Monoclonal Antibody-Based ELISA for Thiabendazole in Liver

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An enzyme-linked immunosorbent assay (ELISA) was developed for analysis of thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ] residues in bovine liver. Monoclonal antibodies which bind TBZ and 5-OH-TBZ were developed using the haptens, 2-(2-succinamido-4-thiazolyl)benzimidazole and 5-succinamido-2-(4-thiazolyl)benzimidazole. The ELISA method uses monoclonal antibody in a competitive format, following a one-step, 10-min aqueous extraction of samples. Monoclonal antibodies with various specificities permit an ELISA specific for TBZ alone or for the group of thiazolyl compounds including TBZ, 5-OH-TBZ, and cambendazole. Tissue samples of 0.1 g can be used, and the limit of detection, determined by analysis of liver samples fortified with TBZ, is 20 ppb. The extraction and assay can be completed within 2 h. The assay materials are sufficiently stable to be packaged as a kit with a shelf life of at least 6 months. ELISA results were validated by comparison to results obtained by high-pressure liquid chromatography with UV detection. Since samples exceeding the U.S. tolerance (100 ppb) can readily be distinguished from negative samples simply by visual inspection of the ELISA wells, the method provides a rapid screening test which could be used for regulatory monitoring of TBZ residues in meat.

INTRODUCTION

Benzimidazoles are widely used as anthelmintics in cattle and swine for the control of gasterointestinal roundworms, tapeworms, liver flukes, and lungworms. One of these compounds, thiabendazole, is also used as a fungicide on many crops. The mechanisms of action of these compounds include disruption of microtubules, inhibition of glycogen uptake, and inhibition of fumarate reductase (reviewed by Davidse, 1986; McKellar and Scott, 1990). Chronic and acute toxicity of benzimidazoles, such as teratogenesis and immunosuppression, have been associated with varying exposures (Delatour and Parish, 1986). Consequently, the Food and Drug Administration has established residue limits for these drugs (or their metabolites) in food animal tissues (for example 21 CFR 556.730), and the Environmental Protection Agency has established tolerances for thiabendazole fungicide residues (40 CFR 180.242). The USDA Food Safety and Inspection Service (FSIS), responsible for implementation of the residue regulations as part of the meat and poultry inspection system (Food Safety and Inspection Service, 1991), is committed to the implementation of rapid, multiresidue screening methods for compounds of interest (Ellis, 1989; Berkowitz, 1990). Standard analytical methods for residues of benzimidazole anthelmintics in meat products utilize liquid chromatography with UV detection, with mass spectrometry as the confirmatory method (Bogan and Marriner, 1980; Barker et al., 1990; Marti et al., 1990; Wilson et al., 1991). An immunoassay for thiabendazole using polyclonal antibodies was reported by Newsome and Collins (1987), who analyzed TBZ residues in produce. A need persists for a fast and simple multiresidue screening test that could be used to test for TBZ residues (the sum of TBZ plus 5-hydroxythiabendazole in meat. In addition, on-site analyses would be facilitated by methods minimizing the use of organic solvents. These needs motivated our development of an ELISA method utilizing monoclonal antibodies for determination of thiazolylbenzimidazoles in bovine liver.

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MATERIALS AND METHODS

Benzimidazoles. Thiabendazole, 5-hydroxythiabendazole, and cambendazole were provided by Merck Sharp and Dohme Research Laboratories (West Point, PA). 5-Aminothiabendazole was obtained from Aldrich Chemical Co. (Milwaukee, WI). Methyl benzimidazolecarbamate was obtained from the EPA Pesticides and Industrial Chemicals Repository (Research Triangle Park, NC). Albendazole was provided by SmithKline Animal Health Products (West Chester, PA). Fenbendazole was provided by Hoechst-Roussel Agri-Vet Co. (Somerville, NJ). Mebendazole was purchased from Janssen (Piscataway, NJ). Stock solutions (typically 1 mg/mL) were prepared in dimethylformamide and stored at 4 °C.

