Monoclonal Antibody for Multiresidue ELISA of Benzimidazole Anthelmintics in Liver

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A monoclonal antibody has been prepared that binds the major benzimidazole anthelmintic drugs, including albendazole, fenbendazole, oxfendazole, and several of their metabolites. In addition, the antibody binds methyl benzimidazolecarbamate, a metabolite and breakdown product of the pesticide benomyl. The antibody was elicited from mice using the novel hapten methyl 5(6)-[(carboxypentyl)-thio]-2-benzimidazolecarbamate and was used to develop an ELISA method that can detect multiple benzimidazole drug and pesticide residues at concentrations between 1 and 8 ppb. The ELISA provided the basis for quantification of drug residues in bovine liver using aqueous extraction. The sulfoxide and sulfone metabolites of albendazole and fenbendazole were readily extractable and quantifiable by this method. ELISA of liver tissue from cows treated with fenbendazole produced excellent agreement with the results of HPLC analysis. In bovine liver samples fortified with equal amounts of benzimidazole drug and sulfoxide and sulfone metabolites, the limits of detection were 58 ppb for the albendazole group and 120 ppb for the fenbendazole compounds. This sensitivity enables rapid identification of samples requiring residue-specific quantitative analysis. Since the ELISA method employs stable nonhazardous materials and reagents, it could be performed in the field for rapid screening of meat products for undesired residues.

Keywords: *ELISA, monoclonal antibody, fenbendazole, albendazole, oxfendazole, drug residues, liver*

INTRODUCTION

Benzimidazole anthelmintic drugs are widely used in both clinical medicine and veterinary practice (Horton, 1990; Campbell, 1990). These broad-spectrum anthelmintics act, at least in part, by selectively binding to the protein tubulin, thereby disrupting functions including cell division in the helminth [reviewed by Lacey (1990)]. Additional mechanisms, such as uncoupling of oxidative phosphorylation (McCracken and Stillwell, 1991), may also be involved. The potential toxicity of the benzimidazoles to the host organisms [reviewed by Delatour and Parish (1986)] is reflected in the regulatory tolerances for drug residues in food animal tissues (Food Safety and Inspection Service, 1993). Methods based on chromatographic separation of the various benzimidazole residues have been described, for example, by Barker et al. (1986, 1990) and Wilson et al. (1991). With their reliance on major laboratory equipment and organic solvents, these methods do not fulfill the needs for rapid screening tests and on-site methodology for regulated residues in foods. In contrast, immunological methods appear ideally suited for this purpose (Newsome, 1986; Office of Technology Assessment, 1988). Immunoassays for several benzimidazole pesticides and drugs have been reported previously (Newsome and Shields, 1981; Newsome and Collins, 1987; Nerenberg et al., 1978, 1982; Michiels et al., 1982; Bushway et al., 1990). We previously reported monoclonal antibodies that bind thiabendazole (TBZ) and other thiazolylbenzimidazoles and described ELISAs suitable for liver, urine, and produce matrices (Brandon et al., 1992, 1993). We now report a monoclonal antibody that binds the other major group of benzimidazoles, of which methyl 2-benzimidazolecarbamate is the parent compound (Figure 1). This monoclonal antibody provides the basis for a multiresidue ELISA that utilizes a simple aqueous extraction procedure and is easily performed on bovine liver tissue.

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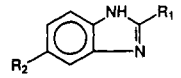
MATERIALS AND METHODS

Benzimidazoles and Related Chemicals. Compounds were either obtained as described previously (Brandon et al., 1992) or as follows. 2-Amino-5-(propylsulfonyl)benzimidazole (the 2-amino sulfone metabolite of albendazole) was provided by SmithKline Animal Health Products (West Chester, PA). Fenbendazole (FBZ) was provided by Hoechst-Roussel Agri-Vet Co. (Somerville, NJ). Oxfendazole and fenbendazole sulfone were provided by Syntex, Inc. (Palo Alto, CA). 4'-Hydroxyfenbendazole was provided by S. A. Barker (Louisiana State University, Baton Rouge, LA). Flubendazole was purchased from Janssen Life Sciences (Piscataway, NJ), and additional quantities were synthesized according to the method of van Gelder et al. (1972). 2-Acetylbenzimidazole was synthesized by oxidation of 2-(1-hydroxyethyl)benzimidazole (Cheeseman, 1964). 2-Aminobenzimidazole, benzophenone, and benzaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI). Oxibendazole was synthesized as described by Smith, Kline, and French Laboratories (1968). 2-(*n*-Butylmercapto)benzimidazole was synthesized as described by Wilson et al. (1991).

