# CHEMICAL AND BIOLOGICAL STABILITY OF ARTEMISININ IN BOVINE RUMEN FLUID AND ITS KINETICS IN GOATS (*Capra hircus*)

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**ABSTRACT**:- FERREIRA, J.F.S.; GONZALEZ, J.M**. Chemical and biological stability of artemisinin in bovine rumen fluid and its kinetics in goats (*Capra hircus*).** [Estabilidade química e biológica da artemisinina no fluído ruminal de bovinos e sua cinética em caprinos]. *Revista Brasileira de Parasitologia Veterinária*, v. 17, supl. 1, p. 103-109, 2008. USDA-ARS, Appalachian Farming Systems Research Center, 1224 Airport Rd., Beaver, WV 25813, USA. E-mail: Jorge.Ferreira@ars.usda.gov

There is a pressing need to develop alternative, natural anthelmintics to control widespread drug-resistant gastrointestinal nematodes in ruminants, such as *Haemonchus contortus*. Artemisinin and its semi-synthetic derivatives are widely used against drug-resistant *Plasmodium falciparum*, but their role in veterinary medicine is only emerging. Artemisinin may be useful in controlling gastrointestinal parasites including *Haemonchus*. However, no ruminant studies involving artemisinin have been reported. The stability of artemisinin in capsules, crystals, or stock solutions in ethanol and dimethyl sulfoxide was evaluated in bovine rumen culture medium incubated for 24 hours at 39°C. A second study established artemisinin kinetics in goats after oral administration of artemisinin capsules at 23 mg/kg of body weight. Artemisinin recovered from rumen culture ranged from 67 to 92% at pH 6.8 and was 95% at pH 3.0. The kinetics data showed that artemisinin was metabolized to dihydroartemisinin by goats, while unabsorbed artemisinin was eliminated in feces. Dihydroartemisinin peaked in the blood (0.7µg/mL) at 12 hours, and decreased to 0.18 µg/mL at 24 hours. At 24 hours, artemisinin concentration in feces was 2.4 µg/g, indicating its poor bioavailability in goats when provided orally and as capsules. These results suggest that the bioavailability of artemisinin to goats can improve by dissolving capsules in ethanol or dimethyl sulfoxide, by using more stable and bioavailable artemisinin-derived drugs, and by using routes of delivery other than oral.

KEY WORDS: *Artemisia annua*, artemisinin, pharmacokinetics, ruminants, biological fate.

**RESUMO**

Embora a artemisinina seja o antimalarial mais usado para combater cepas de *Plasmodium* resistentes ao quinino, seu papel no campo da parasitologia veterinária está apenas iniciando. Existe uma urgência em desenvolver antelmínticos alternativos naturais para o controle de nematódios gastrintestinais resistentes aos anti-helmínticos atuais, tais como o *Haemonchus contortus*. A inexistência de trabalhos envolvendo ruminantes levou ao estudo da estabilidade química, biológica e da cinética da artemisinina (oral) em cabras. Em cultura de rúmen bovino, a artemisinina foi recuperada em 67 a 92% ao pH 6.8 e em 95% em um pH 3.0 após 24 horas de incubação. No estudo cinético em caprinos, a artemisinina foi metabolizada em diidroartemisinina após administração oral de 23 mg/kg de peso vivo, atingindo o pico (0.7µg/mL) em 12 horas. A artemisinina não metabolizada foi encontrada nas fezes (2.4µg/g), 24 horas após administração oral, indicando sua baixa biodisponibilidade quando fornecida em cápsulas e por via oral. O estudo em cultura de rúmen indica que a artemisinina se torna mais disponível se for dissolvida em etanol ou *dimetilsulfóxido*, enquanto que o estudo cinético indica que pode-se conseguir maior atividade biológica em se usando derivativos mais estáveis e outras vias de administraçao que não a oral.

PALAVRAS-CHAVE: *Artemisia annua*, artemisinina, pharmacocinética, ruminantes.

