oxopentanoate, 624-45-3; hexanoic acid, 142-62-1; 2-furfuryl acetate, 623-17-6; 2-furanylpropan-1-one, 3194-15-8; ethyl 2-amino-3-methylbutyrate, 17431-03-7; ethyl 4-oxopentanoate, 539-88-8; acetophenone, 98-86-2; acetylthiophene, 39709-34-7; linalool oxide A, 34995-77-2; 2-phenylethanol, 60-12-8; 3,5,5-trimethylcyclohex-2-enone, 78-59-1; ethyl 2-amino-4-methylpentanoate, 2743-60-4; 2-methyl-5-propionylfuran, 10599-69-6; methyl phenylacetate, 101-41-7; quinoline, 91-22-5; 4-phenylbutan-2-one, 2550-26-7; 2-methyl-4*H*-1-benzopyran-4-one, 5751-48-4; 4-methyl-2-phenylpent-2-enal, 26643-91-4; 3-phenylthiophene, 2404-87-7; 4-methyl-2-phenylhex-2-enal, 26643-92-5.

LITERATURE CITED

Bateman, M. A.; Morton, T. C. The Importance of Ammonia in Proteinaceous Attractants for Fruit Flies (Family: Tephritidae). *Aust. J. Agric. Res.* **1981**, *32*, 883–903.

Buttery, R. G.; Ling, L. C.; Teranishi, R.; Mon, T. R. Insect Attractants: Volatiles of Hydrolyzed Protein Insect Baits. *J. Agric. Food Chem.* **1983**, *31*, 689–692.

Gothilf, S., Volcani Center, Bet-Dagan, Israel, personal communication, 1984.

Hagen, K. S.; Greany, P.; Sawall, E. F.; Tassan, R. L. Tryptophan in Artificial Honeydews as a Source of an Attractant for Adult *Chrysopa carnea*. *Environ. Entomol.* **1976**, *5*, 458–468.

Jackson, D. S.; Lee, B. G. Medfly in California 1980–1982. *Bull. Entomol. Soc. Am.* **1985**, *31*, 29–37.

Landolt, P. J. Insect Attractants, Behavior and Basic Biology Research Laboratory, USDA, Gainesville, FL, personal communication, 1983.

Manley, C. H.; Fagerson, I. S. Major Volatile Neutral and Acid Compounds of Hydrolyzed Soy Protein. *J. Food Sci.* **1970a**, *35*, 286–291.

Manley, C. H.; Fagerson, I. S. Major Volatile Components of the Basic Fraction of Hydrolyzed Soy Protein. *J. Agric. Food Chem.* **1970b**, *18*, 340–342.

Markh, A. T.; Vinnikova, L. G. Volatile Components of Soybean Protein Hydrolyzates. *Appl. Biochem. Microbiol. (Engl. Transl.)* **1973**, *9*, 774–778.

Matsumoto, K. E.; Buttery, R. G.; Flath, R. A.; Mon, T. R.; Teranishi, R. Protein Hydrolysate Volatiles as Insect Attractants. In *Bioregulators for Pest Control*; Hedin, P. A., Ed.; ACS Symposium Series 276; American Chemical Society: Washington, DC, 1985; pp 353–366.

Mazor, M.; Gothilf, S.; Galun, R. The Role of Ammonia in the Attraction of Females of the Mediterranean Fruit Fly to Protein Hydrolysate Baits. *Entomol. Exp. Appl.* **1987**, *43*, 25–29.

Miller, J. R.; Haarer, B. K. Yeast and Corn Hydrolysates and Other Nutritious Materials as Attractants for Onion and Seed Flies. *J. Chem. Ecol.* **1981**, *7*, 555–562.

Morton, T. C.; Bateman, M. A. Chemical Studies on Proteinaceous Attractants for Fruit Flies, Including the Identification of Volatile Constituents. *Aust. J. Agric. Res.* **1981**, *32*, 905–916.

Steiner, L. F. Fruit Fly Control in Hawaii with Poison-Bait Sprays Containing Protein Hydrolysates. *J. Econ. Entomol.* **1952**, *45*, 838–843.

Teranishi, R., Western Regional Research Center, USDA—ARS, Albany, CA, personal communication, 1984.

van Emden, H. F.; Hagen, K. S. Olfactory Reactions of the Green Lacewing, *Chrysopa carnea*, to Tryptophan and Certain Breakdown Products. *Environ. Entomol.* **1976**, *5*, 469–473.

