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# Pharmacokinetic behavior in sheep and cattle of 5-chloro-2-(methylthio)-6-(1-naphthyloxy)-1*H*-benzimidazole, a new fasciolicide agent

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The physicochemical properties, p$K_a$, Log *P* and solubility of compound alpha, (5-chloro-2-(methylthio)-6-(1-naphthyloxy)-1*H*-benzimidazole), a new fasciolicide agent, were characterized using conventional methods. Also, its pharmacokinetics was evaluated in sheep and cattle. In both species an oral dose of 12 mg/kg was administered. Blood samples were collected during 144 h and analyzed by using an HPLC assay. Results showed that compound alpha is a weak base with a p$K_a$ value of 2.87 and log *P* of 1.44. The solubility was very low in aqueous solvents. Pharmacokinetic studies showed that in both species compound alpha could not be detected at any sampling time. The mean half-life ($t_{½}$) values of alpha sulphoxide in sheep and cattle were 19.86 and 29.87 h, while the half-life values of alpha sulphone were 19.43 and 46.32 h respectively. $C_{max}$ values of alpha sulphoxide did not differ between species while alpha sulphone values were higher in cattle. Plasma protein binding of alpha sulphoxide was between 82% and 86%. These results, combined with the previous efficacy studies, suggest that compound alpha could be a promising fasciolicide agent.

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## INTRODUCTION

Fasciolosis is an important helminth infection caused by the trematode *Fasciola hepatica,* a parasite that infests the biliary ducts of many different species (ruminants, horses, humans). This fluke species occurs throughout the world and is a cause of serious economic loss in the animal husbandry industry.

A number of drugs have been used in control fasciolosis in animals. Drugs differ in their safety and mode of action. Fasciolicides fall into five main chemical groups: salicylanilides, halogenated phenols, sulfonamides, phenoxyalkanes and benzimidazoles (Fairweather & Boray, 1999). Salicylanilides and halogenated phenols can be regarded as close analogues and include: clioxanide, oxyclosanide, niclosamide, rafoxanide, closantel, nitroxynil, bithionol, hexaclorophene, and niclofolan. With the exception of niclosamide the above-mentioned compounds are usually marketed as flukicides for sheep and cattle, being highly effective against adult and to a lesser extent, immature flukes. Clorsulon, a compound belonging to the sulphonamides family, is also recommended for the control of adult flukes and frequently used in combination with ivermectin. Benzimidazoles have broad-spectrum of activity against a number of nematode, cestode, and trematode parasites (Lacey, 1990). Common benzimidazoles, such as cambendazole, fenbendazole, mebendazole, and oxfendazole, have little or no activity against *Fasciola hepatica*. Albendazole has been recommended, although its activity is restricted to mature flukes and requires elevated dose rates. Triclabendazole (TCBZ) was introduced in the early 1980s for the treatment of *Fasciola hepatica* infections in livestock. Because of its high activity against adult and juvenile stages of this parasite, it has been established as the principal anti-fluke drug on the market.

Considering that no fasciolicides have been marketed recently, a new benzimidazole derivative, 5-chloro-2-methylthio-6-(1-naphthyloxy)-1*H*-benzimidazole (known as 'alpha'), was synthesized at the Universidad Nacional Autónoma de México (Hernández-Campos *et al.*, 2002). *In vivo*, compound alpha was tested as fasciolicide in sheep and cattle, showing high effectiveness (Rivera *et al.*, 2002). When compound alpha was compared with TCBZ at 12 mg/kg p.o., closantel at 3.5 mg/kg s.c. and clorsulon at 2 mg/kg s.c., in naturally infected cattle, no statistical differences in the efficacy between groups were found (Vera *et al.*, 2003). An additional study showed that the effective dose of compound alpha in naturally and infected cattle was 12 mg/kg (Ibarra *et al.*, 2004). Its safety has also been evaluated (Vera *et al.*, 2006), showing a maximum tolerated dose of


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180 mg/kg with a safety index of 15 times the recommended dose.

