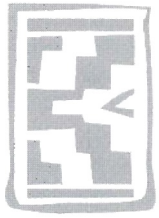

Stability and partitioning of closantel and rafoxanide in ruminal fluid of sheep

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ABSTRACT

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The stability and the partitioning of closantel and rafoxanide in ruminal fluid (RF) was examined *in vitro*. Stability was evaluated in two studies in a ruminal fluid-artificial saliva (RF-AS) mixture containing either drug. Drug concentrations were measured in samples collected sequentially from four batches of RF-AS fortified with either closantel or rafoxanide in one study and in four separately incubated aliquots of a RF-AS mixture of each drug in the second study at the start and at various intervals during a 24 h incubation period. The viability of the *in vitro* RF-AS incubation model was validated by the presence of digoxin degradation (T½ of 39,1 ± 13 h) and by the absence of significant time related differences ($P > 0,5$) in volume of gas produced, pH and methylene blue reduction time of the RF-AS drug mixture. Partitioning of closantel and rafoxanide was determined by measuring the relative drug concentration of the fluid and particulate phases in RF fortified with either drug at different concentrations. Closantel and rafoxanide were shown to be stable in a RF-AS mixture and were not subjected to any significant biodegradation. An initial marked reduction in drug concentration measured in the RF-AS mixture during the first 2 h of incubation was attributed to the attachment of both drugs onto particulate matter. This was subsequently confirmed in the partitioning study. More than 80 % of closantel and rafoxanide was shown to be associated with the particulate phase of RF.

Keywords: Closantel, drug stability, partitioning, rafoxanide, ruminal fluid, salicylanilides, sheep

INTRODUCTION

Substances are subjected to reduction, hydrolytic and fission metabolic reactions by microflora or by the reducing conditions within the rumino-reticulum fluid (Dobson 1967; Prins 1987). Fission reactions are lytic reactions collectively referred to as decarboxylation, dealkylation, dehalogenation, deamination, dehydroxylation and ring fission reactions. Compounds may be unaffected, inactivated or activated by these reactions. They are therefore important considerations in the pharmacokinetics, pharmacodynamics and the toxicity of drugs administered orally in ruminants. Inactivation of chloramphenicol (Davis, Neff, Baggot & Powers 1972) and nitrophenols (Adams 1995) by reduction of the nitro groups, digitalis by hydrolysis (Westermarck 1959) and trimethoprim by demethylation (Nielsen, Romvary & Rasmussen 1978) have been demonstrated. The prodrugs febantel (Beretta, Fadini, Stracciari & Montesissa 1987; Delatour, Tiberghien, Garnier & Benoit 1985) and netobimin (Delatour, Cure, Benoit & Garnier 1986), are

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converted to fenbendazole and albendazole respectively, in the rumen.

The antiparasitic efficacy of the halogenated salicylanilide, clioxanide, is markedly reduced if administered intra-abomasally compared to intraruminally, suggesting the need for activation of the compound in the rumen (Boray & Roseby 1969; Symonds & Roseby 1969). According to Prichard (1978), clioxanide is deacetylated in the rumen to form a hydroxy derivative which is more readily absorbed. Similar intraruminal activation of closantel and rafoxanide has not been reported (Prichard 1978). However, the stability or degradation susceptibility of closantel and rafoxanide in ruminal fluid has not been investigated.

The importance of partitioning of drugs between the fluid and particulate phases of the rumino-reticulum contents on the pharmacokinetics has recently been reported (Hennessy, Ali & Sillince 1995). An increase in the intraruminal mean residence time (MRT) and the reduced rate of passage to the abomasum is found with drugs predominantly adsorbed onto the particulate phase. Delay in the outflow rate of larger particulate particles from the rumino-reticulum and an increase in the intraruminal MRT of drugs have been shown to increase the bioavailability and prolong the systemic availability of the benzimidazole anthelmintics, albendazole and oxfendazole, as well as that of the macrocyclic lactone, ivermectin in sheep and cattle (Ali & Hennessy 1995; Ali & Hennessy 1996; Hennessy, Ali & Tremain 1994; Liftchitz, Virkel, Mastromarino & Lanusse 1997; Sánchez, Alvarez & Lanusse 1997). Improved anthelmintic efficacy is associated with an increase in the bioavailability and prolonged systemic availability for several anthelmintics (Prichard 1978; Hennessy 1994).

In vitro studies were conducted to determine the stability of closantel and rafoxanide in ruminal digesta and to establish the partitioning ratio of both drugs in the fluid and particulate phases of the ruminal contents.