## INTRODUCTION

*Artemisia annua* L. (Asteraceae) is the only commercial source of artemisinin, a sesquiterpene lactone effective against multi-drug resistant *Plasmodium falciparum* malaria (KLAYMAN, 1993). Artemisinin-derived antimalarials (Fi-

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gure 1) are currently in high demand and artemisinin-based combination therapies (ACTs) have been adopted in 56 countries in Africa, Asia, and South America as an alternative to monotherapies against chloroquine-resistant malaria (WHO/RBM, 2006). Despite its worldwide reputation as a fast-acting, safe antimalarial, artemisinin has not been investigated for its potential role as an anti-parasitic drug in animals. Artemisinin and its derivatives were reported as significantly effective against the protozoan parasites *Babesia equi* (KUMAR et al., 2003), *Eimeria* spp. (ALLEN et al., 1997; ARAB et al., 2006), and *Neospora caninum* (KIM et al., 2002). Artemisinin activity against trematodes parasites has been found for the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini* (KEISER et al., 2006), and the blood fluke *Schistosoma mansoni* (immature stages) in mice, (UTZINGER et al., 2001a; LESCANO et al., 2004; XIAO et al., 2004). Although artemether (Figure 1) was not effective in treating canine lung fluke (*Paragonimus westermani)* infection (XUE et al., 2008), artemether alone or combined with praziquantel or hemin, was effective against *Schistosoma* sp. infections in animals and humans (UTZINGER et al., 2001a; UTZINGER et al., 2001b; XIAO et al., 2001; XIAO et al., 2002; XIAO et al., 2003).

The mode of action of Artemisinin-derived drugs against

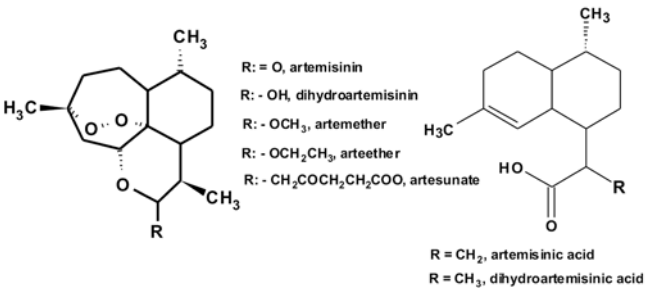


Figure 1. Artemisinin and its semi-synthetic antimalarials (left) are currently available where chloroquine is no longer effective against malaria. Artemisinin and artemether are also effective against protozoan and nematodal infestations in humans and animals. Artemisinin precursors (right) are present in field and greenhouse plants throughout the growing season.

malaria and cancer (EFFERTH et al., 2003), its low toxicity to mammals (KLAYMAN, 1993), and its proposed mode of action against trematodes and the protozoa *Plasmodium* spp. (KEISER; UTZINGER, 2007) suggest that pharmaceutical or plant artemisinin could be effective in fighting some gastrointestinal nematodes (GIN) in small ruminants. Artemisinin is hypothesized to exert its effect by reacting with heme groups from hemoglobin molecules digested by protozoa and by disrupting normal cell structure and functions in the parasite through artemisinin-derived free radicals (WRIGHT; WARHURST, 2002); through free radical damage on cell membranes after artemisinin activation by cytochrome C in the electron transport chain in the mitochondrial membrane (LI et al., 2005). Worms such as *Haemonchus contortus* lack the genes for heme biosynthesis, and require heme from cytochrome C or hemoglobin for growth and reproduction. *Caenorhabditis elegans* has its growth arrested at the L4 stage when grown in the absence of exogenous heme (RAO et al., 2005). This dependence on heme suggests that artemisinin might affect blood-sucking GIN development in a similar way that it affects *Plasmodium*.

The few studies involving artemisinin and its derivatives against parasites other than *Plasmodium* started in 1983 when artemisinin and artemether reduced *Clonorchis sinensis* burden in rats in 100% at 200 mg/kg and 83 to 100% at 30 to 60 mg/kg, respectively (CHEN et al., cited by SHU-HUA et al., 2008). Despite these promising results, it took 16 years to test artemisinin and artemether in humans, but doses were approximately 30 times lower than the successful ones used in rats in 1983 (TINGA et al., 1999). Not surprisingly, artemisinin failed to reduce fecal egg output from patients. Recently, artemether and artesunate at 75 mg/kg (SHU-HUA et al., 2008) were more effective than tribendimidine and praziquantel in reducing *C. sinensis* burden in rats.

In many parts of the world the most important GIN in sheep and goat production systems is *H. contortus*. The few commercial anthelmintics used to eliminate *H. contortus* from small ruminants are becoming less effective due to drug resistance. In addition, residues can be detected in the muscles, fat, and milk if the recommended waiting period is not observed (CHAGAS, 2004). The decreased effectiveness of commercial anthelmintics and consumer aversion to chemical residues in animal products have stimulated research on alternative approaches to control GIN (ATHANASIADOU; KYRIAZAKIS, 2004).