Considering the promising activity exerted, and in order to enhance the ADME information, the main objective of this study was to evaluate some physicochemical properties (ionization, lipophilicity, and solubility) of compound alpha as well as its pharmacokinetic behavior in sheep and in cattle.

## MATERIALS AND METHODS

### *Reagents*

Compound alpha, alpha sulphoxide (α-SO) and alpha sulphone (α-$SO_2$), as well as the internal standard (IS) albendazole sulphoxide, were synthesized at the Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México.

Methanol and acetonitrile (ACN) were of chromatographic grade. Ethanol, dimethylsulphoxide (DMSO), *n*-octanol, hydrochloric acid, sodium hydroxide, sodium hydrogen phosphate, and sodium dihydrogen phosphate were of analytical grade.

### *Physicochemical properties*

The ionization constant (p$K_a$) of compound alpha was determined according to the method previously described by Albert and Serjeant (1984). The absorbance spectra in aqueous solutions over the pH range of 1–7 were measured using a Beckman DU68 UV–Vis Spectrophotometer (Beckman Instruments, Fullerton, CA, USA) to determine the absorption maxima of the neutral and ionized species. Analytical wavelength of 302 nm was selected; briefly, solutions of $10^{-5}$ M were titrated with 0.1 M NaOH. The determination of p$K_a$ value was performed by fitting the obtained data to the equation:

$$A_{obs} = [A_i + A_m(10^{pH-pK_a})]/(1 + 10^{pH-pK_a}),$$

where $A_{obs}$, $A_i$, and $A_m$ are the observed absorbance, the absorbance of the neutral species and the absorbance of the anionic form respectively.

### *Solubility*

Solubility was determined according to the procedure of Yalkowski *et al.* (1983). The solvents used were ACN, methanol, acetone, propylene glycol, DMSO, as well as 0.1 M hydrochloric acid and phosphate buffers (0.1 M) in the pH range 1–7.4. Tubes containing each compound were shaken at 100 strokes/min at 37 °C for 24 h. At this time a sample was collected and filtered through a 0.45 μm nylon filter. Drug concentration was determined by a spectrophotometric method.

### *Partition coefficient*

The octanol–water partition coefficient was determined by the traditional shake-flask technique. To 2 mL of a buffer solution (pH 7.4) containing 100 μM of compound alpha, 2 mL of *n*-octanol, previously saturated with buffer solution was added. Then, the mixture was shaken for 40 min, centrifuged and the aqueous and organic phases were separated. Samples were assayed by a spectrophotometric method. The partition coefficient (log *P*) was calculated according to the following equation:

$$\log P = \log([\text{drug}]_o/[\text{drug}]_{aq})$$

where $[\text{drug}]_o$ is the drug concentration in the organic phase and $[\text{drug}]_{aq}$ is the drug concentration in the aqueous phase.

### *Pharmacokinetic studies*

Extent of binding of α-SO was determined by equilibrium dialysis. For this, 1 mL of plasma was dialyzed against 1 mL phosphate buffer (pH 7.4) in the dialysis cell. α-SO at concentrations of 6.4 and 12.8 μg/mL were added to plasma compartment. After 4 h incubation, samples (0.5 mL) were removed from both compartments and analyzed the same day by HPLC.

Two separate studies in sheep and cattle were carried out at the Centro de Producción e Investigación para la Sanidad Animal (CEPIPSA) and at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) respectively. Protocols were approved by the local Animal Ethics Committee.

Study 1 was performed in eight female sheep, weighing between 50 and 60 kg. They were fed on concentrates and water being provided *ad libitum*. The animals received a single oral dose of 12 mg/kg body weight of compound alpha as a suspension, and blood samples were collected by jugular venipuncture into heparinized glass tubes at 0 (predose), 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 30, 36, 48, 72, 96 and 120 h. Plasma was separated by centrifugation and stored at −20 °C until analysis.

