# Detection of Dimetridazole and Other Nitroimidazole Residues in Turkey Using an Immunoassay

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A series of monoclonal antibodies were generated that can bind dimetridazole, a nitroimidazole drug used in veterinary medicine. A competition enzyme-linked immunosorption assay (cELISA) is described and is used to characterize the binding of these antibodies to a number of nitroimidazole drugs. The 50% inhibition of control occurred for the most sensitive of these antibodies at 12 ng/mL for dimetridazole, 75 ng/mL for hydroxydimetridazole, 25 ng/mL for ipronidazole, 2000 ng/mL for hydroxyipronidazole, and 20 000 ng/mL for metronidazole. An extraction method for these nitroimidazoles that is compatible with the immunoassay is described. Using this method, as little as 1 ng of dimetridazole could be detected in turkey muscle.

## INTRODUCTION

Dimetridazole (DMZ) is a nitroimidazole compound used in veterinary medicine to treat poultry for coccidiosis and histomoniasis ("black head"). The major metabolite of DMZ is 2-(hydroxymethyl)-1-methyl-5-nitroimidazole (DMZOH), formed by hydroxylation of the 2-methyl group. Further oxidation to the 2-carboxylic acid analog has been reported (Law et al., 1963). Dimetridazole and ipronidazole (IPR) were approved by the U.S. Food and Drug Administration (FDA) in 1971 and 1965, respectively, for use in turkeys. Both drugs have how been withdrawn from the market, DMZ in 1987 and IPR in 1989. Many countries, however, including Canada, Australia, and Denmark permit use of these drugs in food animals. Thus, a sensitive rapid screening method for detection of these compounds in food products is desirable. In this paper we describe the development of a monoclonal antibody to DMZ and its application to detection of nitroimidazoles in turkey.

A number of analytical methods have been described for determination of nitroimidazoles. A tandem mass spectrometry method to detect DMZ, IPR, and their alcohol metabolites in the low parts per billion (ppb) range has recently been described by Matusik et al. (1992). Mallison and Henry (1992) have described a liquid chromatographic method that can measure DMZOH with a 2 ppb detection limit. Their method also can detect the hydroxyl metabolite of ipronidazole (IPROH) as well as the parent compounds in swine and turkey muscle tissue. Carignon et al. (1988) have reported a high-pressure liquid chromatographic (HPLC) method to measure DMZ in pork tissue. Gas chromatographic methods for detection of DMZOH in swine muscle have been reported by Newkirk et al. (1990) and by Stone et al. (1978). A polarographic method with a 2 ppb limit of sensitivity for DMZOH in swine muscle tissue has been described by Craine et al. (1974). Garland et al. (1980) described a negative ion chemical ionization method for IPR and IPROH. Both IPR and DMZ were recovered from swine feed and analyzed by Roybal et al. (1987) using an HPLC method, and MacDonald et al. (1971) developed a method to detect IPR in turkey tissue at the 2 ppb level.

A competition enzyme-linked immunosorbent assay (cELISA) is described in this paper. The cELISA is based on a monoclonal antibody and detects DZM, DMZOH, IPR, and IPROH. Turkey muscle tissues fortified with DMZ, DMZOH, and IPR at levels ranging from 0.3 to 50 ppb were evaluated with the cELISA.

## MATERIALS AND METHODS

**Reagents.** Dimetridazole (DMZ) (1,2-dimethyl-5-nitroimidazole), hydroxydimetridazole (DMZOH) [1-methyl-2-(hydroxymethyl)-5-nitroimidazole], ipronidazole (IPR) (1-methyl-2-isopropyl-5-nitroimidazole), and metronidazole (METRON) [1-(hydroxyethyl)-2-methyl-5-nitroimidazole] were all provided by the U.S. Department of Agriculture, Food Safety Inspection Services. The above chemicals were greater than 95% pure for use as analytical standards.

