Depletion study of trimethoprim and sulphadiazine in milk and its relationship with mastitis pathogenic bacteria strains minimum inhibitory concentrations (MICs) in dairy cows

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San Martín Nuñez, B., Cañon, H., Iragüen, D., Espinoza, S., Lillo, J. Depletion study of trimethoprim and sulphadiazine in milk and its relationship with mastitis pathogenic bacteria strains minimum inhibitory concentrations (MICs) in dairy cows *J. vet. Pharmacol. Therap.* **24,** 83–88.

Time-related concentrations in milk of a combination of trimethoprim–sulphadiazine (TMP–SDZ) intramammary formulated infusion and its relationship with pathogenic bacteria strains minimum inhibitory concentrations (MICs) isolated from clinical mastitis cows were analysed. The MICs study was performed for *Escherichia coli*, *Staphylococcus aureus and Streptococcus* sp. strains. The SDZ concentrations in milk were analysed using high-performance liquid chromatography (HPLC) and TMP using a microbiological assay. Ten lactating cows milked three times daily were used in the time–concentration studies of TMP–SDZ. Milk samples (approximately 20 mL) from the treated mammary quarters were taken at 6, 12, 24, 30 and 36 h after first administration. In order to define the withdrawal time, milk samples from the treated mammary quarters were taken at 24, 36, 48, 72, 84 and 96 h, after finishing the therapy. The MICs fluctuated between 1 and 8 μg/mL. Effective therapeutic concentrations lasted for 36 h when intramammary infusion was repeated three times every 12 h. No TMP was detected in milk for 24 h after finishing therapy. Milk SDZ concentrations were below 0.1 μg/mL in all treated cows after 84 h finishing therapy. At 96 h after finishing therapy, no SDZ milk concentrations were found in six animals, although four animals of the experimental group still had concentrations of 0.07 μg/mL.

(Paper received 31 May 2000; accepted for publication 15 December 2000)

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INTRODUCTION

Clinical mastitis is recognized worldwide to cause major economic losses in dairy cattle because of the loss in milk production and quality (De Graves & Fetrow, 1993; Watts *et al.*, 1995). It is estimated that 70% of the economic losses from this disease is caused by lower milk production, 14% to premature animal removal, 7% to milk wasting and 8% to veterinary expenses and treatments (Philpot & Nickerson, 1991).

There is extensive literature about clinical mastitis including aetiology, pathogenesis, therapy and epidemiology. One main objective is towards the search for measures that upgrade the prevention, control and treatment of the clinical case. The selection of therapy must be based on the aetiology, disease acuteness and animal history.

A good antimicrobial therapy for bovine clinical mastitis is aimed at the elimination of the infectious agent by maintaining homogeneous concentrations above the MIC in the mammary gland for an adequate time. The antimicrobial therapy for the more severe cases is based on systemic and/or intramammary treatments with tetracyclines, β-lactams, cephalosporins, macrolides, aminoglycosides and sulphonamides (Watts *et al.*, 1995; Morin *et al.*, 1998). Additionally, it is important to note that in the USA only one sulphonamide is approved for the use in lactating dairy cattle (sulphadimethoxine) and that trimethoprim–sulphonamide combinations are not approved for dairy cattle (Boeckman & Carlson, 1995).

It has been shown that sulphonamides in combination with trimethoprim (TMP) are commonly used for broad spectrum antimicrobial therapy in veterinary medicine. The main indications in cattle are infections of the alimentary and urinary tract, mastitis and metritis.

There is information on the pharmacokinetic behaviour of the trimethoprim–sulphadiazine (TMP–SDZ) combination in

lactating cows after intravenous (i.v.), intramuscular (i.m.) and subcutaneous (s.c.) routes (Kaartinen *et al.*, 1999). The pharmacokinetic properties of this combination have been also investigated in calves (Shoaf *et al.*, 1987) and young cattle (Clarke *et al.*, 1989). Some information exists on the pharmacokinetic behaviour of TMP in lactating cows (Nielsen *et al.*, 1978) and SDZ in adult cattle after i.v. administration (Nouws *et al.*, 1988). However, there are only limited data on the use of intramammary infusion of the (TMP–SDZ) combination in lactating cows.

The selection of an antimicrobial in cows must also consider its elimination time from the milk to avoid residual levels of antimicrobials. There are several factors determining an antimicrobial withdrawal time, such as its physical and chemical structure, excipients, route of administration, age, animal condition or disease, etc. (Prescott & Baggot, 1991). Thus, several studies are needed when a new antimicrobial agent or a new formulation is produced to avoid the presence of its residues in animal products, such as milk.

