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Metabolism of Fluvalinate by a Lactating Dairy Cow

Gary B. Quistad,* Luana E. Staiger, Gene C. Jamieson, and David A. Schooley

When a lactating cow was given a single oral dose (1 mg/kg) of [*trifluoromethyl*-^{14}C]fluvalinate, 53, 42, and 0.9% of the applied dose were excreted in urine, feces, and milk, respectively, after 8 days. The major urinary metabolites consisted of the anilino acid (**2**), which arose from hydrolysis of fluvalinate (**1**), and β-glucuronide conjugates of **2**, representing 6–19 and 63–76% of the urinary ^{14}C, respectively. Fecal ^{14}C-labeled residues consisted of **1**, **2**, and the bile acid conjugates of **2**, which were present as 47, ca. 11, and ca. 13% of the fecal ^{14}C. Although tissues, in general, contained only traces of radiolabel, fluvalinate contributed at least 70% of the ^{14}C-labeled residue in milk and fat.

As part of our continued study of the metabolic fate of fluvalinate [α-cyano-3-phenoxybenzyl 2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate, ZR-3210, MAVRIK], we now report the degradation of this pyrethroid by a lactating cow. For the preceding paper in this series see Quistad et al. (1982a).

EXPERIMENTAL SECTION

Radioassay and Chromatography. Radioactivity was quantitated by liquid scintillation counting (LSC) alone or in conjunction with sample combustion to $^{14}CO_2$ (Quistad et al., 1982a). Thin-layer chromatography (TLC) on silica gel utilized the following solvent systems: SS 1 (hexane–ethyl acetate–acetic acid, 12:9:0.1), SS 2 (hexane–ethyl acetate, 5:1), SS 3 (hexane–ethyl acetate, 1:1), SS 4 (ether–hexane, 2:1), SS 5 (hexane–ethyl acetate–acetic acid, 150:75:1), SS 6 (hexane–ethyl acetate, 2:1), SS 7 (ether–hexane, 1:1), SS 8 (hexane–ethyl acetate, 1:2), and SS 9 (hexane–ethyl acetate, 3:1).

The analysis of ^{14}C-labeled residues by reversed-phase and normal-phase liquid chromatography (LC) has been described (Quistad et al., 1982a). The following mixtures of methanol–0.1% acetic acid were used for reversed-phase LC: SS 10 (gradient 60–70% methanol over 15 min, 70–90% over 10 min, isocratic at 90% for 10 min); SS 11 (gradient 55–75% methanol over 20 min, isocratic at 75% for 5 min, gradient 75–90% over 10 min); SS 12 (65% methanol); SS 13 (80% methanol); SS 14 (isocratic at 75% methanol for 15 min, gradient 75–90% over 5 min, isocratic at 90%); SS 15 (85% methanol); SS 16 (isocratic at 80% methanol for 15 min, 80–90% over 5 min); SS 17 (gradient 40–90% methanol over 30 min). Normal-phase LC employed mixtures of ether–pentane, half-saturated with water: SS 18 (3:97), SS 19 (30:70), SS 20 (15:85), SS 21 (75:25), and SS 22 (90:10).

Synthetic Metabolite Standards. The fluvalinate and synthetic metabolite standards used in this study were racemic unless otherwise noted. The preparations of [*trifluoromethyl*-^{14}C]fluvalinate (**1**), anilino acid (**2**), the amide of **2**, the glycine conjugate of **2** (i.e., **6**), the taurine conjugate of **2**, cis and trans lactones of hydroxy acid **3**, and 4′-hydroxyfluvalinate (**5**) are reported elsewhere (Quistad et al., 1980, 1982a; Staiger et al., 1982). The glutamic acid conjugate of **2** (as its dimethyl ester) was synthesized from dimethyl glutamate and the acid chloride of **2**. 4′-Methoxyfluvalinate was prepared by reacting the cyanohydrin of 3-(4-methoxyphenoxy)benzaldehyde with **2**. The haloaniline **4** was prepared by T. Mastre (Zoecon) and the (*R*)-anilino acid [(*R*)-**2**] was provided by Dr. Ted Baer (Zoecon).

The synthesis of the methylated, peracetylated glucuronides of **2** is summarized in Figure 1. Glucurone (D-glucurono-3,6-lactone, 40 g, 0.23 mol) was stirred 1 h with methanol (300 mL) containing sodium hydroxide (0.11 g, 3 mmol) according to Bowering and Timell (1960). Evaporation of the methanol gave methyl D-glucopyranuronate, which was peracetylated according to Bowering and Timell (1960) to give a 54% yield of methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate (mp 111–115 °C). Methyl 1,2,3,4-tetra-*O*-acetyl-D-glucopyranuronate (5.0 g, 13 mmol) was reacted with 31% HBr in acetic acid (25 mL) for 16 h at room temperature to give methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-D-glucopyranuronate [cf. Bowering and Timell (1960)], which was not purified but was treated immediately with water and Ag_2CO_3–Celite [from $AgNO_3$ (4.5 g), Celite (4.0 g), and Na_2CO_3 (1.5 g) according to Fieser and Fieser (1969)] to give methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate in 88% yield [cf. Pravdic and Keglevic (1964)]. The anilino acid (**2**, 188 mg, 0.64 mmol) reacted with methyl 2,3,4-tri-*O*-acetyl-D-glucopyranuronate (213 mg, 0.64 mmol) in the presence of dicyclohexylcarbodiimide (131 mg, 0.64 mmol) and 4-(dimethylamino)pyridine (8 mg, 0.06 mmol) in CH_2Cl_2 (5 mL) to give **7** in 39% yield. The structure of **7** was confirmed by ^{1}H nuclear magnetic resonance (NMR) and mass spectroscopy. Together with chromatographic data, this evidence showed the synthetic material to consist of a 1:1 mixture of derivatized α- and β-glucuronides. The four diastereomeric glucuronide derivatives were separable by LC (SS 12) and the methylated, peracetylated α-glucuronides resolved as a single peak from the corresponding β-gluc-

Biochemistry Department, Zoecon Corporation, Palo Alto, California 94304.