Synthetic Procedures. *Analytical Instrumentation.* Proton NMR spectra were obtained at 200 MHz on a WB200 spectrometer (Nicolet Instrument Corp., Madison, WI). Mass spectra were obtained with a VG 70/70 HS magnetic spectrometer (Fisons, Inc., Manchester, U.K.).

2-Amino-5(6)-(propylthio)benzimidazole. A solution of 1.61 g (15 mmol) of cyanogen bromide (highly toxic, handle with gloves in hood) in 8 mL of $CHCl_3$ was added dropwise to a solution of 2.8 g (15.4 mmol) of 4-(propylthio)-1,2-phenylenediamine (Gyurik and Theodories, 1975) in 10 mL of $CHCl_3$. After the reaction mixture was shaken with dilute NH_4OH solution, product was extracted with ether and $CHCl_3$. After removal of solvent on a rotary evaporator, the extract residue was mixed with ether and filtered. The filter cake was washed with ether and dried to give



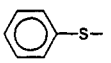
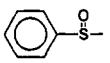
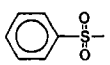
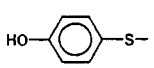
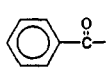
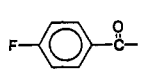
Compound	R_1 =	R_2 =
methyl benzimidazole carbamate	$-NHCOOCH_3$	H—
albendazole	$-NHCOOCH_3$	$CH_3CH_2CH_2S-$
albendazole sulfoxide	$-NHCOOCH_3$	$CH_3CH_2CH_2\overset{O}{S}-$
albendazole sulfone	$-NHCOOCH_3$	$CH_3CH_2CH_2\overset{O}{\underset{O}{S}}-$
2-amino-5-propylsulfonyl-benzimidazole	$-NH_2$	$CH_3CH_2CH_2\overset{O}{\underset{O}{S}}-$
2-amino-5-propylthio-benzimidazole	$-NH_2$	$CH_3CH_2CH_2S-$
oxibendazole	$-NHCOOCH_3$	$CH_3CH_2CH_2O-$
fenbendazole	$-NHCOOCH_3$	
oxfendazole	$-NHCOOCH_3$	
fenbendazole sulfone	$-NHCOOCH_3$	
4'-hydroxyfenbendazole	$-NHCOOCH_3$	
mebendazole	$-NHCOOCH_3$	
flubendazole	$-NHCOOCH_3$	
thiabendazole		H—
cambendazole		$(CH_3)_2CHOCN-$
5(6)-carboxypentylthio-2-methyl benzimidazole carbamate (hapten, I)	$-NHCOOCH_3$	$HOOC(CH_2)_5S-$

Figure 1. Principal benzimidazole anthelmintics and related compounds, including hapten (I).

2.34 g of a tan solid. This was dissolved in the minimal amount of CH_3OH, and hot benzene (potential carcinogen; use adequate ventilation) was added. This solution was concentrated on a steam bath and upon cooling yielded a white precipitate. The precipitate was rinsed with benzene and ether and oven-dried, resulting in 1.66 g of product, mp 175.0–176.0 °C.

Albendazole (ABZ). A 170-mL solution of 4.04 g (22 mmol) of 4-(propylthio)-1,2-phenylenediamine (Gyurik and Theodorides, 1975), 4.54 g (22 mmol) of 1,3-bis(methoxycarbonyl)-*S*-methylisothiourea (Beard et al., 1975), and 2 mL of acetic acid was diluted with 170 mL of water and heated on a steam bath for 45 min. The precipitate that formed was filtered out, washed with water, ethanol, and ether, and then oven-dried to give 4.4 g of methyl 5-(propylthio)-2-benzimidazolecarbamate (albendazole), mp 209.5–210.5 °C [lit. mp 208–210 °C (Gyurik and Theodorides, 1975)].

Albendazole Sulfoxide (ABZ-SO) and Albendazole Sulfone ($ABZ-SO_2$). When albendazole in acetic acid was oxidized by 75% the molar amount of peracetic acid at room temperature, both the sulfoxide and sulfone were formed, as indicated by TLC on silica gel 60H with ethyl acetate as developing solvent. After attempts to improve the purity of the sulfoxide by crystallization from CH_3OH or methyl ethyl ketone proved inadequate, 420 mg of sulfoxide was dissolved in the minimum volume of acetic acid, diluted with CH_3OH, and then mixed with 2 g of oven-dried silica gel 60H. This mixture was warmed and stirred to allow evaporation of CH_3OH, and then the coated silica gel was added to the top of a 15 cm × 2.5 cm column of silica gel 60H. A gradient of 0–5% acetic acid in ethyl acetate eluted the sulfone first, followed by the sulfoxide. The fractions containing sulfoxide were diluted with ether, and the resulting precipitate was washed with more ether and then dried to give albendazole sulfoxide, mp 174 °C (dec): MS (probe) (70 eV) *m/z* (relative intensity) 281 $[M]^+$ (17), 265 (4), 249 (9), 239 (30), 238 (100), 207 (33), 206 (81), 191 (21), 178 (8), 159 (43). The solid obtained by evaporation of solvent from the sulfone fractions was washed with CH_3OH and ether to give albendazole sulfone, mp 285–286 °C: MS (probe) (70 eV) *m/z* (relative intensity) 297 $[M]^+$ (13), 265 (12), 254 (5), 238 (6), 222 (7), 191 (13), 159 (18), 78 (14), 32 (57), 31 (100).