Study 2 was performed in seven bovines, three males and four females, weighing between 186 and 254 kg. They were maintained in tick-free individual pens and were fed with concentrates and alpha. Water was provided *ad libitum*. A single oral suspension dose of 12 mg/kg weight of compound alpha was given. Whole blood was collected by jugular venipuncture into heparinized glass tubes at 0, 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 42, 48, 60, 72, 96, 120 and 144 h postdosing. Plasma was separated immediately by centrifugation and stored at −20 °C until analysis.

### *Sample analysis*

In both studies samples were assayed by using an HPLC method previously reported (Del Rivero *et al.*, 1998) with some modifications. Briefly, 2 mL of plasma were spiked with 150 μL of internal standard (albendazole sulphoxide, 15 μg/mL) and extracted by passing through a preconditioned C18 Sep-Pak cartridge (Waters Associates, Milford, MA, USA). The sample was washed with 10 mL of water and eluted with 3 mL of methanol. The organic phase was evaporated to dryness under nitrogen gas. The residue was reconstituted with 0.5 mL of methanol and 20 μL aliquot was injected into the HPLC system.

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The chromatographic system consisted of a Shimadzu HPLC apparatus set at a wavelength of 304 nm and a C18 10 $\mu$ column (3.9 × 300 mm $\mu$-Bondapak; Waters). The system was run in the isocratic mode using methanol–acetonitrile–acetate buffer, 10 mM and pH 5 (40:30:30) as the mobile phase at a flow rate of 1 mL/min.

Using the described chromatographic conditions compounds alpha, α-SO and α-$SO_2$ were resolved with no interference from endogenous compounds.

The analytical method was validated prior to the start of the study. In sheep, the method was linear in the range of 0.2–12.8 $\mu$g/mL for compound alpha, α-SO and α-$SO_2$. In cattle, method was linear in the range of 0.2–25.6 $\mu$g/mL.

The limit of detection (LOD) was estimated by integrating the baseline threshold at the retention time of each compound in five spiked plasma samples. The LOD was defined as the mean 'noise'/internal standard peak area ratio plus 3 standard deviations (SD). The limit of quantification (LOQ) was estimated as the mean 'noise'/internal standard peak area ratio plus 6 SD. In cattle, the method estimated LOD values were 25 ng/mL for compounds alpha and α-SO as well as 50 ng/mL for α-$SO_2$. In sheep, the LOD values were 30 ng/mL for alpha and α-SO as well as 50 ng/mL for α-$SO_2$. In both species the LOQ values were 0.2 $\mu$g/mL for the three analytes. The recovery of drug analytes from plasma was calculated by comparison of the peak areas from spiked plasma samples with the peak areas resulting from direct injections of standards in mobile phase. In sheep mean absolute recoveries and coefficient of variations (CV) within the concentration range between 0.2 and 12 $\mu$g/mL (triplicate determinations) were 94% (CV: 6%) (alpha), 95% (CV: 8.0%) (α-SO), and 89% (CV: 4.1%) (α-$SO_2$).

In cattle the values obtained in the concentration range of 0.2–22.6 $\mu$g/mL were: 89% (CV: 8%) (alpha), 85% (CV: 7.0%) (α-SO), and 82% (CV: 6%) (α-$SO_2$). Precision (intra- and inter-assay) was determined by analyzing replicates of fortified plasma samples ($n$ = 5) with each compound at three different concentrations: 0.6, 2.4, and 9.6 $\mu$g/mL in sheep and 1.6, 8.4 and 16.8 $\mu$g/mL in cattle. Coefficient of variations ranged from 1.5% to 5.8%.

Data showed that the compounds were stable at −20 °C for at least 1.5 months with an average recovery between 94% and 97% respectively.

### *Pharmacokinetic analysis*

Plasma concentration vs. time curves of α-SO and α-$SO_2$ in each specie were fitted to a one-compartment open model with first order absorption and apparent first-order elimination using WINNONLIN version 4.01 software program (Pharsight Corporation, Mountain, CA, USA).

