Biotransformation of Dimetridazole by Primary Cultures of Pig Hepatocytes

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Monolayer cultures of pig hepatocytes were used to investigate the role of the liver in the biotransformation of the veterinary drug dimetridazole (1,2-dimethyl-5-nitroimidazole). ^{14}C-Labeled dimetridazole (DMZ) was primarily hydroxylated to 1-methyl-2-hydroxymethyl-5-nitroimidazole (up to 90%) and to a minor extent N-demethylated to 2-methyl-(4,5)-nitroimidazole (6−10%). Prolonged incubation with the parent drug but also the 2-hydroxymethyl metabolite resulted in the formation of two other minor metabolites. The major one of these was identified as the glucuronide of the 2-hydroxymethyl metabolite, based on its molecular mass and the succesful hydrolysis with β-glucuronidase. The second showed a molecular mass of 144 and is hypothesized to be 2-hydroxymethyl-5-nitroimidazole. No evidence was obtained for the formation of a cysteine or glutathione conjugate involved in the detoxification of reactive intermediates. In addition to free metabolites, there was a time-related formation of protein-bound metabolites up to a maximum of 30 pmol/mg of protein after exposure to 50 μM DMZ for 48 h. In general, these metabolites accounted for 0.06−0.15% of the metabolized DMZ. Unextractable metabolites were also observed after incubation of cells with the 2-hydroxymethyl and 1-desmethyl metabolites. It is concluded that the 1-desmethyl and, in particular, the 2-hydroxymethyl metabolite are the major metabolites formed by pig hepatocytes. These compounds are thus far the only metabolites identified *in vivo,* but together with the parent compound, they accounted for only a small part (<5%) of the excreted drug. Therefore, the site of formation of the majority of the unknown *in vivo* metabolites may be extrahepatic, and complimentary models are needed to investigate this hypothesis.

Keywords: *Pig hepatocytes; dimetridazole; nitroimidazoles; bound residues*

INTRODUCTION

Dimetridazole is a widely used veterinary drug, active against anaerobic bacteria and protozoal parasites. The compound has been shown to be mutagenic in bacteria (Voogd *et al.,* 1974) and caused benign mammary tumors in rats (Cohen *et al.,* 1973; WHO, 1989). This stresses the need to obtain detailed information on the identity and properties of the metabolites of dimetridazole that may be present as residues in tissues of treated animals.

Both in orally dosed turkey and swine, DMZ has been shown to be extensively metabolized resulting in only very small amounts of the parent compound in urine and feces (Law *et al.,* 1963; Shaw *et al.,* 1990). Two metabolites have been identified in tissues and plasma of pigs (Figure 1). The major one, originally identified in turkeys (Law *et al.,* 1963), is 1-methyl-2-hydroxymethyl-5-nitroimidazole (Carignan *et al.,* 1988; Shaw *et al.,* 1990), resulting from the hydroxylation of the 2-methyl group. A second minor metabolite was shown to be 2-methyl-(4,5)-nitroimidazole (Carignan *et al.,* 1988, 1991), resulting from the N-demethylation of the drug. On the basis of levels in feces and urine, the parent compound and these two metabolites appear to account for only a small part (< 5%) of the excreted drug (Shaw *et al.,* 1991). However, regarding the presence of the intact nitro group, which is a prerequisite for the
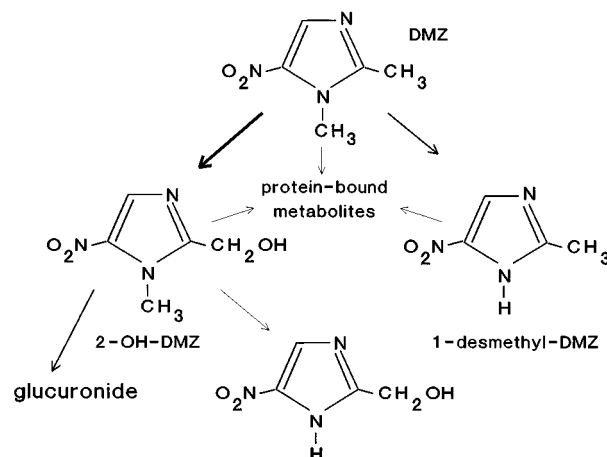
Figure 1. Proposed metabolism of dimetridazole (DMZ) by pig hepatocytes resulting initially in the formation of 1-methyl-2-hydroxymethyl-5-nitroimidazole (2-OH-DMZ) and, to a minor extent, 2-methyl-(4,5)-nitroimidazole (1-desmethyl-DMZ). The 2-hydroxymethyl metabolite is subsequently glucuronidated or demethylated. Both dimetridazole and the 2-hydroxymethyl and 1-desmethyl metabolites are partly metabolized to protein-bound metabolites.

