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Release of Protein-Bound Residues of Thiabendazole from Liver

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Tissue-bound residues of thiabendazole (TBZ), a veterinary anthelmintic and postharvest fungicide, are formed when this compound is incubated with rabbit hepatocytes or administered to mice or pigs. Several pretreatment steps were investigated for removing free TBZ and metabolites prior to the release of bound residues, and three procedures were evaluated for the release of bound residues from solvent-extracted rabbit hepatocytes: incubation under acidic conditions, enzymatic action using cystathionine β-lyase, and Raney nickel desulfurization. Immunoaffinity chromatography utilizes monoclonal antibodies capable of binding TBZ or its 5-hydroxy metabolite enabled isolation of crossreactive residue fractions. Residues released from incurred pig liver and isolated by immunoaffinity included TBZ, as determined by HPLC with photodiode array detection. The methodology described should facilitate food safety assessments of TBZ.

KEYWORDS: Thiabendazole; bound residue; hepatocyte; liver; immunoaffinity

INTRODUCTION

Thiabendazole (TBZ) is an anthelmintic used for the treatment of worm infection in animals (*1*) and a fungicide for the postharvest treatment of crops to prevent fungal infection (*2*). On administration of TBZ to various animal species, the following metabolites have been reported (**Figure 1**): 5-hydroxythiabendazole (5-OHTBZ), 4-hydroxythiabendazole, 2-acetylbenzimidazole, and benzimidazole (*3*, *4*). In addition, low levels of "bound residues" of TBZ, poorly extractable compounds which may be covalently bound to macromolecules, have been reported in animal systems (*3*, *5*−*7*), and recently, in cultured hepatocytes (*8*, *9*). Bound residues of TBZ and other benzimidazole drugs (e.g., mebendazole and cambendazole) (*10*) have proven difficult to release and identify. In animals, TBZ is initially oxidized to 5-OHTBZ by cytochrome P450 1A2 and subsequently to other reactive metabolic intermediates (*9*, *11*). Reactive benzimidazole metabolites may bind to cellular protein covalently via cysteine sulfhydryls (*12*), or in the case of benzimidazol-2-ylglyoxal, through Schiff's base linkages involving lysine residues (*13*) (**Figure 1**, compound III).

To establish the acceptable daily intake and maximum residue levels for regulated veterinary drug residues in food animal tissues, information is required on their nature, quantity, and toxicity. It is particularly difficult to obtain this information for drugs that produce bound residues. Nondestructive release, extraction, and subsequent structural identification of bound residues are necessary to determine the toxicological significance of these xenobiotic-macromolecule complexes. The objective of this study was to release bound TBZ residues from rabbit hepatocytes and liver containing incurred residues and isolate them by immunoaffinity chromatography.

MATERIALS AND METHODS

Chemicals and Biochemicals. TBZ, benzimidazole, and aluminum−nickel catalyst (Raney type alloy, 50/50 powder) were obtained from Sigma-Aldrich (Poole, Dorset, UK). 5-OHTBZ and 2-acetylbenzimidazole were kindly supplied by Dr. W. L. Henckler (Merck, Sharp, & Dohme, Rahway, NJ). ^{14}C-TBZ, the monoclonal antibodies (MAbs) 300 and 430 (which bind TBZ and 5-OHTBZ), and the preparation of immunoaffinity columns were described previously (*14*, *15*). Lactococcal cystathionine β-lyase was a gift from Dr. Arno Alting, The Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands. All other chemicals were reagent-grade commercial products.

Preparation of hepatocytes and liver containing bound residues of ^{14}C-TBZ. Rabbit hepatocytes were prepared, incubated with 25 μM ^{14}C-TBZ, and extracted with methanol/saline (1:1, v/v) as previously described (*8*). To obtain liver samples with incurred residues, the following procedures were used: Mice were dosed orally with ^{14}C-TBZ (4 μCi/mg TBZ, 50 μg/g body weight). After 8 h, the mice were euthanized and the livers were recovered. A pig (33 kg) was treated with thiabendazole (6.6 g in feed, equivalent to 200 mg/kg body weight). The animal was euthanized after 6 h, and the liver was recovered. Samples were stored frozen until prepared for analysis.