Hapten Synthesis. Two haptens were prepared for eliciting TBZ-binding antibodies. 2-(2-succinamido-4-thiazolyl)benzimidazole (2-succinamidothiabendazole, I, Figure 1) was prepared according to the procedure of Newsome and Collins (1987), but the succinylation step was done at 50 °C for 20 h in order to avoid formation of the succinimide derivative, and some acetonitrile and dimethyl sulfoxide were added to the reaction in an attempt to maintain homogeneity. The mixture was brought to pH 5 with HCl solution and stirred well. Solids were collected by filtration, washed with water, methanol, and ether, and then air dried to give a pale jade powder: mp 284 °C; yield 47%. MS (70 eV) *m/z* (rel int) 316 $[M]^+$ (14), 299 (19), 298 (87), 243 (32), 217 (14), 216 (100), 175 (18), 174 (99), 78 (80), 63 (82) (obtained with a VG70/70 HS magnetic spectrometer); ^{1}H NMR (200 MHz, DMSO-d_6, 80 °C) δ 2.61 (2 H, t, J = 6.5 Hz, CH_2CONH), 2.77 (2 H, t, J = 6.5 Hz, CH_2COOH), 7.22 (2 H, dd, J = 4, 6 Hz, H-5,6), 7.60 (2 H, dd, J = 4, 6 Hz, H-4,7), 7.91 (1 H, s, H-5′), and at room temperature 12.34 (1 H, s, H-1).

5-Succinamido-2-(4-thiazolyl)benzimidazole (5-succinamidothiabendazole, II, Figure 1) was prepared as follows: A solution of 1.39 g (6.4 mmol) 5-aminothiabendazole (prepared by the method of Hoff and Fisher, 1969), mp 231.0–231.8 °C (lit. mp 232–233 °C), in 30 mL pyridine was added to a solution of 750 mg (7.49 mmol) succinic anhydride in 75 mL acetonitrile and stirred for 1.5 h at 50 °C and then for 15 h at 22 °C. The mixture was filtered and the filter cake washed with acetonitrile and then ether. After the filter cake was dried on a steam bath, 1.36 g of light tan solid remained. This was dissolved in 60 °C methanol, filtered, and concentrated by boiling off methanol. The chilled solution contained a whitish solid that was collected by filtration, washed with methanol and ether, and oven dried. Yield (46%) was 0.93 g of 5-succinamidothiabendazole: mp 224.0–224.8 °C;

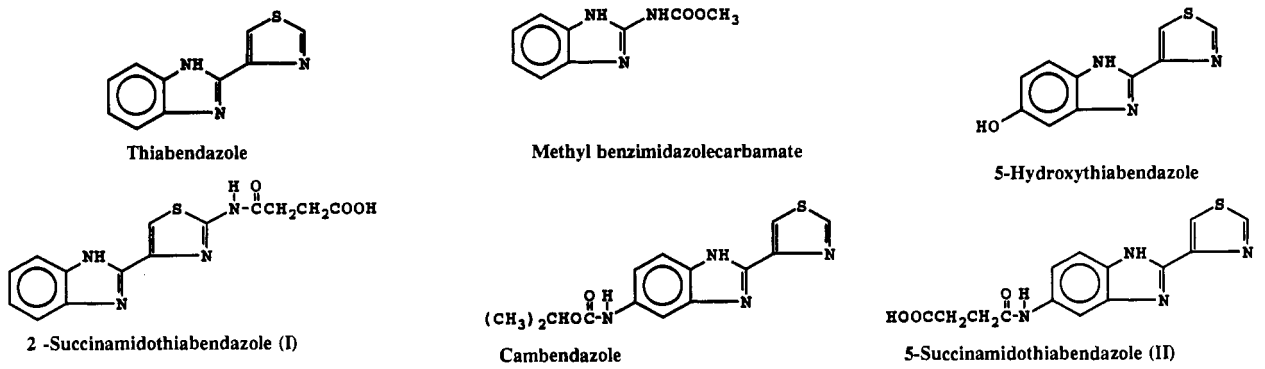

Figure 1. Thiazolylbenzimidazoles and haptens used to elicit antibodies.

MS (70 eV) *m/z* (rel int) 316 $[M]^+$ (10), 298 (100), 269 (16), 216 (54), 215 (16), 149 (10), 107 (11), 44 (53), 32 (50), 28 (66); ^1H NMR (200 MHz, DMSO, 80 °C) δ 2.6 (4 H, m), 7.31 (1 H, dd, J = 8, 1.8 Hz, H-6), 7.50 (1 H, d, J = 8 Hz, H-7), 7.99 (1 H, br s, H-4), 8.34 (1 H, d, J = 2 Hz, H-5'), 9.25 (1 H, d, J = 2 Hz, H-2'), 9.74 (1 H, s, CONH), and at room temperature 12.2 (1 H, br s, COOH), 12.9 (1 H, s, H-1).