mutagenic activity of this type of compounds (Lu *et al.,* 1984; Wislocki *et al.,* 1984b), these metabolites are of major toxicological concern.

There are indications that the metabolism of DMZ results in the formation of protein-bound metabolites (WHO, 1989), as shown in the case of the related nitroimidazole drug ronidazole (Wolf *et al.,* 1983). Furthermore, protein-bound metabolites have been detected following incubation of DMZ with rat liver

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microsomes (Miwa *et al.*, 1982; Lu *et al.*, 1984). Regarding the fact that, 48 h after the last treatment, the parent compound and the two metabolites mentioned above could no longer be detected in plasma and tissues, such bound metabolites may eventually become the most important residues.

Previously, we described the isolation of pig hepatocytes from a sample of pig liver and their successful use for studying the biotransformation of the veterinary drugs furazolidone, furaltadone, and sulfadimidine (Hoogenboom *et al.*, 1989, 1991, 1992, 1994). The aim of the present study was to further evaluate the possibilities of this model for predicting the *in vivo* metabolism of drugs by investigating the biotransformation of DMZ, including the formation of protein-bound metabolites.

MATERIALS AND METHODS

Materials. Dimetridazole and 2-methyl-(4,5)-nitroimidazole were obtained from Sigma (St. Louis, MO); 1-methyl-5-nitroimidazole was from Aldrich (Milwaukee, WI). 1-Methyl-2-hydroxymethyl-5-nitroimidazole and the cysteine and glutathione conjugates of dimetridazole were provided by G. Carignan (Bureau of Drug Research, Health Protection Branch, Ottawa, Canada). The latter two conjugates had been synthesized based on a method described by Wislocki *et al.* (1984a). [^{14}C]Dimetridazole, labeled at C_2 (specific activity, 0.74 GBq/mmol) was a kind gift of Rhone-Poulenc, Essex, U.K. All other chemicals were of analytical grade and were obtained from regular commercial sources.

Purification of Radiolabeled Dimetridazole. Radiolabeled DMZ with an initial purity of 89%, as determined by HPLC, was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 5 mM. This solution was diluted with water to a concentration of 100 μM and subsequently extracted twice with ethyl acetate (EtAc). The organic phase was evaporated and the residue dissolved in DMSO. This procedure resulted in a purity greater than 99%.

Purification of Radiolabeled 2-Hydroxymethyl- and 1-Desmethyldimetridazole. Small amounts of radiolabeled 2-hydroxymethyl and 1-desmethyl metabolites were isolated from the medium obtained from hepatocytes that had been incubated with [^{14}C]DMZ for 24 h. Media were extracted with EtAc. The dried extracts were redissolved in acetonitrile/water 30/70 (v/v) and purified on an HPLC system equipped with a PRP-1 column as described below. The two fractions containing the two metabolites were collected and re-extracted with EtAc. The EtAc was evaporated, and the residue was dissolved in DMSO. Radiochromatograms revealed purities of 98 and 100% for the 2-hydroxymethyl and 1-desmethyl metabolites, respectively.

Incubation Procedures. Pig hepatocytes were isolated from livers of female pigs (Dutch Landrace × Yorkshire) as described previously (Hoogenboom *et al.*, 1989). Hepatocytes from four different pigs were used for these studies. Initial cell viability, as judged by the exclusion of trypan blue, varied between 90 and 95%. Cell suspensions were diluted to a density of 1.25–1.5 million viable cells/mL of Williams' medium E supplemented with 5% fetal calf serum, 0.5 μg/mL insulin, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Cells were seeded into 60-mm tissue culture dishes (2.5 mL/dish) and incubated at 38 °C, 5% CO_2, and high humidity for 14–24 h before the start of the experiments.