The maximum plasma concentration ($C_{max}$) and time to reach the maximum plasma concentration ($t_{max}$) were determined directly from the individual plasma concentration–time profiles. Terminal half-life ($t_{1/2\lambda z}$) was calculated as: $t_{1/2\lambda z}=\ln(2)/\lambda_z$, where $\lambda_z$ represents the first-order rate constant associated with the terminal (log linear) portion of the curve. The area under the plasma concentration–time curve (*AUC*) was calculated to the last sampling point using the trapezoidal rule and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope $\lambda_z$.

Total plasma clearance (*Cl/F*) was calculated as Dose/*AUC*, the volume of distribution ($V_d$/F) was estimated by dividing the clearance by the elimination rate constant, $\lambda_z$. The mean residence time (*MRT*) was determined by the method of (Yamaoka *et al.* 1978) as follows: $MRT = AUMC_{(0-\infty)} / AUC_{(0-\infty)}$ were $AUMC_{(0-\infty)}$ is the total area under the first moment curve.

## RESULTS AND DISCUSSION

### *Physicochemical properties*

The effects of a drug are related to three factors: its physicochemical properties, its pharmacokinetic parameters, and its intrinsic pharmacological activity. With regard to the physicochemical properties, Fig. 1 shows the distribution species diagram of compound alpha. Results showed a p$K_a$ value of 2.87 indicating that compound alpha is a weak base. The log $P$ value was 1.44 denoting the lipophilic feature of the compound. Solubility data ($\mu$g/mL) are listed in Table 1. It can be seen that as with other benzimidazoles, compound alpha is poorly water-soluble. The highest solubility in aqueous solutions was in 0.1 M HCl, probably due to the formation of the conjugated acid. These results suggest that absorption of the drug could be solubility-limited.

### *Pharmacokinetic studies*

Binding of α-SO to plasma proteins was high (82–85%) and similar at both concentrations.

As other benzimidazoles such as TCBZ or albendazole, compound alpha could not be detected at any sampling time in either sheep or cattle. The absence of compound alpha indicates that it was extensively metabolized presystemically either in the intestine or in the liver and oxidized to form the sulphoxide and sulphone metabolites. Like other sulphide benzimidazoles (albendazole, TCBZ) the sulphoxide metabolite, α-SO, has demonstrated to have flukicidal activity (McConville *et al.*, 2006). Sulphide and sulphoxide benzimidazoles are known

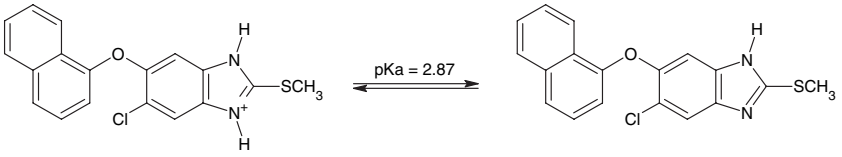


**Fig. 1.** Ionization equilibrium of compound alpha.

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**Table 1.** Solubility of compound alpha in solvents at 25 °C

| Solvent | Solubility ($\mu$g/mL) |
|---|---|
| Phosphate buffer solution pH = 7.4 | >0.02 |
| Phosphate buffer solution pH = 6.0 | 0.47 |
| Phosphate buffer solution pH = 2.2 | 3.80 |
| Phosphate buffer solution pH = 1.3 | 6.20 |
| NaOH 0.1 M | 0.32 |
| HCl 0.1 M | 10 |
| ACN | 9 |
| Acetone | 38 |
| Propylene glycol | 41 |
| Hexane | 62 |
| Methanol | 100 |
| DMSO | 210 |

ACN, acetonitrile; DMSO, dimethylsulphoxide.

to bind nematode tubulin (Lacey *et al.*, 1987) and therefore have activity against nematodes although sulphides exert an inhibitory activity on tubulin at lower concentrations than sulphoxides. In most species examined, the sulphoxide moiety predominates in plasma and is thought to confer activity against gut dwelling nematodes following secretion across the gastrointestinal wall into the gut lumen where it may undergo sulphoreduction (Mckellar & Scott, 1990).

The mean observed plasma metabolite concentrations in sheep and cattle are presented in Fig. 2. The results of the pharmacokinetic analysis for the metabolites in both species are summarized in Table 2.

