Rapid Screening for Benzimidazole Residues in Bovine Liver

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Rapid enzyme immunoassays for benzimidazole anthelmintic and/or fungicide residues were developed using monoclonal antibodies that specifically bind methyl benzimidazolecarbamates (MBCs, including albendazole, fenbendazole, and oxfendazole) and thiazole-containing compounds (thiabendazole, its 5-hydroxy metabolite, and cambendazole). Bovine liver samples were extracted by homogenization in water. Extracts were clarified by centrifugation, and dilutions of the supernatants were analyzed using both a commercialized immunoassay (EnviroGard thiabendazole kit, Strategic Diagnostics, Inc., Newark, DE) and an MBC-specific assay described previously. Two sets of 40 blank, fortified, or incurred samples were analyzed for the thiazole benzimidazoles, and an additional set of 31 samples was analyzed for methyl benzimidazolecarbamates only. Using the thiabendazole kit, all samples were analyzed correctly by ELISA, including all 34 samples containing fortified or incurred residues (60−120 ppb) of thiabendazole or related compounds. Using the method for MBCs, all unknowns were correctly scored. The simple water extraction and rapid ELISAs appear to function well for regulatory screening of bovine liver for residues of fenbendazole, oxfendazole, and thiazole-containing benzimidazoles. Although the marker residue for albendazole is not detected by these procedures, residues can be estimated from the analysis of albendazole sulfoxide and sulfone.

Keywords: *ELISA; drug residues; monoclonal antibody; thiabendazole; fenbendazole*

INTRODUCTION

The occurrence of drug residues in food animal tissues is a continuing regulatory concern, with new demands for streamlined, low-cost testing procedures. Benzimidazole (BNZ) anthelmintics are broad-spectrum drugs that act, in part, by disrupting microtubule function and, possibly, by interfering with oxidative phosphorylation (Lacey, 1990; McCracken and Stillwell, 1991). The older literature on toxicity of the benzimidazoles to host organisms was reviewed by Delatour and Parish (1986), and additional mechanisms have been proposed more recently. Mizutani et al. (1993) showed that thioformamide may be the proximate toxicant in the nephrotoxicity of thiabendazole (TBZ) and other thiazoles in mice depleted of glutathione. TBZ was shown to induce cytochrome P4501A1 in rabbit hepatocytes (Aix et al., 1994) and, along with other BNZ derivatives, to activate the aromatic hydrocarbon (Ah) receptor in hepatocytes by a ligand-independent pathway (Lesca et al., 1995). Methyl benzimidazolecarbamate (MBC) is one of hundreds of compounds suspected of having endocrine disuptor activity (U.S. Environmental Protection Agency, 1997), an emerging food safety issue now being addressed by research and testing initiatives (Cooper and Kavlock, 1997) mandated in the United States by the 1996 Food Quality Protection Act and other legislation. This compound (also known as carbendazim) is a metabolite or breakdown product of some BNZ anthelmintics and related fungicides such as benomyl. Regulatory tolerances for BNZ residues in food animal tissues have been established in the United States and abroad (U.S. Food Safety and Inspection Service, 1990; Heitzman, 1994). Because residues of these compounds occur with low frequencies, it would be useful to have a quick qualitative test to determine which samples need quantitative analysis. To this end, this paper describes how two immunoassay procedures (Brandon et al., 1992, 1994) can be adapted to a regulatory screening program.

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MATERIALS AND METHODS

Standards. BNZ standards were obtained as described previously (Wilson et al., 1991), and several additional compounds were used in this study. Fuberidazole was obtained from E. Merck (Darmstadt, Germany). Oxfendazole (OFZ) and fenbendazole sulfone (FBZ-SO2) were provided by Syntex, Inc. (Palo Alto, CA), and albendazole sulfone (ABZ-SO2) and the marker residue, albendazole 2-aminosulfone metabolite (ALB), were provided by SmithKline Animal Health Products (West Chester, PA). The preparation of albendazole sulfoxide (ABZ-SO) was previously described (Brandon et al., 1994).

