 ml of acetone and allowed to blend for 3 min at ice temperature. A 50-g layer of anhydrous sodium sulfate was prepared on a sintered glass funnel. The mixture was filtered with vacuum, the filtrate transferred into a 1-l. flask, 25–50 ml of benzene added, and the mixture carefully rotary evaporated to a 10–15-ml volume. The entire filter cake was removed and reextracted three times with 150 ml of ethyl acetate. The ethyl acetate extraction was combined with the 10–15 ml acetone extract. Anhydrous sodium sulfate (100 g) was added and vigorously shaken for 1 min, and the mixture filtered through a medium porosity sintered glass funnel. The cake was washed with 75 ml of ethyl acetate and the solvent rotary evaporated.

The residue was partitioned with 300 ml of hexane and 100 ml of acetonitrile. The acetonitrile layer was drained into a 1-l. round-bottomed flask. The hexane was reextracted three times with 100 ml of acetonitrile and the combined acetonitrile phases rotary evaporated. The next step was hexane–water partition.

Extraction Procedure for Fat (Figure 1). Frozen fat (50 g) was placed into a Waring Blendor with 150 ml of methylene chloride and blended for 3 min at low speed. The homogenate was centrifuged in 250-ml centrifuge bottles for 5 min at 2000 rpm (approximately 1060 RCF). The liquid was filtered through a glass-wool plug into a 1-l. flask. The solids were reextracted once with 150 ml of methylene chloride and once with 100 ml of 1:1 methylene chloride–methanol. The funnel was rinsed with 25 ml of methanol and the combined extracts rotary evaporated. Hexane (100 ml) was added, the solution was heated on a steam bath to dissolve the fat, and the fat was transferred to a separatory funnel. The hexane solution was partitioned between 300 ml of hexane and 100 ml of acetonitrile. The acetonitrile was drained into a 1-l. round-bottomed flask. The hexane was reextracted three times with 100 ml of acetonitrile and the combined acetonitrile phases rotary evaporated. The next step was hexane–water partition.

Hexane–Water Partition Step. The residue was transferred to a 1-l. separatory funnel and partitioned between 150 ml of hexane and 500 ml of deionized water (1 ml of saturated Na_2SO_4 solution/500 ml of H_2O). *The empty flask was saved.* The water phase was drained into another 1-l. separatory funnel. The hexane was reextracted twice with 100 ml of water (0.2 ml of saturated Na_2SO_4/100 ml of H_2O); each time the water phase was drained into the second funnel.

To the combined water phases (700 ml) in the 1-l. separatory funnel, 1 ml of saturated Na_2SO_4 solution was added. To the 1-l. round-bottomed flask saved from the previous step, 100 ml of methylene chloride was added and swirled, and the solution was transferred to the separatory funnel. The methylene chloride phase was shaken and drained into a 1-l. round-bottomed flask. The water phase was reextracted four times with 100 ml of methylene chloride. The combined methylene chloride extracts were rotary evaporated.

Florisil Chromatography. The Florisil was removed

Table I. Per Cent Recovery of FΔFAc and FΔF from Fortified Tissue[a]

	Fortification level							
	0		5 ppb		10 ppb		20 ppb	
Tissue	FΔFAc	FΔF	FΔFAc	FΔF	FΔFAc	FΔF	FΔFAc	FΔF
Liver	0	0	100	86	100	100	80	86
Kidney	0	0	100	122	100	100	92	100
Muscle	0	0	100	100	100	56	88	100
Fat	0	0	100	100	100	100	100	100
Blood	0	0	100	100	100	100	100	100
Milk	0	0	96	100	95	100	100	94
Av %[b]			99 ± 2	101 ± 12	99 ± 2	100 ± 0[c]	93 ± 8	97 ± 6

[a] Each tissue was fortified with a methanol solution containing 1 μg/ml of FΔFAc and FΔF directly into the Waring Blendor. Assay order was determined from a randomized series table and assayed with tissue residue samples. [b] Overall average: FΔFAc is 97 ± 6%; FΔF is 99 ± 8% (if one value of 56 is deleted). [c] Average value when the muscle is included is 92 ± 18%.

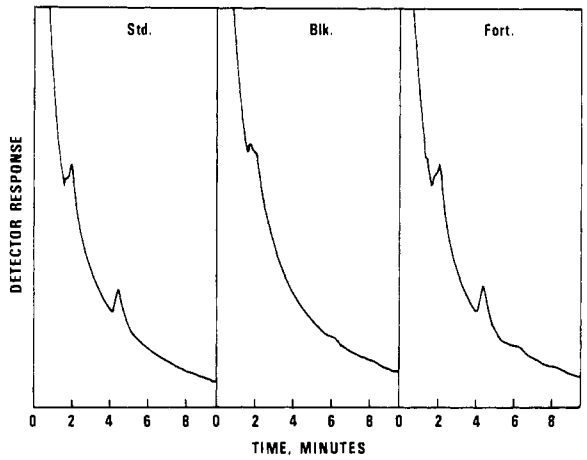

Figure 2. Glc-electron capture chromatograms of a standard, blank, and fortified muscle sample chromatographed on 2-ft 1% OV-17; attenuation, 1 × 32 on an F&M 402 chromatograph; standard, 4 ng of FΔF; blank, 0.2 g of control muscle; fortified, 0.2 g of muscle fortified with 4 ng of FΔF (20 ppb); sample size, 50 g; final volume, 1.0 ml; injection size, 4 μl.

