J. vet. Pharmacol. Therap. **19**, 295–299, 1996.

Stability of ivermectin in rumen fluids

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Andrew, N.W., Halley, B.A. Stability of ivermectin in rumen fluids *J. vet. Pharmacol. Therap.* **19**, 295–299.

To determine whether ivermectin is metabolized in the rumen, *in vitro* studies were conducted with the tritium-labelled H_2B_{1a} component of ivermectin in rumen fluid from sheep and cattle. No detectable metabolism occurred over 24 h in *in vitro* incubations at 38°C. The viability of the microbes in the rumen fluids was demonstrated by the conversion of 17% and 11% of [^{14}C]cellulose to $^{14}CO_2$ in 24 h in the incubations with sheep and steer rumen fluids respectively. The results indicate that ivermectin is not metabolized in the rumen. Based on the lack of *in vitro* metabolism of ivermectin in rumen fluid, the similarity of *in vitro* liver microsomal metabolism with *in vivo* metabolism of the avermectins and the physicochemical properties of the avermectins, any disappearance of ivermectin *in vitro* from rumen fluid is probably a result of binding to solids or surfaces. Apparent discrimination by dung beetles, where observed, between control faeces and faeces from cattle or sheep treated with ivermectin or abamectin therefore must be attributable to chance, to factors unrelated to treatment or to factors such as changes in amino acid composition rather than the production of volatile metabolites of ivermectin.

(Paper received 24 October 1995; accepted for publication 21 February 1996)

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INTRODUCTION

Ivermectin and abamectin, potent endo- and ecto-parasiticides with broad spectra of activity, are used as animal health drugs in a variety of species (Di Netta, 1989). Ivermectin is also the drug of choice for treating onchocerciasis in man (Greene *et al.*, 1989). In a study to determine the pharmacokinetics in sheep, it was reported that ivermectin is rapidly metabolized in the rumen (Prichard *et al.*, 1985). They reported the bioavailability of ivermectin in sheep following intra-abomasal administration to be 100% but only 25.1% following intraruminal administration. To determine the stability of ivermectin in rumen fluid, they incubated ivermectin with filtered rumen fluid and 50% was reportedly metabolized in 2 h. From this, Prichard and co-workers concluded that ivermectin was metabolized in the rumen, although they did not observe chromatographic evidence for any metabolites. Since some species of dung beetles, in some studies, appear to differentiate between dung from untreated animals and dung from cattle or sheep treated with ivermectin or abamectin (Lumaret *et al.*, 1993; Holter *et al.*, 1993; Wardhaugh & Mahon, 1991), investigators have also speculated that ivermectin might be metabolized by gut flora or have cited the work by Prichard and co-workers to support theories that volatile metabolites of ivermectin might be present in faeces and that these metabolites might attract or repel dung fauna (Wardhaugh & Mahon, 1991; Holter et al., 1993; Sommer & Overgaard Nielsen, 1992). However, since all major metabolites identified in sheep and cattle faeces and liver are also produced in *in vitro* incubations with liver microsomes from cattle, sheep and rats (Chiu & Lu, 1989), there is no indication of additional volatile metabolites being produced by rumen flora. Bogan and McKellar (1988) have also determined that ivermectin is more stable than was earlier reported in studies where rumen fluid was collected after subcutaneous dosing of sheep. Apparent changes in attractiveness of dung following treatment with ivermectin or abamectin might be due to changes in gut flora or to changes in the amino acid composition of the dung (Wardhaugh & Mahon, 1991; Lumaret, *et al.*, 1993; Bernal *et al.*, 1994). Any direct effect of avermectins on gut flora is not expected, however, since the avermectins are not anti-microbials (Halley *et al.*, 1993). To clarify whether ivermectin is metabolized in the rumen, we conducted an *in vitro* study to determine its stability in rumen fluid from sheep and cattle.