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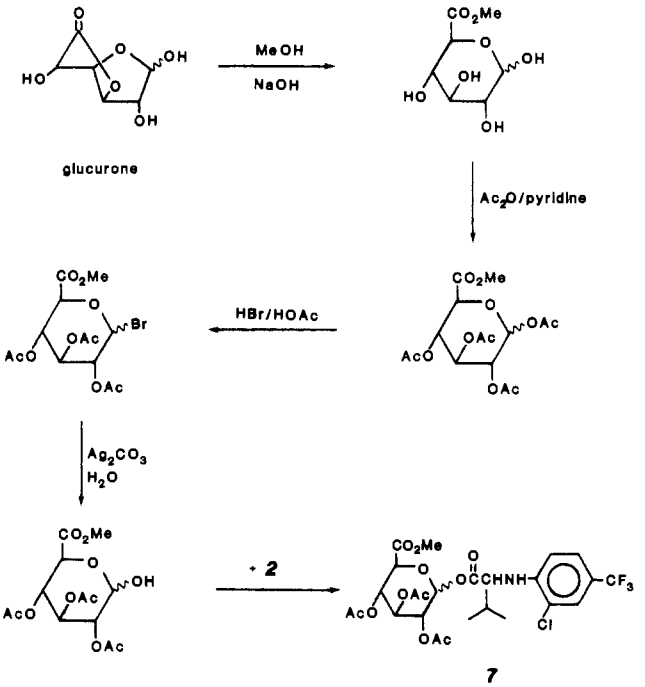


Figure 1. Synthesis of derivatized glucuronides of anilino acid (2).

uronide derivatives by GLC (retention times of 20.3 and 21.9 min, respectively; 3% OV-17 on Chromosorb W-AW-DMCS; 1 m × 2 mm column; programmed from 150 to 300 °C at 5 °C/min; Hewlett-Packard Model 402 gas chromatograph). Likewise, a mixture of methylated, peracetylated (*R*)-acid glucuronides was prepared from (*R*)-2.

The methyl ester of the cholic acid conjugate of (*RS*)-2 was synthesized in 31% yield from 2 and the methyl ester of cholic acid by using the dicyclohexylcarbodiimide method (vide supra). This method was also used to prepare the methyl cholate conjugate of (*R*)-2 and the other bile acid conjugates of (*RS*)-2.

Dosage. [*trifluoromethyl*-^{14}C]Fluvalinate was administered via gelatin capsule to a lactating Holstein cow (497 kg). The dose was prepared by adsorbing [*trifluoromethyl*-^{14}C]fluvalinate (a mixture of $\alpha R,2R$, $\alpha S,2S$, $\alpha R,2S$, and $\alpha S,2R$ stereoisomers; 2.79 mCi/mmol, 2.76 mCi, 498 mg, 99.3% radiochemical purity by LC in SS 10) onto cellulose powder (4 g) that was transferred to a gelatin capsule. The cow was maintained in a metabolism stall for periodic collection of urine, feces, blood, and milk. After 8 days, the cow was sacrificed for removal of tissues that were frozen (−18 °C) for subsequent analysis.

Blood Analysis. At designated intervals, blood was collected into heparinized tubes and immediately transferred to glass bottles for storage. Aliquots (0.5 mL) were directly pipetted into quartz boats for quantitation of radiolabel following combustion. Additional aliquots (~3 mL) were centrifuged, and the plasma (0.1–0.5 mL) was quantitated by direct LSC (Instagel, Packard).

To a sample of 24-h whole blood (24 mL) was added saturated ammonium sulfate in 2.5% H_2SO_4 (ca. 50 mL), methanol (ca. 20 mL), and Celite (~4 g) prior to filtration. The filtrate was evaporated to dryness, then extracted with ether for analysis by LC (SS 10).

Milk Analysis. Aliquots of milk (10–100 mL, 24 and 84 h posttreatment) were extracted with either acetonitrile or ether that was evaporated to dryness for subsequent analysis by TLC (SS 1 or SS 2) and LC (SS 10). The diastereomer ratio of fluvalinate was analyzed on normal-phase LC (SS 18; $k' = 6.2$ for $\alpha R,2R$ and $\alpha S,2S$; $k' = 7.1$ for $\alpha S,2R$ and $\alpha R,2S$).

Tissue Analysis. An aliquot (47 g) of flank muscle was minced and extracted by using a Brinkmann Polytron homogenizer as follows: methanol (150 mL), 2,2,4-trimethylpentane (150 mL, 2×), and methanol (150 mL), filtering between each extraction. The phases of the combined filtrates were separated, the 2,2,4-trimethylpentane was extracted with methanol, and aliquots (5.0 mL) of each phase were quantitated by LSC. The methanol was concentrated on a rotary evaporator until mostly water remained. The aqueous residue was extracted with ether 3 × 30 mL), and aliquots of both phases were quantitated by LSC. After concentration of the ether phase, metabolites were fractionated by TLC (SS 1). Radiolabel coeluting with fluvalinate and anilino acid standards was eluted from the silica gel for additional analysis by LC (SS 10). [^{14}C]Fluvalinate is stable during this analysis sequence and also to storage of tissues at −18 °C. The remaining TLC zones were eluted for quantitation by LSC. Aliquots (~170 mg) of the residual solids were quantitated for unextractable ^{14}C by combustion.

An aliquot (24 g) of renal fat was mixed with Celite (3 g) and homogenized in 2,2,4-trimethylpentane (2 × 150 mL) followed by methanol (2 × 150 mL). The combined filtrates were mixed well and the phases separated. Each phase was reextracted with the other phase, and aliquots (1.0 mL) were quantitated by LSC. An aliquot of the methanol phase (88% of the fat ^{14}C) was concentrated for analysis by LC (SS 10). Aliquots (~170 mg) of the dried residue were quantitated by combustion.