Currently, no study has addressed plant secondary metabolite stability, bioavailability, metabolism, or pharmacokinetics in ruminants. These factors are considered key to understanding the pharmacological and toxicological aspects of any drug and are now being given high priority earlier in the drug research and development process (NAVARATNAM et al., 2000). Reliable methods to quantify artemisinin and its derivatives in body fluids are difficult to develop, but artemisinin and its derivatives are known to be metabolized to the bioactive dihydroartemisinin, which is rapidly eliminated (WHITE, 1994). The objectives of this study were to elucidate the biological stability of artemisinin in bovine rumen fluid and its kinetics and biological fate in goats following oral administration of commercially available artemisinin preparations.

## MATERIAL AND METHODS

### Chemicals and reagents

Artemisinin standards (98% pure), acetonitrile, diethyl ether, dimethyl sulfoxide, and hexane, all reagent or HPLC grade, were purchased from Sigma (Saint Louis, MO). Crystallized artemisinin (98% pure) and Artemin® capsules (100 mg artemisinin + maltodextrin) were donated by Holley Pharmaceuticals (Fullerton, CA), and Nutricology artemisinin 100-mg capsules were donated by Allergy Research Group (Alameda, CA).

**Artemisinin metabolic stability in bovine rumen fluid**

Twelve tubes containing 30 mL of a mixture composed of bovine rumen fluid and McDougall's buffer (MCDOUGALL, 1948) at 60:40 (v/v) ratio, and 1.0 g of powdered alfalfa hay were randomly assigned to 3 replicates each of four treatment groups with the following compounds added: 1) 15 mg crystallized artemisinin (0.5 mg/ml), at pH 6.8 (rumen pH); 2) 100 mg artemisinin (3.3. mg/mL) from 100-mg Nutricology capsules, at pH 6.8; 3) 15 mg of crystallized artemisinin dissolved in ethanol (0.5 mg/ml) at pH 6.8; 4) the same as 2, but acidified with 12 *M* HCl to pH 3.0 (abomasum pH). All tubes were incubated at 39°C for 24 hours, and were shaken manually six times during incubation. The rumen fluid culture medium was vacuum filtered through Whatman #1 filter paper placed in a Buchner funnel attached to an Erlenmeyer flask. Retained alfalfa debris was rinsed with 25 ml of double distilled water. The filtered rumen fluid culture medium and the water rinse were combined into a separatory funnel and partitioned twice with 40 ml of hexane. The aqueous phase was discarded and the hexane phase was kept and named 'Hex fraction'. The alfalfa debris retained in the Buchner funnel was washed twice with 20 ml of hexane and was named 'Hex wash'. The alfalfa debris and the filter paper were refluxed (69°C) with 70 ml of hexane for 60 sec and named 'Hex reflux'. All hexane fractions were evaporated to dryness in a fume hood, reconstituted in acetonitrile, filtered through a 0.45 µm nylon membrane attached to a luer-lock disposable syringe and analyzed by high-performance liquid chromatography (HPLC) with photodiode array detection (PAD) (FERREIRA; GONZALEZ, 2009).

**Artemisinin pharmacokinetics in goats**

Five goats of approximately 30 kg received ten 100-mg Artemin® capsules transferred to a bolus capsule for oral delivery. HPLC-PAD analysis of capsules (data not shown) established that goats received and average 23 mg artemisinin/kg of body weight. A pretreatment (time zero) blood sample was collected from each goat, each animal being its own control. Following artemisinin administration to animals, 4.0 mL of blood was collected in heparinized syringes at 1, 2, 4, 8, 12, and 24 h after treatment. The plasma was collected and extracted for artemisinin analysis immediately or was frozen (-80°C) for extraction within 24 hours. Plasma (2.0 mL) was transferred to a 15-mL screw-top glass centrifuge tube. Following additions of 0.5 mL of a 0.9% NaCl solution, the tube contents were vortexed for 5 to10 s, and then 2.0 mL of ethyl acetate was added for partitioning. The tube was vortexed again, centrifuged for 10 min at 1580*g*, and the top ethyl acetate layer was transferred to a 4.0 mL glass flask. Partitioning with ethyl acetate was repeated, and the two ethyl acetate layers were combined. The flasks were closed with caps lined with polytetrafluoroethylene and stored at -80°C until analyzed by HPLC with a mass spectrometer detector (MS).