Protein Conjugates. Haptens I and II were coupled to amino groups of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) and horseradish peroxidase (HRP, Scripps Laboratories, La Jolla, CA) using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide as carboxyl group activating reagent (Hoare and Koshland, 1967). Conjugates were purified by dialysis against phosphate-buffered saline (PBS, 0.15 M NaCl, 5 mM sodium phosphate, pH 7.0) and analyzed for protein (Redinbaugh and Turley, 1986) and for bound hapten using the following absorptivities (1 cm, PBS, pH 7): HRP, 1 mg/mL, A_{403} = 2.4, A_{280} = 0.74; BSA, 1 mg/mL, A_{280} = 0.69, A_{300} = 0.089; *I*, 1 mM; A_{300} = 16.9; II, 1 mM, A_{310} = 20.3. Conjugates of BSA were stored at a concentration of at least 1 mg/mL at −20 °C. Conjugates of HRP were stored at 4 °C in PBS containing 10 mg/mL BSA and 0.2% Merthiolate. Conjugates have been stable for over 1 year.

Antibody Production. Antibody and hybridoma production were performed as described previously (Brandon et al., 1987). BSA conjugates of I and II containing 5–6 mol of hapten/mole of protein were used as immunogens 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 and were then expanded and cloned.

Screening ELISA. Poly(vinyl chloride) plates (Costar, Inc., Cambridge, MA) were coated with the BSA conjugate immunogen, 50 μL/well at 10 μg/mL in PBS, for 4 h at room temperature or overnight at 4 °C, and remaining protein-binding sites were blocked by incubation for 1 h with BSA (10 mg/mL) in PBS containing 0.05% Tween-20 (PBS-Tween). Supernatants from hybridoma cultures were screened for the presence of antibody by application to the wells of the ELISA plate at a 5-fold dilution in PBS-Tween containing 10 mg/mL BSA, 50 μL/well. The BSA in the diluent minimized binding by BSA-specific antibodies and permitted selective detection of hapten-specific antibodies in the culture supernatants. After an incubation period of 1 h, wells were washed 3 times with PBS-Tween and rinsed with water. HRP-rabbit anti-mouse IgG (Zymed Laboratories, South San Francisco, CA) was used as labeled secondary antibody and was applied to the wells, 50 μL/well, for 1 h. After the wells were washed and rinsed as above, bound HRP was detected with 6.7 mM H_2O_2 + 1 mM 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in 60 mM sodium citrate, pH 4.2, as substrate (50 μL/well). The absorbance was determined at 414 nm on a microplate reader (V_{max}, Molecular Devices, Menlo Park, CA) after an appropriate interval (usually 15 min) or after the reaction was terminated by addition of 10% sodium dodecyl sulfate. Positive supernatants were then serially diluted in PBS-Tween + 10 mg/mL BSA and assayed to determine the dilution factor necessary to produce 80% of maximal absorbance. Use of supernatant antibody at this dilution factor yielded inhibition ELISAs which were most sensitive to changes in hapten concentration. Positive supernatants were then characterized by a second screening using inhibition ELISA as described below.

Inhibition ELISA. ELISAs to confirm hapten specificity and to characterize selected antibodies prior to production of purified IgG were conducted as follows. Polystyrene assay plates (Immulon II, Dynatech, Inc., Chantilly, VA) were used, with 100 μL of coating solution and 200 μL of blocking solution. Stock solutions of benzimidazoles were diluted with water to produce a 5-fold dilution series from 10^{-4} to 10^{-9} M and 250 μL of each solution was mixed in a test tube with an equal volume of diluted antibody-containing supernatant. After a preincubation of 1 h at room temperature, 100 μL of each dilution was applied in triplicate to the wells of assay plates. After a 1-h incubation, assay wells were washed three times with PBS-Tween and rinsed twice with distilled water. Bound monoclonal antibody was detected with HRP-labeled antibody (100 μL, 1-h incubation) and ABTS substrate (100 μL) as described above. The resulting standard curves were fit to a logistic model (Finney, 1978) using the Softmax program (Molecular Devices, Menlo Park, CA). The concentration of analyte giving half-maximal response in the ELISA, called the IC_{50}, was used to assess the relative affinities of antibodies for the different benzimidazoles. Cells producing selected antibodies were grown for production of ascitic fluid in BALB/c mice, and antibody was purified and isotype determined as described previously (Brandon et al., 1987). For quantitative analysis of samples by inhibition ELISA, serially diluted analyte and TBZ standards were mixed with purified antibody, and the assay was conducted as above.