Withycombe, D. A.; Mookerherjee, B. D.; Hruza, A. Isolation of Trace Volatile Constituents of Hydrolyzed Vegetable Protein via Porous Polymer Entrainment. In *Analysis of Food and Beverages*; Charalambous, G., Ed.; Academic Press: New York, 1978; pp 81–94.

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Depletion of [^{14}C]Clorsulon in Cows' Milk

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Radioactive residue levels were determined in milk and plasma of Holstein cows dosed once with an oral suspension of ^{14}C-labeled clorsulon at 7 mg/kg of body weight. Average milk residue levels decreased from 0.54 ppm at 0.9 day postdose to 0.004 ppm at 6.9 days postdose with a half-life of 0.81 day. The drug residue was isolated by a batch adsorption method on an affinity agarose gel of carbonic anhydrase–Sepharose 4B and analyzed by HPLC–reverse isotope dilution assay (RIDA). This novel method greatly simplified the extraction of drug residue from milk and provided pure drug residue isolates. RIDA results of the isolates indicated that the unchanged drug was the major residue component in milk at 0–4 days postdose accounting for 56–99% of the total radioactive residue. Depletion half-life of the parent drug was 0.82 day, in close agreement with that of the total residue in milk. About 0.7% of the dose was recovered in the milk during the 6.9-day period.

Clorsulon [MK-401, 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide] (Figure 1) is a potent fasciolicide, effective against mature and immature *Fascila hepatica* in cattle and sheep (Mrozik, 1976; Mrozik et al., 1977; Ostlind et al., 1977). The drug appears to be extremely safe, since no gross toxicosis was observed in sheep after intraruminal doses as high as 400 mg/kg of body weight. The minimum effective dose for the removal of 14-week-old flukes from beef calves was ≤2 mg/kg parenterally (Wyckoff and Bradley, 1983). In vitro studies indicate that the drug acts by blocking the glycolytic pathway in the flukes, by direct inhibition of 3-phosphoglycerate kinase and phosphoglyceromutase (Schulman and Valentino, 1980). Schulman et al. (1979, 1982) performed pharmacokinetic studies in rats showing the drug is well-absorbed after oral administration. A single oral dose of clorsulon (6.25 or 12.5 mg/kg) produces peak blood concentrations about 4 h after dosing with 75% of the drug found in plasma and the rest bound to carbonic anhydrase in

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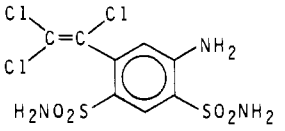


Figure 1. Clorsulon [MK-401, 4-amino-6-(trichloroethenyl)-1,3-benzenedisulfonamide]. Ring-labeled with carbon-14.

erythrocytes. Results from these authors show that the drug probably reaches the flukes by a mechanism partially involving ingestion of erythrocytes containing the drug bound to carbonic anhydrase.

We have carried out metabolism studies with steers dosed intraruminally with ^{14}C-labeled clorsulon at 10 mg/kg of body weight. Our results show that the edible tissue of steers contain extremely low residue levels (100–400 ppb, 7 days after dosing), which deplete at half-lives of 4–5 h. A major portion of the radioactive residue in the liver and kidney tissue is the unchanged drug (Chiu et al., 1985b).

In our metabolism studies, affinity columns prepared by coupling carbonic anhydrase (CA) to Sepharose 4B are successfully used for the isolation of clorsulon residue from tissue hydrolysates (Chiu et al., 1985b). This is possible because the mild inhibitory property of clorsulon on CA ($IC_{50} = 1.2 \times 10^{-6}$ M) enables it to be readily adsorbed on and desorbed from the CA affinity gel.

The study reported in this paper was to evaluate the total drug residue levels and the amount of parent drug in milk from cows dosed once orally at 7 mg/kg of body weight.

For analysis of clorsulon in milk, a batch isolation method was developed using the carbonic anhydrase–Sepharose 4B (CAS) affinity agarose gel. The method and results of the assays are presented in this paper.

