Metabolic and Tissue Residue Studies on Parbendazole in Sheep

C. John DiCuollo,*[1] James A. Miller,[1] Wilford L. Mendelson,[2] and Joseph F. Pagano[2]

Parbendazole is a potent anthelmintic, exhibiting a broad spectrum of activity against a wide variety of gastrointestinal nematodes of ruminants, swine, poultry, and laboratory animals. Parbendazole-^{14}C was orally administered to sheep at 45 mg/kg, resulting in peak plasma levels of carbon-14 activity in approximately 6 hr. Only 26% of the administered dose was recovered in the urine. Seven metabolites were isolated from the urine employing thin-layer and column chromatographic procedures. Metabolic transformation primarily involved side-chain oxidation with ring hydroxylation playing a minor role. By employing autoradiographic techniques, one metabolite was found to have a longer tissue residence time than the others. In a study in which sheep were orally administered 100 mg/kg of parbendazole, it was determined that residues of this metabolite in edible tissues were less than 0.1 ppm after 6 days withdrawal.

Parbendazole (methyl 5(6)-butyl-2-benzimidazolecarbamate) was first demonstrated to exhibit a broad spectrum of anthelmintic activity against a wide variety of gastrointestinal nematodes of ruminants, swine, poultry, and laboratory animals (Actor *et al.*, 1967). Since then, extensive studies in ruminants have proven the usefulness of parbendazole as an anthelmintic agent (Benz, 1968; Rubin, 1969; Bennett, 1968). Its usefulness as an anthelmintic has also been reported for nonruminants, such as swine and monkeys (Chang and Wescott, 1969; Theodorides and Laderman, 1968).

This report describes the absorption, distribution, tissue residence time, and excretion of parbendazole in sheep. In addition, its metabolic fate is described, utilizing ^{14}C-labeled parbendazole.

EXPERIMENTAL SECTION

Radiosynthesis of Parbendazole-^{14}C. Thiourea-^{14}C (Mallinkrodt Nuclear Corp., 50 mCi, 0.635 g, 8.35 mmol) was treated with 6 drops of water and dimethyl sulfate (0.585 g, 4.7 mmol). The mixture was heated in a pear shaped flask at 120° until most of the water had evaporated and a white solid was deposited. The flask was cooled in an ice bath and methyl chloroformate (1.48 g, 15.6 mmol) and 2.5 *N* NaOH (10 ml) were added and the solution stirred at room temperature for 10 min. A solution of 2-amino-4-*n*-butylaniline (1.50 g, 9.1 mmol) in water (4 ml), EtOH (1 ml), and HOAc (1 ml) was added and the temperature was slowly brought to 90–100°. The heating and stirring were continued for 1 hr after gas evolution (methyl mercaptan) had ceased.

The reaction was cooled, and water was added. The product was collected and purified by slurrying in acetone or aqueous EtOH. The resulting white solid, after drying *in vacuo*, had mp 218.5–220°, a yield of 1.44 g (5.8 mmol, 68% from thiourea-^{14}C), and a radiochemical purity of 99.8% *via* thin-layer chromatography. The specific activity of this product (parbendazole) was 6.1 mCi/mmol (Figure 1).

Synthesis of 2-Amino-4-*n*-butylaniline. To a solution of *p*-*n*-butylaniline (30.0 g, 0.22 mol) in glacial HOAc (100 ml) was added acetic anhydride (56 g, 0.55 mol). The reaction mixture was heated and stirred at 65° for 1 hr. After cooling to room temperature, concentrated H_2SO_4 (0.15 g) and HNO_3 (67%, 100 g) were added dropwise (exotherm). The reaction was stirred at 30–35° for 1 hr and poured into a large excess of ice water. Filtration with a water wash gave an orange solid which on drying weighed 47.2 g (0.20 mol, 91% purity, mp 74–76°). This material (2-nitro-4-*n*-butylaniline) was used directly.

A solution of 2-nitro-4-*n*-butylaniline (47.2 g, 0.20 mol) in MeOH (100 ml) containing NaOH (50%, 20 g) was refluxed for 1 hr. While heating, sodium sulfhydrate (45%, 65 ml) was added to maintain a gentle reflux. The reaction was refluxed overnight. Most of the MeOH was removed *in vacuo* at room temperature and water (100 ml) was added. The product was extracted with several portions of benzene, which were combined and washed with H_2O, NaCl, and dried Na_2SO_4. This extract contained 2-amino-4-*n*-butylaniline.