The fetus (~6-week gestation) was removed from membranes and extracted with methanol (2 × 100 mL). Aliquots of the filtrate (5.0 mL) were quantitated by LSC, and dried unextractable residue (100 mg) was quantitated by LSC following combustion. Aliquots of the remaining tissues were quantitated by LSC after combustion (100–600-mg aliquots). The bile (0.2-mL aliquot) was quantitated by LSC also.

Urine Analysis. The urine was briefly refrigerated (4–12 h) prior to quantitation by LSC (0.1–1.0-mL aliquots in Instagel), and then frozen (−20 °C). Samples of urine were periodically thawed for analysis. Aliquots (50 μL) of the 24- and 96-h urine were analyzed by LC (SS 10) and TLC (SS 1). Radioactive TLC zones were separately analyzed by LC for metabolite identification.

Amino acid conjugates of 2 were analyzed by a combination of TLC and reversed-phase LC. Radiolabel comigrating on TLC (SS 1) with the glycine conjugate of 2 (24-h urine, $R_f = 0.14$) was treated with ethereal diazomethane and was examined subsequently by TLC ($R_f = 0.34$ for the methyl ester in SS 1) and LC ($k' = 8.2$, SS 10). An aliquot of the 24-h urine was analyzed for the taurine conjugate of 2 (SS 17). For analysis of the glutamic acid conjugate of 2, an aliquot of the 24-h urine was methylated and then examined by TLC (SS 3) and reversed-phase LC (SS 10).

The hydroxy acid 3 was isolated by TLC ($R_f = 0.14$, SS 1) where it comigrates with the glycine conjugate of 2. Treatment of 3 with CH_2N_2 gave the methyl ester that was characterized by TLC ($R_f = 0.47$, SS 1). When 3 was heated at 80 °C in 1 M HCl for 1 h, it was converted to a mixture of trans and cis lactones that were identified by comparison to authentic standards on LC ($k' = 6.8$ and 9.2 in SS 11, respectively).

Volatile metabolites from the 24-h urine were investigated by first extracting an aliquot (3 mL) with ether and then evaporating the ether to dryness while heating (35 °C) in a flask stoppered with a polyurethane plug. The plug was extracted with ether, an aliquot was quantitated

by LSC, and after gentle evaporation of the solvent, the radiolabel was analyzed and found to coelute on LC with authentic haloaniline 4 (k' = 7.1, SS 11).

Aliquots of the origin zone extracted from TLC plates (SS 1) were subjected to separate treatments: (1) 1 M HCl (100 °C, 1 h), followed by extraction into ether and analysis of the ether phase by TLC (SS 1) and LC (SS 10); (2) β-glucuronidase (5 mg, *Helix pomatia*, Sigma) in citrate–phosphate buffer (5 mL, pH 4.5, 37 °C, 16 h), with analysis of an ether extract by TLC in SS 1; (3) ethereal diazomethane, followed by acetic anhydride (200 μL) and pyridine (100 μL) for 16 h at room temperature. The products (methylated, peracetylated glucuronides) were extracted into ether following addition of water and acidification, and the ether extract was analyzed by TLC (R_f = 0.51, SS 4).

The glucuronide conjugates of **2** were derivatized by methylation and peracetylation for identification by gas–liquid chromatography coupled with mass spectrometry (GLC–MS) and NMR. An aliquot of neat urine (400 mL, 96-h urine) was extracted with ether in a continuous liquid–liquid extraction apparatus for 104 h. The combined ether extracts were evaporated to dryness, and the flask was rinsed with acetone prior to preparative TLC purification (SS 5). The origin zone from TLC was eluted with methanol and derivatized as follows: (1) ethereal diazomethane followed by evaporation to dryness (2) acetic anhydride and pyridine (1 mL each, 16 h, room temperature). Following the addition of water (10 mL) and acidification, the sample was extracted into ether and purified: (1) TLC (SS 4); (2) LC (sample divided into two equal portions for separate injections with SS 12; collection of 1-min fractions in test tubes); (3) re TLC purification (conditions above); (4) repurification by LC (conditions above). Mass spectra were obtained by using a Hewlett-Packard Model 5985 coupled GC/mass spectrometer in the electron-impact (EI) or chemical-ionization (CI) modes: (*R*)-acid β-glucuronide as its methyl, peracetyl derivative, m/z (rel intensity), EI, 613 (0.1, M^+), 611 (0.1, M^+), 252 (37), 250 (100), 155 (62), 139 (24), 127 (28), 43 (92); (*S*)-acid β-glucuronide derivative, 613 (0.2 M^+), 611 (0.6 M^+), 252 (66), 250 (100), 155 (95), 139 (55), 127 (65), 43 (91). The FT ^{1}H NMR spectrum of the combined (*RS*)-acid β-glucuronides (as methyl, peracetyl derivatives) was obtained by Dr. M. Maddox (Syntex Corp.) using 350 μg of purified product with a Bruker WH-90 instrument: 90 MHz ($CDCl_3$) δ 1.03 [d, 6, J = 6 Hz, $CH(CH_3)_2$], 1.75 (s, 1.5, $COCH_3$), 1.86 (s, 1.5, $COCH_3$), 2.00 (s, 6, $COCH_3$), ca. 2.2 [m, 1, $CH(CH_3)_2$], 3.69 (s, 3, OCH_3), ca. 4.0 (m, 2, $CHCO_2$, C-5 pyranose), ca. 5.1 (m, 4, NH, C-2, C-3, and C-4 pyranose), 5.78 (d, 1, J = 8 Hz, pyranose at C-1 β-glucuronide), 6.5 (m, 1, ar), ca. 7.5 (m, 2 ar). The derivatized (*R*)-acid and (*S*)-acid β-glucuronides had k' values of 14.7 and 15.9 by LC in SS 12, respectively. The free, underivatized (*RS*)-acid β-glucuronides migrated as a single broad peak upon LC in SS 11 (k' = ca. 5.1).

