

# Pharmacokinetics of Xylazine, 2,6-Dimethylaniline, and Tolazoline in Tissues from Yearling Cattle and Milk from Mature Dairy Cows after Sedation with Xylazine Hydrochloride and Reversal with Tolazoline Hydrochloride\*

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## ■ ABSTRACT

Xylazine hydrochloride was administered IM at 0.35 mg/kg to 13 steers and 10 lactating dairy cows at Time 0. Ten minutes later, tolazoline hydrochloride was given IV at 4 mg/kg. Tissue and milk samples were analyzed using gas chromatography with nitrogen and phosphorous detection to determine concentrations of xylazine, 2,6-dimethylaniline (a toxic metabolite of xylazine), and tolazoline (at various intervals). Concentrations of xylazine and 2,6-dimethylaniline were below the limit of quantitation (10 µg/kg) by 72 hours in tissues and 12 hours in milk. The concentration of tolazoline was below 10 µg/kg by 96 hours in tissues and 48 hours in milk. Based on the results of these residue studies submitted by the sponsoring agency to the Ministry of Agriculture and Forestry in New Zealand, withholding periods

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for both xylazine hydrochloride and tolazoline hydrochloride injection were established.

## ■ INTRODUCTION

Xylazine is the major  $\alpha_2$ -adrenoreceptor agonist used in veterinary medicine during the past 30 years to modify animal pain. Its sedative and analgesic activity is related to central nervous system depression. Its muscle relaxant effect is based on inhibition of the intraneural transmission of impulses in the central nervous system.<sup>1</sup> The principal pharmacologic activities develop within 10 to 15 minutes after IM injection in cattle.<sup>1</sup> At 0.1 mg/kg IM, xylazine has been shown to produce good sedation and marked muscle relaxation in cattle that ordinarily will leave the animal in a standing position.<sup>1</sup> Xylazine has been shown to be safe for inducing narcosis, analgesia, and muscle relaxation in healthy, fasted ruminants.<sup>2</sup> Due to their highly lipophilic nature, xylazine and oth-

er  $\alpha_2$ -adrenoreceptor agonists are distributed into tissues within 30 to 40 minutes.<sup>3</sup> Smaller doses of xylazine are required for sedation of cattle compared with other domestic species (e.g., 0.05 mg/kg IV in calves versus 0.5 mg/kg IV in horses).<sup>4</sup> The half-life of elimination of xylazine in cattle is about 30 minutes.<sup>1,5</sup> Xylazine is metabolized in animals via biotransformation to metabolically inactive compounds, which are then excreted in the urine.<sup>3</sup>

2,6-dimethylaniline (DMA) is a toxic metabolite of xylazine.<sup>3</sup> Its toxicity has been studied at length because of its many industrial uses in the manufacturing of wood preservatives, dyes, pharmaceuticals, antioxidants, and polymers.<sup>3</sup>

Tolazoline is a mixed  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptor antagonist that competitively inhibits  $\alpha$ -adrenoceptors.<sup>1,6</sup> Administered IV at 2 mg/kg, tolazoline is effective in reversing the pharmacologic effects of xylazine, decreasing the recovery time from xylazine-induced sedation.<sup>2,7</sup> Onset of arousal is usually apparent within 5 minutes of tolazoline administration, depending on the depth and duration of xylazine-induced sedation.<sup>4,8</sup> After IV administration to dogs or humans, tolazoline rapidly enters body tissues other than brain and fat. The tolazoline remaining in tissues at 90 minutes was found predominantly in muscle, kidney, liver, spleen, and plasma.<sup>9</sup> Tolazoline has been shown to be a safe and effective antagonist for sedation induced by xylazine in cattle.<sup>2,3</sup> However, there are few data available regarding residues of tolazoline in bovine tissue.