The aim of the present study was to analyse the time-related concentrations in milk of a combination of (TMP–SDZ) intramammary formulated infusion and its relationship with MICs of clinical mastitis isolated pathogenic bacteria; also suggesting the corresponding withdrawal time in accordance to the established maximum residual limits (MRLs) of different international organizations.

MATERIALS AND METHODS

Isolation and identification of pathogenic bacterial strains and MICs determination

Experimental design. Milk samples were taken from 100 cows with clinical mastitis. The samples were collected aseptically as recommended by National Mastitis Council (1999). The samples were transported to the laboratory at 4 °C within 24 h of collection. The isolation and identification of bacterial strains were performed using standard bacteriological methodology suggested by National Mastitis Council (1999).

The strains were stored at −30 °C until used. When needed, the strains were grown on tryptose agar for *Escherichia coli* and *Staphylococcus aureus*, and blood agar for *Streptococcus* sp.

Antibacterial susceptibility test. Minimum inhibitory concentrations determination was performed following the recommendations of NCCLS (1993), using *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 as control organisms. Stock solution of TMP (400 μg/mL) and SDZ (2000 μg/mL) at a 1:5 ratio was used. From this solution, twofold dilutions in Mueller Hinton pH 7.2 broth were prepared. After 18–24 h incubation at 37 °C, the plates were read. MIC was defined as the lowest concentration of antimicrobial agent that completely prevented the visible growth of the organism. MIC_{90} was defined as the concentration of antimicrobial agent able to inhibit the growth of 90% of a population of microorganisms.

Detection of TMP and SDZ in milk

Experimental design. Ten clinically healthy multiparous Holstein Friesan cows were used. The cows were lactating for at least 1 month and most were in late lactation. During the entire experiment the cows were housed in a stanchion barn, milked three times daily, and fed with a diet of good quality silage, hay and grain, according to the farm milk production system. Water was provided *ad libitum*. One to two weeks before the experiment began, the udders of the cows were examined clinically and milk samples were taken for bacteriological culture and milk somatic cell count (SCC). This procedure was repeated for 2 days before challenge. All cows had low SCC (< 250 000 cells/mL) in bulk milk. The experimental quarters used in the study were culture-negative and had milk SCC of < 150 000 cells/mL. All animals did not receive any treatment or feed containing antimicrobials for at least 8 weeks as described by Reichmuth *et al.* (1997). To confirm the absence of antimicrobial agents, a sample was taken (time 0) prior to the administration of the intramammary infusion.

A commercial intramammary infusion provided by Intervet Chile Ltda. (Avda. Pio × 2460, 8th Floor, Office 808, Providencia, Santiago, Chile), containing TMP (40 mg) and SDZ (200 mg) in oily excipient (up to 8 g), was administered to one mammary quarter of each animal. The udder was massaged to have a uniform distribution through the gland. The treatment was repeated three times at 12 h intervals. In order to avoid interference with the production and management of the milking farm and to maintain the treatment regime, additional milkings were performed as required prior to the intramammary infusion administration.

To assess SDZ and TMP concentrations in milk during therapy, milk samples (approximately 20 mL) from the treated mammary quarters were taken at 6, 12, 24, 30 and 36 h after first administration. If any sampling time coincided with the normal farm milkings, the samples were taken before milking.

In order to define the withdrawal time, milk samples from the treated mammary quarters were taken, before milking, at 24, 36, 48, 72, 84 and 96 h, after finishing the therapy. If any sampling time coincided with the normal farm milkings, the samples were taken before milking.

The samples were taken aseptically and stored at 4 °C until arrival at the laboratory. All samples were kept frozen at −20 °C until analysis. The analysis of samples was performed within 48 h of arrival.

Drug assay. Prior to SDZ and TMP determination in milk samples, the analytical methodologies were validated following the recommendations of Food and Drug Administration (FDA) (1994), and Veterinary International Committee for Harmonization (VICH) (1998), obtaining the limit of detection, quantification, recovery rates and linearity of each methodology.