Cells were incubated for various incubation periods with different concentrations of [^{14}C]DMZ in Williams' medium E without further additions. DMSO was used as the solvent for the addition of DMZ and added to the medium at a final concentration of 0.5%. Following exposure, media samples were collected and stored at −20 °C. Cells were washed twice with 2.5 mL of phosphate-buffered saline (PBS) and scraped from the dishes in 1 mL of PBS using a cell scraper (Costar, Badhoevedorp, The Netherlands). Cells were stored at −20 °C until analysis.

Determination of Protein-Bound Metabolites. For the determination of protein-bound metabolites, samples were thawed and the cellular protein precipitated with 2 mL of ice-cold methanol. The protein was washed another three times with ice-cold methanol and twice with ice-cold ethanol, air-dried, and dissolved in 0.5 mL of 0.8 N NaOH and 0.5 mL of 10% sodium dodecyl sulfate at 50 °C. An aliquot of 0.75 mL was mixed with 15 mL of Minisolve scintillation cocktail (Zinsser Analytical, Maidenhead, U.K.) and counted in a liquid scintillation counter. Aliquots of 25 μL were removed in triplicate for protein measurement by the method of Lowry as modified by Peterson (1977).

A few samples were examined using an alternative method as described by Law and Meng (1996). In short, cells collected in PBS were solubilized with 1% SDS and centrifuged at 17000g for 20 min. The protein in the supernatant was extracted with an equal volume of phenol saturated with Tris buffer (10 mM containing 0.2 mM EDTA, pH 7.5). The phenol extract was washed once with Tris buffer, and the protein was precipitated with 2 vol of acetone, subsequently resolubilized with 10% SDS, and precipitated again with 2 vol of acetone. This was repeated once, and the resulting protein pellet was washed twice with acetone and once with EtAc. Levels of radiolabel and protein were determined as described above.

Metabolite Analysis. HPLC analysis of medium samples was performed on a Waters HPLC system connected to a diode array detector (Waters, Etten-Leur, The Netherlands) and an on-line radioactivity detector (LB506C, Berthold, Wildbad, Germany). Samples were injected on a PRP-1 column (250 × 4.6 mm; Hamilton, Reno, NV) and eluted with a mixture of two eluants [eluant A: formic acid/NaOH (0.1 M; pH 3.6): water:acetonitrile (50/925/25 v/v) and eluant B: formic acid/NaOH (0.1 M; pH 3.6):water:acetonitrile (50/650/300 v/v)], using a 22-min linear gradient starting at 98/2 (A/B) and ending at 0/100 with a flow rate of 0.8 mL/min. Later studies revealed a better separation of metabolites following replacement of the PRP-1 column by an LC-18-DB column (250 × 4.6 mm, Supelco, Zwijndrecht, The Netherlands). Routinely, 100 μL of medium was injected without further pretreatment. Following the passage through the diode array detector and prior to the on-line radioactivity detector, the eluant was mixed with Maxifluor scintillation fluid (Baker, The Netherlands) at a flow rate of 3.5 mL/min.

LC−MS Identification of Metabolites. DMZ and the 2-hydroxymethyl and 1-desmethyl metabolites were extracted with EtAc from the incubation medium. The organic solvent was evaporated under nitrogen, and the residue was dissolved in eluant A before LC−MS analysis. For the purification of the two unknown polar metabolites, cells (pig 3) were incubated with 500 μM 2-hydroxymethyl-DMZ for 48 h. Medium was acidified to pH 3.6 and extracted three times with EtAc. The remaining water phase was filtered through a 0.2-μm filter and injected on the LC-18-DB column as described above. For this purpose, the NaOH used to adjust the pH of the formic acid buffer to pH 3.6 was replaced by NH_4OH. The fraction containing the two unknown metabolites was collected and taken to dryness under vacuum after addition of an equal volume of methanol. The extract was redissolved in eluant A, and the purification on the LC-18-DB column was repeated before LC−MS analysis.