TBZ Assay Kits. EnviroGard assay kits were provided for evaluation under a Cooperative Agreement with EnSys, Inc. (Research Triangle Park, NC), which later became part of Strategic Diagnostics, Inc. The immunochemical reagents used in this kit have been described (Brandon et al., 1992).

BNZ ELISA Kits. Assay materials, including monoclonal antibody 587, were described previously (Brandon et al., 1994). The following modifications in the procedures were employed. Assemblies of 96 wells were prepared from 2 × 8 arrays of Immulon II Dividastrips (Dynatech Laboratories, Chantilly, VA). All antibody-coated plates were treated with sucrose, dried, and stored desiccated at 4 °C. The enzyme conjugate

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of the hapten, 5(6)-(carboxypentylthio) 2-methylbenzimidazolecarbamate (**I**), was provided as a 1000-fold concentrate and stored at 4 °C in PBS-Tween + BSA (0.15 M NaCl, 5 mM sodium phosphate, pH 7.0, containing 10 mg/mL bovine serum albumin and 0.01% merthiolate). The conjugate was diluted in PBS-Tween + BSA on the day of the assay. A one-component commercial solution of tetramethylbenzidine (TMB; ELISA Technologies, Lexington, KY) was used as substrate, and 0.3 N HCl was employed as stop solution/color enhancer. Assay materials were prepared at Western Regional Research Center and shipped to the FSIS Midwestern Laboratory as needed. Materials were stable for at least six months (see below).

Sample Fortification. Liver was obtained from cows used in incurred residue studies and known not to have been dosed with BNZs. The liver samples were negative for BNZs by LC analysis (Wilson et al., 1991). Liver was blended and stored frozen until used in the fortification studies. Samples were fortified by addition of drug or metabolite standards in the range of 1−2 mg/mL in dimethylformamide (DMF). For study of extraction procedures, the following compounds and fortification levels were used: albendazole sulfone [(ABZ-SO2) 50, 100, and 200 ppb], FBZ (400, 800, and 1600 ppb), and fenbendazole sulfone [(FBZ-SO2) 130, 260, and 520 ppb].

Incurred Residue Samples. The dosing of cows with TBZ and FBZ and the subsequent analyses by LC and ELISA were described previously (Wilson et al., 1991; Brandon et al., 1992, 1994). The ABZ incurred sample was from Beltsville study 305.18 (unpublished data, 1989).

Extraction. One gram samples were weighed into plastic centrifuge tubes. Nine milliliters of water was added to each tube, and the tubes were shaken for 1 h on a Model 6010 shaker (Eberbach Corp., Ann Arbor, MI) at 280 oscillations/min. The mixtures were then centrifuged at 4500*g* for 10 min and supernatants decanted for analysis. In some experiments, to reduce the scatter in the blanks in the BNZ ELISA (see below), the centrifugation was at 20000*g* for 20 min. Sample sets labeled as "unknown" were prepared and coded by one member of the team and analyzed without breaking the code.

Analyses. For the TBZ ELISA, instructions provided in the kit were followed. The kit also provided a TBZ standard (1 ppm) in methanol, which was diluted in water for the assay (0, 0.25, 1, and 4 ppb TBZ), which were analyzed on some plates. Additional standards were prepared in methanol and diluted in water for assay. For the BNZ ELISA, stock solutions of standards were prepared in DMF and further diluted in water for working standards. To obtain qualitative results, extracts were analyzed directly, without further dilution. In both assays, incubations with substrate (30 min) were terminated by addition of an equal volume of stop solution and the absorbance was determined at 450 nm using a Bio-Kinetics Model EL312e microplate reader (Bio-Tek Instruments, Winooski, VT). Data were analyzed by importing data files into a spreadsheet. Assays to determine plate stability and limits of detection utilized a Vmax plate reader and the Softmax program (Molecular Devices, Menlo Park, CA). Statistical parameters (*t* tests) were evaluated using one-sample and two-sample analysis methods in Statgraphics Plus, ver. 6.1 (Manugistics, Rockville, MD).