There are currently no food animal labels for xylazine in the United States, and therefore, no residue limits have been set in food animal species. Currently, any detected residues of xylazine, its metabolite DMA, or the  $\alpha$ -adrenergic blocking agent tolazoline in edible bovine tissues offered for sale would be violative. The European Agency for the

Evaluation of Medicinal Products (EMA) concluded that there was no need to establish a maximum residue limit (MRL) for xylazine, including bovine (both beef and dairy cattle) and equidae species.<sup>10</sup> The present study was designed to generate additional data on xylazine and tolazoline residue depletion in bovine tissues and milk. The objective was to determine residues of xylazine, DMA, and tolazoline in tissues of beef steers and in milk of dairy-breed cattle dosed with xylazine hydrochloride (AnaSed Injection, Lloyd Laboratories) and tolazoline hydrochloride (Tolazine Injection, Lloyd Laboratories) by IM injection.

## ■ MATERIALS AND METHODS

### Animals

Twelve yearling beef cattle and 10 mature, lactating (average milk yield of 11.6 L/day), nongestating dairy cows 3 to 10 years of age with a mean weight of 439 kg were used in the study. The dairy cows were randomly selected from a herd of 250 animals. All animals were weighed, identified with a numbered ear tag, and examined by a veterinarian for signs of disease. Grass pasture and meadow hay were fed before and throughout the study and water was available ad libitum. The cows were maintained throughout the study on a typical New Zealand dairy farm and were housed in free-stall barns. No drug supplements were administered for 14 days before or during the study. Before drugs were given, a whole-blood sample was collected from each animal to determine levels of  $\gamma$ -glutamyl transpeptidase as a measure of liver function.

### Treatments

Xylazine hydrochloride (20 mg xylazine base equivalent/ml) was supplied in 20-ml multiple-dose vials (AnaSed Injection) as a sterile solution. Tolazoline hydrochloride (100 mg tolazoline base equivalent/ml) was supplied in 100-ml

multiple-dose vials (Tolazine Injection) as a sterile solution. The drug components as well as the excipients used in manufacturing the drug product were tested and passed according to US Pharmacopeia specifications.

Dosing intervals for xylazine and tolazoline were designed to mimic their use in conjunction with a routine procedure requiring chemical restraint. Individual animal doses were calculated for each residue trial based on animal weight in kg (determined on a calibrated scale) and reported drug concentrations provided on the product labels. Xylazine was administered at 0.35 mg/kg IM in the anterior third of the neck at Time 0 and tolazoline was given at 4 mg/kg IV 10 minutes later. Cows were treated after the morning milking at 8:30 AM.

### Tissue Sample Collection

Four animals were euthanized at each of three time points (72, 96, and 120 hours after administration of tolazoline). A control animal that had received no injections was euthanized at 120 hours as well. Each animal was necropsied, and samples from liver, kidney, muscle, and fat were collected. Samples included three 100-g samples from the midsection of the diaphragmatic lobe of the liver, both kidneys, two separate 100-g samples from the midbelly region of the semimembranosus muscle of the right rear leg, and three 100-g samples of fat from the peritoneal area along the greater curvature of the abomasum. Samples were frozen in plastic containers immediately after collection and transported to the National Chemical Residue Laboratory, Wallaceville Research Centre (New Zealand) where they were stored at  $-80^{\circ}\text{C}$  until they were analyzed within 21 days. Residues for xylazine and tolazoline have been shown to be stable in bovine tissue for 8 months at  $-80^{\circ}\text{C}$ .<sup>11</sup>

The cows were completely milked into individual collection buckets beginning at 5 PM

and at 12-hour intervals thereafter for 3 days. The collection from each cow was measured and the output recorded. The entire milk collection was thoroughly mixed and two 150-ml samples (one for analysis and one as a reserve) were taken from the top portion. Milk samples were handled and stored in a similar manner as for tissue samples.