The diamine was isolated by distillation (115–122° (0.1–0.2 mm)) resulting in a yield of 37 g (0.18 mol, 90% purity). The product was a tan solid with a mp of 60–64° which rapidly darkened at room temperature. The product was isolated as the dihydrochloride having a mp of 238–241°. *Anal.* Calcd for $C_{10}H_{16}N_2 \cdot 2HCl$: C, H, N, Cl.

Animal Studies. Several studies are reported with varying protocols. All animals were orally administered parbendazole-^{14}C by means of a rumen tube. The doses employed represent approximately two to five times the recommended therapeutic dose of 22.5 mg/kg. The specific activity of the radioactive dose was 0.50 μCi/mg.

Three lambs receiving 45 mg/kg were placed in separate metabolism cages where urine samples were collected at varying time intervals through 96 hr after drug administration and assayed for radioactivity. Heparinized blood samples (20 ml) were withdrawn from the jugular vein employing vacutainer tubes at 0, 2, 4, 6, 8, 12, 16, 24, and 48 hr after drug administration. The samples were centrifuged for 20 min at 1500 rpm and the plasma was withdrawn for radioassay.

In order to determine the time at which the drug and its related products were completely cleared from the edible tissues (less than 0.1 ppm) six groups of three animals receiving 100 mg/kg were sacrificed by i.m. administration of succinoylcholine chloride at 5 hr, 2, 4, 6, 16, and 33 days post-drug administration. After sacrifice, 50-g samples of liver, kidney, fat, muscle, and plasma (50 ml) were collected in quadruplicate and frozen immediately for further processing. One undosed animal was also sacrificed and similar tissue samples removed for use as background counting samples.

Determination of Radioactivity in Biological Fluids. Plasma and urine samples were assayed for radioactivity by incorporating 0.1 ml of sample directly into 20 ml of a scintillation solution consisting of 0.8% BBOT and 8% naphthalene dissolved in a solvent consisting of 40% *p*-dioxane and 40% toluene in absolute EtOH. Each of the samples was corrected for quench by employing an internal standardization method (Kerr *et al.*, 1957). An *n*-hex-

[1] Applebrook Research Center, Smith Kline Animal Health Products, West Chester, Pennsylvania 19380.
[2] Research and Development Division, Smith Kline & French Laboratories, Philadelphia, Pennsylvania 19101.

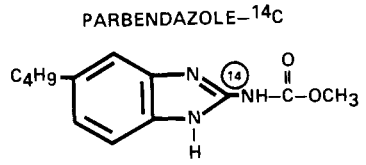


Figure 1. Structure of parbendazole-^{14}C.

adecane-^{14}C reference source obtained from Nuclear-Chicago, Inc., having a specific activity of 1.03 μCi/g, was employed as the internal standard. Background counts were determined from urine and plasma samples obtained from undosed animals. Quantitative recoveries were determined by addition of known amounts of parbendazole-^{14}C to the biological fluids. All counts were performed in a Packard Tri-Carb scintillation spectrometer (No. 3065) at a counting efficiency of 89%. Drug concentrations were determined by correcting the net sample radioactivity for the counting efficiency of the standard added to each vial and by the specific activity of the dosing solution.

Determination of Total Radioactivity in Tissues. Samples weighing 0.5 g were excised from four randomly selected areas from each tissue. Each of the weighed samples was placed in small bags constructed from dialysis tubing and dried *in vacuo* in a desiccator for 48 hr. A modified Schoniger combustion technique was then employed to combust the samples (Kelly, *et al.*, 1961; Oliverio *et al.*, 1962). The resultant $^{14}CO_2$ was absorbed by introducing into each flask 15 ml of liquid scintillation phosphor.

The composition of this phosphor was as follows: 0.8% 1,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (BBOT) and 10% naphthalene dissolved in a solvent consisting of 33.3% MeOH and 27% phenethylamine in toluene. Each of the flasks was placed in a shallow ice–water bath for 30 min at which time an additional 3 ml of phosphor was added. This solution was then counted at 4° in a Packard Tri-Carb =3356 spectrometer set at 8.5% gain with a channel width of 50–1000.

Quantitative recoveries were established by the addition of known quantities of parbendazole-^{14}C to tissues obtained from undosed animals. Quench corrections were determined by the method of internal standardization employing an *n*-hexadecane-^{14}C standard (Kerr *et al.*, 1957). Drug concentrations were determined in the same manner as described previously for biological fluids.