Competitive ELISA. These assays were conducted on assay wells coated with monoclonal mouse IgG at 5 μg/mL (50 μL/well for poly(vinyl chloride) plates or 100 μL/well for polystyrene plates). Analyte was mixed with the HRP conjugate of I or II (for use with antibodies elicited with the BSA conjugate of I or II, respectively) before application to the assay wells. Alternatively, analyte and HRP conjugate could be added sequentially to the assay wells and then mixed. After incubation for 1 h, assay wells were washed and rinsed, and bound HRP conjugate was detected and results analyzed as described above.

For assay of small numbers of samples, Immulon II Dividastrips (Dynatech) were substituted for the 96-well plates. This product enabled multiples of 16 wells to be used in a single assay. Plates or strips coated with IgG could be stored dessicated at 4 °C for at least 6 months, provided the coated and blocked wells were incubated with 2% sucrose in water for 30 min, then drained and dried at 37 °C for 1 h.

Incurred Residue Samples. Bovine tissue and urine 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 TBZ 43% Cattle Wormer Paste (MSD Agvet, Rahway, NJ) to achieve a TBZ dosage of approximately 50 mg/kg. After a withdrawal time of 24 or 48 h, 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

Table I. Specificities of Monoclonal Antibodies to Thiabendazole in Inhibition ELISA

compound	IC_{50},[c] M antibody 300[a]	antibody 430[b]	antibody 448[b]
thiabendazole	3.8×10^{-8}	1.6×10^{-8}	1.2×10^{-8}
5-OH-TBZ	8.1×10^{-7}	8.6×10^{-7}	1.8×10^{-8}
5-NH_2-TBZ	1.1×10^{-6}	1.1×10^{-8}	4.9×10^{-9}
methyl benzimidazole-carbamate	1×10^{-5}	$>10^{-4}$	$>10^{-4}$
cambendazole	3.8×10^{-5}	2.0×10^{-9}	2.0×10^{-9}
albendazole	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

[a] Elicited with 2-succinamidothiabendazole. [b] Elicited with 5-succinamidothiabendazole. [c] Concentration of compound which inhibits binding of the antibody to solid-phase hapten conjugate by 50%.

Table II. Specificities of Monoclonal Antibodies to Thiabendazole Determined by Competitive ELISA

compound	IC_{50},[c] M antibody 300[a]	antibody 430[b]	antibody 448[b]
thiabendazole	6.9×10^{-8}	1.8×10^{-8}	1.3×10^{-8}
5-OH-TBZ	8.1×10^{-7}	1×10^{-7}	2.0×10^{-8}
5-NH_2-TBZ	1.1×10^{-6}	3.2×10^{-8}	1.3×10^{-8}
methyl benzimidazole-carbamate	1×10^{-5}	$>10^{-4}$	$>10^{-4}$
cambendazole	$>10^{-4}$	5.7×10^{-9}	9.7×10^{-10}
2-succinamidothiabendazole	1.1×10^{-8}	$>10^{-4}$	5.6×10^{-6}
5-succinamidothiabendazole	$>10^{-4}$	1.8×10^{-9}	2.9×10^{-9}
albendazole	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
mebendazole	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$
fenbendazole	$>10^{-4}$	$>10^{-4}$	$>10^{-4}$

[a] Elicited with 2-succinamidothiabendazole. [b] Elicited with 5-succinamidothiabendazole. [c] Concentration of compound which inhibits bending of the antibody to solid-phase hapten conjugate by 50%.

for analysis by ELISA. Urine samples, maintained frozen until received in our laboratory, were thawed and refrozen as aliquots.

Fortified Liver Samples. Fresh calf liver from a local retail market was ground in a blender and divided into 10-g portions. To each portion, 1 mL of TBZ in dimethylformamide was added to achieve levels of 20, 40, and 160 ppb. Samples were stored frozen until extracted for analysis.

Extraction of Liver Samples. Homogenized, frozen portions of liver were thawed, and 10 mL of 10% dimethyl sulfoxide in water was added per gram of liver. The mixture was stirred for 1 h at room temperature and then centrifuged for 10 min at 4500*g*. The supernatant extract was decanted and assayed immediately or stored as frozen aliquots. In variations of this procedure water and PBS-Tween were used as solvents and extraction three times were shortened to 10 min.

Analysis of Liver Samples by ELISA. Dilution series of liver extracts in the range 2–256-fold dilution and TBZ standards were prepared in water. The diluted extracts were then mixed with antibody for inhibition ELISA or with HRP conjugate for competitive ELISA, as described above.