Feces Analysis. Portions (50–250 g) of the 36- and 96-h feces were extracted several times with methanol (also with CH_2Cl_2 for the 36-h feces sample). Aliquots of the combined extracts were analyzed by LC (SS 10) for metabolites by collecting timed fractions and radioassay by LSC. The organic extract was purified by silica gel chromatography (silica gel 60, E. Merck; 4.5 × 42 cm column). Elution with hexane–ether (1:1, 200 mL) gave predominantly fluvalinate but also a trace of 4′-hydroxyfluvalinate. The radiolabeled metabolite corresponding to 4′-hydroxyfluvalinate coeluted on LC with an authentic standard (k' = 2.6, SS 13), and methylation (CH_2N_2, 4 h at 0 °C) of the phenolic hydroxyl gave 4′-methoxyfluvalinate, which also coeluted with a synthetic standard (k' = 5.1, SS 13).

When the silica gel column was eluted with ethyl acetate (200 mL), anilino acid **2** and a radiolabeled peak eluting after fluvalinate on LC (k' = 20.6, SS 10) were the major components (61 and 27% of the ethyl acetate eluate). The latter metabolite was methylated (CH_2N_2) and purified twice by TLC (SS 6, R_f = 0.35; SS 7, R_f = 0.28). After a final purification by LC (SS 13), the metabolite was identified as an anilino acid (**2**) conjugate of cholic acid by mass spectrometry: for the methyl ester, m/z (rel intensity) direct inlet, EI, 701 (1.1, M^+), 699 (3, M^+), 612 (19), 381 (8), 369 (6), 355 (4), 252 (97), 251 (81), 250 (100), 249 (56); for the methyl ester, m/z (rel intensity) direct inlet, CI (CH_4), 728 (5, M + 29), 700 (6, M + H), 682 (6, M + H − H_2O), 664 (25, M + H − 2H_2O), 405 (5), 387 (41), 369 (100), 296 (33), 250 (53). GLC–MS of the bis(trimethylsilyl) ether gave the following: EI, 845 (0.2, M^+), 843 (0.3, M^+) 252 (40), 251 (16), 250 (100). A synthetic standard of the methyl cholate conjugate of **2** had the same chromatographic (TLC and LC) and mass spectral characteristics as the methylated metabolite. The methyl cholate conjugate of **2** was resolved into (*R*)-**2** and (*S*)-**2** components by normal-phase LC (k' = 20.6 and 18.9, respectively, SS 19).

For isolation of the remaining bile acid conjugates of **2**, an additional methanol extract of the 96-h feces (340 g) was purified by a silica gel column as described above. Elution of the silica gel column with ether gave (among other products) a mixture of the conjugates of **2** with chenodeoxy- and deoxycholic acids. After methylation (CH_2N_2) this mixture was purified by TLC (single zone at R_f = 0.34, SS 2). The methylated chenodeoxy- and deoxycholate conjugates of **2** were unresolvable from each other but showed coincidence with synthetic standards in two reversed-phase LC solvent systems (k' = 19.2, SS 14; k' = 10.8, SS 15). Although only partial resolution was obtained by normal-phase LC (SS 20), the methylated chenodeoxy- and deoxycholate conjugates of **2** were resolved finally by reversed-phase LC using acetonitrile–0.1% acetic acid, 75:25 (k' = 20.1 and 18.4, respectively).

Elution of the above silica gel column with methanol gave 3.3 g of fecal mass, and after methylation with CH_2N_2, dissolution of this residue in ether gave an 87% recovery of ^{14}C while reducing the mass by 79%. When a portion of this ether-soluble ^{14}C-labeled residue was purified by TLC (SS 8), two bile acid conjugate zones were obtained. The first TLC zone (R_f = 0.62) contained a mixture of methylated glycochenodeoxy- and glycodeoxycholate conjugates of **2** that were resolved by reversed-phase LC (k' = 10.9 and 12.0, respectively, in SS 16). These radiolabeled bile acid conjugates were also coincident with synthetic standards of methylated glycochenodeoxy- and glycodeoxycholate conjugates of **2** by normal-phase LC (SS 21) that resolved these conjugates into their (*R*)- and (*S*)-**2** diastereomers. The second TLC zone from above (R_f = 0.35) contained the methyl glycocholate conjugate of **2**. This ^{14}C-labeled metabolite was coincident with an authentic standard by reversed-phase LC (k' = 6.0 in SS 13) and normal-phase LC (k' = 7.0, SS 22).

Elution of the silica gel column with methanol also gave hydroxy acid **3** and amino acid conjugates of **2**. The hydroxy acid was identified as its methyl ester by TLC (SS 9, R_f = 0.32) and reversed-phase LC (k' = 5.4, SS 12). Possible amino acid conjugates of **2** were collectively methylated (CH_2N_2), fractionated by TLC (SS 8), and analyzed by LC (SS 11).

Table I. Radiolabel Balance for a Lactating Dairy Cow Treated at 1 mg/kg with [*trifluoromethyl*-^{14}C]Fluvalinate

	% of applied dose
urine	53.3
feces	42.3
tissues	1.8[a]
milk	0.88
total recovery	98.3

[a] 0.2% of applied dose was actually recovered from tissues and subsamples of muscle and fat (see Table III). By using the mean value for renal, omental, and subcutaneous fat and the estimated fat content of a dairy cow [17.7% from Morrison (1961)], it is calculated that 1.6% of the applied dose remained in fat at sacrifice.