The absorption rate constant ($k_a$) and the lag time data denote a slow appearance of the metabolites in plasma after oral administration. This fact could be related to the low solubility of the drug or to an association between drug molecules and the particulate material of the digesta which delays the rate of passage of the drug down the gastrointestinal tract and prolongs the duration of drug absorption (Hennessy, 1993).

In cattle the maximum concentration of α-SO and α-$SO_2$ in plasma were attained at 33.43 and 63.43 h respectively, indicating that the α-$SO_2$ remains in the body for a longer period of time. The delay in the appearance of the sulphone could be explained by its dependence on the formation of the α-SO, considering that as with other benzimidazoles this process has been characterized as slow (Lanusse *et al.*, 1993). A similar situation occurs in sheep, where the maximum plasma values of α-SO and α-$SO_2$ were 14.10 and 23.34 h. These data suggest that in sheep compound alpha was oxidized to its sulphoxide and released to the systemic circulation faster than in cattle, however, $C_{max}$ values were comparable in sheep (5.64 ± 1.33 $\mu$g/mL) and in cattle (5.11 ± 1.02 $\mu$g/mL).

Although in the absence of *F*, the values of *V/F* give only relative information about the magnitude of the distribution, the large value obtained for α-SO suggests an extensive tissue distribution. No significant differences were found in V/F, which indicates that distribution is similar in both species.

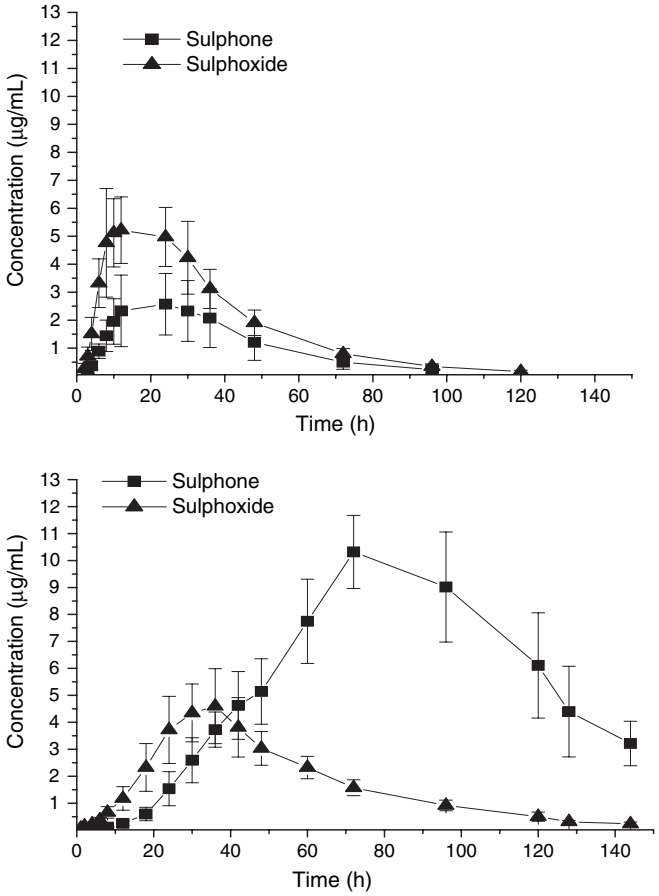


**Fig. 2.** Plasma concentration *vs.* time profile of the metabolites of compound alpha in sheep and in cattle.

Elimination half-life values confirmed the long-lasting α-SO concentrations in plasma. Half-life was longer in cattle than in sheep which could be due to a lower capacity of elimination in cattle.

Mean residence time is the mean time required for an intact drug molecule to transit through the body, including absorption, metabolism, distribution, and elimination. It can be estimated for any route of administration, and after oral administration the *MRT* would represent the residence time of the drug in the body plus the time required for absorption or metabolite formation. Although in both species *MRT* was long, a prolonged plasma detection period for α-SO was found in cattle resulting in significantly longer *MRT* (60%).