MATERIALS AND METHODS

Radiolabeled Clorsulon. ^{14}C-Labeled clorsulon [4-amino-6-(trichloroethenyl)-1,3-[U-*ring*-^{14}C]benzenedisulfonamide] was synthesized and purified by the Labeled Compound Synthesis Group, Merck Sharp and Dohme Research Laboratories. The compound was prepared by using ^{14}C-ring-labeled-benzoic acid as the starting material in a five-step synthesis (Gatto and Mertel, 1981, unpublished data). The specific activity was 5.18 μCi/mg, and radiopurity was 99.6%. The drug was prepared as an oral suspension (113 mL) at 8.5% (w/v) in aqueous propylene glycol.

Unlabeled Clorsulon. A standard solution of clorsulon at 0.9958 mg/mL was prepared in methanol. This solution was used as the unlabeled (cold) carrier in all reverse isotope dilution assay (RIDA) experiments. The average absorption coefficient (*A*%) of the standard was determined to be 435.3 ± 4.4. The value of 435 was used in all RIDA calculations.

Animal Handling. Four lactating Holstein dairy cows from Verona, WI, were used in this study. The average milk production from these animals was between 28.55 and 33.00 kg/day at dosing, and they were tested and determined to be free of tuberculosis and brucellosis. Physical examinations of the cows were performed at the beginning and late acclimation period and at termination of the study. The animals were confined to tie stalls during the acclimation and test periods. Treated and control animals were housed with spatial separation and a divider so that cross contamination of the animals and milk could not occur. Roughage was available to the animals ad libitum. Long hay was offered at approximately 25% of the total roughage. Concentrate diet (Purina Milk Generator) was fed at the rate of 1.0 kg for each 2.5 kg of milk produced, based on the average daily milk production for the previous week. Half of the concentrate diet was fed at the a.m. milking (about 6:30 a.m.) and the remainder at the p.m. milking (about 4:00 p.m.). All animals were acclimated to the test facility, diet, and management program for 30 days. On the day before dosing (i.e., day −1) the treatment groups were assigned randomly such that each treatment was approximately balanced with respect to body weight and milk production. The total dose given to each animal was based on the average of two consecutive weighings obtained on the 2 days before dosing (i.e., day −2 and day −1). For dosing, the test material formulation was measured into gelatin capsules. The capsules were then sealed and immediately administered with a balling gun. Control animals received neither test material nor placebo.

Milk Sample Collection. The animals were milked twice daily at about 6 a.m. and between 4 and 5 p.m. The milk was weighed and mixed, and three subsamples of milk (100–200 mL) from each animal were taken at each sampling interval. These samples were frozen immediately.

Blood Sample Collection. Blood samples were drawn from a jugular vein immediately before dosing, at 6, 12, 24, and 30 h postdosing and at 2, 3, 4, and 7 days postdosing. Following centrifugation, the plasma samples were frozen immediately.

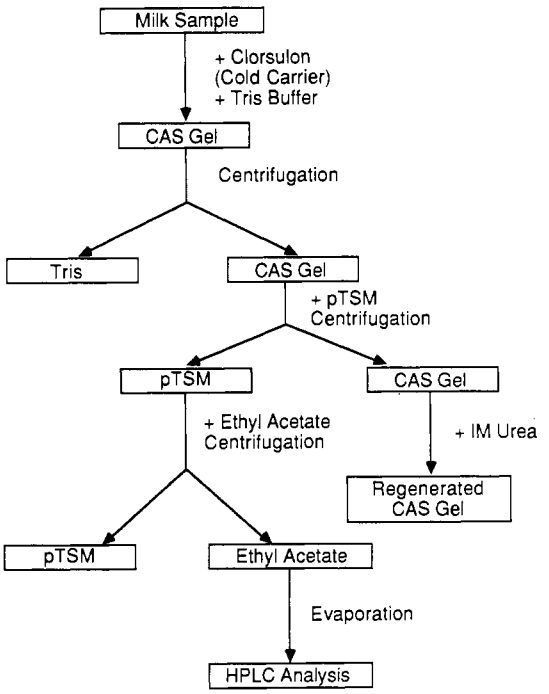
Reagents and Solutions. All organic solvents were HPLC grade, from EM Science or Fisher Co. The CN–Br-activated Sepharose 4B was from Pharmacia Co. (Piscataway, NJ). Carbonic anhydrase (CA) (EC 4.2.1.1. from bovine erythrocytes) was from Sigma Chemical Co. (St. Louis, MO). Buffer solutions for coupling and elution of CA-Sepharose 4B were as reported previously (Chiu et al., 1985b). Coupling buffer was 0.1 M sodium bicarbonate at pH 8.3 containing 0.5 M sodium chloride. Elution buffer was 0.01 M *p*-toluenesulfonamide (pTSM) containing 0.5 M sodium chloride and 0.01 M Tris–phosphate, pH 7.5. For regeneration of the gel, 1 M urea in 0.2 M sodium acetate, pH 5.5, was used.