### Test Solutions

A mixed spiking solution containing xylazine, DMA, and tolazoline in acetonitrile (10  $\mu\text{g}/\text{ml}$ ) was prepared. This solution and the gas chromatography (GC) standard solution were determined to be stable for 7 days. Each milliliter of the GC standard ethyl acetate solution contained 1  $\mu\text{g}$  each of xylazine, DMA, N,N-dimethylaniline, and tolazoline, and 5  $\mu\text{g}$  of 2,4-dichloroaniline (DCA).

### Sample Preparation

In preparation for analysis, tissue and milk samples were removed from the freezer and allowed to thaw slightly. Liver, muscle, and fat samples were chopped and 100 g of each tissue was removed for analysis. Kidney samples were prepared by thawing an entire kidney, removing all external fat, and homogenizing the kidney in a blender. A 100-g sample was taken from the homogenized tissue for analysis. All samples were returned to the freezer if not analyzed immediately after sample preparation. The entire 150-ml milk sample from each collection time was homogenized, extracted, and analyzed for drug residues.

### Sample Analysis

Concentrations of xylazine and tolazoline in milk and tissues were measured by GC with nitrogen and phosphorus detection NPD. The analytical method was determined to be precise for xylazine, DMA, and tolazoline in the concentration range of 10 to 200  $\mu\text{g}/\text{kg}$ .<sup>11</sup> The

linearity of response was determined with spiked tissue and milk samples; correlation values for xylazine, DMA, and tolazoline were all greater than 0.998.

Tissue and milk samples were extracted with acetonitrile at a pH greater than 9. Water was then removed by the addition of dichloromethane and sodium chloride. The extract was dried using sodium sulfate; 1N hydrochloric acid and toluene were added, and the extract was reduced in volume under nitrogen. Sample extracts were then partitioned with 1N hydrochloric acid and a mixture of diethyl ether and hexane (30:70 v:v). The organic phase was then discarded and the water phase made basic by the addition of sodium carbonate. Xylazine and DMA were then partitioned into a mixture of ethyl acetate and hexane (30:70 v:v).

For liver samples, the xylazine and DMA fractions were cleaned up further using a Sep-Pak (Waters) cartridge system. Sample volumes were then reduced to 200  $\mu$ l for GC-NPD analysis. The tolazoline was recovered from the water phase by the addition of sodium hydroxide solution and enough sodium chloride to saturate the solution, which was then extracted with a mixture of ethyl acetate and hexane (30:70, v:v). Sample volumes were then reduced to 200  $\mu$ l for analysis by GC-NPD. Two internal standards were used in this method. Because DMA has a volatile nature, it is easily lost at critical steps in the method. N,N-dimethylaniline internal standard was added to every tissue and milk sample before the extraction process. This standard mimics the behavior of DMA and is indicative of losses of DMA due to the treatment of samples by the procedure. Fat tissue samples were first dissolved in toluene, and then precipitated by the addition of acetonitrile. The solvent was placed in a laboratory freezer for 1 hour, filtered, and reduced to a small volume. Fat samples were then extracted and analyzed in the same manner

as other samples. The DCA internal standard was added at the final step following the extraction process to adjust samples to a constant volume of 200  $\mu$ l for analysis by GC-NPD.

Quantitation of drug residues was based on extracted spiked tissue and milk recoveries and not on external standards. Ten replicates for each tissue matrix were spiked with 10  $\mu$ g/kg of the analyte from the mixed spiking solution and were analyzed by the procedure. The percentage recovery data obtained with the standard additions and a batch of samples were used to determine the normal range within which the percentage recovery should lie. Milk samples were assayed separately by the GC-NPD procedure in batches of 14 samples, including a four-point standard recovery curve. The recovery curve consisted of a blank sample of milk and three samples each extracted from a portion of the blank milk sample spiked with 10, 50, and 100  $\mu$ g/kg of the analytes from the mixed spiking solution. Blank tissue and milk samples showed no evidence of interfering peaks in GC-NPD analyses related to sampling and storage procedures.