In cattle, the mean *AUC* value of α-$SO_2$ was 3.4 times higher than α-SO which suggests that in this species the first metabolite suffers an extensive oxidation. By contrast, in sheep the *AUC* for α-$SO_2$ was about two times lower than the *AUC* for α-SO. When the pharmacokinetic parameters of α-SO were compared with those obtained for TCBZ (Table 3), we found that in sheep, the half-life was longer than sulphoxide of TCBZ (16.0 ± 1.7 h), while in cattle, values were similar.

In conclusion, in the course of the present study we found that compound alpha is a weak basic lipophilic substance, which is

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**Table 2.** Pharmacokinetic parameters (mean ± SD) for alpha metabolites obtained after oral administration of a single dose of 12 mg/kg of alpha in sheep and cattle

| | Sheep | | Cattle | |
|---|---|---|---|---|
| Parameters | α-SO | α-$SO_2$ | α-SO | α-$SO_2$ |
| $K_a$ ($h^{-1}$) | 0.124 ± 0.02 | 0.098 ± 0.018 | 0.01 ± 0.0016* | 0.04 ± 0.026** |
| $T_{lag}$ (h) | 2.4 ± 0.51 | 3.6 ± 0.18 | 9.7 ± 1.5* | 17 ± 4.1** |
| $t_{½}$ (h) | 19.86 ± 1.18 | 19.43 ± 3.33 | 29.87 ± 8.84* | 46.32 ± 15.23** |
| $t_{max}$ (h) | 14.10 ± 6.44 | 23.34 ± 7.42 | 33.43 ± 4.72* | 65.14 ± 6.41** |
| $C_{max}$ (μg/mL) | 5.64 ± 1.33 | 2.32 ± 0.85 | 5.11 ± 0.83 | 10.05 ± 1.87** |
| $AUC_{0-\infty}$ (μg·h/mL) | 232.8 ± 50.5 | 101.32 ± 39.6 | 256.16 ± 19.6 * | 877.5 ± 295.9** |
| *V/F* (L/kg) | 1.54 ± 0.38 | 3.24 ± 1.4 | 1.72 ± 0.4 | 0.65 ± 0.23** |
| *Cl/F*(L/h·kg) | 0.053 ± 0.011 | 0.11 ± 0.05 | 0.04 ± 0.009 | 0.01 ± 0.0018** |

$C_{max}$, peak plasma concentration; $t_{max}$, time of the peak plasma concentration; $t_{½}$, elimination half life; *AUC*, area under the concentration vs. time curve; *MRT*, mean residence time; *V/*F, distribution volume/*F*; *Cl/F*, Clearance/*F*.
Significant differences between species (*) α-SO, (**) α-$SO_2$, ($P < 0.05$).

**Table 3.** Pharmacokinetic parameters of triclabendazole metabolites in sheep and cattle

| Specie | Dosage (mg/kg) | Metabolite | $C_{max}$ (μg/mL) | $t_{max}$ (h) | $t_{½}$ (h) | References |
|---|---|---|---|---|---|---|
| Sheep | 10 | TCBZ-SO | 13.1 ± 1.4 | 34.1 ± 1.8 | 16.0 ± 1.7 | Mohammed-Ali *et al.*, (1986) |
| | | TCBZ-$SO_2$ | 13.7 ± 1.0 | 42.0 ± 2.4 | 25.4 ± 1.0 | |
| Cattle | 12 | TCBZ-SO | 10.7 ± 2.32 | 30.00 ± 6.57 | 23.8 ± 3.85 | Mestorino *et al.*, (2007) |
| | | TCBZ-$SO_2$ | 15.6 ± 2.3 | 42.00 ± 6.6 | 52.9 ± 8.5 | |

TCBZ-SO, triclabendazole sulphoxide; TCBZ-$SO_2$, triclabendazole sulphone.

extensively metabolized. The α-SO metabolite demonstrates extensive protein binding and a prolonged half-life. The results obtained in both animal species combined with the previous efficacy findings suggest that this drug could be a promising fasciolicide agent.

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