Hapten Synthesis. *2-Amino-5(6)-(propylthio)benzimidazole.* 2-Amino-4-(propylthio)aniline (2.8 g), prepared as described by Gyurik and Theodorides (1975), was dissolved in 10 mL of chloroform. A solution of 1.63 g of CNBr in 10 mL of chloroform was added dropwise. After standing overnight, the solution was washed with dilute ammonia and water. Ether was used to extract the wash mixtures. The combined ether and $CHCl_3$ solvents were removed on a rotary evaporator, and the residue was dissolved in a small volume of methanol. Benzene was added, and the solution was put on a steam bath to boil out the methanol and concentrate the benzene solution. After cooling, 1.67 g of 2-amino-5(6)-(propylthio)benzimidazole, mp 175.0–176.0 °C, was obtained: ^1H NMR (200 MHz, DMSO-d_6) δ 0.93 (3H, t, J = 7.5 Hz, CH_3), 1.51 (2H, m, J = 7, 7.5 Hz, $CH_3CH_2CH_2$), 2.78 (2H, t, J = 7 Hz, CH_2CH_2S), 6.26 (2H, s, NH_2), 6.94 (1H, dd, J = 1.5, 7.5 Hz, H-6), 7.05 (1H, d, J = 7.5 Hz, H-7), 7.15 (1H, d, J = 1.5 Hz, H-4), 10.73 (1H, s, H-1).

2-Succinamido-5(6)-(propylthio)benzimidazole. A solution of 1.5 g of 2-amino-5(6)-(propylthio)benzimidazole and 0.83 g of succinic anhydride in dry pyridine was diluted with acetonitrile and stirred for 20 h. Solids were filtered out, rinsed with acetonitrile and ether, and air-dried to give 1.4 g of a grayish-white solid. This was dissolved in hot pyridine and then concentrated on a rotary evaporator. Addition of ether forced out a white solid. This was collected by filtration, washed with ether, and oven-dried to give 2-succinamido-5(6)-(propylthio)-benzimidazole, mp coalesces at 200 °C, melts with decomposition at 245 °C: MS (probe) (70 eV) *m/z* (relative intensity) 307 $[M^+]$ (41), 289 $[M - H_2O]^+$ (100), 260 (12), 247 (12), 234 (20), 207 (42), 192 (20), 165 (36), 164 (43); ^1H NMR (200 MHz, DMSO-d_6) δ 0.93 (3H, t, J = 7.3 Hz, CH_3), 1.52 (2H, m, J = 7, 7.5 Hz, $CH_2CH_2CH_3$), 2.45–2.75 (4H, m, $COCH_2CH_2COOH$), 2.84 (2H, t, J = 7 Hz, CH_2CH_2S), 7.12 (1H, d, J = 8 Hz), H-6), 7.38 (1H, d, J = 8 Hz, H-7), 7.46 (1H, s, H-4), 11.97 (1H, br s, H-1).

Methyl 6-Mercaptohexanoate. 6-Bromohexanoic acid (Aldrich Chemical Co.) was esterified with methanol containing 3% concentrated sulfuric acid at 60 °C. The methyl ester was converted to the 6-mercapto derivative by reaction with sodium hydrosulfide hydrate at room temperature for 5 h. The solution was acidified to pH 3 and steam-distilled to yield methyl 6-mercaptohexanoate of 87% purity.

5-[(Carboxypentyl)thio]-2-nitroaniline. A solution of 26 mmol of sodium hydroxide in 30 mL of water was added to a mixture of 12 mmol each of 5-chloro-2-nitroaniline (Fuson et al., 1947) and methyl 6-mercaptohexanoate and put on a steam bath for 4 h. The solution was cooled and brought to pH 5 with 1.2 mL

of concentrated hydrochloric acid. The yellow solid that formed was collected by filtration, washed well with water, oven-dried, and dissolved in hot ethanol. Solids precipitating from the chilled solution were washed with methanol and with ether and oven-dried to give a yellow product, mp 153.0–154.5 °C.