**Quality control of artemisinin capsules**

Three 100-mg Artemin® capsules (Holley Pharmaceuticals) were randomly removed from each of three bottles used in the pharmacokinetics experiment, the contents were dispensed in a 50-mL volumetric flask and the volume was completed with acetonitrile, to produce an expected concentration of 2.0 mg/mL artemisinin (n = 9). Three capsules were also removed from one bottle of Nutricology artemisinin (Allergy Research Group) used for the cow rumen fluid stability study and dissolved as described above (n = 3). All dissolved capsules were analyzed by HPLC-PAD in the same day.

**HPLC analysis**

HPLC-PAD analyses were performed according to Ferreira and Gonzalez (2008), using a Shimadzu HPLC system consisting of an in-line degasser (DGU14A), two pumps (LC-10ADvp), a temperature-controlled (4°C) autosampler (SIL-10ADvp), a photodiode array detector (SPD-M10ADvp), and a controller (SCL-10Avp) interfaced to a computer running LC Solutions v. 3.0 chromatography manager software (Shimadzu, Japan). The mobile phase consisted of (A) 0.1% acetic acid/methanol/acetonitrile (38.0:46.5:15.5 v/v) and (B) acetonitrile under isocratic (75% A and 25% B) conditions for 8 minutes. After each run, the column was rinsed for 2 minutes with 50% A and 50% B and then reequilibrated for 2 minutes with 75% A and 25% B before the next injection. Separations were performed on a 50 × 4.6 mm i.d., 3 µm, Gemini C18 110A° analytical column (Phenomenex, Torrance, CA, USA) operating at 30°C with a flow rate of 0.2 mL $min^{-1}$. An injection value of 20 µL was used for standards and samples. A calibration curve was generated for artemisinin with standards ranging from 0.125 to 2.0 mg/mL with a $R^2 = 0.999$ for HPLC-PAD. Detection was accomplished at 215 nm.

**Mass spectrometer conditions**

The HPLC-MS system consisted of the above HPLC Shimadzu system interfaced with a 2010A MS Shimadzu detector using an electrospray ionization probe in the positive mode as ion source. The temperatures for the probe, curved desolvation line, and heat block for the analysis were set using the values for the tuning file (250, 250, and 200°C, respectively). Nitrogen gas was used for the nebulizer at flow rate of 1.5 L/min; the detector voltage was set at 1.5 eV. A scan from *m/z* 100 to 500 was performed to identify the most abundant ions for each compound. Quantification for artemisinin and dihydroartemisinin was performed using selected ion monitoring of *m/z* 283.1 and 307.10, respectively. Retention time for dihydroartemisinin and artemisinin were 7.1 and 7.8 min, respectively.

**Statistics.** Statistical analyses were performed using SAS version 8.0 (SAS Institute Inc., Cary, NC). Means were compared using one-way analysis of variance and the Duncan's test of significance.

## RESULTS AND DISCUSSION

**Artemisinin metabolic stability in rumen fluid**

Artemisinin content of Nutricology capsules was confirmed

by HPLC-PAD (FERREIRA; GONZALEZ, 2009). The treatments in which the capsule contents were dispensed into the rumen fluid culture medium mimics the way artemisinin was administered to goats in the pharmacokinetics study. Artemisinin recoveries for treatment 1 to 4 were 67.2, 92.4, 70.4, and 95.0%, respectively (Figure 2A). For treatments 2 and 4, 82.4 to 85.3% of the recovered artemisinin was associated with the alfalfa debris extracted with hexane refluxing. Only 2 to 4% was dissolved in the rumen aqueous environment and 1.3 to 8.6% was removed with the hexane washes ('Hex wash'), suggesting that artemisinin was not irreversibly sorbed to the alfalfa hay, but was unsolubilized in rumen fluid culture medium. In treatment 1, 63.2% of the recovered artemisinin was probably retained in the filter paper or sorbed to the alfalfa debris, and was recovered by hexane refluxing. Only 2.7% was dissolved in rumen fluid, indicating poor solubility of artemisinin in aqueous medium. In treatment 3, approximately 30% was found in the hexane fraction (Figure 2B), resulting from the partitioning of the rumen fluid with hexane, while 26.0% was recovered from the alfalfa debris and 14.5% was unbound to alfalfa hay and recovered with the hexane washes. This suggests that although artemisinin was dissolved in ethanol before mixing with rumen fluid culture medium, approximately 15% of it precipitated from the solution in the presence of the aqueous medium. HPLC-PAD analysis of the ruminal fluid showed that the dissolved artemisinin was not metabolized to dihydroartemisinin by rumen microflora. Artemisinin was probably sorbed to the alfalfa hay and only partially extracted by hexane refluxing. Although artemisinin is structurally unrelated to currently used anthelmintics, absorption of fenbendazole to rumen contents was also observed after oral administration (KELLER, 2004), while moxidectin and ivermectins were found extensively (>90%) bound to sheep ruminal and abomasal contents when animals were fed on Lucerne hay (LIFSCHITZ et al., 2005). In addition, although no detectable metabolism occurred in ivermectin incubated in sheep and bovine rumen fluid at 38°C, its disappearance from rumen fluid in vitro was attributed to its binding to solids or surfaces (ANDREW; HALLEY, 1996). Thus, it seems that oral delivery of drugs to ruminants is less effective than other routes of administration that bypass the rumen.