Analysis of TMP. Trimethoprim was assayed microbiologically, using *Bacillus pumilus* as test organism. Specificity for TMP was derived by the addition of *para*-aminobenzoic acid (100 μg/mL) to the agar medium in order to negate the effect of the sulphonamide (Norbrook Laboratories, 1992b). The concentration of TMP was determined by measuring the zone of inhibition with a Vernier calliper. The limit of quantification was 0.025 μg/mL with a mean inhibition halo of 1.3 ± 0.08 cm. Standard curves were plotted using intervals of 0.025–0.4 μg TMP/mL milk with inhibition halos between 1.3 and 4.8 cm, respectively; the response was found to be linear and a good correlation was obtained between TMP concentrations and zone of inhibition ($r = 0.98$). The recovery rate was 78%. Milk samples and standards were tested on plates in duplicate. When the inhibition halos of samples were bigger than those of the standard curve (> 4.8 cm), appropriate dilutions were prepared to fall in the range of the standard curve; the results were then multiplied by the corresponding dilution factors.

Analysis of SDZ. Sulphadiazine was analysed by reverse-phase, high-performance liquid chromatography (HPLC) (Norbrook Laboratories, 1992a). The apparatus consisted of a Waters 746 Data Module integrator, a Waters 410 HPLC Pump, a Waters 996 Photoarray Diode Detector adjusted at 270 nm, a Waters 717 plus Autosampler injector. The analytical column (Waters Symmetry C18; 25 cm × 4.6 mm ID) and guard column (Waters Sentry Guard μBondapack C18; 2 cm × 3.9 mm ID) were packed with 5 and 10 μm particles of dimetiloctadecilsylil silica, respectively. The eluant at a flow rate of 1.0 mL/min was a mixture of acetonitrile–0.017 M phosphoric acid (24–76) with a pH of 2.3 ± 0.5.

The limit of detection was 0.05 μg/mL and the limit of quantification was 0.07 μg/mL. Standard curves were plotted using intervals of 0.05–80 μg/mL SDZ/mL milk; the response was found to be linear and a good correlation was obtained between concentrations of SDZ and chromatographic area ($r = 0.98$). The recovery rate was 81%. Milk samples and standards were tested in duplicate.

Data analysis. The drug concentrations in milk were analysed with a software based on statistical theory. The actual concentration of drugs in the milk sample for SDZ and TMP were calculated using the following formula:

$$C = A \times 1/R$$

where C is the drug concentration in the sample (μg/mL), A the drug concentration from the validated curves (μg/mL) and R the recovery rate.

RESULTS AND DISCUSSION

From 100 milk samples collected from clinical mastitis cows, 36 *E. coli*, 23 *S. aureus and* 14 *Streptococcus* sp. strains were isolated. These results are similar to those of previously published works, as clinical mastitis can be caused by bacteria that cannot be

Table 1. Minimum inhibitory concentrations (μg/mL) of TMP:SDZ combination at 1:5 ratio against pathogenic bacteria isolated from dairy cows with clinical mastitis

Organism	*n*	TMP:SDZ concentrations (μg/mL)										MIC_{90} (μg/mL)
		0.025:0.125	0.05:0.25	0.1:0.5	0.2:1	0.4:2	0.8:4	1.6:8	3.2:16	6.4:32	12.8:64	>25.6:128	
E. coli	36	1	1	11	20	2	–	–	–	–	–	1*	0.2:1
Streptococcus sp.	14	1	3	1	9	–	–	–	–	–	–	–	0.2:1
S. aureus	23	–	–	1	–	2	6	13	–	–	–	1*	1.6:8

*One resistant bacteria strain.

detected by routine microbiological identification procedures (Kirk, 1991; Ruegg *et al.*, 1992; Erskine *et al.*, 1995), by fungi (Pianta, 1987; Schoonderwoerd & Plante-Jenkins, 1988) or other causes such as traumas.

The MIC determination was carried out before determining the TMP and SDZ concentration in milk. Prior to MICs determination, we defined the TMP:SDZ stock solution ratio in which we should work. Although, the literature points out that the optimal plasma ratio is 1:20, we thought that the pharmacokinetic behaviour of these drugs should not be the same when administered intramammary compared with when they are used parenterally. Thus, we used a stock solution at the ratio of 1:5 considering the ratio of the drugs in the commercial intramammary infusion. Based upon the results obtained in the time-related TMP and SDZ concentration study, as it will be explained later on, the stock solution ratio of 1:5 made sense.

The MIC ranges for the TMP:SDZ association are presented in Table 1. These were between 0.025:0.125 and 0.4:2 μg/mL for *E. coli*, 0.025:0.125 and 0.2:1 μg/mL for *Streptococcus* sp. and 0.1:0.5 and 1.6:8 μg/mL for *S. aureus* with MIC_{90} of 0.2:1, 0.2:1 and 1.6:8 μg/mL, respectively. One *E. coli* and one *S. aureus* strain were found to be resistant to this combination.