5-(Carboxypentyl)-1,2-phenylenediamine. A solution of 5-[(carboxypentyl)thio]-2-nitroaniline in acetic acid plus 5% Pd on C was hydrogenated under 165 kPa (24 lb/in.^{2}) of pressure for 16 h to yield the diamino derivative.

5(6)-[(Carboxypentyl)thio]-2-[(methoxycarbonyl)amino]-benzimidazole (I, Figure 1). A solution of 5 mmol of 1,3-bis-(methoxycarbonyl)-*S*-methylisothiourea (Beard et al., 1975) in 40 mL of 1:1 ethanol–water was added to 5.5 mmol of 5-(carboxypentyl)-1,2-phenylenediamine and heated on a steam bath for 19 h. The solid that formed was filtered and then successively washed with water, methanol, and ether. After oven-drying, there was 1.3 g of a dull coral solid, mp 163.0–164.0 °C: MS calcd for $C_{15}H_{19}N_3O_4S$, 337.1096; MS found, 337.1083; ^{1}H NMR (200 MHz, DMSO-d_6) 1.3–1.6 [6H, m, $CH_2(CH_2)_3CH_2$], 2.19 (2H, t, J = 7 Hz, CH_2COOH), 2.88 (2H, t, J = 7 Hz, CH_2S), 3.78 (3H, s, OCH_3), 7.11 (1H, dd, J = 1.5, 8 Hz, H-6), 7.37 (1H, d, J = 8 Hz, H-7), 7.45 (1H, d, J = 1.5 Hz, H-4).

Synthesis of Conjugates. Hapten I was conjugated to amino groups of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide as carboxy group activating reagent (Hoare and Koshland, 1967). The hapten was coupled to Kunitz trypsin inhibitor from soybeans (KTI, Sigma) and horseradish peroxidase (HRP, Scripps Laboratories, La Jolla, CA) using the 2-morpholinoethyl isocyanide (Fluka, Ronkonkoma, NY) as described by Aigner et al. (1982). Conjugates were purified by dialysis against phosphate-buffered saline (PBS, 0.15 M NaCl, 5 mM sodium phosphate, pH 7.0), and protein concentration was determined colorimetrically (Redinbaugh and Turley, 1986). Coupling ratios were determined by UV–visible spectrophotometry using absorptivities for the proteins stated previously (Brandon et al., 1992; Kassell, 1970) and A_{300} = 11.8 for compound I (1 cm, PBS, pH 7, 1 mM). The coupling ratios obtained were 8 mol of I/mol of BSA, 5.0 mol of I/mol of KTI, and 3.0 mol of I/mol of HRP.

Antibody Production. Antibody and hybridoma production was performed as described previously (Brandon et al., 1987). The KTI conjugate of I was used as immunogen in BALB/c mice. Fusions were conducted using spleen cells from responding mice and P3-X65-Ag8.653 myeloma cells. Desired hybridomas were identified by screening and inhibition ELISAs using the BSA–hapten conjugate and were then expanded and cloned. Immunoglobin was obtained in quantity by growth of cell line 587 as an ascites tumor, followed by purification of the IgG_1 (kappa) immunoglobulin by precipitation with ammonium sulfate and chromatography on (diethylaminoethyl)cellulose.

Competitive ELISA. Assays were conducted on polystyrene assay wells (Immulon II 96-well plates or Dividastrips, Dynatech, Chantilly, VA) coated by incubating with 100 μL of monoclonal mouse IgG from clone 587 at 5 μg/mL (4 h; all incubations conducted with shaking at room temperature, 20–25 °C). Wells were washed three times with phosphate-buffered saline (PBS) containing Tween 20 detergent (PBS–Tween, 0.15 M NaCl, 5 mM sodium phosphate, 0.05% Tween 20, pH 7.0) and rinsed twice with distilled water. Remaining protein-binding sites were blocked by incubation for 1 h with a solution of 10 mg/mL BSA in PBS–Tween, containing 0.01% merthiolate (PBS–Tween + BSA). Plates or strips coated with IgG could be stored desiccated at 4 °C for at least 6 months, provided the coated and blocked wells were incubated with 2% sucrose in water for 30 min and then drained and dried at 37 °C for 1 h. Dilution series of liver extracts (see below) were prepared in water. Standard solutions of FBZ or ABZ were prepared as 10 ppm in dimethylformamide (DMF) and diluted in water to 100 ppb and then in a 5-fold dilution series to cover the working range of the assay (0.8–100 ppb). Diluted extracts or standard solutions (50 μL/well) and HRP conjugate dissolved in PBS–Tween + BSA (0.3 μg/mL, 50 μL/well) were added sequentially to the assay wells and then mixed. After incubation for 1 h, assay wells were washed and rinsed as described above, bound HRP conjugate was detected by reaction with substrate for 20–30 min, and results were analyzed as previously described (Brandon et al., 1992). The use of DMF as extractant was explored (see below), and this solvent produced a significant matrix effect when present at a final concentration > 1%. For these samples alone, results were corrected by subtracting the ELISA value obtained for a negative control liver extracted under identical conditions.