### Data Analyses

The limit of detection (LOD) for each analyte in individual tissues and milk has been conservatively estimated, taking into account the response of the 0.01-mg/kg standard additions that were analyzed, the linearity of the response observed for the standard curves, blank response, and the variability of detector response. These limits were conservative due to the variability of the detector sensitivity and are set to ensure that under the worst conditions these limits will be detectable. The LOD for xylazine, DMA, and tolazoline in liver, kidney, muscle, and milk is 5  $\mu$ g/kg. In fat, the LOD for xylazine and DMA is 5  $\mu$ g/kg, but for tolazoline it is 10  $\mu$ g/kg. Where analytical data points were reported as zero, nil, or negative in

the study report, the data points were set at one-half of the limit of quantitation (LOQ) for the purposes of statistical analysis. The LOQ for these compounds by the validated method corresponds to the lowest level of tissue recovery tested with spiked samples (10 µg/kg).

The linear regression of drug concentration on drug response ratio was determined by least squares for the standard samples analyzed by GC-NPD. The correlation coefficient for analytical data calculated using DCA as an internal standard ranged from 0.9982 for the xylazine standard curve to 0.9998 for the DMA standard curve.

A Student's *t*-test was used to compare mean milk yields before and during the milk residue trial. Differences were declared significant when  $P < .05$ .

## ■ RESULTS

The method functioned in a satisfactory manner for determination of the concentrations of xylazine, DMA, and tolazoline in bovine tissue samples. Three of the four animals euthanized at 72 hours had tolazoline residues above the LOQ in liver samples (14, 15, and 17 µg/kg) and two of the four animals had tolazoline residues above the LOQ in kidney samples (12 and 20 µg/kg). A third animal had detectable tolazoline residues in kidney tissue at 72 hours. The concentrations of xylazine, DMA, and tolazoline residues observed in these studies were below the LOQ for the analytical method (10 µg/kg) when measured in tissue samples collected at 96 and 120 hours. Concentrations of tolazoline were above the LOD (5 µg/kg) in all four liver samples and one kidney sample at 96 hours. At 120 hours, concen-

**TABLE 1. Residues of Xylazine and Tolazoline in Milk (µg/kg) Following Administration of Xylazine Hydrochloride (0.35 mg/kg IM) and Tolazoline Hydrochloride (4 mg/kg IV) to Lactating Dairy Cows**

Animal	Xylazine*			Tolazoline*		
	12 hr	24 hr	36 hr	12 hr	24 hr	36 hr
17	ND	ND	ND	>200	57	D
69	D	ND	ND	>200	20	D
93	ND	ND	ND	>200	64	11
124	12	ND	ND	>200	113	22
141	ND	ND	ND	>200	58	11
146	D	ND	ND	>200	35	D
181	ND	ND	ND	>200	10	D
281	11	ND	ND	>200	39	D
294	18	ND	ND	>200	D	D
324	D	ND	ND	>200	260	40

\*Additional samples were collected at 48, 60, and 84 hr after treatment; no residues were detected for either drug at those times.

D = detected but the residue concentration was below the limit of quantitation of the method (10 µg/kg); ND = not detected (below the limit of detection).

trations of tolazoline were above the LOD in all four liver samples and three kidney samples.

Mean milk yields per milking were statistically similar ( $P = 0.37$ ) before (5.6 L/milking) and during (5.8 L/milking) the trial. This finding indicated there was no effect of the xylazine and tolazoline hydrochloride injections on milk production.

Xylazine residues were found in milk from three of the 10 test animals at 12 hours (mean of  $14 \pm 3.8$   $\mu\text{g}/\text{kg}$ ), but at no other time point (Table 1). No DMA residues were detected in milk samples at any sampling time. Tolazoline residues detected in milk samples from all 10 test animals were above the LOQ at the first milking after treatment (Table 1). Tolazoline residues were still detected in milk from four animals at the third milking (36 hours) with a mean concentration of  $21 \pm 14$   $\mu\text{g}/\text{kg}$  of milk. No tolazoline residues were found in milk samples collected at 48, 60, or 84 hours.