**Hapten Preparation.** The immunogen was prepared as follows. Hydroxydimetridazole was reacted with succinic anhydride to form the hemisuccinate and then conjugated to keyhole limpet hemocyanin (KLH) (DMZ-KLH) and to bovine serum albumin (BSA) (DMZ-BSA) using the mixed anhydride method (Erlanger et al., 1959).

**Monoclonal Antibody Production.** Six-month-old BALB/cBkl mice (Bantin and Kingman Laboratories, Fremont, CA) were injected intraperitoneally (ip) with 100 μg of the DMZ-KLH conjugate mixed 1:1 with complete Freund's adjuvant. Mice received a single ip injection every other week for a total of three injections. Four days prior to fusion, each mouse was given an intrasplenic injection of 100 μg of DMZ-BSA conjugate in sterile saline. The spleen was removed, and the splenocytes were fused with SP2/0 myeloma cells and grown under conditions described by Stanker et al. (1986).

A direct-binding ELISA, described by Stanker et al. (1986) and modified as described below, was used to screen culture the fluids from the growing hybridomas for antibodies to DMZ. DMZOH linked to BSA (DMZ-BSA) served as antigen in both the direct-binding ELISA and the competition ELISA (cELISA) (described below). The coating antigen was prepared as follows. Fifty milligrams of the BSA dissolved in 5 mL of distilled water was added to 50 mg of the hemisuccinate. The pH was adjusted to 7.0 by addition of 1 N sodium hydroxide. Then, 50 mg of

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1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Pierce Chemical Co., Rockford, IL) was added, and the mixture was stirred overnight at ambient temperature and dialyzed for 48 h against four changes of PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2). Microtiter plates (Nunc Maxisorb, Denmark) were coated with DMZ–BSA by addition of 100 μL/well of a 200 ng/mL solution of DMZ–BSA in carbonate buffer. The DMZ–BSA was incubated in uncovered plates at 37 °C for 18 h to evaporate the liquid and allow the DMZ–BSA to coat the bottom of the microtiter wells. The "coated" plates were then stored in sealed plastic bags at 4 °C and used within 2 weeks. Nonreacted sites on the plastic microtiter plates were blocked by adding 400 μL of assay buffer (AB) [0.1 M Tris, 0.15 M NaCl, 0.001% Tween 20, 0.005% Thimerosal, and calf serum (1 mL/100 mL of AB), pH 7.2] and then incubating the plates at room temperature for 1 h. The blocking solution was removed, 50 μL of the hybridoma supernatant(s) or the anti-dimetridazole Mab was added, and the plates were incubated for 1 h at 37 °C. The plates were carefully washed with a solution of 0.05% Tween 20 in water, and peroxidase conjugated goat anti-mouse antiserum (Sigma Chemical Co., St. Louis, MO) diluted 1:500 in AB was added to each well. Following a second 1-h incubation at 37 °C, the plates were washed again and the substrate 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) was added. Absorbance measurements at 405 nm were taken and the resulting data transferred to a Macintosh computer and analyzed with the Cyberdoma ELISA software described by Slezak et al. (1983).

Hybridoma cells from wells showing a positive response in the ELISA screen were expanded and subcloned twice by limiting dilution to ensure their monoclonal origin. Ascites fluid was prepared in irradiated mice (Stanker et al., 1986), and the monoclonal antibodies were purified from the ascites by hydroxyapatite chromatography (Stanker et al., 1985). Isotype determination was done by ELISA using mouse heavy- and light-chain-specific antisera (Southern Biotechnology Associates, Birmingham, AL).