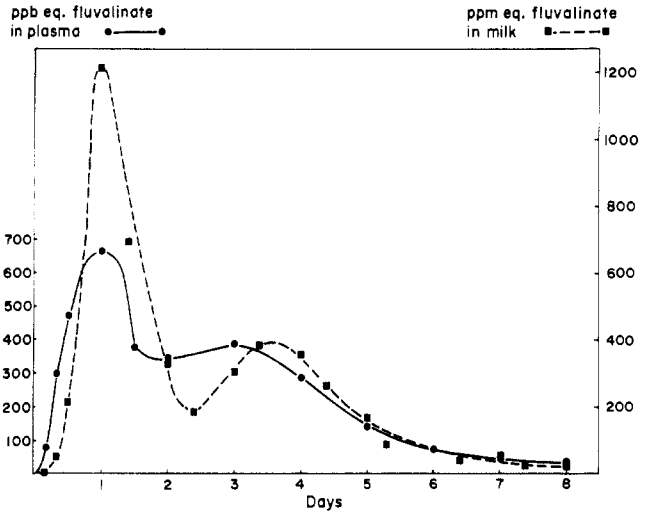


Figure 2. Pharmacokinetics of radiolabel in the blood plasma and milk of a lactating cow dosed at 1 mg/kg with [*trifluoromethyl*-^{14}C]fluvalinate.

Unextractable residual ^{14}C in fecal solids was combusted to $^{14}CO_2$ for quantitation. Duplicate aliquots of all fecal samples were also combusted for quantitation of radiolabel.

Fluvalinate Isomer Analysis. The $\alpha R,2R$, $\alpha S,2S$, $\alpha R,2S$, and $\alpha S,2R$ stereoisomers of fluvalinate were assayed as menthyl ester derivatives (Quistad et al., 1982a).

RESULTS AND DISCUSSION

When a lactating dairy cow was treated at 1 mg/kg with [*trifluoromethyl*-^{14}C]fluvalinate, radiolabel was excreted readily within 8 days in urine and feces (53 and 42% of the applied dose, respectively). Less than 1% of the applied radioactivity was excreted in milk and about 98% of the applied dose was recovered (Table I). Although production of $^{14}CO_2$ from the $^{14}CF_3$ moiety was not monitored, the high recovery of applied radiolabel suggests negligible production of $^{14}CO_2$ from [*trifluoromethyl*-^{14}C]fluvalinate. This result would be in agreement with previous work on rat metabolism of **1**, in which monitoring for $^{14}CO_2$ evolution showed this to be a minor metabolic process (Quistad et al., 1980).

Blood. The absorption into and subsequent dissipation of radiolabel from blood following oral administration of fluvalinate were both rather slow (Figure 2). Analysis of the ^{14}C in the 24-h (peak) whole blood showed that anilino acid **2** and fluvalinate contributed 55 and 3% of the toal radiolabel in the blood. Hence, fluvalinate is either poorly absorbed into blood relative to **2** or it is readily hydrolyzed by blood esterases.

Milk. The concentration profile of ^{14}C in milk closely paralleled that of blood (Figure 2). Analysis of 24- and 84-h milk revealed that fluvalinate constituted at least 68 and 70%, respectively, of the total ^{14}C in the milk (Table II). The anilino acid (**2**) and haloaniline (**4**) were minor residues in milk (both ≤2% of milk ^{14}C). Milk is also a relatively unimportant route of excretion for fluvalinate and its metabolites since only 0.9% of the applied dose appeared in milk. This limited excretion of fluvalinate and/or its metabolites in milk agrees with data for the metabolism by cows for the pyrethroids permethrin and fenvalerate (Gaughan et al., 1978; Wszolek et al., 1980).

Table II. Residues from Metabolism of [*trifluoromethyl*-^{14}C]Fluvalinate by a Lactating Cow

	% of total ^{14}C, ppb		
	fluvalinate (1)	anilino acid (2)	haloaniline (4)
milk			
24 h	68 (700)	⩽2 (⩽2)	⩽2 (⩽1)
84 h	70 (41)	0.6 (0.2)	⩽0.1 (⩽0.02)
muscle (flank)	⩾27 (5)	4 (0.4)	⩽2 (⩽0.2)
fat (renal)	80 (112)	2 (1)	0.2 (0.1)

Table III. Tissue Residues from a Cow Treated Orally with [*trifluoromethyl*-^{14}C]Fluvalinate

tissue	ppb equiv as fluvalinate	% of applied dose
muscle		
leg	10	
flank	18	
shoulder	22	
kidney	29	0.008
liver	49	0.069
fat		
renal	160	
subcutaneous	27	
omental	83	
pancreas	30	0.0023
lung	18	0.014
tongue	6	0.0019
udder	32	0.062
hide	13	
heart	18	0.016
ovaries	23	0.00011
adrenal	14	0.000091
spleen	10	0.0026
uterus	11	0.0014
brain	<1	<0.001
bladder	16	0.001
thyroid	8	0.000064
fetus	6	0.00001
reticulum	7	
omasum	9	
small intestine	21	
cecum	17	
large intestine	15	
rumen	13	
gall bladder	60	0.00045
bile	160	0.0031
milk	see Figure 2	0.9 (total)

Tissues. Eight days after a single oral dose of fluvalinate at 1 mg/kg, tissues contained only trace levels of fluvalinate and its metabolites (Table III). The sum of the ^{14}C in all whole organs analyzed was only 0.2% of the applied dose. Relatively more ^{14}C was found in fat. If one averages the ^{14}C-labeled residue levels in renal, subcutaneous, and omental fat and assumes a dairy cow is about 18% fat (Morrison, 1961), then by calculation about 1.6% of the applied ^{14}C was retained in fat after 8 days. This figure, however, is only an approximation since the averaged fat samples may not be entirely representative of total body fat. Analysis of renal fat demonstrated that fluvalinate represented at least 80% of the ^{14}C in the sample and the anilino acid **2** contributed only 2%.