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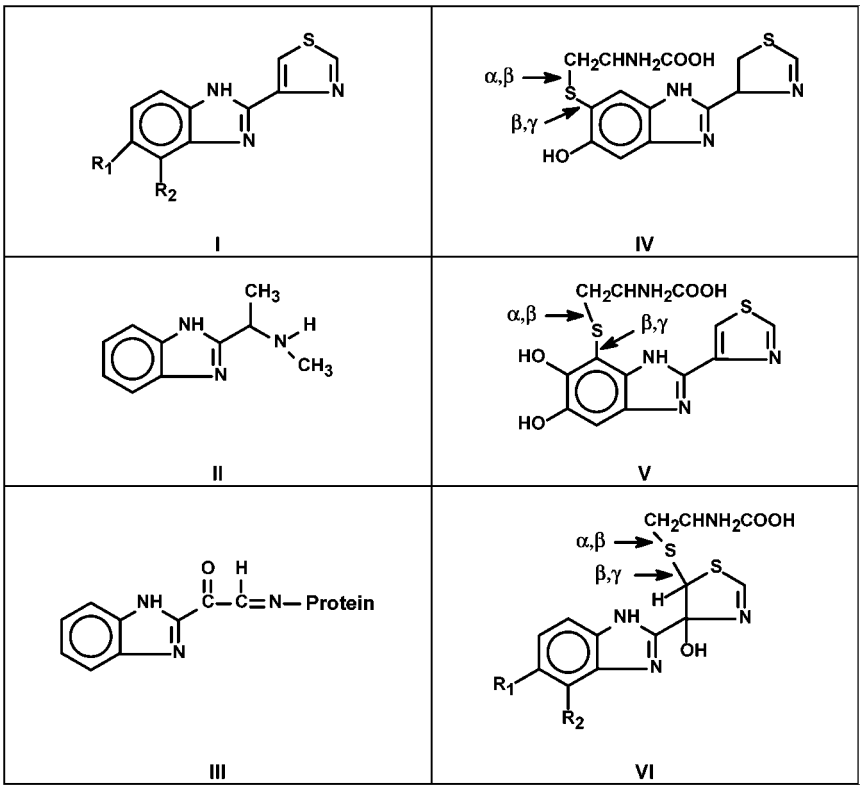

Figure 1. Chemical structures of thiabendazole and metabolites. (I) thiabendazole (R_1, R_2 = H), 5-hydroxythiabendazole (R_1 = OH, R_2 = H), 4-hydroxythiabendazole (R_1 = H, R_2 = OH); (II) product of Raney nickel desulfurization of TBZ; (III) a bound residue that could be formed from benzimidazol-2-ylglyoxal, a metabolite identified in mouse urine (13); (IV–VI) adducts which might be formed after TBZ is metabolized by cytochrome P450 and interacts with protein cysteine groups. Compounds depicted would result from protein hydrolysis. Arrows indicate bonds that could be hydrolyzed by cystathionine β-lyase.

Analysis of Extractable Residues. Livers were homogenized and extracted with ethyl acetate. The extracts were subjected to liquid/liquid partitioning and silica column cleanup, followed by LC determination (*16*).

Analysis of Bound Residues. Extractable residues were first removed, as follows. Hepatocyte or liver samples were thawed and homogenized and suspended in methanol/water (2:1, hepatocytes; 1:1, liver, v/v), 4 mL/g tissue. Following 5 min treatment in an ultrasonic bath, suspensions were centrifuged at 1000*g* for 10 min, and the resulting pellets were washed and recentrifuged three times with methanol and two times with ethanol. Liver samples containing incurred residues were additionally incubated in water for 16 h at 37 °C and recentrifuged. Samples were then dried under a stream of nitrogen, and the dried pellets were then subjected to various procedures for release of bound residues (see below). For quantification of residues that were not released, the remaining pellet was dissolved by incubating in a solution of Na dodecylsulfate (50 mg/mL) containing 0.5 M NaOH (37 °C, 48 h). A portion of the resulting mixture was diluted with water, treated with hydrogen peroxide to remove interference, and analyzed for ^{14}C by liquid scintillation counting.

Chromatography. The LC system consisted of a model 616 HPLC pump, model 600S system controller, model 717 autosampler, model 996 photodiode array detector, and Millennium Chromatography Manager software from Waters (Milford, MA). A stainless steel analytical column (250 × 4.6 mm i.d.) packed with Hypersil ODS (5 μm, Capital HPLC, West Lothian, UK) together with a guard column containing μBondapak C_{18} material (Waters) were used. The mobile phase was CH_3CN/0.05 M NH_4CO_3, 1:4 (v/v), pumped at 1 mL/min and degassed online. With the analytical column maintained at 30 °C, 5-OHTBZ, benzimidazole, 2-acetylbenzimidazole, and TBZ eluted at 5.7, 6.5, 9.7, and 13.9 min, respectively. Immunoaffinity chromatography columns utilized MAb 300, which binds TBZ, and MAb 430, which binds TBZ and 5-OHTBZ. Each column bound up to 100 ng TBZ quantitatively, indicating that the antibodies retained their binding affinity on immobilization. To isolate TBZ residues, columns were prewashed with 5 mL of water, and the sample was applied. Columns were then washed with 12 mL of water and eluted with 70% ethanol in water. One mL fractions were collected for assay of radioactivity.