Distribution of Drug- and Non-Drug-Related Radioactivity in the Liver. In order to determine the presence of drug-related residues in the liver, the following extraction procedures were employed. Samples of liver (10 g) obtained from each of the drug treated animals were homogenized for 5 min in a Sorvall Omni Homogenizer. The homogenized liver was extracted with chloroform in a Soxhlet extractor for 24 hr, followed by extraction with ethyl acetate for an additional 24 hr. This procedure quantitatively removed all nonconjugated drug-related products as indicated by examination of liver tissue from nondosed animals to which known amounts of parbendazole metabolites were added, including conjugated and nonconjugated metabolites.

Tissue remaining after soxhlet extraction was suspended in 10 ml of distilled H_2O. The resulting suspensions were adjusted to pH 0.75 with 6 *N* HCl and placed in a boiling water bath for 1 hr in order to hydrolyze conjugates of drug-related products and to aid in liberating any protein bound drug products. After hydrolysis, samples were cooled to room temperature and adjusted to pH 3.5 with 18 *N* NaOH. This aqueous layer was filtered and lyophilized. Both the lyophilized filtrate and residue were subjected to exhaustive extraction with chloroform and ethyl acetate, respectively, in a soxhlet extractor for 24 hr.

The remaining residue was further extracted twice with 5 vol of H_2O for 2 hr at pH 7.6 at 25° to remove soluble protein, nucleic acids, and other water-soluble components (referred to as aqueous-soluble fraction). This aqueous fraction was centrifuged to remove insoluble residues, which were dried *in vacuo* at 25°, and aliquots were removed for radioassay. Larger aliquots of the dried residue (as well as the water-soluble components) were hydrolyzed with 6 *N* HCl in sealed tubes at 100° for 18 hr to hydrolyze protein.

The protein hydrolysates were neutralized, desalted by column chromatography (Amberlite XA-2), concentrated, and applied to a two-dimensional paper chromatographic system for the identification of amino acids according to the procedures of Benson *et al.* (1950). The first solvent system employed was water-saturated phenol (39% phenol–H_2O, w/v) and the second solvent system was *n*-BuOH–propionic acid–H_2O (10:5:7). Amino acids were identified with a ninhydrin spray reagent and radiolabeled components were identified by autoradiographic procedures. No attempt was made to identify or quantitate the amino acids detected.

Nucleic acids and polysaccharide components were precipitated from the aqueous-soluble fraction with 3 vol of ethanol at 4°. The EtOH precipitate was washed with cold EtOH and redissolved in 0.01 *M* phosphate buffer (pH 7.6) followed by extraction twice with an equal volume of phenol (90% phenol, 10% H_2O). The nonprotein components, which remain in the aqueous layer, were concentrated by precipitation with 3 vol of EtOH and the resulting precipitate redissolved in 0.10 *M* phosphate buffer (pH 7.6) and assayed for radioactivity. The radioassay procedure was sensitive to 3 cpm and was only qualitative in nature.

The residue remaining after the preparation of the aqueous-soluble fraction was extracted with chloroform–MeOH (2:1) to remove lipoidal components. These lipid extracts were dried and subsequently extracted with $CHCl_3$–MeOH–H_2O (8:4:3), according to the methods of Foch *et al.* (1957). The radioactivity in the lower phase was indicative of lipoidal components exclusive of parbendazole metabolites. The radioassay procedure was qualitative only, with a lower sensitivity limit of 3 cpm.

Procedures for the Extraction of Parbendazole and Its Metabolites from Tissue. The following tissues were excised from slaughtered animals at 5 hr, 2, 4, 6, 16, and 33 days post-drug administration: muscle, liver, kidney, plasma, and fat. Fresh liver, kidney, and muscle tissues were homogenized (50 g) in 200 ml of distilled water for 2–3 min in a Waring Blendor at full speed. Homogenates were then adjusted to pH 5.0 with 9 *M* H_3PO_4 and incubated at 37° for 24 hr in the presence of 100,000 units of glucuronidase (Ketodase, Warner Chilcott) and 5 ml of pH 5.0 1 *M* phosphate buffer. After enzymatic hydrolysis, each of the samples was saturated with NaCl and exhaustively extracted with ethyl acetate. The solvent extract was concentrated *in vacuo* at 30° to approximately 30 ml and assayed for radioactivity. This fraction contained all of the parbendazole metabolites.