In a previous experiment, artemisinin was dissolved in dimethyl sulfoxide and mixed with rumen fluid culture medium at 0.5, 1.0, and 2.0 mg/mL (n=3) before incubation (data not shown). Artemisinin recovery from rumen culture medium after partitioning with hexane was 36.4%. This recovery was slightly higher than that of treatment 3 using ethanol (29.7%) because in this dimethyl sulfoxide study, the hexane fraction and hexane washes were combined before partitioning. This suggests that dilution of artemisinin in ethanol or dimethyl sulfoxide makes artemisinin more miscible in an aqueous environment than crystalline or formulated (capsules) artemisinin.

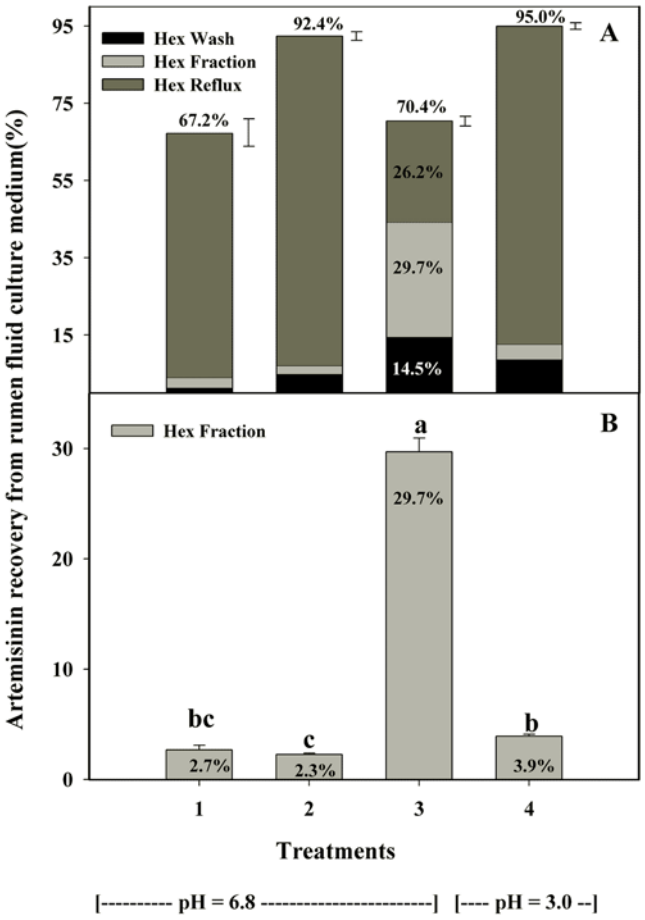


Figure 2. A) Artemisinin recoveries (HPLC-PAD) from rumen fluid culture medium incubated at 39°C for 24 hours. Rumen culture medium tubes (n=3) were added of artemisinin crystals (Treatment 1), artemisinin from capsules (Treatment 2), artemisinin crystals dissolved in ethanol (Treatment 3), or artemisinin capsules + HCl (Treatment 4) under rumen (6.8) pH (treatments 1-3) and abomasum (3.0) pH (treatment 4). Numbers on the top of columns represent total average recovery after all fractions were added. B) Isolated recovery of artemisinin dissolved in ethanol and recovered from rumen fluid culture medium after hexane partitioning. Different letters represent significant differences at *a*= 0.05 by Duncan's test. Bars represent standard deviation of the means.