Incurred Residue Samples. Bovine liver samples, kindly provided by Carolyn Henry (FSIS Midwestern Laboratory, St. Louis), had been prepared as part of a previous study (Wilson et al., 1991). Animals, maintained and treated by Loyd D. Rowe (USDA–ARS, Food Animal Protection Research Laboratory, College Station, TX), were dosed orally with Panacur Paste 10% (American Hoechst Corp., Somerville, NJ), to achieve a FBZ dosage of approximately 10 mg/kg. After a withdrawal time of 5 or 6 days, animals were killed and organs removed. Liver samples were shipped frozen to the FSIS Midwestern Laboratory where they were thawed, homogenized, and stored frozen until analyzed. Upon receipt in our laboratory, these frozen samples were thawed, divided into portions, and refrozen until extracted for analysis by ELISA.

Fortified Liver Samples. Fresh calf liver from a local retail market was ground in a blender and divided into portions (10–25 g). To each portion were added solutions of benzimidazole drug or metabolite in DMF to achieve the desired levels. In addition, some samples were fortified with a mixture of ABZ and its sulfoxide and sulfone metabolites to achieve a mixture more comparable to that found in the liver of a dosed animal. Samples were stored frozen until extracted for analysis by ELISA. To verify the absence of benzimidazole residues in the unfortified liver, control samples were also analyzed by HPLC, using 2-(butyrylmercapto)benzimidazole as internal standard. Extraction and sample preparation were done essentially as described by Barker et al. (1986), and their chromatographic system was adapted as follows. The chromatograph consisted of a SP8700 solvent delivery system and pump module and a Spectro Monitor III UV detector (Spectra-Physics, San Jose, CA). Chromatography was performed on a PRP-1 10-μm poly(styrene-divinylbenzene) reversed-phase column, 305 × 7 mm (Hamilton, Reno, NV). Elution conditions were as follows: linear gradient of CH_3CN–H_2O from 38:62 (v/v) to 63:37 (v/v), 20.5 min, 0.5 mL/min; CH_3CN–0.05 N H_3PO_4 (63:37 (v/v), 30 min, 0.5 mL/min; then 10 min, 1.0 mL/min.

Extraction of Incurred Residue Samples for ELISA. Samples were thawed, and 10 mL of distilled water was added per gram of tissue. Samples were stirred at room temperature for 1 h and then centrifuged at 4500*g* for 10 min to remove the tissue.

Extraction of Fortified Liver Samples for ELISA. Liver samples fortified with fenbendazole, albendazole, or their metabolites were extracted with water or 0.05 M citric acid, pH 3.0 (10 mL/g of tissue). The suspension was stirred for 1 h at room temperature, and particulates were removed by centrifugation for 10 min at 4500*g*. The citrate extracts were neutralized by addition of a small volume of NaOH solution. As an alternative extraction procedure for the poorly soluble FBZ, portions of FBZ-fortified liver were thawed, and 1 mL of DMF was added per gram of liver. The mixture was stirred for 20 min at room temperature, and then 9 mL of water was added per gram of tissue. Stirring was continued for an additional 40 min. The suspension was then centrifuged for 10 min at 4500*g*. The supernatant extract was decanted and assayed immediately or stored as frozen aliquots.

RESULTS

Characterization of Antibody Specificity. Three independent cell fusions (632 cultures) were screened for antibodies that bound albendazole and methyl benzimidazolecarbamate with acceptable affinity, as judged by an IC_{50} (concentration of analyte inhibiting the maximal response by 50%) < 10^{-7} M in ELISA. Twelve cultures that grew robustly and also met this criterion were expanded, and four independent clones were eventually obtained and recloned. Antibody from clone 587 was selected for further characterization by competitive ELISA, as shown in Table 1.

Table 1. Specificities of Monoclonal Antibody 587 in Competitive ELISA

compound	IC_{50}[a] (ppb)
5(6)-alkylbenzimidazoles	
albendazole	1.4
albendazole sulfoxide	1.5
albendazole sulfone	1.8
2-amino-5-(propylthio)benzimidazole	>10000
2-amino-5-(propylsulfonyl)benzimidazole	>10000
oxibendazole	1.4
5(6)-arylbenzimidazoles	
fenbendazole	3.8
fenbendazole sulfone	8.3
4′-hydroxyfenbendazole	5.3
oxfendazole	0.62
mebendazole	2.4
flubendazole	0.63
thiazolylbenzimidazoles	
thiabendazole	>100
cambendazole	>100
5-hydroxythiabendazole	>100
other compounds	
methyl benzimidazolecarbamate	2.4
2-aminobenzimidazole	>10000
5-aminobenzimidazole	>100
2-acetylbenzimidazole	>1000
benzophenone	>10000
benzaldehyde	>100

[a] Concentration of compound that inhibits binding of the HRP–hapten conjugate to solid-phase antibody by 50%.