In the case of fat tissue, a preliminary acid hydrolysis was required before extraction to obtain quantitative removal of drug-related substances. Fat (50 g) was hydrolyzed with 100 ml of 1 *M* H_2SO_4 in a steam bath at approximately 110°. This hydrolysate was transferred to a separatory funnel where it separated into two phases; the lower aqueous phase was quantitatively removed. This acid extract was washed several times with petroleum ether (bp 30–45°) and ethyl acetate. The wash solution was exhaustively extracted with ethyl acetate at pH 10.5 and at pH 5.0 to remove major metabolites of parbendazole. These procedures were found to quantitatively remove parbendazole-^{14}C and its metabolites, as indicated by recoveries obtained from the addition of radioac-

tive standards to control tissues obtained from undosed animals. These radioactive standards contained proportionately the total complex of parbendazole metabolites excreted by sheep. Extraction recoveries from muscle, liver, kidney, and fat were determined to be 77, 86.5, 78, and 62.7%, respectively. All tissue assays were corrected for these extraction efficiencies.

Procedures for Determining Drug-Related Conjugates in Sheep Urine. In order to determine the presence of glucuronide or ethereal sulfate conjugates of parbendazole in the urine, the following experiments were performed. Six aliquots of 5 ml each were withdrawn from a pooled 96-hr urine collection. The samples were divided into three equal groups and adjusted to pH 5.0 with 9 *M* H_3PO_4. To one group was added 5 ml of phosphate buffer at pH 5.0; this was the control group. The second group received 5.0 ml of 1 *M* phosphate buffer and 2.5 ml of Ketodase containing 25,000 units of glucuronidase. The remaining group was treated as above except that 1 ml of Gluslase was added (containing 50,000 sulfatase units and 100,000 glucuronidase units) instead of Ketodase. All samples were incubated for 32 hr at 37°.

After incubation, sufficient NaCl (0.5 g/ml of aqueous solution) was added to the point of saturation to each of the samples. They were then extracted four times with 75 ml of ethyl acetate and the pooled ethyl acetate fractions dried with 10–20 g of anhydrous Na_2SO_4, filtered, and concentrated to 30–50 ml *in vacuo* at 30°. These extracts were assayed for total radioactivity according to the procedures described for the radioassay of biological fluids. Increases in activity above the control samples reflected the extent of conjugate formation. Differences in radioactivity resulting from Glusulase and Ketodase treatment reflected the extent of ethereal sulfate formation.

Thin-Layer Chromatographic Techniques. The following thin-layer chromatographic procedures were developed for the detection of urinary and tissue metabolites and for the validation of the isolation, quantitation, and purification procedures. All thin-layer chromatographic methods were employed utilizing silica gel G as an adsorbant. The following solvent systems were employed ascending for 1 hr: $CHCl_3$–MeOH–H_2O (70:20:2); $CHCl_3$–MeOH (9:1); methyl ethyl ketone–benzene–MeOH (10:10:1); methyl ethyl ketone–benzene–MeOH (10:10:4). Parbendazole and its metabolites were detected employing a KI–starch spray reagent following exposure to chlorine vapors, and by autoradiographic techniques.

The KI–starch spray method involved exposure of the plates to chlorine vapors from a solution of sodium hypochlorite for 3–5 min and then spraying with an aqueous solution containing 2% starch and 1% KI. This procedure is suitable for the detection of imides for which *N*-chloro derivatives are formed (Smith, 1960). Drug-related metabolites gave rise to blue colored spots.

Autoradiographic techniques were also employed as a means of detecting radioactive substances in these tissues. After the solvent development of the chromatograms, they were allowed to dry and then exposed to medical X-ray (General Electric, No Screen) for approximately 1–4 weeks, depending on the level of radioactivity on the plates (Schwarz BioResearch, Inc., 1967). After exposure, films were developed in a General Electric photographic developer solution, diluted with 5 parts water. After a 2-min development period, films were washed with 1% HOAc and cleared with General Electric Fixer Solution diluted with 5 parts water. Spots detected on the plates utilizing the spray reagent were compared to those obtained by autoradiography. Radioactive areas were located and scraped from the plates and counted for radioactivity by incorporating directly into the liquid scintillation phosphor described under counting methods for biological fluids. This technique served to identify the major metabolites and was found to be quantitative, as determined through recoveries of known radioactive metabolite standards isolated from the urine of dosed animals.