**Competition ELISA (cELISA) for Dimetridazole.** A cELISA for dimetridazole and other related nitroimidazoles was as follows. To each well of an antigen-coated, assay-buffer-blocked, microtiter plate was added 100 μL of sample buffer (SB) [0.5 M Tris, 0.15 M NaCl, 0.001% Tween 20, 0.005% thimerosal, and calf serum (1 mL/100 mL AB), pH 7.2]. Competitors dissolved in 100 μL of SB were added to the microassay plate and serially diluted (a 2-fold series). Next, 100 μL of SB containing a predetermined amount of anti-dimetridazole monoclonal antibody (for Mab Di-7, 100 μL of a 300 ng/mL solution) was added to each well. Thus, each well contained 200 μL of sample buffer containing antibody and competitor. The amount of anti-dimetridazole antibody used is approximately that concentration of antibody that results in 50% of maximum activity in a direct binding ELISA where no computer was present. The plates were sealed with plastic wrap, incubated for 1 h at 37 °C, and then processed with peroxidase-conjugated anti-mouse antibody (diluted 1/50) and substrate as described above.

In each experiment, microtiter wells containing all components except competitor were prepared and the activity in these wells was taken to represent 100% activity (control wells). The test wells, each containing different amounts of competitor, were normalized to the 100% activity wells, and percent inhibition was calculated as follows.

$$\% \text{ inhibition of control} = [1 - (OD_{405nm} \text{ of test})/(OD_{405nm} \text{ of no competitor wells})] \times 100$$

**Dimetridazole Extraction.** Dimetridazole and other nitroimidazoles were extracted from turkey as follows: A 50-g sample of turkey breast (purchased from local supermarkets) was placed in a 250-mL conical polypropylene centrifuge tube; 20 g of NaCl and 20 g of $K_2HPO_4$ were added, followed by 90 mL of ethyl acetate. The sample was then homogenized in a polytron (Brinkman Instruments, Westbury, NY) at medium-high speed (setting 6) for 1.5 min. The resulting slurry was centrifuged at 1000*g* for 2 min in a countertop centrifuge to sediment the particulate. The particulate was subjected to two additional extractions (90 mL of ethyl acetate was used for each extraction). The ethyl acetate fractions from each extraction were pooled (approximately 270 mL) into a separatory funnel. Distilled water (5 mL) was then added and the sample shaken by hand for 30 s. Next, 5 mL of 1 N HCl was added and the sample shaken for an additional 30 s. The phases were allowed to separate, and the aqueous fraction (bottom) was collected into a 50-mL centrifuge tube. Three additional extractions each using 5 mL of 1 N HCl were performed and the aqueous fractions collected and pooled. The pooled aqueous fraction was cooled on ice, 10 g of $K_2HPO_4$ added, and the tube again allowed to cool on ice. An additional 10 g of $K_2HPO_4$ was then added and the sample cooled (not all of the salt is dissolved at this time). Methylene chloride (10 mL) was then added and the tube capped, shaken, and centrifuged for 1 min at approximately 1000*g* in a tabletop centrifuge to separate the phases. The methylene chloride fraction (top) was recovered to a separatory funnel. Three additional methylene chloride extractions were performed. Next, 10 mL of alkaline wash buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 8.0) was added to the pooled methylene chloride fraction and shaken for 10 s. The phases were allowed to separate, and the aqueous phase was discarded. The methylene chloride fraction was partitioned a second time against alkaline wash buffer and evaporated to dryness at room temperature under a stream of nitrogen. Prolonged drying was avoided since these nitroimidazoles can be volatilized. Finally, the residue was dissolved in 1 mL of sample buffer and used in the cELISA.

Turkey breast samples were spiked with nitroimidazole standards, in assay buffer, by delivering between 100 and 500 μL of standard to a coarsely ground meat sample at three or four locations. Assay buffer alone was added to the unspiked samples.