For LC−MS analysis, an Alltima C18 column (100 × 2.1 mm, 5 μm, Alltech, Deerfield, MI), was used, eluted with an eluant consisting of a mixture of a formic acid/NH_4OH buffer (0.1 M, pH 3.6):water:acetonitrile (50/925/25 v/v) at a flow rate of 0.2 mL/min. Post-column, the eluant was mixed with methanol at a flow rate of 0.2 mL/min and without splitting directed to a Finnigan MAT (San Jose, CA) TSQ 700 mass spectrometer, equipped with a Finnigan MAT atmospheric pressure ionization (API) interface. The interface was operated in electrospray mode at 5 kV with a sheath gas pressure of 70 psi and an auxiliary gas reading of 20 psi. The transfer capillary was heated at 200 °C. Tandem MS was performed on the protonated molecular ions, using argon as collision gas at 2 mTorr.
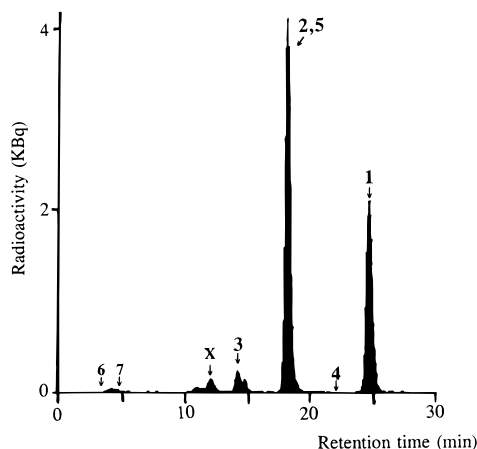
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Figure 2. HPLC chromatogram of a sample of medium following incubation of pig hepatocytes with radiolabeled dimetridazole for 24 h. Retention times of dimetridazole (**1**), 1-methyl-2-hydroxymethyl-5-nitroimidazole (**2**), 2-methyl-(4,5)-nitroimidazole (**3**), 1-methyl-5-nitroimidazole (**4**), 2-methyl-aldehyde-5-nitroimidazole (**5**), and the cysteine (**6**) and glutathione conjugates (**7**) are indicated.

RESULTS AND DISCUSSION

Identification of Metabolites in Incubation Medium of Pig Hepatocytes. Incubation of pig hepatocytes with different concentrations of ^{14}C-labeled DMZ resulted in the formation of one major metabolite and several minor metabolites. A typical HPLC radiochromatogram of a medium sample taken after incubation of cells with DMZ for 24 h is shown in Figure 2 (retention times of the reference standards are indicated with arrows). The major metabolite (**2**) had an identical retention time and UV spectrum as a standard of 1-methyl-2-hydroxymethyl-5-nitroimidazole. Based on the same criteria, one of the minor metabolites (**3**) was identified as 2-methyl-(4,5)-nitroimidazole, the N-demethylated metabolite. In particular after prolonged incubation (24–48 h), some unknown metabolites were observed, especially one with a retention time of 12.1 min (metabolite X). The UV spectrum of this metabolite showed an absorption maximum around 315 nm, indicating the presence of an intact nitroimidazole ring. None of the unknown metabolites coeluted with standards of 1-methyl-5-nitroimidazole (retention time 22.5 min) or the cysteine and glutathione conjugates with retention times of 3.9 and 4.6 min, respectively.

Extracts of medium were prepared to confirm the identity of the two major metabolites by LC–MS. Reference standards of DMZ and the 2-hydroxymethyl and 1-desmethyl metabolites yielded retention times of respectively 22.1, 10.3, and 7.1 min and masses (M$^+$) of respectively 142, 158, and 128, corresponding with the protonated molecular ions. Tandem-mass spectra for DMZ and 1-desmethyl-DMZ showed for both compounds one major daughter ion at m/z 96 and 82, respectively, indicating the loss of the NO$_2$ moiety (M − 46). The mass spectrum of 2-hydroxymethyl-DMZ showed a major daughter ion at 140, corresponding with the loss of water (M − 18). This has been reported for other primary alcohols. An EtAc extract from the medium obtained from pig hepatocytes incubated with 50 μM DMZ for 24 h was further investigated by LC–MS. The extract contained compounds with identical retention times as DMZ, 2-hydroxymethyl-DMZ, and 1-desmethyl-DMZ as well as the corresponding molecular masses and major daughter ion peaks. In combination with the identical UV spectrum, this confirms the identity of

2-hydroxymethyl-DMZ and 1-desmethyl-DMZ as the two major metabolites of DMZ.