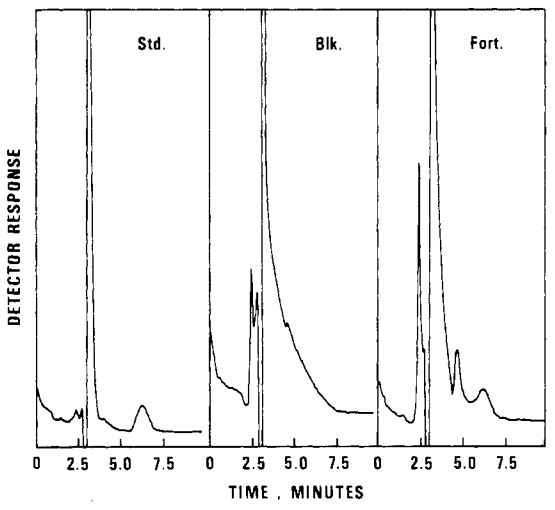

Figure 3. High-pressure liquid chromatograms of a standard, blank, and fortified sample chromatographed on a 1-m HCP (hydrocarbon polymer) column with 3:7 methanol–water; uv detector, 1 × 10^{-2} absorbance units full scale on the Du Pont 820; standard, 6 ng of FΔFAc; blank, 0.3 g of control muscle; fortified, 0.3 g of muscle fortified with 6 ng of FΔFAc (20 ppb); sample size, 50 g; final volume, 1.0 ml; injection size, 6 μl.

from the oven and cooled to room temperature, 4.0 ml of deionized water was added per each 100 g, stoppered, and placed on a "tumbling" mill for 1 hr, and then allowed to equilibrate for 16 hr or more. The Florisil (10 g) was slurried in 25 ml of chloroform and poured into a glass tube

Table II. Effect of Storage on Recovery[a]

		% recovery	
Tissue	Fort. level[b]	FΔFAc[c]	FΔF[d]
Liver	10	0	120
	20	0	120
Kidney	10	0	200
	20	0	190
Muscle	10	0	180
	20	0	175
Fat	10	0	180
	20	88	100
Blood	10	0	180
	20	0	200

[a] Approximately 10 weeks. [b] Fortified with FΔFAc and FΔF. [c] Values less than 5 ppb are reported as 0%. [d] Overall average recovery for FΔFAc and FΔF in all tissue after storage was 173 ± 29%. However, if the low liver values are excluded from the average, the overall recovery is 187 ± 10% or 93% for each.

(300 mm × 10 mm i.d., with a Teflon stopcock, standard taper top, and a sintered glass plug). The Florisil was topped with a glass wool plug. A 250-ml reservoir was placed on top of the glass tube and the column washed with 25 ml of chloroform (faster flow can be obtained with nitrogen pressure at 4 psi). The sample was transferred to the column with three 10-ml portions of chloroform. Chloroform (400 ml) was added to the sample flask, swirled, and transferred to the column. The FΔFAc was eluted with 50 ml of 5:70 methanol–chloroform and the FΔF with 350 ml of 2:8 methanol–chloroform (0.35 ml of concentrated ammonium hydroxide per 350 ml of the mixture). Both fractions were rotary evaporated. Each residue was transferred to a separate 50-ml round-bottomed flask with five 2-ml portions of methanol and the solvent was rotary evaporated.

Liquid–Liquid Chromatography (FΔFAc). Operating conditions were: first stage air, 50 psi; second stage air, 22–25 psi; flow rate, 0.5 ml/min; uv-detector setting, 1 × 10^{-2}; *low ground* switch in *open* position; *filter-normal* switch in *normal* position; *column flow* control in *drain position.* Methanol–water (3:7) was placed in the reservoir and the mobile phase degassed as follows: operated with vacuum and recycle valves open for 3 min, vacuum valve open for 2 min, and vacuum and recycle valves open for 1 min. The FΔFAc eluate from the Florisil column was dissolved in 1.0 ml of methanol. The high pressume pump was turned off, 4 μl was injected onto the column, and the pump was turned on again. The peak height of the standard and sample was measured to the nearest 0.5 mm using the base-line technique. The amount of FΔFAc was calculated from the peak height of a 1.0-ppm standard injected under identical conditions.