These results confirm that the *in vitro* MICs fluctuate considerably depending on the pathogen under study. Limited information regarding the MICs of this combination exists. However, there is some information on MICs for TMP and SDZ separately, ranging from 0.1 to 0.5 and 3 to 10 μg/mL, respectively, against a variety of pathogens, such as *E. coli*, *Salmonella* sp., *Pasteurella* sp., *Haemophillus* sp. (Bushby, 1980).

The time-related SDZ and TMP concentrations in milk during therapy and after finishing therapy are shown in Tables 2 and 3.

Table 2. Sulphadiazine concentrations (μg/mL) in milk analysed by HPLC in each sampling time and cow

	Sampling time (h)											
	During therapy						After finishing therapy					
Cow	0*	6	12	24	30	36	24	36	48	72	84	96
1	0.00	25.72	2.64	2.00	7.49	5.02	1.03	0.11	0.07	0.07	0.07	ND†
2	0.00	19.94	6.49	2.40	5.67	3.51	1.73	0.36	0.13	0.07	0.07	0.07
3	0.00	26.43	2.39	2.60	5.24	3.15	1.41	0.07	0.19	0.07	0.07	0.07
4	0.00	19.82	1.87	2.42	3.78	2.78	1.46	0.07	0.16	0.07	0.08	0.07
5	0.00	16.31	2.11	1.91	3.45	2.85	0.18	0.25	0.19	0.07	0.07	0.07
6	0.00	14.56	2.00	1.47	4.11	3.54	0.35	0.44	0.07	0.07	0.07	ND†
7	0.00	16.85	2.53	1.79	5.50	5.23	2.00	0.13	0.09	0.07	0.07	ND†
8	0.00	19.44	3.48	2.16	4.16	2.57	1.32	0.31	0.18	0.07	0.07	ND†
9	0.00	33.17	2.13	2.11	3.33	1.73	0.07	0.35	0.07	ND†	ND†	ND†
10	0.00	20.85	2.17	1.36	6.63	5.19	0.36	0.27	0.23	0.19	0.07	ND†

The treatment was repeated three times at 12 h intervals. Additional milkings were performed as required prior to the intramammary infusion administration. * Samples taken before intramammary infusion administration to confirm the absence of antimicrobials agents. † ND: not detected.

Table 3. Trimethoprim concentrations (μg/mL) in milk analysed by microbiological assay in each sampling time and cow

	Sampling time (h)								
	During therapy						After finishing therapy		
Cow	0*	6	12	24	30	36	24	36	48
1	0.00	4.30	0.63	0.53	0.73	0.25	ND†	ND†	ND†
2	0.00	4.39	0.67	0.47	0.72	0.50	ND†	ND†	ND†
3	0.00	4.44	0.87	0.57	0.69	2.73	ND†	ND†	ND†
4	0.00	3.59	0.70	0.70	0.69	1.19	ND†	ND†	ND†
5	0.00	3.42	0.90	0.50	0.72	0.50	ND†	ND†	ND†
6	0.00	4.79	0.73	0.40	0.72	0.25	ND†	ND†	ND†
7	0.00	4.62	0.70	0.60	0.73	0.67	ND†	ND†	ND†
8	0.00	3.93	0.80	0.64	0.65	1.02	ND†	ND†	ND†
9	0.00	2.90	0.91	0.54	0.71	0.25	ND†	ND†	ND†
10	0.00	3.42	0.94	0.53	0.72	0.26	ND†	ND†	ND†

The treatment was repeated three times at 12 h. Additional milkings were performed as required prior to the intramammary infusion administration. *Samples taken before intramammary infusion administration to confirm the absence of antimicrobial agents. †ND: not detected.

Table 4. Trimethoprim : sulphadiazine ratios at sampling times during therapy

Mean concentration (μg/mL)	Sampling time (h)					
	0	6	12	24	30	36
TMP	0.00*	3.98	0.79	0.55	0.71	0.76
SDZ	0.00*	21.31	2.78	2.02	4.94	3.56
TMP : SDZ	0.00*	1:5.3	1:3.5	1:3.7	1:6.9	1:4.6

*Samples taken before intramammary infusion administration to confirm the absence of antimicrobial agents.