## RESULTS

**Hybridoma Production.** Spleen cells from a BALB/c mouse immunized with DMZ–KLH were fused with SP 2/0 myeloma cells, and the resulting hybridomas were cultured in 30 96-well microculture dishes. Greater than 90% of the wells had growing hybridomas at 10 days after the fusion. Those wells containing anti-dimetridazole antibodies were identified by evaluating the supernatant from each well for antibodies reactive to DMZ–BSA in an ELISA. (BSA represents an extraneous protein because the immunogen was DMZ–KLH; therefore, anti-KLH antibodies were not detected.) Approximately 115 wells were observed to be secreting antibody that recognized the DMZ–BSA conjugate but not BSA itself. Cells from these wells were expanded and tested against DMZ–BSA, DMZ–KLH, BSA, and KLH. Forty-eight wells were observed to contain cells that secreted antibody that recognized both hapten conjugates but did not bind either of the unconjugated carrier proteins. These were next evaluated for their ability to produce antibodies that bound dimetridazole in the cELISA. Only 11 wells were observed to produce antibodies that bound dimetridazole in the cELISA. These 11 were subcloned twice to ensure their monoclonal origin, and their isotypes were determined. The antibodies, referred to as Di-1–Di-11, were found to have either IgM, IgG2a, or IgG2b heavy chains, and all had kappa light chains (Table I).

**Antibody Characterization.** The amount of dimetridazole necessary to cause 50% inhibition of antibody binding (the $IC_{50}$) for each of the 11 Mabs isolated is shown in Table I. The $IC_{50}$ ranged from 2 to 260 ng, indicating the range in relative affinity that these antibodies have for dimetridazole. Typical cELISA data obtained with Mab Di-7 and DMZ, DMZOH, IPR, IPROH, and metronidazole are shown in Figure 1. Di-7 differentially binds these compounds and serves as an example for the other Mabs. Di-7 had the greatest sensitivity for DMZ ($IC_{50}$ of 1.0 ± 0.6 ng) followed by IPR ($IC_{50}$ of 2.5 ± 1.0 ng). There was approximately a 6-fold reduction in sensitivity to DMZOH. A 166-fold reduction in binding to the hydroxyl metabolite of IPR was observed. Met-

Table I. $IC_{50}$ Values (Nanograms per Well)[a]

| antibody | isotype[b] | DMZ | DMZOH | IPR | IRPOH | METRON |
|---|---|---|---|---|---|---|
| Di-1 | IgM | 3 | 11 | 11 | 390 | >10000[c] |
| Di-2 | IgM | 18 | 160 | 50 | 5000 | >10000 |
| Di-3 | IgM | 18 | 160 | 150 | >10000 | >10000 |
| Di-4 | IgM | 120 | 190 | 50 | 5000 | >10000 |
| Di-5 | IgM | 200 | 1000 | 350 | >5000 | >10000 |
| Di-6 | $IgG_{2b}$ | 200 | >1000 | >1000 | 8000 | >10000 |
| Di-7 | $IgG_{2a}$ | 1.2 | 7.5 | 2.5 | 200 | 2000 |
| Di-8 | IgGM | 20 | 100 | 18 | 6000 | 10000 |
| Di-9 | $IgG_{2b}$ | 260 | >1000 | 4500 | >10000 | 10000 |
| Di-10 | $IgG_{2a}$ | 100 | 250 | 80 | 10000 | 5000 |
| Di-11 | IgM | 30 | 90 | 35 | >10000 | 10000 |

[a] ng/well is converted to ppb simply by multiplying by 10. [b] All utilize kappa light chains. [c] > indicates that no inhibition was detected at the amount indicated.

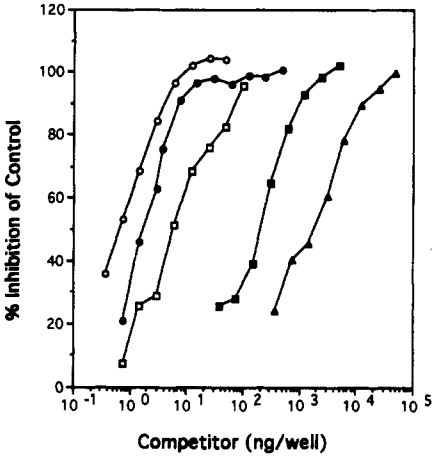


**Figure 1.** Typical cELISA showing the ability of Mab Di-7 to bind DMZ (open circles), IPRON (solid circles), DMZOH (open squares), IPRONOH (solid squares), and METRON (open triangles). These reactions were in 100-μL volumes.