Carbonic Anhydrase–Sepharose 4B (CAS) Affinity Gel. Carbonic anhydrase solution (625 mg in 100 mL of coupling buffer) was mixed with 25 g of CN–Br-activated Sepharose 4B (prewashed with 100 mL of 1 mM HCl). The mixture was transferred to six 50-mL polystyrene centrifuge tubes and agitated end to end for 2 h at room temperature. A glycine solution (0.2 M, pH 8, 35 mL/tube) was added to the gel to block the unreacted sites, and the mixture was agitated overnight. The gel suspension was filtered, washed with 2 L of coupling buffer, followed by 500 mL of 0.1 M sodium acetate, pH 5.5, and finally eluted with 1 L of coupling buffer. The gel was stored at 4 °C.

Reverse Isotope Dilution Assay. *1. Isolation of* [^{14}C]*-Clorsulon Residue from Milk by CAS.* A 2-g sample of milk (12 000 dpm) to which 199.2 μg (200 μL) of unlabeled clorsulon (carrier) had been added was mixed with 2 mL of Tris buffer. The mixture was added to 2.5 g of CAS gel, vortexed briefly, and centrifuged, and the supernatant was pipetted off. The gel was washed with 0.1 M Tris–phosphate, pH 7.5 (2 × 10 mL), until the final eluate was clear. The gel was then vortexed with 3 × 5 mL of pTSM elution buffer and centrifuged, and the supernatant was pipetted into a graduated cylinder. An aliquot of 0.5 mL was removed for scintillation counting. The pTSM eluate was extracted with 2 × 10 mL of ethyl acetate. In order to obtain a good separation, the pTSM–ethyl acetate mixture was filtered through a sintered glass funnel. The filtrate was transferred to a tube and centrifuged, and the ethyl acetate layer was pipetted into a graduated cylinder. An aliquot of 0.1 mL was scintillation counted. The ethyl acetate layer was rotary-evaporated to dryness. The residue was redissolved in a small amount of ethyl acetate, transferred to a centrifuge tube, and evaporated to dryness. This residue was redissolved in about 300 μL of HPLC mobile phase for HPLC analysis. Used affinity gel was regenerated by washing with 0.2 M sodium acetate, pH 5.5, containing 1 M urea. The isolation scheme is shown in Figure 2.

2. Extraction of [^{14}C]*Clorsulon from Control Milk.* To a 10-g milk sample from a control animal was added with an aliquot of [^{14}C]clorsulon containing 60 317 dpm radioactivity. From this, a 2-g sample was mixed with 298.7 μg (300 μL) of standard clorsulon solution. Tris buffer (3 mL) was added and mixed. The mixture was added to 2.5 g of CAS gel, vortexed, and centrifuged. The elution of the sample was continued as described above.

HPLC Analysis. A liquid chromatograph (LC) from Spectra Physics (Model 8700) was used in all assays. The LC was equipped with a sample injection valve (Rheodyne Model 7125), a syringe-loading 200-μL sample loop, and an ultraviolet detector (Schoeffel Instruments, Model 770). A recorder–integrator (Hewlett-Packard Model 3388A) and a fraction collector (LKB 2111) were connected to the LC. Fractions on the chromatograms were marked by an event-marking device installed between the



CAS Gel: Carbonic Anhydrase-Sepharose 4B
pTSM = p-Toluenesulfonamide

Figure 2. Extraction scheme of [^{14}C]clorsulon residue from cows' milk.

detector and the fraction collector. All chromatographs were monitored at 265 nm. Reversed-phase Zorbax ODS columns (4.6 mm × 25 cm or 4.6 mm × 15 cm; Du Pont) with CO:Pell ODS packed guard columns (Whatman Co.) were used in all assays. The mobile phase was acetonitrile–0.01 M sodium phosphate (pH 4), 25:75, at a flow rate of 1 mL/min.

UV Spectroscopy. UV absorption spectra were obtained with a UV spectrophotometer (Perkin-Elmer Model 559).