RESULTS AND DISCUSSION

Standard Curve for TBZ Kit. Figure 1 (top) shows the standard curve for the TBZ assay, indicating sensitivity in the 1 ppb range. The results are consistent with the kit specifications, which indicate the following concentrations for 50% conjugate bound (also referred to as $B/B_0 = 0.5$ or inhibitory concentration at 50%, IC_{50}) and the lower limit of detection [(LLD) estimated as the concentration for 85% conjugate bound or $B/B_0 = 0.85$)]: TBZ, 1 and 0.2 ppb; 5-OHTBZ, 1 and 0.2 ppb; cambendazole (CBZ), 0.12 ppb and <0.1 ppb. Because the U.S. federal tolerance for TBZ is established as the sum of TBZ + 5-OHTBZ metabolite (40CFR 180.242), the kit specificity is well matched to the regulatory need. CBZ has a zero tolerance in the United States. No other regulated BNZs were detected by this method. The fungicide, fuberidazole, not registered in the United States, gave a weak, positive response, as expected (Bushway et al., 1995).

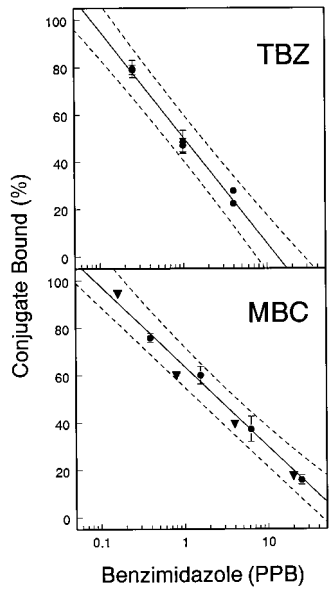

Figure 1. (Top) Standard curve for TBZ assay kit, using standards provided with the kits, on different days. The linear regression ($r = 0.98$) and 95% confidence interval are shown. (Bottom) Standard curve for the BNZ assay kit. The regression line for an assay performed on freshly prepared plates ($r = 0.98$) and the 95% prediction interval are shown. The points are the values obtained with the same standards on plates that had been prepared 9 months earlier.

Standard Curve for BNZ Kit and Plate Stability. Figure 1 (bottom) shows the standard curve for the BNZ assay, with MBC as standard. Sensitivity is in the 2 ppb range. The following concentrations were determined for $B/B_0 = 0.5$ and the lower limit of detection: MBC, 2.7 and 0.7 ppb. Also shown in this figure are values obtained for standards analyzed on plates stored for 9 months. All values fell within the 95% prediction interval computed for the original standard curve.

Analysis of Unknowns Using the TBZ Kit. Eighty unknowns were grouped into two sets of 40 samples and extracted and analyzed without further dilution. The unknowns included 12 blank liver samples, 4 incurred TBZ samples, 30 samples fortified with the thiazole BNZs, 8 incurred ABZ or FBZ samples, and 26 samples fortified with MBCs. The results are summarized in Table 1. All samples were correctly analyzed by this method. The blank liver samples had a coefficient of variation (CV) of 4%. All positive samples were easily differentiated from blanks by visual observation as well as by quantitative analysis. Results from positive samples analyzed with at least three replicates are shown in Table 2. At the time of last analysis, incurred TBZ sample 275 contained 106 ppb of TBZ + 5-OHTBZ, just over tolerance of 100 ppb. The ELISA yielded a B/B_0 of 18%, an unequivocal, strongly positive test. The incurred and fortified samples containing MBCs all yielded analyses similar to the blanks, readily distinguishable from the samples containing TBZ-like compounds. The assay results are consistent with the known specficity of the antibody (monoclonal antibody 448) used in the ELISA (Brandon et al., 1992).