MATERIALS AND METHOD

Stability studies

Two *in vitro* stability studies with closantel and rafoxanide in ruminal fluid were performed. For both studies, ruminal content was collected from a healthy, adult, rumen-cannulated, donor sheep fed on a mixture of chopped lucern hay.

Study 1

Five hunderd mℓ of ruminal fluid (RF) were collected in a 1 ℓ glass schott bottle, warmed and maintained at *c.* 40 °C. The RF was filtered through a 2,5 mm sieve and 400 mℓ of the filtrate added to 1 600 mℓ prewarmed artificial saliva (0,47 g NaCl; 0,57 g KCl; 0,25 g $MgSO_4.7H_2O$; 3,66 g Na_2HPO_4; 1,09 g urea; 9,8 g $NaHCO_3$; 0,05 g $CaCl_2$; 1 000 ℓ distilled water), prepared as previously described (Bailey 1961; Engels & Van der Merwe 1976), to maintain the mixture at a pH of *c.* 6,9. All reagents used were of analytical grade. Ten grams of the same feed fed to the donor sheep were suspended in 50 mℓ of artificial saliva and added to the filtrate mixture as a source of nutrition. The ruminal fluid-artificial saliva (RF-AS) mixture was then sub-divided into 4 x 500 mℓ aliquots in 1 000 mℓ Erlenmeyer glass flasks, the air in each flask purged with CO_2, the flask stoppered and placed in a stationary warmwater bath at 40 °C. One milligram digoxin powder (Sigma, South Africa) was dissolved in 10 mℓ of artificial saliva and also added to each flask. The flask was stoppered using a rubber plug containing a collection port and an outlet for measurement of gas production.

The viability and activity of the microbial population in the RF-AS mixture within each flask were monitored by measuring changes in gas production, pH, methylene blue reduction time (MBRT) and digoxin degradation throughout the duration the study. The volume of gas produced (mℓ) was measured by the amount of water displaced in an inverted measuring cylinder connected to the outlet of the rubber stopper, while pH was measured with a pH meter (PHM 82 Standard, Radiometer, Copenhagen). MBRT was measured following the addition of 0,5 mℓ of a solution of 0,01 % m/v methylene blue (British Drug House) to 10 mℓ of filtrate mixture kept in a warmbath at 40 °C and was used as a measure to indicate the proteolytic activity of the microbial population (Dirksen 1979). Digoxin concentrations were measured by fluorescence polarization immunoassay (FPIA) (Dandliker, Kelly, Dandliker, Farquhar & Levin 1973) using a TDX analyzer (Abbott Laboratories) and digoxin degradation was used as an index of the hydrolytic capacity (Adams 1995) of the ruminal microorganisms.

Drug incubation commenced immediately following preparation of the filtrate incubation system, within approximately 40 min from the time of collection of the RF. Twelve-and-a-half milligrams of closantel (0,5 mℓ of a 2,5 % m/v solution, Seponver, Janssen Animal Health, South Africa) or 15 mg of rafoxanide (0,5 mℓ of a 3 % m/v suspension, Ranide, Logos Agvet) were added to each of two flasks and mixed throughly. RF-AS samples (20 mℓ) from each flask were collected via the collection port of the rubber stopper 10 min after mixing of the drugs as well as after 2, 4, 8, 12 and 24 h incubation. Two 1 mℓ samples, one for the determination of either closantel or rafoxanide concentration and the other for digoxin analysis, were transferred to clean polycarbonate tubes and placed on ice immediately after collection. The pH and MBRT of the RF-AS mixture were meas-

ured on the remainder of the sample collected. The volume of gas produced in each flask was measured just prior to the collection of the RF-AS samples. Ten millilitres of the first sample collected from each flask was quenched by the addition of 1 mℓ of methanol (analytical grade, Merck NT Laboratories) and kept as a control sample for concurrent analysis of drug content at subsequent sample collection times.

The samples collected for drug analyses were centrifuged at 1 663 *g* for 15 min and the supernatant transferred to clean polycarbonate tubes. Digoxin analyses were performed on the same day of sample collection, whereas the remainder of each sample was stored at –20 °C until analyzed for closantel and rafoxanide content. The same experimental procedure and drug incubations were repeated the following day.

Study 2

Study 2 was designed to measure the total concentration of closantel and rafoxanide following incubation in ruminal fluid samples spiked with known amounts of either drug. Ruminal fluid (100 mℓ) was collected and prepared in the same way as Study 1. The filtered ruminal fluid was then divided into 40 aliquots of 1 mℓ each and transferred to clean 10 mℓ glass tubes. Artificial saliva (4 mℓ), fortified with either rafoxanide or closantel at 10 $\mu g.m\ell^{-1}$, was added to each tube (20 tubes per drug). The air in the tubes was purged with CO_2, and the tubes were stoppered and placed in a warmwater bath at 40 °C. The volume of gas production and pH were measured in each tube as in Study 1.