RIDA Calculation. The percent of unchanged [^{14}C]clorsulon in the total radioactive residue of a milk sample was calculated as

$$\% \text{ clorsulon} = \frac{\text{total radioact in isolated clorsulon } (R)}{\text{total radioact in milk sample } (R_T)} \times 100$$

where R = SA × W. SA designates the specific activity (dpm/μg) of [^{14}C]clorsulon recovered from HPLC purification, and W is the weight of standard clorsulon (μg) added to the sample. R_T represents the total radioactivity (dpm) in the milk sample based on combustion assay.

Measurement of Total Radioactivity. Approximately 0.5 g of milk and plasma samples were weighed and combusted directly in a Packard Model 306 sample oxidizer (Packard Instrument Co., Downers Grove, IL). The $^{14}CO_2$ was trapped in 8 mL of Carbosorb and 13 mL of Permafluor V (Packard). All radioactivity measurements were made in a Packard Tri-Carb scintillation spectrometer (Model 3255), with quenching corrections based on the external standard method. Tissue blanks were combusted with duplicate samples from control or predose tissues. All blanks and samples were counted for 10 min. The standard deviation due to radiochemical statistics was calculated by the computer using the following relationships:

$$\sigma = [\sigma_{bkg}^2 + \sigma_{sample}^2]^{1/2}$$

$$\sigma_{bkg} = \frac{(\text{total bkg count (all samples)})^{1/2}}{\text{total time counted in min}}$$

$$\sigma_{sample} = \frac{(\text{total count of sample})^{1/2}}{\text{total time counted in min}}$$

Samples were reassayed if the replicate values differed by (a) more than $\pm 3\sigma$ from their mean, *and* (b) more than ±5% from their mean, *and* (c) more than 1 ppb from each other. Tissue duplicate samples were repeated until the above criteria were satisfied.

RESULTS AND DISCUSSION

Radiochemical Statistics and Replication and Detection Limits. The standard deviation due to radiochemical statistics is generally the smallest source of error in a radiochemical analysis. However, as the level of radioactivity is lowered, the total sample count approaches the background count until, at some level, the sample count is not statistically different from the background count. This is sometimes taken as the detection limit. In this experiment, the radiochemical statistics were used to evaluate the replicate determinations and to establish the validity of any set of replicates. Samples were reassayed if the replicate values differed by the three criteria described under Materials and Methods. These differences would have indicated an error in the experiment due to some factor other than radiochemical statistics.

Radioactive residue detection limits were determined by burning and counting respective control samples of milk and plasma and calculated according to

$$\text{detection limit} = \frac{4\sigma \text{ (tissue blanks, dpm)}}{(\text{dpm}/\mu\text{g})(\text{av sample wt in g})\ (\text{fraction recd})}$$

The detection limits for both the milk and plasma assays were 0.002 ppm in this study.

Total Radioactive Residue in Milk and Plasma. Residue levels were determined in milk and plasma of Holstein dairy cows dosed once 3 h following the morning milking (6:30 a.m.) on day 0, with an oral suspension of ^{14}C-labeled clorsulon at 7 mg/kg of body weight. Milk (0.3–6.9 days postdose) and plasma (6–168 h postdose) were assayed for total radioactivity by combustion. Milk and plasma were also taken from control animals for radioactivity background determinations.

The total radioactive residue levels in milk from two experimental animals are presented in Table I. The average residue level in milk decreased from 0.543 ppm at 0.9 day postdose to 0.004 ppm at 6.9 days postdose. The individual residue levels in milk with the exception of 0.3 day postdose levels were fit to a simple exponential equation, to give a depletion half-life of 0.81 day (correlation coefficient −0.985).

The residue levels in plasma are shown in Table II. The residue levels in plasma plateaued at 2.62, 2.53, and 2.40 ppm, respectively, at 12, 24, and 30 h postdose, and then started to deplete at 48 h, dropping from 1.15 to 0.03 ppm at 168 h (Table II). The individual residue levels from 30 to 168 h were fit to a simple exponential equation, affording a half-life of 0.903 day (R = −0.992). The residue levels in plasma indicated that the drug was well absorbed.

The amount of radioactive drug residue recovered in the milk during the 6.9-day period was 29.2 and 27.8 mg equiv from animals 119 and 121, respectively. These recoveries corresponded to 0.8 and 0.6% of the dose, respectively.