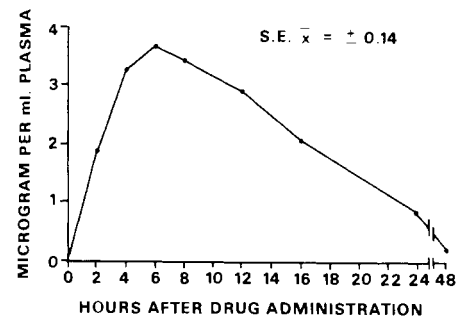


Figure 2. Plasma levels of parbendazole-^{14}C and its metabolites after oral administration of 45 mg/kg to sheep.

Column Chromatographic Procedures. In order to isolate and purify the acidic and neutral metabolites, the following column chromatographic procedures were employed. A silica gel G column (with binder), 3 × 40 cm, containing 5 g of adsorbant was employed for the purification of acid metabolites of parbendazole. After application of the sample, the column was eluted with a $CHCl_3$–MeOH–H_2O system (70:20:2). Fractions were collected and the isolated metabolites identified by thin-layer chromatographic methods.

Neutral urinary metabolites were isolated employing a silicic acid column. Silicic acid (Wills Scientific) was activated by prewashing with acetone and drying for 18 hr at 105°. The activated silicic acid was mixed with $CHCl_3$ and the resultant slurry transferred to a column having bed dimensions of 6 × 98 cm. After loading the neutral metabolites on the column, increasingly polar conditions were achieved by employing $CHCl_3$ as the elution solvent containing increasing percentages of MeOH as follows: 99:1, 98:2, 97:3, and 96:4. Eluted metabolites were detected by thin-layer chromatographic methods and the KI–starch spray reagent. After detection, identical fractions were pooled, evaporated to dryness *in vacuo* at 30°, and recrystallized from hot absolute MeOH.

RESULTS AND DISCUSSION

Absorption of Parbendazole. After a single oral dose of 45 mg/kg of parbendazole, absorption occurred rapidly with peak plasma levels obtained 6 hr after drug administration. After this peak was obtained, plasma levels dropped continuously until 48 hr at which time concentrations less than 0.1 μg/ml were detected (Figure 2).

Analysis of urine specimens for radioactivity indicated 26% of the administered dose was excreted in the urine after 96 hr. Most of the drug and its metabolites were excreted in the first 24 hr after drug administration, at which time 19% of the administered dose was detected (Figure 3).

Urinary Metabolites. Thin-layer chromatographic analysis of urine extracts indicated the presence of a number of drug-related spots, as described in Table I. Components 1, 2, and 3 were found to be the major conjugated substances in the urine. Four additional radioactive components were present as minor metabolites. Component 7 had a mobility similar to the parent compound (parbendazole, R_f 0.95) but was not identical as proven by mixed spot chromatography. In fact, residual parbendazole was not detected in the urine.

Glucuronidase Studies. Treatment of the urine with Ketodase (glucuronidase) resulted in 38% hydrolysis of the total radioactivity. Extraction with ethyl acetate at pH 5.0 and subsequent thin-layer chromatographic analysis of the extracts, employing silica gel G and a $CHCl_3$–MeOH–H_2O (70:20:2) solvent system, resulted in the separations

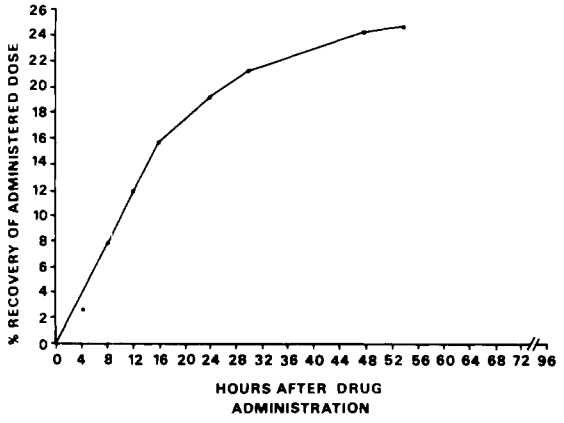


Figure 3. Urinary excretion of parbendazole-^{14}C and its metabolites after oral administration to sheep at 45 mg/kg.

described in Table I. These results indicated that, after glucuronidase treatment, components 1, 2, and 3 significantly decreased in their relative concentrations while the remaining components increased. These data support the fact that components 1, 2, and 3 are excreted in the urine primarily in the unconjugated form while components 4, 5, 6, and 7 are excreted predominately as their glucuronide conjugates. Studies with glusulase indicated that little of the drug-related metabolites were excreted as ethereal sulfates.