MATERIALS AND METHODS

Test compounds

[^{14}C]Cellulose was obtained from American Radiolabeled Chemicals Inc., St Louis, MO, USA, and had a specific activity of 20 μCi/mg. The [22,23-^{3}H]H_2B_{1a} component of ivermectin was supplied by the Labeled Compound Synthesis group, Department of Drug Metabolism, Merck Research Laboratories (MRL).

Separate batches had specific activities of 51.92 mCi/mg and 43.89 mCi/mg and radiochemical purities of 98.5% and 99.7%. H_2B_{1a} is the major component of ivermectin and differs from the minor component by only a methylene group in an aliphatic sidechain. For cattle, swine, sheep and rat, *in vivo* and *in vitro* metabolism of the two components is identical (Chiu *et al.*, 1987). For this reason, only the H_2B_{1a} component was radiolabelled. Unlabelled ivermectin, which is comprised of H_2B_{1a} and H_2B_{1b} components, was obtained from the Chemical Data Department, MRL. The percentage of the two components, H_2B_{1a} and H_2B_{1b}, was determined by high performance liquid chromatography (HPLC) analysis to be 91.7% and 5.8% respectively.

Collection of rumen fluid

Rumen fluid was collected from one sheep and one steer. The sheep was a 6-month old, 40 kg, mixed-breed male fed on alfalfa cubes. The Hereford steer, approximately 6 months old and 300 kg, was fed on alfalfa cubes and a small amount of corn and soybean concentrate. Animals were euthanized using penetrating captive bolt followed by exsanguination. Rumen fluid was collected following dissection, filtered through two layers of cheesecloth and placed into a 500 mL plastic bottle pre-warmed to approximately 40°C. The fluid was purged with CO_2 for approximately 3 min and the bottle was tightly closed. The fluid was used within 2 h of collection.

Assay for ivermectin and metabolites

Reverse-phase high performance liquid chromatography (RP-HPLC) was performed using a Shimadzu (Columbia, MD, USA) dual pump gradient system. A Zorbax ODS analytical column (4.6 mm × 25 cm, Mac-Mod Analytical, Chadds Ford, PA, USA) was used and the eluate was monitored at 245 nm using a flow-through ultraviolet detector. Fractions (1 min) of the eluate were collected, mixed with Instagel XF scintillation fluid (Packard Instrument Company, Meriden, CT, USA) and the radioactivity was determined using a Packard 1900 TR scintillation spectrometer. The mobile phase flow rate was 1 mL/min 80% methanol –20% water for 50 min, followed by a methanol wash.

Incubations

Incubations contained 875 ng (1 μM) of ivermectin ([22,23-^{3}H] H_2 B_{1a} component, 0.70 μCi) in 1 mL of rumen fluid and were conducted in 16 × 100 mm glass culture tubes that were purged with N_2, capped and placed in a pre-equilibrated 38°C water bath. Incubations were conducted over 0, 2, 4 and 24 h and quenched by the addition of 1 mL of methanol. The contents were vortexed and centrifuged and the supernatant was removed. The precipitate was washed with an additional 1 mL of methanol, vortexed and centrifuged. The supernatants were combined and aliquots were removed for recovery determination (Table 1) and analysis by HPLC (Table 2).

To demonstrate the viability of the microbes in the rumen fluid, incubations with [^{14}C] cellulose were conducted as follows.

Table 1. Percentage recovery of radioactivity following *in vitro* incubations in rumen fluid

Animal	Incubation	Recovery (%)*†
Sheep	Active rumen fluid	101, 96
	Control	118, 107
Steer	Active rumen fluid	99, 94
	Control	102, 99

*Results of duplicate incubations; †% recovery = net d.p.m. following work-up divided by net d.p.m. of [^{3}H]ivermectin added to each tube before incubation.