Metabolite X could not be extracted from the medium with EtAc and was therefore purified by HPLC. Purification of medium samples on the LC-18-DB column instead of the initially used PRP-1 column revealed that metabolite X was contaminated with about 10−20% of a minor metabolite (X$_2$). These metabolites, both showing maximal UV absorption around 315 nm, were also observed after incubation of cells with 2-hydroxymethyl-DMZ. When the purified fraction containing metabolites X and X$_2$ was incubated with β-glucuronidase from *Escherichia coli* for 60 min, the major metabolite disappeared with the simultaneous reappearance of a compound coeluting with 2-hydroxymethyl-DMZ. The minor metabolite was not hydrolyzed by β-glucuronidase. Subsequent analysis by LC−MS revealed that the major metabolite (RT 5.4 min) showed a major mass peak of m/z 334 and minor peaks at m/z 356 and 158 concomitant with the glucuronide of 2-hydroxymethyl-DMZ, the corresponding sodium adduct, and 2-hydroxymethyl-DMZ itself. The minor metabolite (RT 4.3 min) showed a molecular mass of 144 and a M − 46 major daughter ion at m/z 98, indicating the presence of the NO$_2$ moiety. This minor metabolite is also observed after incubation of cells with 2-hydroxymethyl-DMZ and retained the UV absorption at 315 nm. It is hypothesized that this metabolite is 1-demethylated 2-hydroxymethyl-DMZ (Figure 1). The metabolite was not observed after incubation of cells with the 1-desmethyl metabolite.

Protein-Bound Metabolites. Despite extensive washing of the cellular protein with methanol and ethanol, a small part of the radiolabel could not be removed from the proteins and is therefore assumed to be protein bound. In order to confirm the non-extractability of these metabolites, a second method was used based on extraction of the protein with phenol, repeated solubilization with sodium dodecyl sulfate, subsequent precipitation, and extraction with acetone, and washing of the protein with organic solvents (Law and Meng, 1996). Application of this theoretically more exhaustive method on pig hepatocytes incubated with 50 μM DMZ for 24 h resulted in bound metabolite levels of 25 ± 1 pmol/mg of protein as compared to 25 ± 1 pmol/mg of protein for the method based on the precipitation and extraction with methanol and ethanol.

Time- and Dose-Related Formation of Metabolites. A number of quantitative studies were carried out to investigate the time- and dose-related metabolism of DMZ by pig hepatocytes. Table 1 shows the formation of the various metabolites for two batches of pig hepatocytes incubated with 50 μM of the parent drug for different time periods. DMZ was primarily metabolized during the first 24 h. In the next 24 h (pig 3), only a small part of the remaining drug was further metabolized, accompanied by a clear increase in the levels of non-extractable protein-bound radioactivity and a minor increase in the concentrations of the 2-hydroxymethyl and 1-desmethyl metabolites. There was also an increase in the concentrations of the unknown metabolites X and X$_2$ (RT 12.1 min). A similar time-related pattern was observed with hepatocytes from another pig (pig 1) incubated with 100 μM DMZ (data not shown).

Table 2 shows the dose-related biotransformation of DMZ by two batches of pig hepatocytes. The overall biotransformation of DMZ tended to decrease slightly with increasing concentrations. At all concentrations, the 2-hydroxymethyl metabolite accounted for about

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Table 1. Relative Amounts of Dimetridazole, and the 2-Hydroxymethyl, 1-Desmethyl, and Unknown Metabolites (RT 12.1 min) in the Culture Medium and Protein-Bound Metabolites in Two Batches of Pig Hepatocytes Following Incubation with 50 μM of the Drug for 6, 24, or 48 (Pig 3) h[a]

incubation time (h)	fraction of total radioactivity added (%)				
	DMZ	2-hydroxymethyl-DMZ	1-desmethyl-DMZ	unknown RT 12.1 min	protein-bound metabolites
			Pig 2		
6	66.1 ± 3.5	31.0 ± 2.9	2.0 ± 0.2	0.0 ± 0.0	0.025 ± 0.004
24	37.5 ± 1.2	52.5 ± 0.9	4.2 ± 0.6	2.8 ± 0.2	0.037 ± 0.001
			Pig 3		
6	72.8 ± 0.6	26.9 ± 0.4	1.0 ± 0.4	0.0 ± 0.0	0.021 ± 0.000
24	42.1 ± 0.3	51.0 ± 0.2	3.0 ± 0.0	2.7 ± 0.1	0.035 ± 0.001
48	33.7 ± 0.5	54.7 ± 0.3	3.2 ± 0.2	4.6 ± 0.1	0.044 ± 0.001

[a] Results are expressed as relative proportion of the total radioactivity (mean ± SEM; n = 3). Dishes contained 1.3 and 1.8 mg of cellular protein for hepatocytes from pigs 2 and 3, respectively.