Isolation and Purification of the Urinary Metabolites. Approximately 8 l. of urine representing a 96-hr collection from sheep administered parbendazole-^{14}C was treated with sufficient Ketodase to hydrolyze conjugates of parbendazole. A concentration of approximately 5000 units/ml of glucuronidase was employed and hydrolysis carried out at pH 5.0 for 32 hr at 37°. After hydrolysis, the procedure outlined by the diagram in Figure 4 was followed to fractionate the parbendazole metabolites from urine.

Fraction B (containing neutral metabolites) was concentrated to dryness *in vacuo* at 30° and the residue dissolved in hot MeOH and allowed to cool. On cooling, a white crystalline substance precipitated from solution. Thin-layer chromatography both on single and mixed spots confirmed this crystalline material to be pure and identical with component 3 of the urinary metabolites.

Fractionation of the components in fraction A was accomplished in the following manner. The fraction was evaporated to dryness *in vacuo* at 30° and redissolved in $CHCl_3$ containing a few drops of MeOH. This fraction was transferred to a glass column (1 × 2 cm) containing 10 g of silica gel and allowed to adsorb onto the column to the

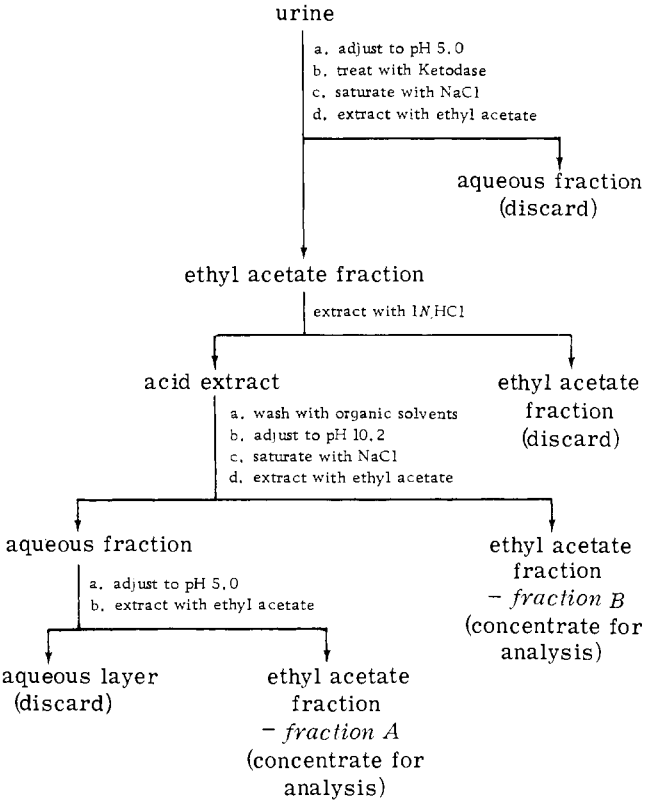


Figure 4. Schematic diagram for the fractionation of sheep urinary metabolites of parbendazole.

level of the adsorbant bed. Elution with 200 ml of $CHCl_3$ removed most of the nonpolar urinary components, as indicated by the presence of only trace quantities of radioactivity. Continued elution with 400 ml of $CHCl_3$–MeOH (99:1, v/v) eluted the remaining nonpolar substances. These conditions were also suitable for the elution of parbendazole. The addition of 400 ml of $CHCl_3$–MeOH (98:2, v/v) eluted a second band of radioactivity while elution with $CHCl_3$–MeOH (96:6, v/v) produced still a third radioactive band. Fractions in each major band were compared by thin-layer chromatography using either the $CHCl_3$–MeOH–H_2O (70:20:2, v/v/v) or the methyl ethyl ketone–MeOH–H_2O system (10:10:4, v/v/v). Results of these chromatographic studies indicated that the major urinary component was eluted in a relatively pure form in band 3 while a minor component was eluted in band 2. Fractions containing the major band were pooled and concentrated to dryness *in vacuo* at 30° and redissolved in 3–5

Table I. Thin-Layer Chromatographic Separation of Urinary Metabolites of Parbendazole-^{14}C

Component	R_f	% of total radioact. before glucuronidase	% of total radioact. after glucuronidase	Fractional increase due to glucuronidase,[a] %
1	0.2	26	17	4
2	0.4	27	18	7
3	0.6	36	27	21
4	0.7	3	3	60
5	0.75	1	6	960
6	0.8	2	22	1780
7	0.9	5	7	126

[a] Calculated utilizing the following formula: (% total radioactivity after glucuronidase/% total radioactivity before glucuronidase) × [1/(100 − 38)] × 100% = fractional increase due to glucuronides.