Treatment with Acid. Dried hepatocyte or liver pellets were incubated in 0.1 or 0.3 M HCl at 37 °C for 16 h. After neutralization with potassium phosphate or NaOH, the mixture was extracted three times with ethyl acetate, and the dried, combined extracts were dissolved in 30% aqueous methanol for fractionation by immunoaffinity chromatography.

Treatment with Raney Nickel. Dried hepatocyte or liver pellets from a 1-g sample were stirred overnight in 8 mL of NaOH, 100 mg/mL. Under nitrogen, 0.2 g of Ni Al alloy was added gradually over 1 h (*17*), preventing excessive foaming due to the generation of hydrogen, and the reaction was allowed to proceed for 7 h. The reaction mixture was then adjusted to pH 1 with phosphoric acid and refluxed under nitrogen for 1 h. The mixture was cooled to room temperature, adjusted to pH 6.5 with NaOH, extracted with ethyl acetate, and fractionated by immunoaffinity chromatography, as above.

Enzymatic Incubation of Rabbit Hepatocytes Containing Bound TBZ Residues with Proteinase K and Cystathionine Lyase. The dried pellet from solvent-extracted rabbit hepatocytes was suspended in water and incubated for 16 h at 37 °C in the presence of 3 mM $CaCl_2$ and 40 μg/mL proteinase K to hydrolyze protein-bound residues to cysteine derivatives (IV−VI, **Figure 1**). These compounds could be substrates for cystathione β-lyase. Two aliquots of the proteinase K digest were removed. Each was adjusted to 3.6 mM ethylenediaminetetraacetic acid (EDTA) and 3.6 mM potassium phosphate, pH 7.4, and heated at 80 °C for 3 min. Further incubation (overnight, 37 °C) was conducted after the addition of pyridoxal-5-phosphate cofactor, in the presence or absence of 7 μg/mL cystathionine β-lyase (*18*). Radioactivity was analyzed in the ethyl acetate extracts, aqueous fraction, and pellet. The ethyl acetate extracts were prepared for immunoaffinity chromatography, as above.

Release of TBZ and Metabolites from Incurred Pig Liver. An additional protease enzyme digestion step was incorporated into the

Table 1. Release of Bound TBZ Residue from Rabbit Hepatocytes

conditions	extractable residue[a]	TBZ-like residue[b]	
	pmol TBZ/mg protein	% of bound residue	% of extractable residue
0.1 M HCl	5.2	2.4	53
0.3 M HCl	5.8	2.1	21
Raney nickel	10.2	8.5	0

[a] Residue released and extracted into ethyl acetate. [b] Residue eluted from MAb 430 immunoaffinity column with 70% aqueous ethanol (v/v), but not eluted with water.

procedure developed for the more readily extracted hepatocytes and mouse liver. Incurred pig liver (1 g) was extracted with solvent and water, then centrifuged, as described above. The resulting pellet was dried, resuspended in 11.25 mL of 0.33 M HCl, containing 0.7 mg/mL pepsin, and incubated at 37 °C for 16 h. The suspension was neutralized with NaOH, extracted, and fractionated by immunoaffinity chromatography, as described above.

Statistics. Unpaired *t*-tests were performed using two-sample analysis in Statgraphics Plus (Manugistics, Rockville, MD).

RESULTS AND DISCUSSION

Extraction and Isolation Conditions. (a) *Effects of Acid*. For mouse liver, incubation of the solvent-washed pellets with acid was effective in releasing ethyl acetate-extractable residues. Levels went from 0.3% of the total radioactivity (water) to 1.3% (0.1 M HCl) to 3.0% (0.3 M HCl). Following incubation of rabbit hepatocytes with either 0.1 or 0.3 M HCl, the levels of released residue extractable with ethyl acetate were similar (**Table 1**). The difference between mouse and rabbit could possibly be related to the very rapid conversion of TBZ to 5-OHTBZ in the rabbit hepatocyte system (*8*). Further characterization of the radioactive material by immunoaffinity chromatography on MAb 430 showed that most of the residue released under acidic conditions eluted with 70% aqueous ethanol, but not with water. This elution profile is characteristic of TBZ and 5-OHTBZ.

(b) *Effects of Raney Nickel Catalysis.* Treatment with Raney nickel resulted in a 2-fold increase in extractable, released residues, compared to acid-incubated samples (**Table 1**). Radioactive residues released and extracted under these conditions were then subjected to immunoaffinity chromatography. All radioactivity was eluted in the water fraction, indicating that there was no specific recognition by the antibody. This result suggests that the residue has a modified thiazole ring. Raney nickel treatment could result in desulfurization of the thiazole ring (*19*), in addition to breakage of the presumptive linkages to cysteine residues, thereby eliminating the immunoreactivity of released compounds (**Figure 1**, compound II).