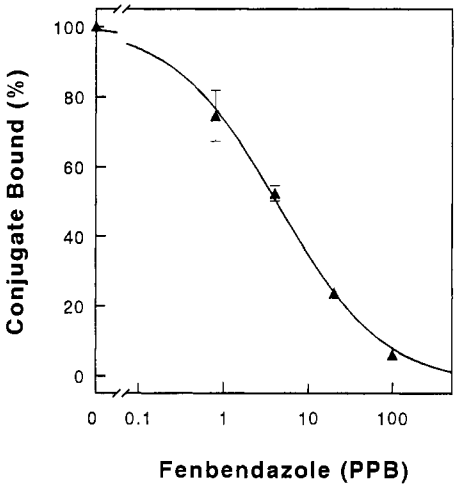


Figure 2. Standard curve for fenbendazole in water matrix in the ELISA using antibody 587. The mean and standard deviations are shown for six data sets obtained on different days (n = 6).

ELISA. The standard curve for the competitive ELISA using antibody 587 is shown in Figure 2. The working range of the assay for fenbendazole is 1–20 ppb. Water extract of liver produced no observable matrix effect; the standard curve for buffer matrix was identical to that obtained using undiluted liver extract.

Analysis of Albendazole-Fortified Liver Samples. Samples, fortified with ABZ, ABZ-SO, or ABZ-SO_2, were extracted with water or citric acid solution prior to ELISA analysis. The water extraction procedure resulted in recoveries in the order ABZ-SO > ABZ-SO_2 > ABZ (Table 2), with the differences significant at the $p < 0.05$ level by the Bonferroni t-test method (SAS Institute Inc., 1987). Results of F tests from two-way analyses of variance of extract × dose show that the use of citric acid improved the recovery of albendazole ($p < 0.05$) but not of the more polar sulfone metabolite ($p > 0.87$). The limits of detection, defined by the 95% one-sided lower confidence limit, ranged from 10 to 114 ppb, depending on the compound and extractant (Table 3). There was no matrix effect due to the citric acid in the working range of the assay (neutralized citric acid ≤25% final concentration). Liver samples fortified with equal quantities of ABZ, ABZ-SO, and ABZ-SO_2 were also analyzed by ELISA, as shown in Figure 3. The limit of detection for this analysis was 58 ppb of albendazole plus metabolites.

Table 2. Recovery of Albendazole and Metabolites in Fortified Samples

	% recovery by ELISA	
amt added (ppb)	water extract	citric acid extract
	Albendazole	
50	28	64
100	14	35
200	15	35
400	14	45
800	18	33
	Albendazole Sulfoxide	
50	129	not done
100	90	
200	75	
400	68	
800	71	
	Albendazole Sulfone	
50	74	80
100	60	59
200	60	66
400	66	60
800	50	47

Table 3. Detection Limits for Albendazole, Fenbendazole, and Metabolites in Fortified Liver by ELISA

compound	extractant	detection limit[a] (ppb)
ABZ	water	110
ABZ	citric acid	51
ABZ-SO	water	10
ABZ-SO	citric acid	not done
ABZ-SO_2	water	18
ABZ-SO_2	citric acid	54
FBZ	water	640
FBZ	DMF–water	29
FBZ-SO	water	120
FBZ-SO_2	water	140

[a] 95% one-sided lower confidence limit of linear regression of ELISA value on amount of compound added.

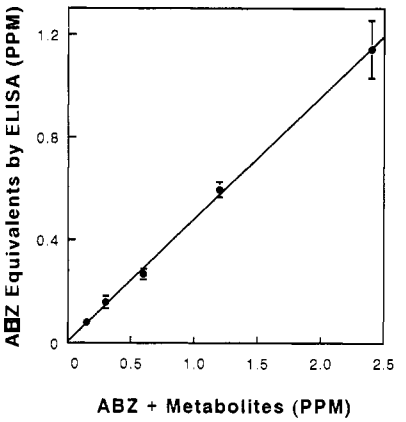


Figure 3. ELISA analysis of beef liver fortified with albendazole plus oxidized metabolites (mean and average deviation of two replicate determinations; r = 0.992).