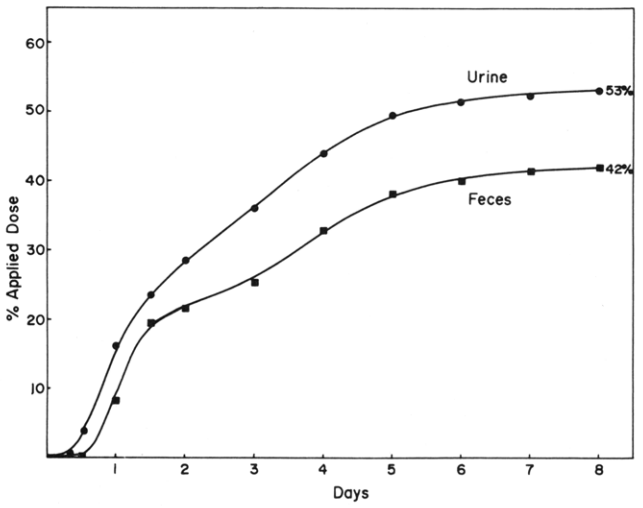


Figure 3. Excretion of radiolabel from a cow dosed orally with [*trifluoromethyl*-^{14}C]fluvalinate.

The low levels of ^{14}C-labeled residues in muscle (Table II) made analysis somewhat difficult. The major nonpolar ^{14}C-labeled residue in muscle was fluvalinate which represented at least 27% of the total muscle radiolabel. The anilino acid **2** contributed 4% of the muscle ^{14}C while polar metabolites contributed 36%.

Analysis of a developing fetus (about 6 weeks into gestation) demonstrated minimal transfer of fluvalinate (or metabolites) from the cow (Table III). In fact, the fetus contained some of the lowest tissue ^{14}C-labeled residues in the whole cow (~0.00001% of the applied dose).

Urine. The urinary excretion of fluvalinate metabolites is shown in Figure 3. The major nonpolar metabolite in urine was the anilino acid **2** (19% of 24-h urine ^{14}C) with lesser amounts of the glycine conjugate of **2** (i.e., **6**), hydroxy acid **3**, and haloaniline **4** (0.8, 2.4, and 1.5% of the 24-h urine ^{14}C, respectively (Table IV). In contrast to the metabolism of [*acid*-^{14}C]permethrin (Gaughan et al., 1978), the glutamic acid conjugate of **2** could not be identified (<0.5% of urinary ^{14}C).

Table IV. Urinary Metabolites of [*trifluoromethyl*-^{14}C]Fluvalinate from a Lactating Cow

	% total ^{14}C in urine	
	12–24 h (12.3% of applied dose)	72–96 h (8.0% of applied dose)
anilino acid (**2**)	19	6
glycine conjugate of **2** (i.e., **6**)	0.8	
taurine conjugate of **2**	⩽0.9	
glutamic acid conjugate of **2**	⩽0.5	
hydroxy acid (**3**)	2.4	
haloaniline (**4**)	1.5	
glucuronides of **2**	63	76

The majority of the urinary ^{14}C-labeled residues were polar, remaining at the origin after TLC; however, reversed-phase LC of these polar conjugates gave a single broad peak (k' = 5.1, SS 11). Treatment of the conjugates with 1 M HCl gave **2** in 91% yield while treatment with β-glucuronidase gave a quantitative cleavage to **2** (no cleavage in a buffer blank without enzyme).

In order to facilitate chromatographic manipulation for the eventual structure proofs of these suspected glucuronides, the polar ^{14}C-labeled residue was methylated and peracetylated. The derivatized conjugates were compared by LC to an authentic mixture (also derivatized) of α- and β-glucuronides (Figure 4) where radiolabel eluted with the last two standard peaks. When the two metabolites were purified by a combination of TLC and LC, they each gave mass spectra consistent with the assigned glucuronide structure. The stereochemistry at the anomeric carbon of

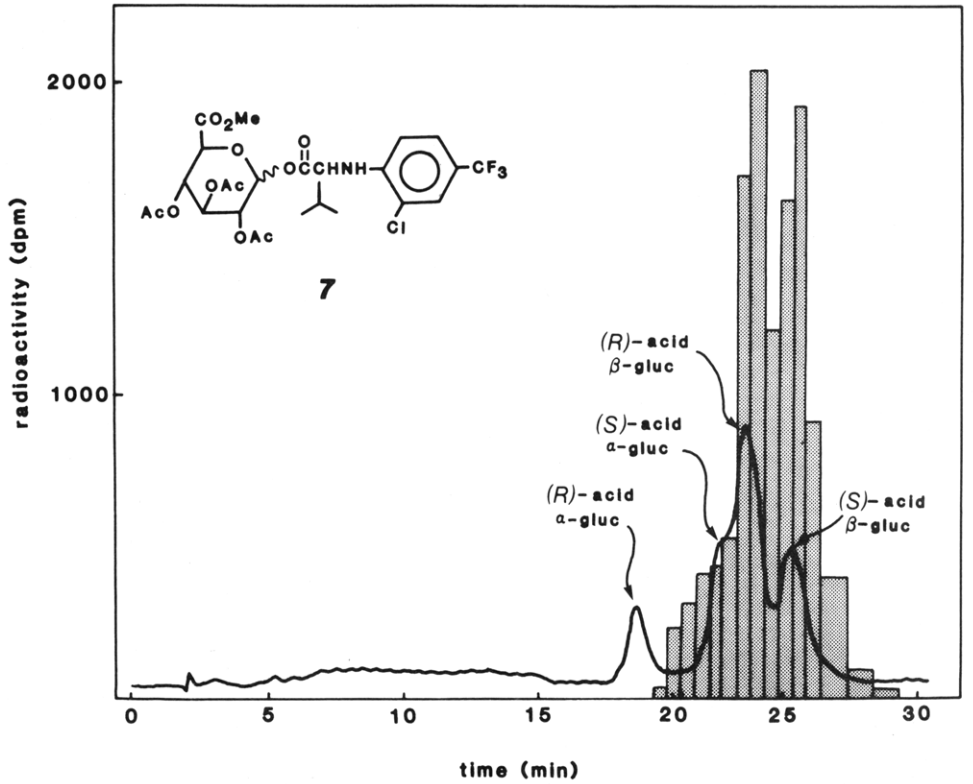


Figure 4. Reversed-phase liquid chromatogram of methylated, peracetylated β-glucuronides: Spectra-Physics 8000A liquid chromatograph; LiChrosorb RP-8 column, 5 μm, 25 × 0.46 cm; SP Model 8310 UV detector at 254 nm; methanol–0.1% acetic acid, 65:35; 1.6 mL/min; 35 °C. The solid line represents UV detector response. The hatched bars represent radioactivity in timed fractions collected from the column and quantitated by liquid scintillation counting.