Carbonic Anhydrase–Sepharose 4B Affinity Gel. Previous metabolism studies in edible tissues of steers dosed intraruminally with [^{14}C]clorsulon at 10 mg/kg of body weight showed that the parent drug was the major residue component in liver and kidney tissues. The levels of the unchanged parent drug in these tissues were quantitated by an HPLC–RIDA method developed in our laboratories (Chiu et al, 1985a). This RIDA method was also applied in the present study to assay for the unchanged clorsulon in milk.

Clorsulon has been known to be a mild inhibitor of carbonic anhydrase (CA) (H. Schwam, 1985, personal communication) and had been shown to inhibit CA in

Table I. Total [^{14}C]Clorsulon Residue (Drug Equivalents, ppm) in Milk of Lactating Cows Dosed with an Oral Suspension at 7 mg/kg of Body Weight[a]

days postdose	animal no.	milk residue[a] ppm	milk residue[a] av ppm
0.3	119	0.196	0.183
	121	0.170	
0.9	119	0.564	0.543
	121	0.523	
1.3	119	0.485	0.477
	121	0.468	
1.9	119	0.290	0.276
	121	0.262	
2.3	119	0.238	0.207
	121	0.176	
2.9	119	0.139	0.116
	121	0.093	
3.3	119	0.099	0.082
	121	0.065	
3.9	119	0.058	0.046
	121	0.034	
4.3	119	0.041	0.033
	121	0.024	
4.9	119	0.026	0.020
	121	0.013	
5.3	119	0.019	0.014
	121	0.010	
5.9	119	0.012	0.009
	121	0.005	
6.3	119	0.010	0.007
	121	0.005	
6.9	119	0.006	0.004
	121	0.002	

[a] Detection limit was 0.002 ppm.

Table II. Total [^{14}C]Clorsulon Residue (Drug Equivalents, ppm) in Plasma of Lactating Cows Dosed with an Oral Suspension at 7 mg/kg of Body Weight[a]

hour postdose	animal no.	plasma residue[a] ppm	plasma residue[a] av ppm
6	119	1.074	1.14
	121	1.201	
12	119	2.296	2.62
	121	2.946	
24	119	2.336	2.53
	121	2.724	
30	119	2.165	2.40
	121	2.644	
48	119	1.076	1.15
	121	1.225	
72	119	0.548	0.50
	121	0.445	
96	119	0.233	0.20
	121	0.162	
168	119	0.034	0.03
	121	0.023	

[a] Detection limit was 0.002 ppm.

host's blood posttreatment with the drug (Schulman et al., 1979). By taking advantage of this property, an affinity agarose gel of CA–Sepharose 4B (CAS) was prepared and was applied in an affinity column for the isolation of unchanged clorsulon in tissue hydrolysates (Chiu et al., 1985b). This method was modified in the present study to a batch operation to isolate [^{14}C]clorsulon residue from milk. When a milk sample was mixed with CAS gel, followed by centrifugation to remove the "unbound" milk constituents, the "bound" radioactivity was then released by treating the gel with *p*-toluenesulfonamide (pTSM), another inhibitor of CA. From the pTSM solution, radioactive clorsulon was readily extracted into an organic solvent such as ethyl acetate. The extract obtained from this three-step procedure was free from lipids and sufficiently pure for HPLC analysis without further purification. The extraction scheme is shown in Figure 2. This stepwise extraction method can be modified so that the adsorption/desorption process is achieved in one batch, i.e., by filtering the CAS–milk suspension on a sintered glass funnel followed by desorption with pTSM on the funnel. This method will be advantageous if a large number of samples are to be analyzed.

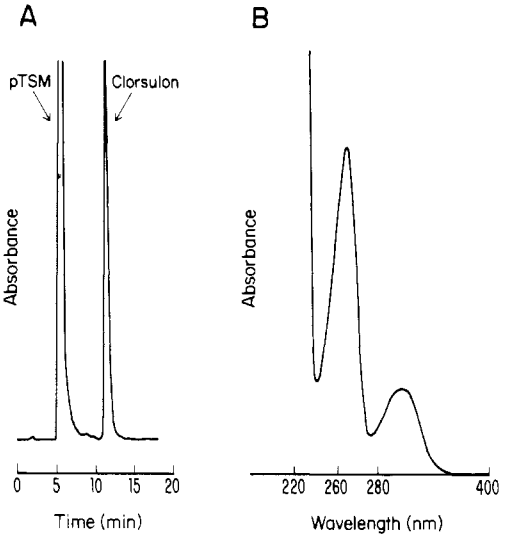


Figure 3. (A) UV tracing (at 265 nm) of HPLC chromatogram of [^{14}C]clorsulon in milk after desorption from CAS agarose gel by pTSM. Reversed-phase HPLC condition was as described in Materials and Methods. (B) UV spectrum of clorsulon isolated from (A), showing λ_{max} at 265 nm.