**Artemisinin pharmacokinetic in goats**

Dyhydroartemisinin, the main metabolite of artemisinin-type compounds, is known to peak in human plasma within two hours of oral intake (NAVARATNAM et al., 2000), but started to appear in goat plasma at four hours after oral intake and peaked at 12 hours (Figure 3). Variation in mean plasma concentrations of dyhydroartemisinin (Figure 3) could be the result of individual differences added to the variation inherent to oral dosing. Coefficients of variation ranging from 30 to 40% were reported following oral dosing of Thai males with artemether (KARBWANG et al., 1997). Artemisinin is also known to have a short half life if taken orally and to have an extensive first pass metabolism in animals (WHITE, 1994). Our results show that the concentrations in plasma decreased almost by a factor of 4 from 12 to 24 h (0.7 μg/mL vs. 0.18 μg/mL). On the other hand, the concentration of unabsorbed artemisinin in feces at 24 h was 2.41 μg/g, decreasing sharply

at 30 h (Figure 3). High fecal artemisinin indicates poor bioavailability in the goat and possible adhesion to gastrointestinal contents, if taken orally. Previous data regarding metabolism of artemisinin-type compounds is scanty. In humans, only negligible amounts have been found in urine and feces (NAVARATNAM et al., 2000). Also, these authors reported that the bioavailability of artemisinin-type drugs was only 19 to 35% after oral administration to animals. However, bioavailabilities higher than 85% were achieved with intramuscular injections. Karbwang et al. (1997) reported that the bioavailability of oral artemether to animals or people was only 43.2% of that achieved intramuscularly. The fecal data indicate that most of the artemisinin is being eliminated by goats, which may prevent orally administered artemisinin from reaching therapeutic blood levels. Other anthelmintic drugs have lower bioavailability and altered pharmacokinetics in polygastric animals after oral administration, such as ivermectin (FLAJS; GRABNAR, 2002) and fenbendazole, the latter being only 25% absorbed by sheep, with up to 90% eliminated through feces within 3 days (KELLER, 2004). It is not known if artemisinin, even at higher doses will affect gastrointestinal nematodes. Furthermore, the effects of high fecal concentrations of artemisinin on GIN egg hatchability need to be tested. Studies with artemisia tea in humans indicate that artemisinin absorption is faster from tea than from oral solid formulations, but with a similar bioavailability (RATH et al., 2004). However, the clinical efficacy of the more stable artemether in humans is dependent on formulation, dosing scheme, duration of treatment, and the severity of disease (KARBWANG et al., 1997). This indicates that oral dosing of goats with the less stable artemisinin might not be as effective as other available alternative dosing strategies.

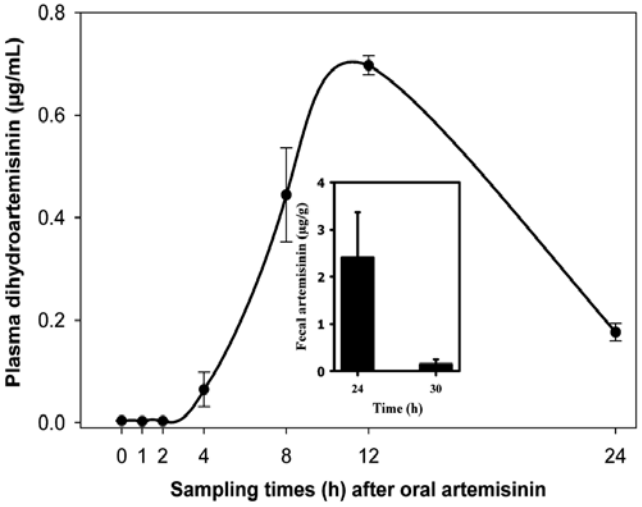


Figure 3. Pharmacokinetics of artemisinin, monitored through dihydroartemisinin concentration in plasma of goats that received 23.0 mg artemisinin/kg body weight. Inserted graph reflects artemisinin concentration from fecal samples after 24 and 30 hours of administration. Bars for plasma are standard errors (n=5, except for time 12 h, where n=4), while for feces bars represent standard deviation (n=5)

**Quality control of artemisinin capsules**

Analysis of 9 capsules from Holley Pharmaceutical artemisinin and from three capsules from Nutricology artemisinin by HPLC-PAD (Figure 4) resulted in 70.3% and 103.1%, respectively, of the artemisinin concentration stated on labels. This indicates the need for quality control in the capsules, before their use in animal studies, to secure that the right dosage will be delivered with minimal error possible. The lower artemisinin concentration from Holley capsules does not indicate adulteration of the product, but simply the existence of different manufacturing systems, which result in varying concentrations of the target compound in the final product. Both artemisinin crystals used as raw material for the capsules, provided by Holley and the Allergy Research Group, were analyzed by HPLC-PAD in our lab and were 98% pure artemisinin.