Table V. Identification of Metabolites in Feces from a Cow Dosed with [*trifluoromethyl*-^{14}C]Fluvalinate at 1 mg/kg

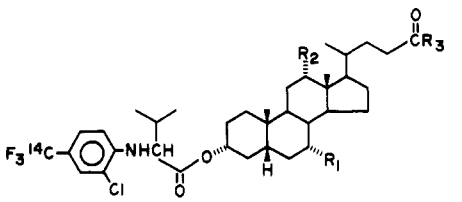
	% of total ^{14}C in sample	
	36 h (11.4% of applied dose)	96 h (7.5% of applied dose)
methanol extract	77	92
fluvalinate (1)	47	44
4'-hydroxyfluvalinate (5)	0.4	0.9
hydroxy acid (3)		1.7
anilino acid (2)	10.0	12.0
anilino acid conjugate of[a]		
cholic acid	5.2	8.1
chenodeoxycholic acid		1.3
deoxycholic acid		2.2
taurocholic acid		<0.5[b]
taurochenodeoxycholic acid		<0.5[b]
taurodeoxycholic acid		<0.5[b]
glycocholic acid		0.3
glycochenodeoxycholic acid		0.2
glycodeoxycholic acid		0.6
residual solids	23	8

[a] See Figure 5 for structures. [b] None detected.

the sugar was determined by 90-MHz ^{1}H NMR. An NMR spectrum of the combined metabolites gave the expected resonance for β-glucuronides (δ 5.8). By synthesizing an authentic sample of derivatized α- and β-glucuronides of the (*R*)-acid, it was then possible to identify the first radiolabeled peak to elute during LC (Figure 4) as the (*R*)-acid β-glucuronide. Hence, the second LC peak is the (*S*)-acid β-glucuronide, and these metabolites are present in cow urine in a 53:47 ratio. Since the corresponding derivatized α-glucuronides of **2** are separable from β-glucuronides of **2** by GLC, we determined that α-glucuronides of **2** represented <10% of the metabolite glucuronides.

Feces. Slightly less radiolabel was excreted in feces than in urine (Figure 3). The ^{14}C-labeled residues in feces (Table V) were quite different from those in urine with fluvalinate constituting about half of the fecal radiolabel. The most abundant metabolite was again the anilino acid **2**. According to TLC and LC, only traces (<1% of fecal ^{14}C) of amino acid (glycine, serine, threonine, and valine) conjugates of **2** were present in the 96-h extract although such conjugates were fecal metabolites of fluvalinate in rats (Quistad et al., 1980). The amide of **2** represented 0.4% of the 96-h fecal ^{14}C (according to TLC and LC).

Bile acid conjugates of anilino acid **2** represented a substantial proportion of the fecal ^{14}C-labeled residue (13% for the 96-h sample, Table V and Figure 5). The cholic acid conjugate alone contributed 5–8% of the radiolabel in feces. By analogy with other work (Quistad et al., 1982b; Staiger et al., 1982) we presume that these conjugates consist of **2** esterified at the 3α-hydroxyl substituent of the bile salt. In contrast with the bile acids in fresh cow bile (Fieser and Fieser, 1959), the excreted conjugates of **2** were largely free of amino acid (i.e., glycine and taurine) adducts. Although three glycine-containing bile acid conjugates of **2** were characterized as minor constituents (Table V), the corresponding taurine adducts were undetectable. The apparent absence of taurine-containing bile acid conjugates of **2** is intriguing since the ratio of tauro to glyco bile acids in fresh cow bile is reported to be 5:1 (Fieser and Fieser, 1959). However, the preponderance of the cholate conjugate of **2** over other bile acid conjugates is consistent with the role of cholic acid as the major bile acid in cow bile (Fieser and Fieser, 1959). A summary of the structures



Conjugate	R1	R2	R3
cholate	OH	OH	OH
chenodeoxycholate	OH	H	OH
deoxycholate	H	OH	OH
taurocholate	OH	OH	$NHCH_2CH_2SO_3H$
taurochenodeoxycholate	OH	H	$NHCH_2CH_2SO_3H$
taurodeoxycholate	H	OH	$NHCH_2CH_2SO_3H$
glycocholate	OH	OH	$NHCH_2CO_2H$
glycochenodeoxycholate	OH	H	$NHCH_2CO_2H$
glycodeoxycholate	H	OH	$NHCH_2CO_2H$

Figure 5. Structures of bile acid conjugates of anilino acid (**2**).