Reverse Isotope Dilution Assay. Unchanged Drug in Milk. For application of the CAS affinity gel in the RIDA of [^{14}C]clorsulon, a 10-g milk sample from a control cow was spiked with the radiolabeled drug. From this, a 2-g sample was mixed with 298.7 μg of unlabeled carrier and treated as described above. The final ethyl acetate extract was analyzed for clorsulon by RIDA. Calculation based of specific activity of the recovered [^{14}C]clorsulon showed 101% drug, indicating the validity of the method. In this control sample, about 50% of radioactivity was desorbed by 22.2 mL of pTSM. With an additional 14.7 mL of pTSM, the total desorbed radioactivity was 86%. Ethyl acetate extraction of the pTSM solution recovered 78% of the desorbed radioactivity.

For experimental milk samples, desorption of the bound drug was carried out twice each with 10 mL of pTSM. The amount of radioactivity desorbed varied, ranging from 32 to 76%, except for the two 2.9-days postdose samples in which only 21 and 24% of radioactivity were recovered. There was no correlation between desorption recovery and the postdose duration of the milk samples. Since quantitation of the drug by RIDA is independent of extraction recovery, expending additional time and solvents to achieve greater extraction recovery was not necessary.

Extraction of the drug from the pTSM solution by ethyl acetate was usually above 85%. HPLC of the ethyl acetate extract showed a clean UV absorption base line and well-resolved peaks of the drug and pTSM (coextracted into ethyl acetate). The UV spectrum and HPLC chromatogram of a 1.3 days postdose milk sample are shown in Figure 3. Results of the unchanged drug assays are shown in Table III. The drug accounted for 71–99% of the total residue for all samples assayed except those from 2.9 days postdose animals. In these samples the drug was 56 and 67% of the total residue, from animals 121 and 119, respectively. Although the reason for the relatively low

Table III. Unchanged [^{14}C]Clorsulon in Cows' Milk by Reverse Isotope Dilution Assay (RIDA)

days post-dose	animal no. 119 total residue, ppb	animal no. 119 unchanged drug %	animal no. 119 unchanged drug ppb	animal no. 121 total residue, ppb	animal no. 121 unchanged drug %	animal no. 121 unchanged drug ppb
0.3	196	*a*	*a*	170	99	168[b]
0.9	564	93	525	523	88	460
1.3	485	96	466	468	71	332
1.9	290	*a*	*a*	262	82	215
2.3	238	84	200	176	96	169
2.9	139	67	93	93	56	52
3.3	99	82	81	65	77	50
3.9	58	72	42	34	*a*	*a*
4.3	41	88	36	24	*a*	*a*

[a] Not assayed. [b] Data not included in half-life calculation.

percentage of parent drug in the 2.9-day milk samples is not known, the low extraction recovery (about 15% less than all other samples) suggests that perhaps some degradation had taken place before analysis (e.g., during milk collection and storage). This probably resulted in a decrease of radioactive species (parent drug or metabolites) that would be adsorbed by the affinity gel. Degradation of clorsulon by conjugation with solvent contaminant or tissue endogenous aldehydes (e.g., acetaldehyde) during tissue sample extraction and storage had been observed previously (Chiu et al., 1984, unpublished data).

It should be noted that although we had stressed the necessity of repetitive HPLC purification for the verification of RIDA results in our previous publication (Chiu et al., 1985a), this was not done in the milk assays. We have observed in the metabolism studies of clorsulon in animal tissues that repetitive HPLC using alternating reversed-phase and normal-phase columns was necessary in order to isolate pure parent drug from the tissue extracts. This is because the residues recovered after a lengthy solvent extraction procedure had undergone substantial degradation (e.g., conjugate formation). In the case of the milk assays, the affinity gel provided an efficient and specific purification step with minimal degradation before HPLC analysis. The resulting UV tracings of the clorsulon residue chromatograms were exceptionally clean, and repetitive HPLC purifications were unnecessary. We have verified this by the analysis of control milk sample with added parent drug and also by comparing the specific activities of parent drug recovered from one RP-HPLC purification with that from an additional NP-HPLC. Since the control milk assay was satisfactory (101%) and no change in specific activity was observed after sequential HPLC purifications, we concluded that multiple chromatographic purification would not be necessary in this study.