ronidazole was the most poorly bound nitroimidazole tested, resulting in an $IC_{50}$ at least 1666-fold less sensitive than DMZ. The other 10 Mabs had relative affinities similar to that of Di-7 but with lower absolute binding. These data (Table I) suggest that the relative rankings of the compounds by these antibodies are similar but that the relative affinities of the antibodies vary. Monoclonal antibody Di-7 was chosen for further study since it is an IgG immunoglobin and has the greatest relative sensitivity for dimetridazole. Di-7 was produced as an ascites fluid, purified as described, and stored at −20 °C until used.

**Competition ELISA Characterization.** The sensitivity of a cELISA is influenced by the amounts of both the primary antibody and the coating antigen (Pesce et al., 1981). Therefore, the amounts of both the anti-dimetridazole antibody (Di-7) and the coating antigen were varied in an effort to determine the optimum conditions for the assay. In these experiments the dimetridazole coating antigen, DMZ–BSA, was used at three levels: 100, 25, and 10 ng/well. At each coating antigen level cELISAs were performed using the Di-7 antibody at concentrations of 240, 120, 60, and 30 ng/well. Coating antigen levels of 6 and 3 ng/well also were evaluated but gave minimal signals that were difficult to detect over background. Antibody concentrations below 30 ng/well were not evaluated. In these experiments, the peroxidase-conjugated antibody was used at a 1/100 dilution of the stock material as supplied by the manufacturer. As expected, sensitivity was increased by reducing the level of antigen used to coat the microtiter wells and by reducing the Di-7 antibody concentration. The results from these experiments are summarized in Figure 2. To obtain the

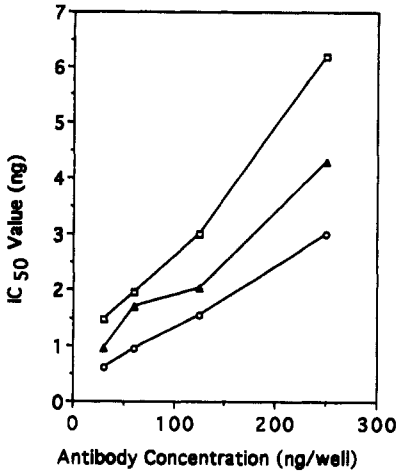


**Figure 2.** Effects of antigen concentration and antibody concentration on the sensitivity of the cELISA for dimetridazole. Squares represent coating antigen at 100 ng/well, triangles 25 ng/well, and circles 10 ng well.

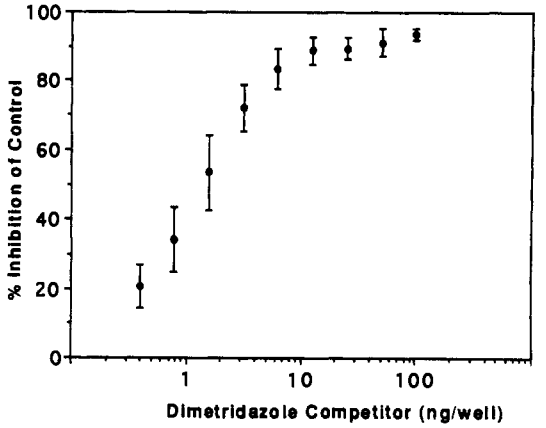


**Figure 3.** Competition curve obtained with dimetridazole standard and monoclonal antibody Di-7. Bars = ±standard deviation ($N$ = 9).

sensitivity necessary to measure dimetridazole near the tolerance level (2 ppb) in all subsequent cELISA experiments, the Di-7 antibody was used at 30 ng/well with a coating antigen concentration of 20 ng/well. Figure 3 shows typical cELISA curves obtained with the DMZ standard under these conditions (bars = standard deviation). The relatively large standard deviation observed in the experiments shown in Figure 3 most likely reflects the need to run the assay under conditions that result in maximum sensitivity (i.e., detection in the nanogram range).