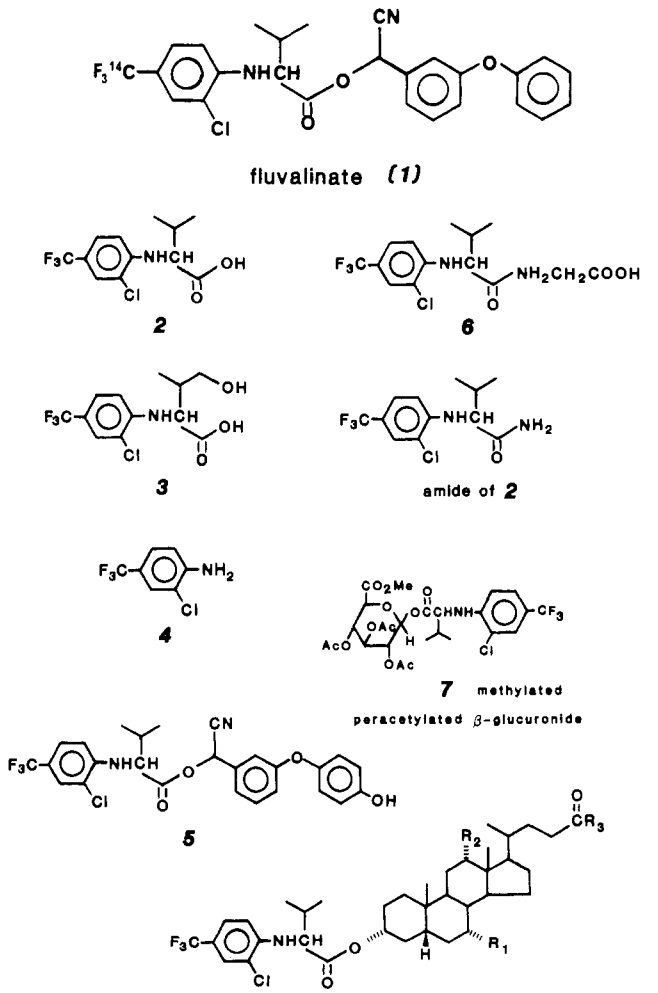


Figure 6. Summary of metabolites of fluvalinate from a lactating dairy cow.

of metabolites from fluvalinate is given in Figure 6.

Fluvalinate Stereoisomers. In order to assess the possible selective dissipation of fluvalinate stereoisomers, fluvalinate was isolated from renal fat, feces, and milk. Formation of menthyl ester derivatives of fluvalinate [cf. Horiba et al. (1980)] allowed assay of the fate of individual (α*R*,2*R*)-, (α*S*,2*S*)-, (α*R*,2*S*)-, and (α*S*,2*R*)-fluvalinate stereoisomers. In general, the α*S*,2*R* isomer content of fat,

Table VI. Analysis of Fluvalinate Stereoisomers as Menthyl Ester Derivatives

fluvalinate sample	% yield of menthylation	% of isomers			
		αR,2R	αS,2S	αR,2S	αS,2R
standard[a]	40	21	17	34	28
renal fat	39	31	14	42	13
feces (24–36 h)	35	37	13	41	10
milk (12–24 h)	25	25	3	50	12

[a] That is, [*trifluoromethyl*-^{14}C]fluvalinate.

milk, and feces was considerably reduced whereas the αR,2R and αR,2S isomers were more abundant (Table VI). Hence, it appears that the αS,2R isomer of fluvalinate is degraded more rapidly than the other stereoisomers; coincidentally, this is by far the most potent insecticidal isomer (Anderson et al., 1980). Since the cholate conjugate of **2** consisted of a 91:9 ratio for (*R*)-**2**:(*S*)-**2**, bile acids appear to selectively remove (*R*)-**2**, which may offer a partial explanation for enhanced disappearance of (αS,2R)-fluvalinate.

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Metabolism of Fluvalinate by Laying Hens

Luana E. Staiger, Gary B. Quistad,* Steven K. Duddy, and David A. Schooley

[*trifluoromethyl*-^{14}C]Fluvalinate (**1**) is rapidly metabolized when given as a single oral dose to laying hens. Within 2 days, 88–100% of the applied dose is excreted with the major excretion products being fluvalinate, the anilino acid (**2**), methylhydroxylated **2**, the taurine conjugate of **2**, and the taurochenodeoxycholic acid conjugate of **2**. Eggs collected for 2 weeks after dosing accounted for ~0.6% of the applied dose with the majority of the radiolabel found in the yolk. Major yolk products included fluvalinate, the anilino acid, and the taurochenodeoxycholic acid conjugate of **2**. Radioactivity did not accumulate significantly in any tissue examined.

Fluvalinate [α-cyano-3-phenoxybenzyl 2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate] is an insecticide with pyrethroid-like activity that is currently being developed by Zoecon Corp. for pest control on numerous field crops. Due to the potential exposure of chickens to fluvalinate residues in processed agricultural products used as feed, we now report the metabolic fate of fluvalinate in laying hens [for the preceding report in this series, see Quistad et al. (1982a)].

EXPERIMENTAL SECTION

Radioassay and Chromatography. Radioactivity was quantitated by liquid scintillation counting (LSC) alone or in conjunction with sample combustion to $^{14}CO_2$ (Quistad et al., 1982a). The extraction of excreta, yolks, and tissues utilized a Polytron homogenizer (Brinkmann). The quantitation of radiolabeled residues in extracts was achieved by using gradient-elution reversed-phase liquid chromatography (LC) by coinjection of a known amount of radiolabeled extract together with authentic metabolite standards and collection of timed fractions for subsequent assay by LSC. The conditions for reversed-phase and normal-phase LC have been described (Quistad et al., 1982a). The following mixtures of methanol–0.1% acetic acid were used for reversed-phase LC: SS 1 (gradient 60–70% methanol over 15 min, 70–90% over 10 min, isocratic at 90% for 10 min); SS 2 (gradient 65–90% methanol over 25 min); SS 3 (isocratic at 80% methanol); SS 4 (gradient 70–90% methanol over 20 min). For normal-phase LC, a Zorbax SIL column (25 × 0.46 cm, 5 μm) was used with elution at ca. 1.7 mL/min with half-water-saturated ether–pentane: 75:25 (SS 5), 80:20 (SS 6), and 2:98 (SS 7). The following solvents were used for thin-layer chromatography (TLC) on silica gel GF (Analtech, Newark, DE): SS 8 (hexane–ethyl acetate, 1:2), SS 9 (ethyl acetate–isobutyl alcohol–acetic acid, 10:3:1), SS 10 (hexane–ethyl acetate–acetic acid, 10:30:0.3), SS 11 (hexane–ethyl acetate, 8:1), SS 12 (hexane–ethyl acetate, 2:1), SS 13 (ethyl acetate), SS 14 (hexane–ethyl acetate, 5:1), SS 15 (hexane–ethyl acetate, 4:1), SS 16 (hexane–ethyl

Biochemistry Department, Zoecon Corporation, Palo Alto, California 94304.

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