RESULTS

Characterization of Antibodies. Three antibodies were selected for characterization. Each was IgG_1 isotype, with kappa light chain. Their specificities are summarized in Table I (inhibition ELISA) and Table II (competitive ELISA); see also Figure 2. Antibodies elicited with the conjugate of I are referred to as type 1 antibodies. They preferentially bound compounds containing a thiazolyl ring without substitution in the benzimidazole nucleus. Thus, antibody 300 bound thiabendazole strongly, while methyl benzimidazolecarbamate and cambendazole were bound weakly. Antibodies elicited with the conjugate of II, referred to as types 2 and 3, differed greatly in specificity from type 1. Type 2 antibodies were more tolerant of substitutions in the benzimidazole nucleus, and these

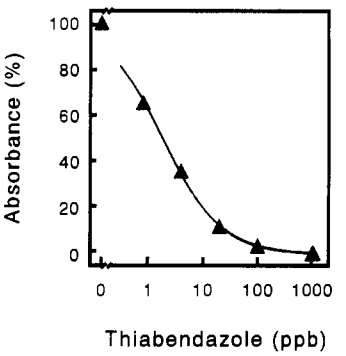

Figure 2. Standard curve for competitive ELISA using the type 3 antibody 448.

Table III. Analysis of Incurred Residues of Thiabendazole in Liver[a]

sample no. of calf liver	inhibition ELISA[b] antibody 300	430	448	HPLC[c] TBZ	5-OH-TBZ
275	175 ± 37	86 ± 12	104 ± 13	71	<50
280	756 ± 114	872 ± 195	742 ± 159	677	259
282 (untreated)	nd[d]	nd	nd	<50	<50

[a] Results in ppb. [b] TBZ equivalents, mean ± sd ($n = 3$). [c] Results of Wilson et al. (1991). [d] Not detected.

Table IV. Thiabendazole Analysis by Competitive ELISA[a]

sample no.	extraction solvent 10% DMSO	PBS + Tween	water
275	125 ± 40 (7)[b]	100 ± 26 (6)	98 ± 31 (6)
280	1119 ± 267 (3)	891 ± 193 (2)	828 (1)
282 (untreated)	nd[c] (1)	nd (2)	nd (1)

[a] Results in ppb, using antibody 448. [b] Mean ± sd (n). [c] Not detected.

antibodies had high affinity for cambendazole and lower affinity for 5-hydroxythiabendazole. There was essentially no binding of bendimidazoles lacking the thiazolyl ring. Type 3 antibodies, however, bound TBZ and 5-hydroxythiabendazole nearly equally well, and cambendazole even more strongly. None of the antibodies bound thiazole.

Analysis of Liver Samples. Table III shows the results of inhibition ELISA analyses of incurred TBZ residues in calf liver, using 10% DMSO as extractant. For comparison, data calculated from the HPLC analysis of Wilson et al. (1991) are given. These authors did not quantitate residue levels of either analyte below 50 ppb.

Since the competitive ELISA format is faster than the inhibition assay, further studies were conducted using this method. A standard curve is illustrated in Figure 2. Nonlinear, least-squares analyses were performed on seven standard curves. Using the variance–covariance matrix of the parameter estimates obtained from the least squares fit, 95% confidence intervals on ppb's were constructed. The lowest concentration significant at the 5% level was <0.2 ppb for all the curves. Table IV shows the results of the competitive ELISA, using the type 3 antibody (448) and three alternative extractants. The small differences between extraction methods were not significant ($p > 0.1$, two-tailed version of Student's t test).

Calf liver samples, fortified with TBZ at three levels (20, 40, and 160 ppb) were analyzed by competitive ELISA, with three replicate extractions at each level. The results were analyzed by linear regression, and the 95% one-sided lower confidence limit was determined to be 2 ppb. Therefore, the limit of detection in a liver matrix based on these samples is <20 ppb.

To determine the efficacy of different antibodies, calf liver samples 275 and 280 were analyzed using both

Table V. TBZ Determination by Competitive ELISA with Two Monoclonal Antibodies[a]

sample no.	antibody 430	antibody 448
275	131 ± 53[b]	180 ± 86
280	939 ± 130	1069 ± 301

[a] Results in ppb. [b] Mean ± sd, $n = 6$.