Table 2. Relative Amounts of Dimetridazole and the 2-Hydroxymethyl, 1-Desmethyl, and Unknown (RT 12.1 min) Metabolites in Medium and Protein-Bound Metabolites in Two Batches of Pig Hepatocytes Following Incubation for 24 h[a]

concn (μM)	fraction of total radioactivity added (%)				
	DMZ	2-hydroxymethyl-DMZ	1-desmethyl-DMZ	unknown RT 12.1 min	protein-bound metabolites
			Pig 2		
1.5	27.1 ± 1.5	65.7 ± 2.9	ND[b]	ND	0.070 ± 0.007
5.0	26.8 ± 1.5	63.4 ± 0.7	5.1 ± 0.5	3.7 ± 0.4	0.046 ± 0.001
15.0	33.3 ± 1.9	56.6 ± 1.1	5.2 ± 0.2	2.9 ± 0.3	0.036 ± 0.001
50.0	37.5 ± 1.2	52.5 ± 0.9	4.2 ± 0.6	2.8 ± 0.2	0.037 ± 0.001
			Pig 3		
1.5	33.6 ± 1.4	66.1 ± 1.1	0.3 ± 0.3	ND	0.018 ± 0.003
5.0	30.0 ± 2.1	65.4 ± 1.7	2.8 ± 0.1	1.8 ± 0.5	0.041 ± 0.004
15.0	35.7 ± 1.2	58.6 ± 1.2	2.9 ± 0.1	2.7 ± 0.1	0.039 ± 0.001
50.0	42.1 ± 0.3	51.1 ± 0.2	3.0 ± 0.0	2.7 ± 0.1	0.035 ± 0.001

[a] Results are expressed as relative proportion of the total radioactivity (mean ± SEM; n = 3). Dishes contained 1.3 and 1.8 mg of cellular protein for hepatocytes from pigs 2 and 3, respectively. [b] ND, not detectable.

Table 3. Relative Amounts of the 2-Hydroxymethyl, 1-Desmethyl, and Unknown Metabolites (RT 12.1 min) in Culture Medium and Protein-Bound Metabolites in Pig Hepatocytes (Pig 3) Following Incubation with 5 μM of 2-Hydroxymethyl Metabolite for 6 or 24 h or 1 μM 1-Desmethyl Metabolite for 24 h[a]

incubation time (h)	fraction of total radioactivity added (%)			
	2-hydroxymethyl-DMZ	1-desmethyl-DMZ	unknown RT 12.1 min	protein-bound metabolites
		2-Hydroxymethyl-DMZ		
0	98.0	ND[b]		
6	96.2 ± 0.8	ND	0.1 ± 0.1	0.013 ± 0.000
24	88.0 ± 0.8	ND	5.3 ± 0.9	0.025 ± 0.003
		1-Desmethyl-DMZ		
0		100.0		
24	ND	100.0 ± 0.0	ND	0.008 ± 0.001

[a] Results are expressed as relative proportion of the total radioactivity (mean ± SEM; n = 3). Dishes contained on average 1.8 of mg cellular protein. [b] ND, not detectable.

80−90% of the metabolized DMZ, and the 1-desmethyl metabolite accounted for 4−8%. At the concentration of 1.5 μM, the N-demethylated metabolite could not be detected due to the limit of detection. Levels of protein-bound metabolites accounted for about 0.04% of the total radioactivity or 0.06% of the metabolites at 5, 15, and 50 μM. The relatively high variation observed between the two batches of hepatocytes incubated with 1.5 μM might be explained by the low levels of non-extractable radioactivity, approaching the limit of detection. Figure 3 shows the time- (A) and dose- (B) related formation of absolute levels of protein-bound metabolites as corrected for the amount of cellular protein. There was a clear linear correlation between the concentration and the formation of bound metabolites over the dose range 5−50 μM. The time-related increase (A), including the very low levels in cells incubated for only a few minutes

(time zero), strongly indicates that these unextractable protein-bound metabolites are formed by active metabolism.