Table 1. Analysis of Unknowns with TBZ ELISA Kit

analyte	level or range (ppb)	*n*	+/−	% $B/B_0 \pm$ SD	CV (%)
blanks		12	−	97 ± 4	4.1
MBCs and metabolite					
ALB	120−190	8	−		
ABZ-SO2	160−1700	2	−		
FBZ	400−600	8	−		
OFZ	440−600	8	−		
all MBCs (and ALB)	120−600	26	−	100 ± 6	5.5
incurred ABZ 305.18		4	−	97 ± 5	5.4
incurred FBZ 277		4	−	97 ± 12	13
TBZ and derivatives					
CBZ	60−120	10	+		
	90	3	+	10 ± 1	10
TBZ	60−120	10	+		
	90	3	+	31 ± 4	13
5-OHTBZ	60−120	10	+		
	90	4	+	30 ± 3	10
incurred TBZ 275		4	+	18 ± 2	11

Table 2. Effect of Extraction Technique on BNZ ELISA Sensitivity

compd/fortifn level	% of analysis that detected BNZ in fortified sample[a] for the given sample preparation method		
	water (*n* = 4)	citric acid (*n* = 2)	water/20000*g* (*n* = 3)
ABZ-SO2			
50 ppb	50	100	100
100 ppb	100	100	100
200 ppb	100	100	100
FBZ			
400 ppb	50	0	67
800 ppb	50	0	100
1600 ppb	25	100	100
FBZ-SO2			
130 ppb	50	0	100
260 ppb	50	100	100
520 ppb	75	100	100

[a] Positive samples defined by the 90% confidence interval based on the blanks.

Choice of Extraction Method for BNZ ELISA. In choosing a sample preparation method, we wished to exploit the potential advantages of ELISA technology, including minimal need for specialized equipment and avoidance of hazardous materials. Water extraction and a low-speed centrifugation were adequate for the thiazole compounds, but the MBCs include compounds with extractability lower than that of the thiazole compounds. In addition, the BNZ ELISA was severalfold less sensitive than the TBZ ELISA. Two of the methods described previously (Brandon et al., 1994) were tested, simple water extraction and extraction with citrate buffer (0.05 M citric acid, pH 3.0). Following preliminary studies using a 10 min extraction time and clarification by centrifugation at 4500*g* for 10 min, the extraction time was extended to 1 h and the extracts were clarified by further centrifugation. The percent conjugate bound values for blank liver samples compared to a water blank were as follows: water, 75 ± 12 ($n = 4$); citric acid, 102 ± 14 ($n = 2$); water + 20000*g*, 69 ± 3 ($n = 3$). The differences between control (water + 4500*g* centrifugation) and the other methods were not significant ($p > 0.1$), although the high-speed centrifugation resulted in the lowest CV. To evaluate these methods, three compounds were selected for fortification studies—ABZ-SO2, FBZ, and FBZ-SO2. The compounds had relatively high LLDs in previous work (Brandon et al., 1994) and in preliminary experiments in this study. The absorbances from ELISA, expressed as percent conjugate bound, were compared to those of liver blanks. Table 2 shows the number of analyses resulting in detection of selected analytes by the different procedures. The "water + 20000*g*" method produced the most sensitive assay. This result follows, in part, from the smaller CV for the blanks, facilitating the distinction between positive and negative samples, whether by reading absorbances or by inspection. Second, both the low-speed centrifugation and the neutralization of citrate extracts resulted in a turbid extract. Clarification may improve the results by eliminating interference by particles.