During therapy (up to 36 h from first administration) TMP concentrations were detected in ranges of 0.25–4.79 μg/mL (Table 3) and SDZ in ranges of 1.36–33.17 μg/mL (Table 2). As mentioned, TMP concentrations were lower than those of SDZ which is expected because lower concentrations of TMP were administered.

As we compare the MICs from isolated strains with SDZ concentrations in milk, these were above MIC_{90} for *E. coli* and *Streptococcus* sp. The case is different with *S. aureus* because the concentrations were below the MIC_{90} at some sampling times during therapy.

According to these results, this combination may be effective against *Streptococcus* sp. isolated from clinical mastitis. For *E. coli* isolated from clinical mastitis, although some works point out that antibiotic treatment may not always be necessary because a spontaneous cure can occur (Pyörälä & Syväjärvi, 1987; Erskine *et al.*, 1990; Pyörälä *et al.*, 1992; Smith & Hogan, 1993), this combination could be used as an alternative if the veterinarian decides to begin a treatment.

The use of this combination, according to our results, is not recommended in the case of *S. aureus* clinical mastitis because, as it was mentioned before, at some sampling times during therapy SDZ concentrations in milk were below the MIC_{90} of this pathogen (Table 3, 4).

No TMP was found in milk at 24 h after finishing therapy. Although the SDZ concentrations at each sampling time in each cow were different, at 84 h after finishing therapy all cows presented SDZ concentrations below 0.1 μg/mL. At 96 h no SDZ concentration was found in six animals although four animals of the experimental group still had concentrations of 0.07 μg/mL.

In order to define the withdrawal time, it is important to consider the MRLs given by different international organizations. In the present work, we consider the MRL of 0.1 μg/mL given by The European Agency for the Evaluation of Medicinal Products (1999) for sulphonamides in different matrices (meat and milk). Based on our results and the before mentioned MRL (Fig. 1) we suggest a withdrawal time, as an average value, of 84 h for this TMP:SDZ combination administered three times by the intramammary route every 12 h and in cows milked three times daily.

Independently from our results and suggestions we have to mention that the Food and Drug Administration (1999) gives tolerances of 0.01 μg/mL to sulphonamides in milk. On the other hand, Food and Agricultural Organization/World Health Organization (1999) gives an MRL of 0.025 μg/mL only for sulphamethazine unable to establish MRLs for other sulphonamides. If we consider these values, future work is needed to establish at which time SDZ concentrations are below those suggested by these organizations.

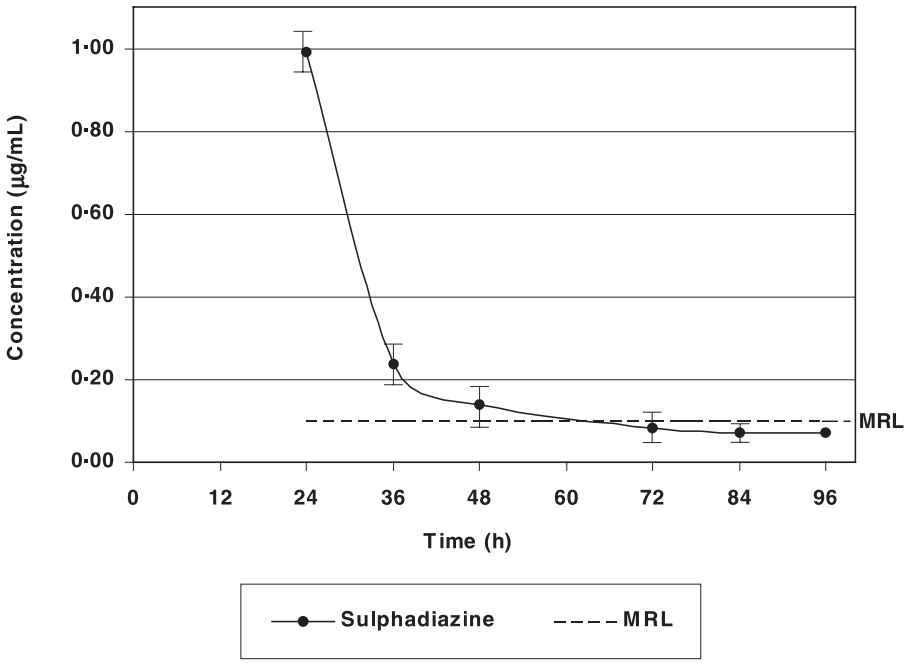

Fig. 1. Mean SDZ concentrations after finishing therapy and its relationship with the MRL (0.1 μg/mL). The bars about the mean points refer to standard deviations.

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