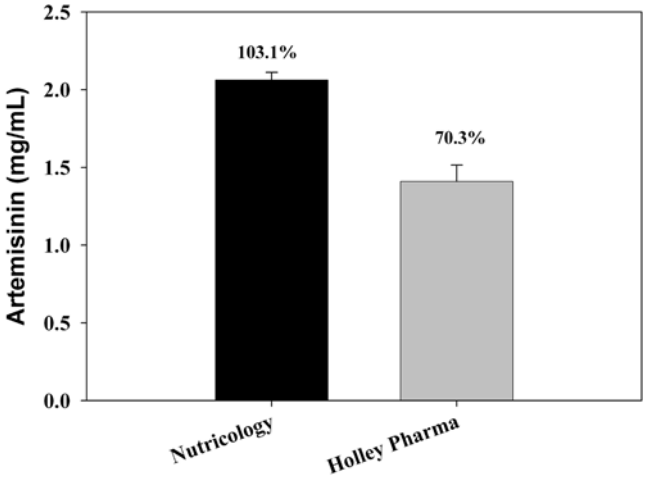


Figure 4. Artemisinin true concentration in 100-mg capsules from Nutricology (n=3) and Holey Pharma (n=9). Capsules were dissolved in acetonitrile to a final expected concentration of 2.0 mg/mL, filtered through a 0.45 µm nylon membrane luer-lock syringe filter, and immediately analyzed by HPLC-PAD.

In conclusion, the key to controlling blood-sucking parasites rests in the ability to deliver biologically active agents to the blood stream where they can disrupt the functioning of the parasite. When we rely on oral dosing, we have to deliver an agent that can survive rumen transformation or degradation or that can be transformed in products that are more biologically active than the parent compound. Artemisinin was reasonably stable after incubation in rumen fluid culture at pH 6.8, and very stable at pH 3.0. Thus, it can survive rumen fermentation to reach the gut, where it is absorbed into the blood, fact proved by its kinetics in goats. However, it is currently unknown if artemisinin and its precursors can be transferred from ingested dry *A. annua* leaves to the blood, where it would be most effective against blood-sucking parasites. The metabolic conversion of artemisinin to the bioactive dihydroartemisinin indicates that goats will absorb it even if fed as a solid formulation, but it is unknown if the concentrations of 0.4 to 0.7 µg/mL found in this study would

be pharmacologically effective as an anthelmintic. More effective GIN control might be achieved by combining artemisinin with flavonoids or other compounds that may act as synergists and potentiate the effect of artemisinin. The poor solubility of artemisinin in water and oil can be circumvented by using low-toxicity organic solvents like ethanol and dimethyl sulfoxide. The spread of GIN resistant to commercially available anthelmintics makes the discovery and development of alternative anthelmintics a priority.

While research on artemisinin as a potential anthelmintic is ongoing, the low toxicity and efficacy of artemisinin-derived drugs to control several parasitic protozoan species suggests that artemisinin drugs could provide effective control of protozoan parasites that afflict goats, such as *Eimeria* sp. More stable artemisinin derivatives need to be evaluated as anthelmintics through routes that both increase bioavailability, such as intramuscular and intravenous injection, and circumvent problems associated with the digestive tract.

**Acknowledgements:** We acknowledge John P. Snuffer, Jeffrey B. Ellison, and Kenneth N. Harless for the collection of animal fluids and set up of the in vitro study; Sarah Coffey for help in artemisinin extractions, Michael Liu (Holley Pharma) and Luba Volosko (Allergy Research Group) for providing artemisinin capsules and crystals, and Barry Harter and Diane Snuffer for their technical help. Thanks are also due to LABEX and the Brazilian College of Veterinary Parasitology for sponsoring the presentation of this work at the XV Brazilian Congress of Veterinary Parasitology in Curitiba, Brazil.

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Received on April 30, 2008.
Accepted for publication on September 14, 2008.