The depletion half-life of clorsulon in milk was 0.82 day based on data from Table III, calculated by linear regression. This is in close agreement with that of the total radioactive residue, 0.81 day. Data from the 0.3-day time point are not included in the depletion calculation since that was before the peak level of residues. The high proportion of the unchanged drug, up to 4 days, suggested that minimal metabolism had taken place and that the parent drug would be an appropriate marker substance for residue assays in milk of cows dosed with clorsulon at 7 mg/kg of body weight.

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LITERATURE CITED

Chiu, S. H. L.; Sestokas, E.; Taub, R.; Lu, A. Y. H., Merck Sharp & Dohme Research Laboratories, unpublished data, 1984.

Chiu, S. H. L.; Buhs, R. P.; Sestokas, E.; Taub, R.; Jacob, T. A. Determination of ivermectin residue in animal tissues by high-performance liquid chromatography reverse isotope dilution assay. *J. Agric. Food Chem.* **1985a**, *33*, 99–102.

Chiu, S. H. L.; Sestokas, E.; Taub, R.; Walker, R.; Lu, A. Y. H. Application of carbonic anhydrase affinity chromatography in study of tissue-bound residue of 4-animo-6-trichloroethenyl-1,3-benzenedisulfonamide (MK-401, clorsulon). *Drug Metab. Disp.* **1985b**, *13*, 374–376.

Gatto, G.; Mertel, H., Merck Sharp & Dohme Research Laboratories, unpublished information, 1981.

Mrozik, H. H., Merck & Co. Substituted benzenedisulfonamide. German Patent DE 2556122, June 24, 1976.

Mrozik, H.; Bochis, R. J.; Eskola, P.; Matzuk, A.; Waksmunski, T. S.; Olen, L. E.; Schwartzkopf, G.; Grodski, A.; Linn, B. O.; Lusi, A.; Wu, M. T.; Shunk, C. H.; Peterson, L. H.; Milkowski, J. D.; Hoff, R. D.; Kulsa, P.; Ostlind, D. A.; Campbell, W. C.; Riek, R. F.; Harmon, R. E. 4-Amino-6(trichloroethenyl)-1,3-benzenedisulfonamide, a new, potent fasciolicide. *J. Med. Chem.* **1977**, *20*, 1225–1227.

Ostlind, D. A.; Campbell, W. C.; Riek, R. F.; Baylis, F. P.; Cifelli, S.; Hartman, R. K.; Lang, H. K.; Butler, R. W.; Mrozik, H.; Bochis, R. J.; Eskola, P.; Matzuk, A.; Waksmunski, T. S.; Olen, L. E.; Schwartzkopf, G.; Grodski, A.; Linn, B. O., Lusi, A.; Wu, M. T.; Shunk, C.; Peterson, L. H.; Milkowski, J. D.; Hoff, R. D.; Kulsa, P.; Harmon, R. E. The efficacy of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide against liver fluke in sheep. *Br. Vet. J.* **1977**, *133*, 211–213.

Schulman, M. D.; Valentino, D. *Fasciola hepatica*: Effects of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide on glycolysis *in vitro*. *Exp. Parasitol.* **1980**, *49*(2), 206–215.

Schulman, M. D.; Valentino, D.; Cifelli, C.; Lang, R.; Ostlind, D. A. A pharmacokinetic basis for the efficacy of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide against *Fasciola hepatica* in the rat. *J. Parasitol.* **1979**, *65*, 555–561.

Schulman, M. D.; Valentino, D.; Cifelli, S.; Ostlind, D. A. Dose-dependent pharmacokinetics and efficacy of MK-401 against old and young-mature infections of *Fasciola hepatica* in the rat. *J. Parasitol.* **1982**, *68*, 603–608.

Schwam, H., Merck Sharp & Dohme Research Laboratories, personal communication, 1985.

Wyckoff, J. H.; Bradley, R. E. Efficacy of a benezenedisulfonamide against experimental *Fasciola hepatica* infections in calves. *Am. J. Vet. Res.* **1983**, *44*, 2203–2204.

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