Table 2. Percentage conversion of [^{14}C]cellulose to [$^{14}CO_2$] and the percentage of the [^{3}H]H_2B_{1a} component of ivermectin remaining following 24-h incubations in sheep and cattle rumen fluids

	Percentage of $^{14}CO_2$ formed from [^{14}C] cellulose		Percentage of [^{3}H]H_2B_{1a} component of ivermectin remaining	
	Active rumen fluid*	Control rumen fluid†	Active rumen fluid*	Control rumen fluid†
Sheep	9.5, 10.8	1.8	93, 94	94
Cattle	17.8, 15.3	0.02	90, 90	92, 92*

*Results of duplicate incubations; †Heated at 80°C for 10 min before incubations.

Aliquots (10 mL) of rumen fluid were added to 20 mL glass scintillation vials containing [^{14}C]cellulose (2.16 μCi, 108 μg, sheep; 4.22 μCi, 211 μg, steer). The vials were sealed with septa and placed in a water bath maintained at 40°C. A needle was used to bubble nitrogen through the fluid and a second double-tipped needle was used to transfer the CO_2 generated to an overflow vial. The overflow vial containing a silicon septum was connected by another double-tipped needle to a 16 × 100 mm glass culture tube containing 6 mL Carbosorb (Packard Instrument Company). The Carbosorb was changed after 0.5, 2, 4, 7 and 24 h, mixed with 14 mL Permafluor E (Packard Instrument Company) scintillation fluid and radioactivity was determined by scintillation spectrometry. Duplicate incubations were conducted for each species along with controls containing ruminal fluid heated at 80°C for 10 min.

RESULTS

Figure 1 illustrates the HPLC radiochromatograms following *in vitro* incubations of ivermectin with cattle and sheep rumen fluid for 24 hours. There was quantitative recovery of radioactivity following processing (Table 1) and no detectable metabolism occurred (Table 2). With the exception of the eluate fractions containing the H_2B_{1a} component, approximately 33–39 min, no eluate fraction contained more than 0.7% of the eluted radioactivity. In addition, since the incubations were conducted under a closed system and the samples were not concentrated before analysis, any volatile radioactivity formed would be detected by

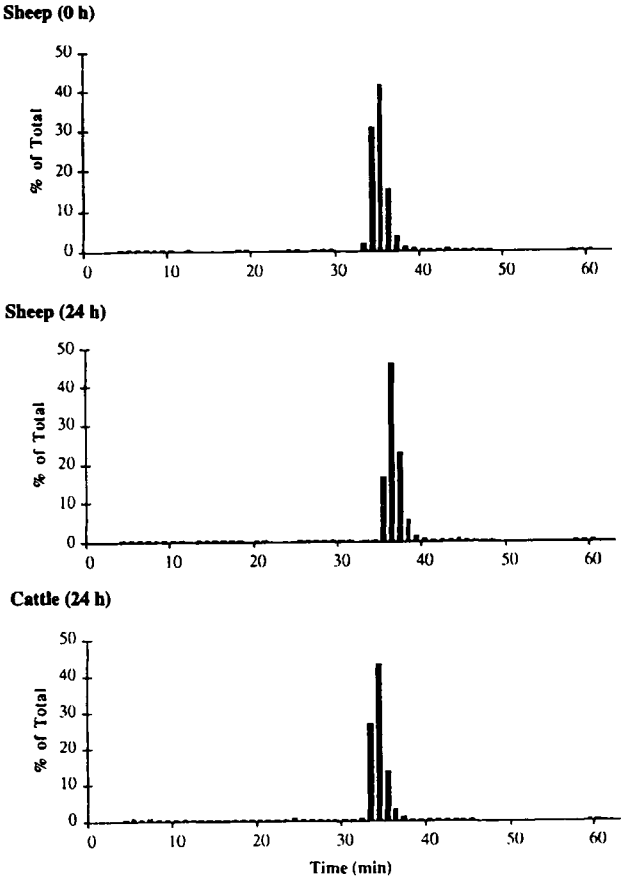

Fig. 1. HPLC Radiochromatograms of Extracts from 24-Hour Incubations of the $^{3}[H]H_2B_{1a}$ Component of Ivermectin at 1 μM with Sheep and Cattle Rumen Fluids.