Table II. Silicic Acid Column Chromatography of Neutral Parbendazole Urinary Metabolites

Elution solvent $CHCl_3$–MeOH	Eluate vol, l.	R_f value of radioact. peak	Metabolite ident.
100:0	8		
99:1	4	0.8	6
98:2	4	0.75	5
97:3	4	0.7	4
96:4	8	0.6	3

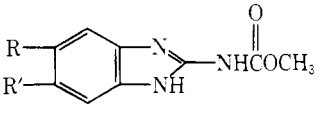
ml of hot MeOH. After cooling, a white crystalline substance precipitated from solution, with a second crop recovered after addition of acetone. Chromatographic comparison of these precipitates as single and mixed spots confirmed the isolation of urinary component 2. Pooling and recrystallizing the fractions containing the second elution band in an analogous fashion confirmed the isolation of urinary component 1.

In order to isolate sufficient quantities of other neutral metabolites from fraction B, large scale silica gel fractionation procedures were employed. Fraction B was suspended in 4–5 ml of $CHCl_3$ and solubilized by the dropwise addition of methanol. This solution was transferred to a silica gel column (5 × 75 cm) and allowed to adsorb onto the bed. The sample was then eluted by the procedure shown in Table II. To monitor the elution pattern, 500-ml fractions were concentrated to dryness *in vacuo* at 30° and subjected to thin-layer chromatographic analysis. Identical fractions were pooled and crystallized from hot MeOH. Thin-layer chromatography confirmed the purity for each of the isolated metabolites, as previously described. Four major metabolites were isolated and identified as urinary metabolites 4–7.

Structure Determination of the Urinary Metabolites. Structures for each of the urinary metabolites were determined utilizing ultraviolet, infrared, proton magnetic resonance, and mass spectrometric techniques. Confirmation of these structures was achieved by chemical synthesis as reported by Dunn *et al.* (1973). Results of the analytical studies are presented in Table III. The metabolic transformation of parbendazole-^{14}C in sheep was postulated to occur as described in Figure 5.

Tissue Depletion of Parbendazole Residues. The depletion of parbendazole-^{14}C residues from muscle, liver, kidney, and fat was determined over varying periods of withdrawal. Table IV presents the comparative results ob-

Table III. Structure of Sheep Urinary Metabolites of Parbendazole[a]



Component no.	R	R′	Chemical name
1	$HOOCCH_2C(OH)HCH_2$	H	5-(2′-Hydroxy-4′-carboxyl-*n*-butyl)-2-carbomethoxyaminobenzimidazole
2	$HOOCCH_2CH_2CH_2$	H	5-(4′-Carboxyl-*n*-butyl)-2-carbomethoxy-aminobenzimidazole
3	$CH_3CH_2C(OH)HC(OH)H$	H	5-(4′-Hydroxy-*n*-butyl)-2-carbomethoxy-aminobenzimidazole
4	$HOCHCH_2CH_2CH_2$	H	5-(4′-Hydroxy-*n*-butyl)-2-carbomethoxy-aminobenzimidazole
5	$CH_3CH_2C(OH)HCH_2$	H	5-(2′-Hydroxy-*n*-butyl)-2-carbomethoxy-aminobenzimidazole
6	$CH_3CH_2CH_2C(OH)H$	H	5-(1′-Hydroxy-*n*-butyl)-2-carbomethoxy-aminobenzimidazole
7	$CH_3CH_2CH_2CH_2$	HO	5-(*n*-Butyl)-6-(hydroxy)-2-carbomethoxy-aminobenzimidazole

[a] Ultraviolet, infrared, proton magnetic resonance, and mass spectrometric techniques were all utilized for determination of each of the above structures.

Table IV. Comparative Radioactive Residues of Parbendazole-^{14}C Remaining in Sheep Tissues after an Oral 100-mg/kg Dose Employing Extraction and Combustion Techniques

Time of sacrifice	Parbendazole-^{14}C residues in ppm[a]									
	Extraction[c]					Combustion				
	Muscle	Liver	Kidney	Plasma	Fat	Muscle	Liver	Kidney	Plasma	Fat
5 hr	1.09	6.69	12.66	2.44	1.46	*b*				
2 days	0.22	2.37	2.39	0.37	0.11					
4 days	0.01	0.33	0.04	0.05	0.00					
6 days	0.00	0.08	0.02	0.00	0.00					
16 days	0.00	0.03	0.00	0.00	0.00	0.011	1.410	0.087	0.012	0.016
33 days	0.00	0.00	0.00	0.00	0.00	0.000	0.227	0.037	0.000	0.000

[a] Less than 0.1 ppm considered insignificant. [b] No determinations performed for these time periods. [c] Results corrected for extraction efficiencies.