**Detection in Turkey Muscle.** To detect DMZ and other nitroimidazole compounds in turkey, the analyte must be extracted and presented to the antibody in a medium compatible with antibody function. Briefly, 50 g of ground turkey breast muscle was fortified with various levels of DMZ, DMZOH, and IPR as described above. The sample was then extracted with ethyl acetate. Analysis of the extracts, following dry-down and resuspension into buffer, by cELISA resulted in unacceptably high background levels in both control and spiked samples. It was necessary to further purify the samples by extraction of the ethyl acetate fraction against 1 M aqueous hydrochloric acid, neutralize the acid, and then extract with methylene chloride. Samples from the methylene chloride fraction were dried and resuspended in methanol for analysis in the cELISA. Again, control turkey samples gave unacceptable levels of inhibition in the cELISA. Inhibitions as high as 40% of control were often observed (data not

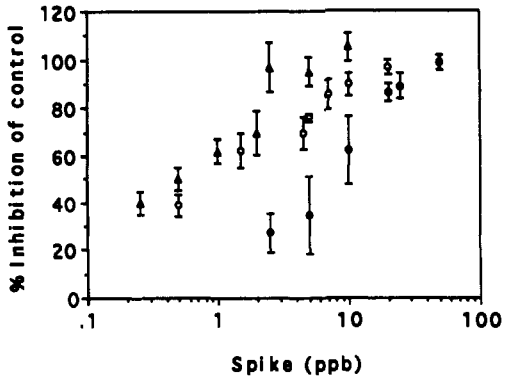


**Figure 4.** Inhibition curves obtained in fortified turkey breast muscle. Samples were fortified as indicated and then extracted. (Triangles) DMZ; (open circles) IPR; (solid circles) DMZOH. Bars = ±standard deviation ($N \geq 3$).

shown). The methylene chloride fraction was next washed by partitioning against alkaline buffer. Finally, the alkaline-washed methylene chloride fraction was dried and the residue resuspended in methanol and analyzed with the cELISA. Nonspiked control turkey samples gave background levels of inhibition between 10% and 20% of control. These levels were sufficiently low to allow detection of dimetridazole in samples spiked at parts per billion levels. Figure 4 summarizes the data obtained when ground turkey muscle samples were fortified before extraction with DMZ, DMZOH, and IPR. In each case the bars represent the standard deviation.

## DISCUSSION

Dimetridazole is a small nitroimidazole molecule that must first be conjugated to a carrier molecule to render the dimetridazole immunogenic. The linkage strategy employed clearly resulted in a potent immunogen. The screening system used allowed quick elimination of the majority of the hybridomas obtained following a cell fusion from those that recognized dimetridazole but only if conjugated to a protein. The 115 hybridomas detected in the initial screen represented approximately 1–4% of the total number of hybridomas observed. This value is consistent with previous studies (Stanker et al., 1987, 1989). However, only 11 hybridomas (approximately 0.2%) were observed to bind free dimetridazole. Analysis of the 11 monoclonals indicated that 7 were IgM antibodies. The $IC_{50}$ for DMZ ranged from 1.2 to 260 ng for these antibodies, implying a large range in relative affinity and suggesting that they represent multiple clones. Only 1 of the 11 antibodies, Di-7, had a relative affinity great enough to be useful for analyte detection in the parts per billion range. These data point out the necessity to screen large numbers of hybridomas to quickly detect the few monoclonals that bind the "free" hapten with the affinity desired. The high number of IgM antibodies and the low relative affinity of many of the IgG antibodies may reflect the limitations inherent in the rapid immunization schedule used.