Four tubes each of the RF-AS mixture containing closantel or rafoxanide were collected at the start of the study and after 3, 6, 12 and 24 h of incubation. The pH of the RF-AS mixture was measured in the tubes which were then placed on ice until storage at –20 °C. The volume of gas produced was measured only for the incubation periods before collection of each tube. Total drug content was measured in each tube.

Drug partitioning in ruminal fluid and particulate phase

Ruminal fluid (100 mℓ) was collected and prepared as for the stability studies. The filtered RF was centrifuged at 20 000 *g* for 12 min after which the fluid phase was decanted into a separate glass tube and the proportion of fluid to particulate recorded. Four 1 mℓ aliquots of fluid were fortified with either closantel or rafoxanide to give a final concentration of 6,3, 12,5, 25 and 50 $\mu g.m\ell^{-1}$ or 7,5, 15, 30 and 60 $\mu g.m\ell^{-1}$, respectively. The fortified aliquots were transferred to 10 mℓ glass tubes and ruminal particulate matter from the particulate phase then added to each tube at the same ratio of fluid:particulate phase measured for the filtered ruminal fluid. The tubes were stoppered and the contents thoroughly mixed in a box shaker for 4 h. After mixing, the tubes were centrifuged at 1 663 *g* for 30 min and the fluid phase transferred to clean polycarbonate tubes. Both the particulate and fluid phases were stored at –20 °C until analysed. Total drug content of either closantel or rafoxanide were measured in both the particulate and fluid phases.

Drug analyses

The concentrations of closantel and rafoxanide in the samples were determined using a high performance liquid chromatographic (HPLC) method described for rafoxanide in plasma (Blanchflower, Kennedy & Taylor 1990). The method was modified and validated for the determination of both drugs in various matrices, including ruminal content. Each drug was interchanged as an internal standard in the determination of the other.

Statistical analysis

The means and standard deviations were calculated for pH; MBRT and digoxin concentrations measured in all RF-AS samples collected; the volumes of gas produced per hour per flask for the interval between each evaluation; the concentration of closantel and rafoxanide in filtrate sample collected from the appropriate flasks; and the concentrations of digoxin, closantel and rafoxanide in all control samples for each time interval and for both drugs in Study 1. Linear regression analysis of digoxin, closantel and rafoxanide in RF-AS versus time was used to calculate the relevant depletion half-lives. Differences in pH, MBRT, volume of gas produced per hour and percentage reduction in concentration of each drug in the RF-AS measured over time were statistically analysed. In Study 2, the percentage reduction in the mean total drug content following incubation at the different examination periods relative to the mean concentration at the start of the study was calculated and statistically compared.

The data were evaluated by analysis of variance (ANOVA), followed by repeated measures analysis (MANOVA) for differences over time and interactions. The MANOVA Test Criteria and Exact *F*-statistics were applied to test for time effects and interaction between time and treatment using Wilks' Lambda procedure. Repeated measures for analysis of variance were performed to test for differences between flasks. The Greenhouse and Huynh-Feldt test procedures were also applied to determine differences within flasks. Statistical procedures were performed using SAS statistical software package (SAS Institute Inc. 1988).

RESULTS

Stability

The viability of microbial activity in the RF-AS mixture was maintained throughout the study period of both studies (Table 1).

TABLE 1 Mean pH, MBRT and volume of gas produced at the various sample collection intervals during the incubation of closantel and rafoxanide in the RF-AS mixture

Time of observation (h)	Mean ± SD		
	pH	MBRT (min)	Gas (mℓ/h/mℓ)
Study 1			
0	6,89 ± 0,28[a]	1,5 ± 0,8[a]	–
2	7,09 ± 0,05[a]	2,4 ± 0,9[a]	0,11 ± 0,07[a]
4	7,17 ± 0,02[a]	2,1 ± 0,9[a]	0,09 ± 0,03[a]
8	7,15 ± 0,07[a]	2,4 ± 0,7[a]	0,07 ± 0,04[a]
12	7,08 ± 0,07[a]	2,7 ± 0,9[a]	0,07 ± 0,04[a]
24	6,96 ± 0,04[a]	2,7 ± 0,6[a]	0,07 ± 0,02[a]
Study 2			
0	7,13 ± 0,01[a]	–	–
3	7,37 ± 0,02[a]	–	0,22 ± 0,01[a]
6	7,38 ± 0,04[a]	–	0,49 ± 0,08[b]
12	7,32 ± 0,02[a]	–	0,33 ± 0,09[c]
24	7,27 ± 0,03[a]	–	0,10 ± 0,05[d]

[a, b, c, d] Values with different superscript are significantly different ($P \leq 0,05$)

No significant ($P > 0,05$) time related effects were observed for pH, MBRT and volume of gas produced ($m\ell.h^{-1}.m\ell^{-1}$ of ruminal fluid) in Study 1 or for pH in Study 2.