When hepatocytes were incubated with 5 μM 2-hydroxymethyl metabolite, this compound was slowly metabolized resulting in the appearance of metabolite X after 24 h (Table 3). As with DMZ, part of the radiolabel became non-extractably bound to the protein. Absolute levels of bound metabolites after 6 and 24 h of incubation were respectively 0.85 ± 0.02 and 1.71 ± 0.22 pmol/mg of protein as compared to 1.36 ± 0.12 and 2.81 ± 0.14 pmol/mg of protein in the case of cells incubated with 5 μM DMZ for 6 and 24 h. Similar results were obtained by Lu *et al.* (1984) using rat liver microsomes.

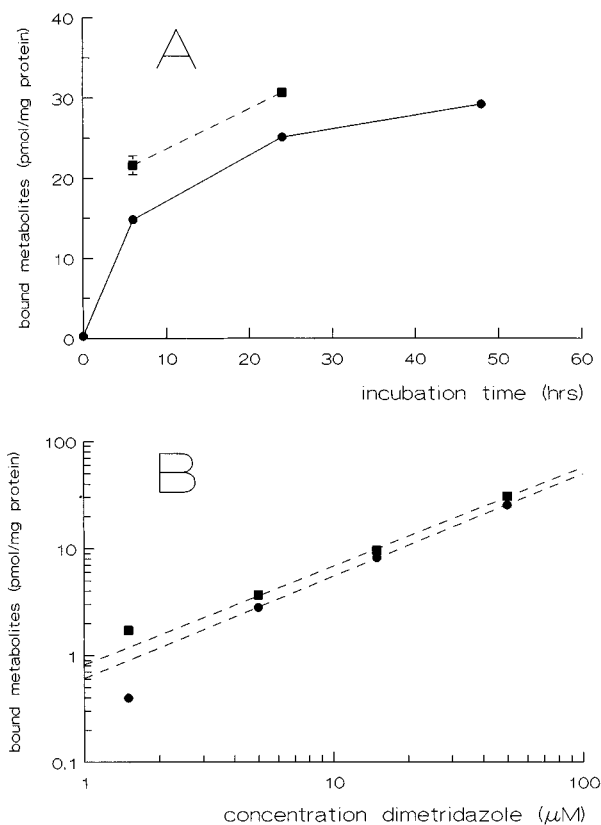Incubation of cells with 1 μM of the 1-desmethyl metabolite for 24 h did not result in the appearance of

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Figure 3. Time- (A) and dose- (B) related formation of non-extractable protein-bound metabolites of dimetridazole by two different batches of pig hepatocytes (■, pig 2; ●, pig 3). Cells were either incubated with 50 μM DMZ for different time periods (A) or with 1.5, 5, 15, or 50 μM DMZ for 24 h (B). Regression lines for the dose-related formation were calculated over the range 5–50 μM. Results are expressed as the mean $\pm$ SEM ($n = 3$).

metabolites in the medium. However, part of the radiolabel was not extractable, resulting in absolute levels of 0.50 $\pm$ 0.07 pmol/mg of protein, as compared to 0.40 $\pm$ 0.03 pmol/mg of protein in the case of cells incubated with 1.5 μM DMZ for 24 h. Although at these low levels the amount of unextractable radioactivity approached the limit of detection, they were clearly elevated 2–3 times over the background. The formation of protein-bound metabolites after incubation of cells with the 1-desmethyl metabolite could be expected based on the hypothesis that the nitro group is a prerequisite for the formation of bound metabolites from nitroimidazole drugs (Miwa *et al.*, 1982; Lu *et al.*, 1984). This may also imply that the 1-desmethyl metabolite has mutagenic properties like DMZ and the 2-hydroxy metabolite and should therefore be included in the residue analysis for DMZ.