Electron Capture Gas Chromatography (FΔF). Operating conditions were: oven temperature, 240–250°; detec-

Table III. Residues in Calf Tissue after FΔFAc Treatment[a]

	FΔFAc and FΔF in calf tissue,[b] ppb, at days after last treatment															
	0[c]		1		3		5		7		10		13		Control	
Tissue	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF	FΔF-Ac	FΔF
Liver	15	40	0	20	0	11	0	0	0	0	0				0	0
	0	34	0	37	0	13	0	0	0	0	0				0	0
	0	17	0	34	12	9	0	6	0	0					0	0
Kidney	6	35	0	25	0	0	0	0	0						0	0
	0	43	0	20	0	7	0	0	0	0					0	0
	0	40	0	16	0	0	0	0	0	0					0	0
Muscle	0	28	0	18	0	0	0	0	0	0	0	0	0	0	0	0
	0	7	0	7	0	0	0	0	0	0	0	0	0	0	0	0
	0	10	0	10	0	0	0	0	0	0			0	0	0	0
Inj. site	7,850	11,200	18,150	7750	925	940	32	40	0	0	15	0	0	0		
	11,300	3,886	5,000	2438	4400	740	0	6	10	6	0	0	0	0		
	42,900	16,600	288	124	18	228	72	40	0	0			0	0		
Fat	0	0	0	0	0	0	0	0	0	0					0	0
	0	8	0	0	0	0	0	0	0	0					0	0
	0	0	0	0	0	0	0	0	0	0					0	0
Blood	0	0	0	0	0	0	0	0	0	0					0	0
	0	0	0	0	0	0	0	0	0	0					0	0
	0	0	0	0	0	0	0	0	0	0					0	0

[a] Residue after three daily injections (20 mg of experimental formulation of S.A.S. Predef 2 × per day). [b] Zero represents residues less than 5 ppb (limit of the method). [c] An on-treatment value.

tor temperature, 290–300°; carrier gas flow, helium, 40–60 ml/min; purge gas, argon–methane (95:5), 130–150 ml/min; attenuation, 10 × 4 or 1 × 64 with a pulse interval of 150. The FΔF eluate was dissolved in 1.0 ml of redistilled ethylene glycol monomethyl ether (Cellosolve). Four microliters was injected onto the OV-17 column. The resolved peak was measured to the nearest 0.5 mm and the amount calculated from the peak height of a 1.0-ppm standard injected under identical conditions.

Glc–Mass Spectra of FΔF. A sample of 9α-fluoroprednisolone was chromatographed on a 3-ft 1% OV-22 column. The column was connected to a Varian CH-7 mass spectrometer *via* a two-stage Biemann–Watson molecular separator. The ion source was maintained at 250°. All spectra were taken at 70 eV. The spectra were collected by repetitive scanning (complete mass spectrum every 8 sec) by our PDP8I data system. All scans were stored on magnetic tape. At the end of the glc run a plot of the total ion current *vs.* the scan number was made. This plot is essentially the same as the typical "gas chromatograph."

RESULTS AND DISCUSSION

Recovery Study. The results were based on the amount of FΔFAc and FΔF recovered after milk, blood serum, fat, muscle, liver, and kidney were fortified at 0, 5, 10, and 20 ppb (Table I) and randomly dispersed among the residue samples. The average recovery in tissues at the different levels of fortification ranged from 93 to 101%. The largest standard deviation at the 5-ppb level was ±12% for FΔF. Since stability studies had shown that FΔFAc was converted to FΔF after 10 weeks of storage (Table II), these recovery studies were carried out by direct addition of both steroids to the Waring Blendor prior to the extraction step.

Method. The column profile was dependent on the lot of chloroform used. Proper elution of both steroids was obtained when the chloroform had a refractive index of 1.444 or larger (Bausch and Lomb (B & L) reading of 7.0). Those lots of chloroform with B & L values of 5.0 eluted the steroids prematurely. Although the reason for the lower B & L value was not pursued, it was presumed to be associated with lots of chloroform stored for 1 year since fresh chloroform did not cause these problems.

Chromatograms for all tissues were quite clean in the retention time for FΔFAc and FΔF. Typical chromatograms (Figures 2 and 3) for muscle are shown for the 20-ppb fortification level.

Glc–mass spectrometry data showed the peak coming through the chromatograph had a molecular ion at *m/e* 318, which corresponded to the thermally degraded product, the 17-ketone analog derived from loss of betene and water. Its reproducible peak height and retention time made it a reliable indicator for detection and quantitation of FΔF by glc–electron capture techniques.

In some partition steps, four extractions are specified. This number was found necessary from our studies. As much as 6% loss was encountered when one less extraction was used.

Residue Data. Milk from five Holstein cows treated with three 20-mg FΔFAc injections at 12-hr intervals showed no residues of FΔFAc or FΔF in milk samples collected while on treatment (−24, −12, and 0 hr) and +12 and +24 hr posttreatment. Consequently, no additional samples beyond 24 hr were assayed although samples were collected through 132 hr. Since all values, even while on treatment, were 0 (<5 ppb) a table of data is not presented.

Tissues from calves treated with three 20-mg FΔFAc injections at 24-hr intervals showed residual FΔF and FΔFAc in tissues at 6 days, but not at 7 days posttreatment (Table III). The only exception was the injection site which persisted for 10 days and dropped below the detectable limits at 13 days. Blood was negative even during treatment.

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