Significant ($P < 0,05$) differences in the volume of gas produced were found between observations in Study 2. The RF-AS mixture in Study 2 was slightly more alkaline and a larger volume of gas per mℓ of ruminal filtrate was produced than in Study 1.

The mean digoxin concentration versus time in the RF-AS mixture decreased gradually over 24 h (T½ of 39,1 ± 13,0 h) (Fig. 1). At 24 h the mean digoxin concentration in the RF-AS mixture had significantly ($P < 0,05$) reduced by 37,8 % relative to initial concentrations.

The mean concentrations of closantel and rafoxanide declined markedly (46 % and 87 %) during the first 2 h of incubation in the RF-AS mixture in Study 1 (Table 2), after which the rate appeared to slow down considerably.

There was no significant ($P > 0,05$) difference in closantel and rafoxanide concentration over time in the RF-AS mixture and in the control, methanol quenched samples, for the 2–24 h of incubation periods.

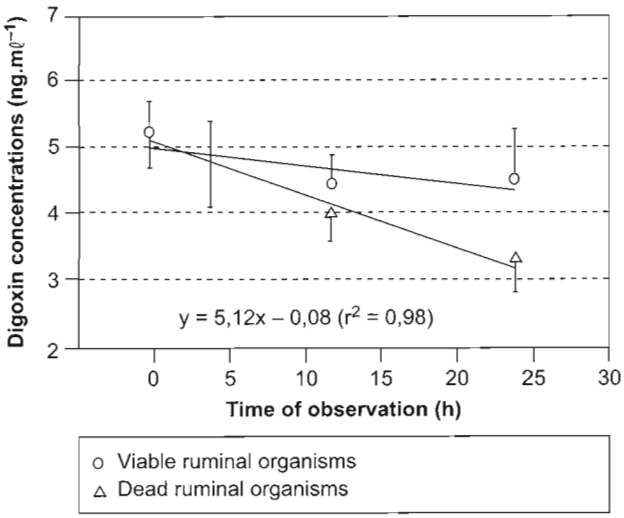

FIG. 1 Mean concentrations of digoxin in a RF-AS mixture at various intervals of incubation in the presence of live and killed (methanol quenched) ruminal organisms

After 24 h of incubation, 33 % of the initial amount added of either closantel or rafoxanide was recovered from the extraction mixture.

Although the mean closantel and rafoxanide concentrations in RF-AS mixture appeared to decline during the incubation of 3–24 h, this was not significant ($P > 0,1$) between test and control samples and between test samples over time in Study 2 (Table 3).

Partitioning of closantel and rafoxanide in ruminal contents

The mean concentrations of closantel and rafoxanide determined in the fluid and particulate phases of ruminal contents and ratio of fluid-particulate phases for each of the drug concentrations added to the reconstituted rumen content samples are given in Table 4. Both closantel and rafoxanide were predominantly (> 80 % for rafoxanide and > 90 % for closantel) associated with the particulate phase of ruminal contents. There were no significant ($P > 0,05$) differences in the ratio of partitioning between fluid and particulate phases of ruminal fluid for both drugs or at the different concentrations. The total recovery of closantel and rafoxanide from both phases was low (*c.* 50 %).

DISCUSSION

Viability of the *in vitro* incubation model used in the stability studies was confirmed by digoxin degradation (Westermark 1959), reduction of methylene blue (Dirksen 1979), consistency of pH of the ruminal fluid-artificial saliva mixture and volume of CO_2 pro-

TABLE 2 Mean concentration of closantel and rafoxanide measured in RF-AS mixture and control samples at various times after incubation in Study 1

Time of observation (h)	Mean ± SD drug concentration (µg/mℓ)			
	Rafoxanide		Closantel	
	RF-AS	Control	RF-AS	Control
0	36,5 ± 34,5[a]	–	7,0 ± 2,4[a]	–
2	5,2 ± 1,5[a]	24,3 ± 19,7[a]	3,8 ± 1,5[a]	6,5 ± 3,0[a]
4	4,1 ± 1,1[a]	3,4 ± 1,4[a]	3,2 ± 1,0[a]	6,3 ± 3,2[a]
8	2,8 ± 0,4[a]	5,0 ± 2,7[a]	2,6 ± 0,9[a]	4,6 ± 2,5[a]
12	1,7 ± 0,4[a]	6,7 ± 4,2[a]	2,1 ± 0,8[a]	5,5 ± 4,3[a]
24	2,5 ± 0,6[a]	2,6 ± 1,2[a]	2,1 ± 0,4[a]	5,2 ± 4,6[a]