Significance of Protein-Bound Metabolites of DMZ. In the case of the nitrofuran drugs furazolidone and furaltadone, protein-bound metabolites were shown to account for up to 5% of the drug metabolized by pig hepatocytes (Hoogenboom *et al.*, 1991, 1994). A similar figure has been reported for the metabolism of ronidazole by rat liver microsomes (Miwa *et al.*, 1982). The more than 10-fold lower levels of protein-bound metabolites of DMZ in the present study indicates that DMZ is less effectively transformed into reactive intermediates capable of binding to cellular proteins. This is in agreement with previous studies by Lu *et al.* (1984), demonstrating a 6-fold lower formation of bound metabolites in the case of DMZ as compared to ronidazole.

Both in the case of nitrofurans and nitroimidazoles, reduction of the nitro group is thought to be involved in the formation of these type of metabolites (Swaminathan and Lower, 1978; Vroomen *et al.*, 1987; Alvaro *et al.*, 1992). The present study demonstrates that in pig hepatocytes DMZ is primarily transformed by oxidative pathways like hydroxylation and N-demethylation and that nitroreduction, if present at all, is only of minor importance. This is supported by the fact that conjugates of DMZ with the thiols cysteine and glutathione are at best only very minor metabolites. These compounds have been shown to prevent the formation of protein-bound metabolites of ronidazole with rat liver microsomes (Miwa *et al.*, 1982; West *et al.*, 1982), most likely due to scavenging of reactive intermediates.

However, despite their relatively low formation rate, protein-bound metabolites may comprise the most significant residues in tissues of treated pigs. This is due to their slow excretion rate and inherent accumulation and the relatively short half-lives (2–7 h) of the parent drug and the 2-hydroxymethyl and 1-desmethyl metabolites. In general, the non-extractable radiolabel is thought to arise from reactive metabolites that are covalently bound to proteins, although it has been suggested that *in vivo*, due to a more extensive metabolism, part of the label may be incorporated into amino acids and proteins. In the case of ronidazole, it was demonstrated that the formation of the major part of the protein-bound metabolites, both *in vitro* and *in vivo*, is due to binding of reactive intermediates to proteins (Alvaro *et al.*, 1992). In this case, acid hydrolysis of the protein resulted in the release of fragments that could clearly be assigned to the protein–drug adduct. Based on a similar principle, drug-related–protein adducts were initially detected in proteins of pig hepatocytes incubated with the nitrofuran drugs furazolidone, furaltadone, nitrofurantoin, and nitrofurazone (Hoogenboom *et al.*, 1991, 1993, 1994), and subsequently in tissues of furazolidone-treated pigs (Hoogenboom *et al.*, 1991; Gottschall and Wang, 1995). Therefore, the results from the present study strongly indicate that protein adducts of DMZ are formed in the target animal, stressing the need to further study their identity and toxicity.

Evaluation of the Hepatocyte Model. Both the 2-hydroxymethyl and 1-desmethyl metabolites have previously been detected in blood and tissues of pigs treated with DMZ (Carignan *et al.*, 1988, 1991; Shaw *et al.*, 1990). Typical plasma levels reported for DMZ, the 2-hydroxymethyl, and the 1-desmethyl metabolite in orally treated pigs reached plateau levels of respectively 48, 888, and 84 ppb (Carignan *et al.*, 1991), with half-lives varying from 2 to 7 h. The fact that in the present study the hydroxylation at the 2-methyl group is much more important than the N-demethylation is in agreement with these data. However, since the parent compound and these two metabolites have been reported to account for less than 5% of the excreted drug in pigs (Shaw *et al.*, 1990; Carignan *et al.*, 1991), other organs and tissues might be involved in the biotransformation of these types of drugs. In particular bacteria in the gastrointestinal tract may play an active role in the overall biotransformation of this drug, regarding their high capacity for reducing nitro groups. Complementary models may further improve the value of this *in vitro* approach for predicting the biotransformation of drugs in target animals.

The results from the present study support the use of *in vitro* models for predicting the *in vivo* formation

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of protein-bound residues. In addition, as shown in the case of furazolidone, *in vitro* models are helpful for the production of protein-bound metabolites, required for the development of methods for the detection of these type of residues in meat. Such methods are essential to check the maintenance of withdrawal periods but also the illegal use of drugs, in particular when the parent compounds are rapidly degraded and excreted. In the case of dimetridazole, this has become very interesting regarding the recent ban by the EC of the use of dimetridazole in food-producing animals (Anonymous, 1995).

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