HPLC analysis. Since no radioactive peaks were detected in the void region of the radiochromatogram and there was quantitative recovery of radioactivity after processing, no volatiles were formed in the incubations. An average of 17% and 10% of the [^{14}C]cellulose was converted to $^{14}CO_2$ in 24 h in the sheep and cattle incubations (Table 2), respectively, demonstrating the activity of the microbes in the rumen liquor.

DISCUSSION

The *in vitro* incubations described in this experiment with cattle and sheep rumen fluid indicate that no metabolism of ivermectin occurred over 24 h. In a previous study to determine the pharmacokinetics in sheep, it was reported that ivermectin is rapidly metabolised in the rumen (Prichard *et al*, 1985). This conclusion was based on the disappearance of ivermectin from rumen fluid in *in vitro* and *in vivo* studies following intravenous, intra-abomasal and intraruminal administration. Since no metabolites were detected using their analytical methods, and since we have found that ivermectin is capable of binding to a variety of surfaces such as glass and plastic (unpublished results), the disapperance of ivermectin in their assay system could have been due to binding of the compound to surfaces, rather than to metabolism. An explanation for the low bioavailability of ivermectin administered intraruminally compared with the intra-abomasal route might be a result of binding of the drug to digesta. Binding to digesta influences the pharmacokinetics of some drugs (Bogan *et al.*, 1984; Hennessy, 1994) and more than 95% of the radioactivity following intraruminal administration of tritiated ivermectin is bound to abomasal particulate material (Steel, 1993).

Some species of dung beetles, in some studies, appear to differentiate between dung from untreated animals and dung from cattle or sheep treated with ivermectin or abamectin (Lumaret *et al.*, 1993; Holter *et al.*, 1993; Wardhaugh & Mahon, 1991). Cattle dung, excreted 2 or 4 days after dose, when ivermectin was present, was not preferred relative to dung from untreated cattle by beetles in Spain (Lumaret *et al.*, 1993). However, beetles belonging mostly to the genera *Euoniticellus*, *Onthophagus*, *Caccobius* and *Aphodius* preferred dung excreted on days 7, 10 and 17 after dose, when ivermectin levels in the dung were not detectable (below 0.02 mg/kg), over control dung. No differentiation by the beetles was observed for dung excreted on days 24 or 31 after dose. *Aphodius* spp. in Denmark were less attracted ($P < 0.01$) in one out of three studies to pitfall traps baited with dung from cattle treated 3, 10, 20 or 30 days previously with ivermectin (Holter *et al.*, 1993). No preferences were seen in the other two studies in Denmark. Dung beetles, mostly *Onthophagus* spp., in Tanzania did not differentiate between control cattle dung or dung excreted from cattle 3, 6, 10, 15 or 21 days after ivermectin treatment. There was also no differentiation by five species of dung beetles in Zimbabwe between control cattle dung and dung excreted 2, 8 or 16 days after ivermectin treatment. However, when numbers of beetles were summed by species in traps containing dung excreted either 2 or 8 days after dosing and compared with the pooled sums of numbers of beetles in traps containing either dung excreted 16 days after dosing or control dung, the sums of *Liatongus militaris* and *Euoniticellus intermedius* beetles were greater ($P < 0.025$) in traps containing dung excreted on days 2 and 8 after dose. Based on this, the authors concluded that these species, both belonging to the tribe Oniticellini, seem to prefer dung with a high concentration of ivermectin. The other species showed no such trend. Greater numbers, relative to controls, of *Onthophagus australis* were observed in pitfall traps baited with cattle dung excreted 3 or 25 days after abamectin treatment, but not in traps baited with 35 day after dose dung at two locations in south-eastern Australia (Wardhaugh & Mahon, 1991). Numbers of *O. pexatus* were greater in the traps baited with 25 day after dose dung at only one of the two sites, and numbers of exotics, pooled catches of *E. fulvus* and *O. taurus*, were greater in the traps baited with 3 day after dose dung also at only one of the sites. In a similar trial, but at a single site and using dung from sheep treated orally with ivermectin, numbers of *O. australis* were greater ($P < 0.01$) in traps baited with 1 day after dose dung, but not in traps baited with dung excreted 2 or 6 days after treatment. Numbers of *O. posticus* were not different, relative to numbers in the traps baited with control dung, in any of the groups of traps containing the dung from treated sheep.