[a] Values not significantly different ($P > 0,05$)

TABLE 3 Mean concentration of closantel and rafoxanide measured in RF-AS mixture at various times after incubation in Study 2

Time of observation (h)	Mean ± SD drug concentration (µg/mℓ) in RF-AS mixture	
	Rafoxanide	Closantel
0	5,8 ± 2,1[a]	9,2 ± 1,6[a]
3	4,8 ± 1,2[a]	9,1 ± 1,0[a]
6	5,8 ± 1,8[a]	7,3 ± 1,7[a]
12	2,8 ± 0,8[a]	6,8 ± 0,5[a]
24	3,7 ± 1,1[a]	5,9 ± 0,7[a]

[a] Values not significantly different ($P > 0,01$)

TABLE 4 Partitioning of closantel and rafoxanide into the fluid and particulate phases of ruminal content

Fortified concentration (µg.mℓ$^{-1}$)	Mean ± SD concentration ($n = 4$)		Ratio of fluid: particulate phases
	Fluid phase	Particulate phase	
Closantel			
6,25	0,52 ± 0,25	4,83 ± 0,86	10,8:100[a]
12,50	0,57 ± 0,10	10,85 ± 0,97	5,2:100[a]
25,00	1,27 ± 0,12	22,92 ± 0,64	5,2:100[a]
50,00	2,68 ± 0,15	35,90 ± 2,50	7,4:100[a]
Rafoxanide			
7,50	0,68 ± 0,25	3,82 ± 1,54	17,8:100[a]
15,00	0,80 ± 0,21	9,40 ± 2,53	8,5:100[a]
30,00	0,46 ± 0,09	14,30 ± 3,00	3,2:100[a]
60,00	0,81 ± 0,17	25,57 ± 2,20	3,2:100[a]

[a] Values not significantly different ($P > 0,05$)

duced. The 24 h examination period used in the stability studies is consistent with the mean residence time of digesta in the rumen under normal conditions when similar diets are fed (Downes & McDonald 1964).

In vitro incubations described in these studies with sheep ruminal fluid indicate that neither closantel nor rafoxanide showed significant biodegradation over 24 h. Disappearance of rafoxanide and closantel from ruminal fluid, particularly noted at the start of the incubations, was associated with adsorption of the drugs onto the particulate portion of ruminal content. Extensive partitioning of closantel and rafoxanide onto the particulate matter in ruminal content was confirmed in the partitioning study. Binding of

drugs to ruminal digesta may give a false impression of its biodegradation in ruminal fluid (Andrew & Halley 1996).

Low concentrations of closantel and rafoxanide in the fluid phase of ruminal content is consistent with the known poor water solubility and high lipid–water partition coefficient of the salicylanilides. This will also account for the poor recovery of the drugs from the RF-AS samples in the current studies. The chromatograms obtained during the HPLC analysis of the ruminal fluid showed no evidence of any degradation products.

The results of the study indicate that the bioavailability of closantel and rafoxanide administered orally to ruminants will not be affected by biodegradation through changes in the MRT in the rumino-reticulum. On the other hand, variation in the outflow rate of ruminal digesta due to changes in feed intake, the chemical and physical nature of the diet, the physiological state of the animal and climatic conditions (Faichney 1986; Grovum & Williams 1973; Kay 1986) may have an important effect on the MRT and thus on the oral bioavailability of both drugs. The affect of the MRT on the bioavailability of several anthelmintics has been reported (Ali & Hennessy 1995, Ali & Hennessy 1996; Hennessy *et al.* 1995; Liftchez *et al.* 1997; Sanchez *et al.* 1997). It is therefore expected that the MRT in the rumino-reticulum of closantel and rafoxanide will be affected proportional to any changes in outflow rate of ruminal digesta. The MRT of these drugs in particular, which is associated mainly with the particulate phase, will be affected by the physical nature of the diet. Particles that pass through a 2,36 mm sieve but are retained on a 1,18 mm sieve (*c.* 4–5 mm long) generally remain in the rumen (Faichney 1986). Further studies are therefore required to examine the influence on the level of feeding and type of feed on the pharmacokinetics of closantel and rafoxanide.

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