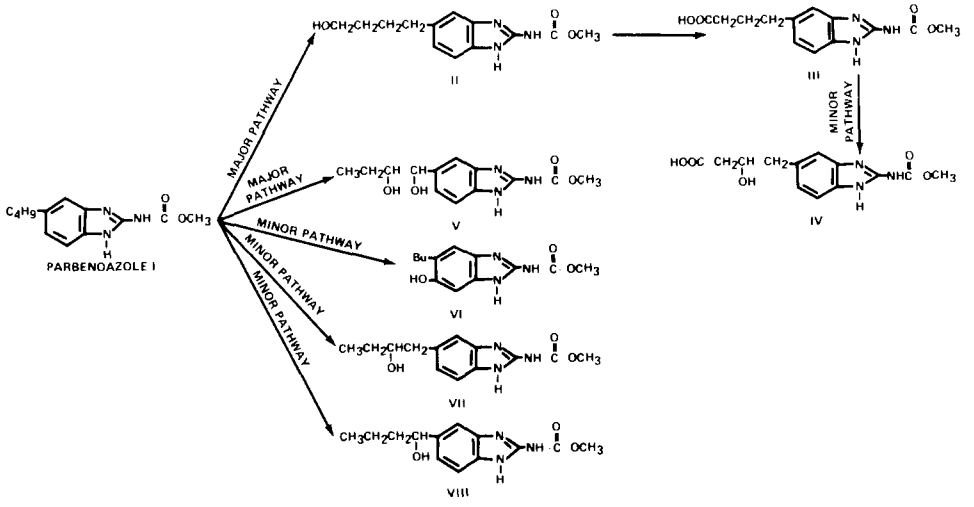


Figure 5. Proposed metabolic pathway for parbendazole in sheep.

tained for the combustion and extraction methods. Utilizing the extraction techniques previously described in the Experimental Section, tissue residues were insignificant (less than 0.1 ppm) between 4 and 6 days. According to the results obtained by combustion, however, liver residues remained elevated at 1.41 ppm even after 16 days.

The inconsistency in these results utilizing both the extraction and combustion techniques was pursued further. Previously described fortification studies demonstrated the efficiency of the extraction procedure in removing from tissues drug-related substances (Experimental Section). The problem of persisting radioactivity, therefore, was attributed to the presence of nonmetabolite residues. A number of investigators have alluded to this problem in the performance of radioactive residue studies. Rosenblum *et al.* (1971) reported that prolonged thiabendazole radioactive residues in sheep tissues were due to nonmetabolites. Thiabendazole is a benzimidazole anthelmintic, similar in structure to parbendazole. In another report, Rosenblum *et al.* (1972) reported on persistent nonmetabolite residues in turkeys receiving ronidazole-^{14}C, an imidazole antiparasitic agent. Similar problems were reported by Potter *et al.* (1973), who detected nonmetabolite radioactive residues when dichlorvos-^{14}C was administered to pregnant sows. To explore this possibility, the following limited studies were performed.

Liver tissue obtained from sheep 16 days after administration of parbendazole was fractionated into protein, polysaccharide, nucleic acids, and lipids. Radioassay of each of the fractions resulted in the detection of radioactivity in the protein fraction only. To qualitatively determine the presence of radioactive amino acids, the protein fraction was hydrolyzed with 6 *N* HCl to its constituent amino acids and submitted to two-dimensional chromatography. Separated components were identified by spraying with ninhydrin and autoradiographic techniques. Five radioactive and ninhydrin positive spots were detected. These spots were considered to be amino acids. None of the known parbendazole metabolites or analogs exhibit a positive reaction toward ninhydrin. Although a number of other ninhydrin positive spots were detected, none were radioactive. This was probably due to the low specific activity of these spots and the lack of sensitivity attributed to the autoradiographic technique.

Although this study is limited in nature and does not conclusively prove the existence of radioactive amino acids, it is suggestive of their presence. If so, one would have also expected to detect radioactivity in the lipid fraction, which was not the case. This could be explained, however, by the rapid turnover of liver lipids, in contrast to the slow metabolic turnover of proteins. Since these were liver samples obtained from animals on 16-day withdrawal, the biological decay of radiolabeled lipids could well have been completed by this time.

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