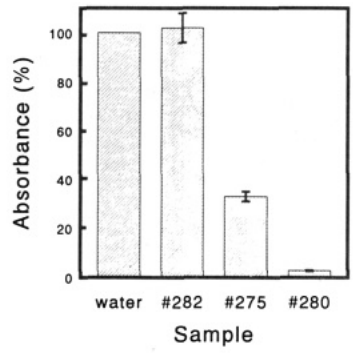

Figure 3. Results of competitive ELISA on bovine urine samples diluted 8-fold with water. Sample 282 was obtained from an untreated animal.

antibody 448 and antibody 430. The data, given in Table V, indicate that the two antibodies gave comparable results. Antibody 430, less reactive with 5-hydroxythiabendazole than antibody 448 (Tables I and II), produced lower values in all six replicate analyses, but the difference of the means was not significant ($p > 0.1$, two-tailed version of Student's t test). Because the speed of a screening test can be an important determinant of its usefulness, it was also determined whether the standard extraction time of 1 h could be reduced. A 10-min extraction produced a recovery of 97% ± 17% (mean ± sd, $n = 4$) compared to the results with a 1-h extraction. Thus, the time can be adjusted to suit the needs of the analyst.

Assays were also conducted on urine collected at the time of sacrifice of animals in the study of Wilson et al. (1991). The three samples corresponded to control, near-tolerance, and highly violative levels of TBZ residues in liver. They were diluted with water and assayed by competitive ELISA. The results of this preliminary study are shown in Figure 3, which indicates that each of the two residue-containing samples produced much lower absorbances in the assay than the control sample (from an untreated animal), with coefficients of variation of 7% ($n = 3$).

DISCUSSION

The assay procedure described above provides a simple screening method, with the required sensitivity for monitoring residues of thiabendazole. For routine laboratory use, the analysis of at least 10 samples can be performed on one standard 96-well assay plate or, for few samples, assays can be conducted using strips. A 10-min extraction of liver samples with water sufficed for good recovery of TBZ, and these conditions appear ideal for a rapid screening method. The use of the 5-succinamidothiabendazole hapten (II) led to the production of group-specific antibodies with more versatility than previously reported antibodies (Newsome and Collins, 1987). On the basis of the binding data for antibodies 430 and 448 (Tables I and II), the potential sensitivity for cambendazole is even greater than for TBZ, but adaptation of the extraction method for this drug (with a zero tolerance in food animal tissues) remains to be done.

The ELISA results agreed with HPLC determination of thiabendazole residues in calf liver (Wilson et al., 1991), and the assay is effective in pinpointing levels of TBZ plus 5-hydroxythiabendazole exceeding regulatory standards in calf liver. At a TBZ + 5-OH-TBZ level near tolerance (sample 275), the immunoassay using the water extraction procedure resulted in a coefficient of variation of 32%, compared to 20–21% for the HPLC method of Wilson et al. (1991). Employing a 10-min extraction with distilled water, the method can be used to detect positive samples exceeding 20 ppb, with visual inspection of the ELISA plate adequate for samples near tolerance (100 ppb). Since the sample preparation is facile and the assay rapid, the method could probably be incorporated into existing residue monitoring systems. Other assay formats such as the use of membranes as solid phases have been successful in our laboratory and could possibly provide more rapid and rugged assay kits.

Residues of thiabendazole decline rapidly after administration of the drug (eliminated mostly by hydroxylation and conjugation to form the 5-glucuronide or sulfate ester) and the highest concentrations of persistent residues are found in liver and kidney (Tocco et al., 1964, 1965, and 1966; McManus et al., 1966; Prichard et al., 1985). However, screening assays performed on a "surrogate" fluid such as urine, blood, or ocular fluid would eliminate the extraction of solid tissue. Our preliminary observations indicated that the TBZ screening assay, performed on diluted bovine urine, could differentiate between control and near-tolerance samples with visual readout. Further studies of the relationship between tissue and fluid concentrations are needed for adequate validation of a residue screening test on urine or other surrogate fluid.

In its present form, the ELISA method can quantify thiazolylbenzimidazoles in liver rapidly, without the use of volatile or toxic solvents. This screening method, incorporated into a residue monitoring program, could permit analysis of residue content before commodities reach consumers and result in considerable cost savings even with increased sample numbers (Newsome, 1986; Office of Technology Assessment, 1988). Our current work is directed toward extending the library of stable hybridoma cell lines, producing additional group-specific monoclonal antibodies for the remaining benzimidazoles, and formatting a multiresidue ELISA suitable for a variety of matrices.

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