Analysis of Fenbendazole-Fortified Liver Samples. The limits of detection (95% one-sided lower confidence limit) for FBZ and its oxidized forms are summarized in Table 3. The use of citrate did not improve the extraction of FBZ or its metabolites (data not shown). An alternative extraction protocol using DMF resulted in lowering the

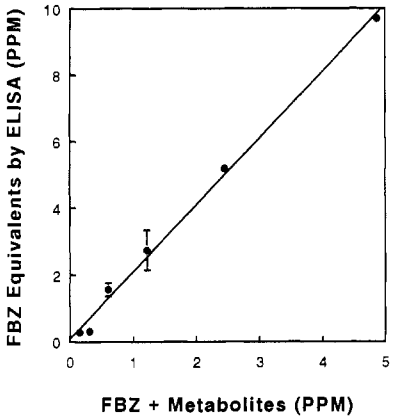


Figure 4. ELISA analysis of beef liver fortified with fenbendazole plus oxidized metabolites (for 0.3, 0.6, and 1.2 ppm levels, mean and average deviation of two replicate determinations; $r = 0.997$).

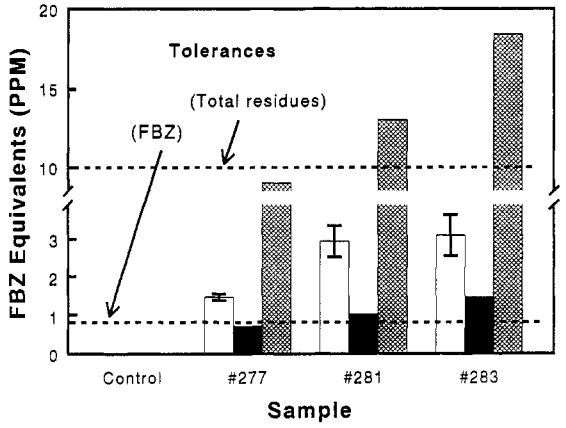


Figure 5. Analysis of incurred residues of fenbendazole in beef liver. Open bars indicate the ELISA analysis; solid bars, the HPLC analysis (Wilson et al., 1991); and cross-hatched bars, the total predicted fenbendazole residues calculated from the HPLC value for FBZ. The U.S. federal tolerances for FBZ and total residues of parent drug FBZ are indicated by the dashed lines.

detection limit of FBZ from 640 to 29 ppb. Liver samples fortified with equal quantities of FBZ, FBZ-SO, and FBZ-SO_2 were also extracted with water and analyzed by ELISA, as shown in Figure 4. The ELISA values are expressed as FBZ equivalents and are higher than the corresponding fortification levels because the assay is much more sensitive to FBZ-SO than to FBZ (see Table 1). The limit of detection for this analysis was 120 ppb of fenbendazole plus metabolites.

Analysis of Incurred Residues of Fenbendazole in Liver. Figure 5 summarizes the analyses of three samples of bovine liver containing incurred residues of fenbendazole and a control sample. The ELISA indicated a strong positive analysis for each of the three residue-containing samples. Using the conversion factor of 10 ppm of total FBZ residues per 0.8 ppm of parent drug FBZ, as used in the U.S. Code of Federal Regulations (21 CFR 556.275), the expected total FBZ residue content of the livers was calculated from the HPLC data (Wilson et al., 1991) and is illustrated as the cross-hatched bars in the graph. For each sample, the ELISA value fell between the HPLC result for FBZ and the calculated total residues of FBZ.

DISCUSSION

Antibody 587 described above provides a good example of the relationship between hapten structure and resulting antibody specificity. Although the exact pattern of hapten conjugation to protein was not studied, we used a method devised for efficient peptide synthesis (Aigner et al., 1982) and expect that hapten was conjugated to the epsilon amino groups of lysyl residues. This would place the 2-benzimidazolecarbamate portion of the conjugate exposed on the outside surface of the carrier protein, available for interaction with receptors of the immune system. As shown in Table 1, substituents in the 5(6)-position had relatively little influence on binding by antibody (12-fold range on a ppb basis), whereas alteration of the carbamate substituent in the 2-position reduced the affinity of binding by at least 100-fold. The data on the 5(6)-alkylbenzimidazoles most clearly illustrate that the substituent in the 2-position had the predominant influence on antibody binding. Oxidation of the sulfur of albendazole, or substitution by an oxygen atom, had no affect on binding. However, the data for binding of 5(6)-arylbenzimidazoles indicate that some bulky 5(6)-position substituents can reduce binding, as in the case of fenbendazole sulfone. The same hapten has recently been used to elicit MBC group-specific polyclonal antibodies in sheep (in collaboration with R. Jackman, Central Veterinary Laboratory, U.K., unpublished results), indicating the general applicability of this hapten strategy. Some alternative haptens reported by Mount et al. (1992) should also be capable of eliciting group-specific antibodies for the methyl benzimidazolecarbamates.