Table 3. Analysis of Unknowns with BNZ ELISA Kit

analyte	level or range (ppb)	*n*	+/−	% $B/B_0 \pm$ dev[a]	CV (%)
blanks		4	−	71 ± 3	4.1
MBC	100	2	+	55 ± 5	8.2
MBC	200, 400		+		
ABZ-SO	50, 100		+		
ABZ-SO2	100	2	+	26 ± 0	0
ABZ-SO2	50, 200, 400		+		
FBZ	400	2	+	53 ± 6	10
FBZ	600, 800		+		
OFZ	400	2	+	10 ± 0	0
OFZ	600, 800		+		
FBZ-SO2	400	2	+	38 ± 4	9.2
FBZ-SO2	300, 800		+		
incurred FBZ 277		4	+	16 ± 1	6.3

[a] Standard deviation for $n = 4$; average deviation for $n = 2$.

Analyses Using BNZ Kit. Preliminary studies (data not shown) indicated that all thiazole BNZs produced negative results in this assay. Table 3 shows the analysis of 31 unknowns, including 4 blank liver samples and 4 incurred residue samples. ELISA values were expressed relative to the average water blank in the assay. Using a value of <60% bound as the criterion of a positive test, all 25 BNZ-containing samples were correctly scored. Detection of FBZ at 600 ppb or less and MBC at 100 ppb was marginal, yielding values between 50 and 60% bound. However, at tolerance levels (800 and 200 ppb, respectively), both compounds could be readily distinguished from blanks. Table 3 also shows that the BNZ ELISA results were highly reproducible, with CVs of 4.1% for blanks and 6.3% for the incurred FBZ samples.

CONCLUSIONS

The ELISA methods studied in this paper have been used previously for quantitative analysis of samples (Brandon et al., 1992, 1994, 1995; Bushway et al., 1995). For regulatory screening of liver samples for BNZs, it is most important to flag potentially positive samples, which typically occur with a frequency of <1%. These kit methods were capable of providing rapid qualitative analyses of the major BNZs of regulatory concern in the United States and abroad. When used with a water extraction as described in this paper, thiazolyl BNZs were readily detected at 60 ppb, MBC at 100 ppb; FBZ at 400 ppb, FBZ-SO2 at 300 ppb; OFZ at <50 ppb (based on the standard curve and on data from related experiments); and ABZ-SO and ABZ-SO2 at 50 ppb. Samples

containing incurred residues of FBZ or TBZ near tolerance were readily identified. Fortified samples demonstrated the feasibility of detecting other analytes either directly (FBZ, MBC, OFZ, CBZ) or by detecting the oxidized metabolites that are produced rapidly after dosing and which are extracted more completely (ABZ-SO, ABZ-SO2, FBZ-SO2). Although ALB, the marker residue for ABZ, is not detected by the BNZ method, the Code of Federal Regulations ascribes >80% of ABZ residues to compounds other than ALB (21 CFR 556.34). Studies of ABZ residues indicated that the oxidized ABZ metabolites, ABZ-SO and ABZ-SO2, exceed the level of the 2-aminosulfone marker metabolite 12 days after dosing, when ALB was approximately half tolerance level (U.S. Food and Drug Administration, 1989). It appears likely that the BNZ kit has suitable specificity and sufficient sensitivity for screening of bovine liver for ABZ residues, but the precise cutoff values for regulatory use of the BNZ ELISA method remain to be determined.

ABBREVIATIONS USED

ABZ, albendazole; ABZ-SO, albendazole sulfoxide; ABZ-SO2, albendazole sulfone; ALB, albendazole 2-aminosulfone metabolite, 2-amino-5(6)-(propylsulfonyl)benzimidazole; B/B_0, ratio of ligand bound to ligand bound at 0 concentration of analyte; BNZ, benzimidazole; CBZ, cambendazole; CV, coefficient of variation; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; FBZ, fenbendazole; FBZ-SO2, fenbendazole sulfone; IC_{50}, concentration of analyte resulting in 50% inhibition of binding; LLD, lower limit of detection; MBC, methyl benzimidazolecarbamate; OFZ, oxfendazole (fenbendazole sulfoxide); PBS-Tween + BSA, phosphate-buffered saline containing Tween 20 and bovine serum albumin; TBZ, thiabendazole; TMB, tetramethylbenzidine.

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