The authors concluded that differentiation between control dung and dung from treated animals was not caused by nutritional differences between the groups. They proposed that some volatile metabolite or change in the gut flora initiated by ivermectin therapy might be the cause.

As discussed above, in the study in Spain, there was an increase in the attraction of dung beetles to 1-kg pats formed from cattle dung voided on days 7, 10 and 17 after subcutaneous treatment with ivermectin compared with the controls, but no increased attraction to pats voided on days 2, 4, 24 or 31 after treatment (Lumaret *et al.*, 1993). The concentration of ivermectin in these samples reached its highest level 5 days after dose and then decreased rapidly. After 12 days, levels of ivermectin were below the level of detection. Bernal *et al.* (1994) determined the amino acid composition in dung from these control and treated cattle. The amino acid profiles revealed that the amino acid composition changed over time and was different between dung from treated and control cattle. The authors suggested that the change in the amino acid composition after 7 days increased the attractiveness of the dung, perhaps by altering the odour.

Our studies indicate that no metabolites of ivermectin were formed in the rumen fluid. Since the incubations were conducted in a closed system, any volatile metabolites would have been trapped and susequently detected upon analysis. Other metabolism studies (Halley *et al.*, 1992) have demonstrated that metabolic profiles in extracts from livers of cattle dosed intraruminally with ivermectin were similar to the profiles from an *in vitro* study using cattle liver microsomes. The profiles of metabolites in liver and faeces following subcutaneous administration of ivermectin to cattle and sheep correlates with those obtained by *in vitro* liver metabolism from the same species (Chiu & Lu, 1989; Halley et al., 1989). Similarly, the same metabolites of abamectin are produced following dosing orally to the goat, a ruminant (Maynard *et al.*, 1989) or orally to rats, a non-ruminant (Maynard *et al.*, 1990), or subcutaneously to cattle (Halley *et al.*, 1992), as are produced *in vitro* by cattle, rat or swine liver microsomes (Halley *et al.*, 1992). Therefore, no additional metabolites of avermectins are formed in the rumen.

Ivermectin, as followed by the tritium-labelled, H_2B_{1a} component, was not metabolized in 24 h in active cultures of rumen fluid from sheep or cattle. The viable microbial activities of these cultures was demonstrated by the conversion of [^{14}C]cellulose to [^{14}C]CO_2. The rapid disappearance, reported by Prichard *et al.* (1985), of ivermectin in rumen fluid was therefore probably a result of to binding of the compound to solids or surfaces, not to metabolism. This is consistent with the lack of observed metabolites in that study, and with the low water solubility, the high K_{oc} and the demonstrated binding of ivermectin to surfaces. Ivermectin and abamectin metabolism products in liver and faeces from *in vivo* studies in ruminants and non-ruminants correlate closely with the metabolism products from *in vitro* incubations with liver microsomes from the same animals. Combined with the lack of metabolism seen in *in vitro* incubations of tritiated ivermectin with sheep and cattle rumen fluid in this study, there is no evidence that these compounds are metabolized in the rumen. Any differences, where they have been observed, in attractiveness or repulsiveness to dung beetles of faeces from cattle or sheep treated with ivermectin or abamectin, relative to control faeces, therefore must be attributable to chance, to factors unrelated to treatment or to factors such as changes in amino acid composition rather than to the production of volatile metabolites of ivermectin.

ACKNOWLEDGMENTS

The authors would like to thank Mr Terry Murphy, Mr Herb Haines and Dr Terry Faidley for assistance in obtaining rumen fluid.

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