The sample preparation procedure for the ELISA is simple and eliminates the need for nonaqueous solvents. For improved recovery of FBZ, DMF can be used to help solubilize the parent compound in liver samples, resulting in a lower detection limit (Table 3). However, the use of DMF entailed subtraction of a blank when the DMF concentration in the assay exceeded 1% and added unwanted complexity to the method. The increased sensitivity afforded by the use of alternative solvents (DMF for FBZ and citric acid for albendazole) should not be needed for samples derived from animals at least several days after dosing, since most of the drug would be in the form of readily extractable and detectable polar metabolites (Delatour and Parish, 1986). The method using water extraction detected various analytes with sensitivity in the order ABZ-SO > ABZ-SO_2 > ABZ and FBZ-SO > FBZ-SO_2 > FBZ (Table 3). The ELISA analysis of incurred residues of FBZ (Figure 5), using only water as solvent, agreed well with previous analyses (Wilson et al., 1991). In these samples, obtained 5 or 6 days after FBZ administration, the more polar sulfoxide and sulfone metabolites would constitute a major part of the total FBZ residues (Delatour and Parrish, 1986; Gottschall et al., 1990). In a study of liver residues in the cow, the percent of extractable FBZ residue found as the parent compound in liver declined from a level of 79–83% 1 day after drug administration to 13% after 7 days (American Hoechst Corp., 1983). The U.S. Code of Federal Regulations has set the tolerance for FBZ at 800 ppb, corresponding to 10 ppm of total residues of FBZ (21 CFR 556.275). Thus, the Code attributes over 90% of FBZ residues in the liver to metabolites, rather than parent compound. The same tolerance and marker compound are used for regulatory monitoring of oxfendazole (21 CFR 556.495), in which case the tolerance corresponds to 1.7 ppm of total residues of FBZ-SO. Although the ELISA method relies on detection of the more readily extracted oxidized compounds to achieve greater sensitivity, it should be noted that even the poorly extractable FBZ is detected at a level below the federal tolerance. Thus, using only water as extractant, the ELISA method can flag potentially violative FBZ-containing samples for further analysis.

Despite the simplicity of the ELISA employing water extraction, the detection limit was 58 ppb for albendazole and its metabolites. On the basis of the solubility of these compounds in water and the recoveries determined in this study (Table 2), we expect that most of the ELISA response resulted from detection of the oxidized metabolites which retained the 2-carbamate substituent. Many other metabolites are, of course, present in tissues containing incurred residues. For albendazole, the U.S. Code of Federal Regulations has established the 2-amino sulfone metabolite as the marker residue in liver (21 CFR 556.34), but the Code attributes >80% of albendazole residues (1.2 ppm at tolerance) to compounds other than the 2-amino sulfone marker residue. Bovine liver obtained 12 days after administration of albendazole suspension had more oxidized albendazole (ALB-SO plus ALB-SO_2) than 2-amino sulfone metabolite (SmithKline Animal Health Products, 1989). It has been reported that the majority of drug residue violations result from failure to observe withdrawal times (Guest and Paige, 1991). Therefore, one would expect that potentially violative liver samples would have relatively high levels of the oxidized 2-carbamate metabolites of ABZ and by detectable by the screening method described in this paper. The limits of detection for albendazole sulfoxide (10 ppb) and the sulfone (18 ppb) documented in this paper indicate that the assay has sufficient sensitivity and appropriate specificity to serve as an effective screening tool, even though it does not detect the marker residue.

It seems likely that this assay could be adapted to provide a "cowside" test. Since liver biopsy of a food animal would be impractical to perform, a surrogate fluid such as urine or blood or, for dairy cows, milk, would have to be analyzed. We envision that the most productive use of such an assay would be for the farmer or rancher to test treated animals 5–10 days following administration of the drug. The precise timing would depend on the established pharmacokinetics of the specific drug used. If the drug residue concentration were consistent with levels previously established for normal metabolism [e.g., Pritchard et al. (1985)], the producer could observe the established withdrawal time with confidence. Although we have not yet studied the assay with all of these matrices, the direct testing of milk appears practical, since we have observed no significant matrix effects in bovine milk (unpublished observations).

In summary, this paper describes a monoclonal antibody specific for residues of a group of benzimidazole anthelmintics having a 2-carbamate substituent. It can be used in a simple competitive ELISA format to produce a sensitive assay applicable to the bovine liver matrix. The method employs a simple one-step water extraction and provides adequate sensitivity and specificity to discriminate control liver samples from samples containing residues of FBZ or ABZ near the tolerance level.

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