Analysis of the binding properties of the 11 hybridomas to related nitroimidazole compounds suggests that the antibodies can be divided into at least two epitope groups. The first group is composed of Di-1–Di-4, Di-7, Di-8, Di-10, and Di-11. These antibodies generally Bind DMZ with the greatest relative affinity followed by DMZOH, IPR, and IPROH and with little or no binding to METRON. Within this group, however, there may be subgroups based on the ability of the antibodies to differentially bind

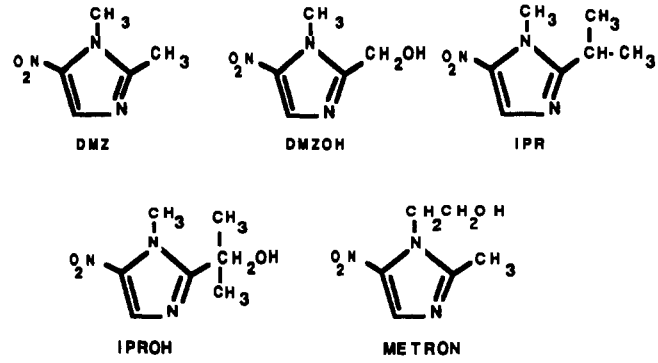


**Figure 5.** Chemical structures for DMZ, DMZOH, IPR, IPROH, and METRON.

IPROH and METRON. The second group is composed of Di-5, Di-6, and Di-9. These antibodies have a lower relative affinity than those in the first group on the basis of their $IC_{50}$ values and are most specific for DMZ with little binding to the other compounds. The antibodies in this latter group may simply represent antibodies derived from cells that were in different compartments of the affinity maturation process that occurs during an immune response. Nevertheless, the presence of different binding properties for these antibodies points out that even though these nitroimidazole compounds are small molecules (e.g., 141.13 Da for DMZ), numerous antibodies can be generated in a single immune response, and that binding for each of these antibodies is influenced by different parameters of the molecule. Differential binding of monoclonal antibodies to small molecules also has been observed by others (Hill et al., 1991; Stanker et al., 1991; Newsome et al., 1990), and the results presented here point out the power of selecting a specific monoclonal antibody.

The specificity of binding for the antibodies described here is difficult to elucidate, in part because of the limited number of related analytes that were available for study (Figure 5). Clearly, substitution of an ethanol group on the no. 1 nitrogen as seen in METRON inhibits binding and suggests that this position is important for antibody binding. However, it is not known if these antibodies interact directly with the no. 1 nitrogen or if it is simply that substitution of an ethanol group on the no. 1 nitrogen vs a methyl group alters the molecule in such a way that antibody binding is inhibited. Molecular modeling of DMZ, DMZOH, IPR, IPROH, and METRON was performed using the modeling programs interfaced by the CAche Molecular Modeling System (CAche-Tectronics, Beaverton, OR). These calculations suggest that the substitutions in IPROH and METRON facilitate rotation of the molecule around the no. 2 carbon, resulting in a significantly altered electron density. This might explain the differential antibody binding results. Regardless of the exact nature of the binding, the antibodies described here are able to bind, rather effectively, a related group of nitroimidazole compounds.

The data shown here using fortified turkey muscle samples demonstrate that Mab Di-7 can be configured into an immunoassay capable of detecting these compounds. A clear dose–response curve was obtained in turkey muscle with the immunoassay, after extraction and cleanup of the material. Simple extractions in aqueous buffers or in ethyl acetate were unsuccessful; in both cases the extracts appeared to contain interfering substances that resulted in apparent competition (approaching 40%) in control tissues. Further cleanup by extracting the sample against alkaline buffer was required to remove most of the interfering substances. The data clearly

demonstrate that the cleanup methods coupled with the immunoassay described here is capable of detecting DMZ, DMZOH, and IPR in extracts of turkey muscle at low parts per billion sensitivity. The relative sensitivity of the immunoassay using fortified turkey breast samples parallels that seen with standards. The assay is most sensitive for DMZ and IPR and least sensitive for their alcohol metabolites. It is equally clear that the length of the cleanup method detracts from the usefulness of the immunoassay. Further studies are needed to streamline the cleanup method and reduce the use of organic solvents.

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