## DISCUSSION

Following IV administration to either dogs or humans, residues of tolazoline detected at 90 minutes were found predominantly in muscle, kidney, liver, spleen, and plasma.<sup>9</sup> Tolazoline reverses the pharmacologic effects of xylazine<sup>3</sup>; however, data were lacking for the fate of tolazoline in milk and tissues of cattle. The residue trials were of sufficient duration to establish a point at which residues were no longer measurable in tissues and milk.

Because tolazoline is excreted intact in the urine, qualitative differences in biotransformation should not influence interspecies extrapolation of drug disposition. However, differences in secretory processes affecting elimination of drug may exist in bovine species versus canines. Assuming drug clearance is a function of basal metabolic rate, cattle would eliminate tolazoline slower than would dogs.<sup>12</sup> The distribution of tolazoline to peripheral tis-

sue sites is dependent on its physiochemical properties, the concentration gradient established between blood and tissue, the ratio of blood flow to tissue mass, and the affinity of tolazoline for tissue constituents.<sup>12</sup> Tolazoline is largely bound to serum protein, mainly albumin.<sup>12</sup> Only free, unbound drug would be capable of leaving the vascular compartment to be active in the tissues.

Many drugs, such as xylazine and tolazoline, are rapidly distributed to the liver and kidneys because of the large percentage of cardiac output perfusing these organs. Organs with a high blood flow:mass ratio include the brain, heart, liver, kidneys, and endocrine glands.<sup>12</sup> Tissues collected and analyzed in the present residue study were muscle, liver, kidney, and fat. Therefore, the limitations of the present study are acknowledged, and replication of the study with some design differences is recognized as essential for a better understanding of the potential residue risk in bovine tissues and milk. For instance, residues in tissues were not determined at times before 72 hours in the present study. In future studies, the design should provide for tissue and milk samples at Time 0 (immediately following injection) as well as the collection of tissue from the injection site. The samples at Time 0 would verify that initial drug residue levels in tissues and milk were high, that these levels decreased rapidly, and that the methodology used in the present study was appropriate for monitoring the decline in these levels over time. Another consideration for future studies should be to verify the potency of the xylazine and tolazoline products by standard assay methods.

The recommendation by the EMEA that there was no need to establish MRL for xylazine in bovine or equidae species was based on several considerations, including that the drug is used only in a small number of animals for infrequent treatments; animals treated with

xylazine are unlikely to be sent for slaughter during or immediately following treatment; xylazine is very rapidly and extensively metabolized and depleted in cattle tissues and milk; residues in cattle were well below doses that could be of concern for consumers by the first day after dosing; DMA was not found in cattle urine, tissues, or milk; and no metabolites derived from cleavage of the thiazine and phenol ring or from decomposition of the phenol ring are present in cattle tissues or milk.<sup>10</sup>

## ■ CONCLUSIONS

Based on the results of these residue studies submitted by the sponsoring agency, withholding periods for both xylazine hydrochloride and tolazoline hydrochloride injection were established by the Ministry of Agriculture and Forestry in New Zealand.<sup>13</sup> The withholding period for xylazine is 3 days for food animals of all species and one milking (or 12 hours) for milk from cattle. Residues were not determined in milk from goats or sheep treated with xylazine or tolazoline. Therefore, milk from goats or sheep treated with xylazine or tolazoline must not be used for human consumption during or within 35 days of treatment. The withholding period for tolazoline is 7 days for cattle meat and offal and 30 days for meat of other species. Milk from tolazoline-treated cattle must not be used for human consumption during or within 36 hours of the last treatment.<sup>13</sup> Administration of a single treatment of xylazine hydrochloride (0.35 mg/kg IM) followed by a single treatment with tolazoline hydrochloride (4 mg/kg IV) did not have any apparent adverse effects on milk production in this sample of dairy cows.

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