(c) *Enzymatic Treatment.* An alternative method to hydrolyze the presumptive cysteine linkages was treatment with cystathionine β-lyase (*18*). However, no substantial differences in the level of released radioactivity or % immunoreactivity were observed in comparison to results obtained using HCl (data not shown). These results suggest that the acid treatment released radioactivity that was not covalently bound via cysteine residues. Covalent binding may have occurred through acid-labile Schiff's base bonds, as in **Figure 1**, compound III. This hypothesis is supported by the decrease in the percent of TBZ-like crossreactive residue released under strong acid conditions (0.3 M HCl), conditions that could reverse Schiff's base formation. The released benzimidazol-2-ylglyoxal lacks a thiazole ring and would not be expected to crossreact with MAb 430.

Table 2. Distribution of Radioactivity Following Incubation of Rabbit Hepatocytes under Different Conditions[a]

fraction	25 °C	37 °C	55 °C	37 °C + inhibitors[b]
released residues (total)	8.4	16.9	13.9	14.0
ethyl acetate extract	4.5	7.3	3.3	4.8
aqueous phase	3.9	9.6	10.6	9.2
pellet	91.6	83.1	86.1	86.1

[a] Four 25-mg samples of homogenized rabbit hepatocytes, solvent-washed and dried, were suspended in 1 mL of water for 16 h, and the distribution of radioactivity was then analyzed. [b] 10 mM EDTA, 0.5 mg/mL PMSF, and 0.1 mg/mL phenylhydrazine, pH 7.4.

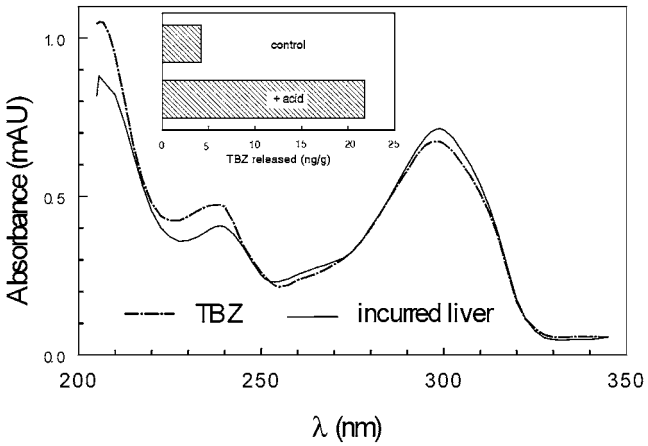

Figure 2. Photodiode-array spectra for thiabendazole standard and for immunoaffinity-isolated, released residue, following incubation of incurred pig liver in the presence of 0.33 M HCl. The inset shows the enhanced release of TBZ from extracted liver pellet in the presence of acid compared to water.

(d) *Effects of Temperature and Enzyme Inhibitors.* The effect of temperature on the release of radioactive residues was studied by the incubation of solvent-washed rabbit hepatocytes in water at various temperatures. Because endogenous liver enzymes could release bound residues during the extraction of free residues, the following enzyme inhibitors were included in some incubations: phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor; EDTA, chelator of divalent cations; and phenylhydrazine, inhibitor of several oxidative enzymes including cytochrome P450 (*20*). **Table 2** shows that the best temperature for residue release by sample incubation in water was 37 °C, and that the overall extractability of residues was not significantly influenced by the presence of enzyme inhibitors.

Analysis of Porcine Liver. Significant levels of free TBZ (4.1 ± 0.4 μg/g) and 5-OHTBZ (11 ± 2 μg/g) were found in the analysis of liver from the TBZ-dosed pig. The preponderance of the oxidized metabolite after 6 h is consistent with the rapid oxidation of TBZ found in pig hepatocytes (*8*). Following the extraction of free residues, bound residues were released by acid/enzyme digestion of the liver protein pellet and were fractionated by immunoaffinity chromatography (**Figure 2**). Although acid treatment was effective in releasing small quantities of TBZ, no immunoreactive metabolites of TBZ were detected by this procedure.

CONCLUSIONS

The results indicate that there is a greater release of bound TBZ residues under acidic conditions compared to water in

model hepatocyte and mouse liver systems ($n = 11$, $p < 0.05$), and similar results were obtained in a food animal system. Immunoaffinity chromatography offers a means to separate unidentified residues into crossreactive and non-crossreactive fractions. However, bound residues were not fully released, even when enzymatic digestion was combined with aqueous and solvent extractions, and the structures of bound residues that are immunochemically unrelated to TBZ remain to be determined. Monoclonal antibodies elicited using a 5-carboxybenzimidazole hapten have recently been reported (*21*) and may prove effective in isolation and characterization of additional TBZ bound residues.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic; MAb, monoclonal antibody; 5-OHTBZ, 5-hydroxythiabendazole; PMSF, phenymethylsulfonyl fluoride; TBZ, thiabendazole.

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