Comparative Disposition and Metabolism of Paraherquamide in Sheep, Gerbils, and Dogs

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ABSTRACT:
The disposition and metabolism of paraherquamide (PHQ), a potent and broad-spectrum anthelmintic, were examined in sheep, dogs, and gerbils. The metabolism of PHQ in these species was extensive and marked by significant species differences both in vitro and in vivo. In sheep and gerbils, PHQ metabolism occurs mainly at the pyrrolidine moiety, generating several metabolites that, for the most part, retained nematocidal activity in vitro. In dogs, the dioxepine group was also extensively metabolized, ultimately resulting in formation of a catechol and loss of pharmacological activity. After oral administration of [3H]PHQ to intact sheep, gerbils, and dogs, the majority of the administered radioactivity was recovered in feces. Intact PHQ accounted for 0% (dogs) to ~30% (sheep and gerbils) of drug-related material in feces. A detailed investigation of the composition of the intestinal content of sheep indicated that a significant amount of the dose was still present in the rumen 24 h after dose and that PHQ underwent significant dehydrogenation in the cecum. The oral pharmacokinetic parameters of PHQ in sheep and dogs suggest that its absorption is rapid in both species but that its apparent elimination rate is significantly higher in the dog ($t_{1/2}$ = 1.5 h) than it is in sheep ($t_{1/2}$ = 6.5 h). The short elimination half-life and the absence of PHQ or other active components in the dog gastrointestinal tract provide a potential explanation of the lack of efficacy of PHQ in this species.

Paraherquamide (PHQ; Fig. 1) is an oxindole alkaloid anthelmintic that was originally isolated from cultures of Penicillium paraherquei (Yamazaki et al., 1981). Its potent antiparasitic activity was first discovered in a rodent model (Ostlind et al., 1990) in which gerbils were infected with immature Trichostrongylus colubriformis. PHQ was found to be effective orally at 1.5 mg/kg and well tolerated at doses up to 200 mg/kg. Subsequent studies in our laboratories revealed that PHQ has broad-spectrum nematocidal activity in sheep and that a single 0.5-mg/kg oral dose was effective against six common nematodes from the ovine gastrointestinal tract, including an ivermectin-resistant strain of Hemonchus contortus, a parasite known to cause significant losses in affected flocks (Shoop et al., 1990; Sargison et al., 2001). Only Oesophagostomum columbianum, a parasitic residing in the lower bowel, showed some resistance and required doses above 4 mg/kg for efficacy. No adverse effects were observed in sheep at oral doses up to 20 mg/kg. PHQ was likewise effective and well tolerated in calves (Shoop et al., 1992a).

In dogs, however, PHQ was not only inactive against most of the nematode species examined but also produced adverse effects within 1 h of treatment that included depression, ataxia, and protrusion of the nictitating membrane at doses ranging from 0.5 to 2 mg/kg (Shoop et al., 1991). These adverse effects were only marginally reversible over 7 days, and in subsequent studies in our laboratories, they were also accompanied by occasional deaths (Shoop et al., 1992a). More recent studies have shown that PHQ is an antagonist of nicotinic cholinergic receptors in nematodes and mammals (Robertson et al., 2002; Zinser et al., 2002) and suggest that PHQ exerts its nematocidal action by inducing nematode paralysis through blockade of cholinergic neuro-muscular transmission. The adverse effects observed in dogs may result from the same mechanism.

The notable differences in the pharmacological and toxicological effects of PHQ in sheep and gerbils, in which it is effective and safe, and that in dogs, in which it is largely ineffective and toxic, are intriguing. The underlying cause for such differences is likely to be multifactorial and may involve differences in the affinity of PHQ to cholinergic receptors in these species as well as species-specific pharmacokinetics, metabolism, or both. The objectives of the present study were to compare the pharmacokinetics and metabolism of PHQ in sheep, gerbils, and dogs.

Materials and Methods

Chemicals. PHQ was isolated as described previously (Yamazaki et al., 1981), and [5-3H]PHQ was prepared by the Merck Labeled Compound Synthesis Group (Rahway, NJ) (Blizzard et al., 1990). All other chemicals were of the highest analytical purity available.

In Vitro Metabolism. Liver microsomes were prepared according to a standard protocol (Raucy and Lasker, 1991). [3H]PHQ (10 μM) was incubated with liver microsomes (2 mg/ml) at 37°C for 60 min in 100 mM phosphate buffer with liver microsomes (2 mg/ml) at 37°C for 60 min in 100 mM phosphate buffer with liver microsomes (2 mg/ml) at 37°C for 60 min in 100 mM phosphate buffer.
buffer containing 5 mM MgCl₂ and 1 mM NADPH in total incubation volume of 2 ml. Disappearance of the parent was monitored by taking 50-μl aliquots and quenching the reaction with 100 μl of acetonitrile containing an internal standard. After protein removal by centrifugation, samples were analyzed as described under Plasma Pharmacokinetics. After 60 min, the remainder of the reaction mixture was treated using 2 volumes of acetonitrile, stored at 4°C for 1 h, and then centrifuged to remove the precipitated protein. The supernatant was concentrated under a stream of nitrogen and analyzed by liquid chromatography-mass spectrometry coupled with online radiometric detection (Metabolite Identification). For NMR analysis of the metabolites produced from sheep liver microsomes, the reaction volume was increased to 100 ml, and products were extracted by solid phase extraction using a 12-ml OASIS HLB cartridge (Waters, Milford, MA) eluted with acetonitrile. Isolation of the metabolites was performed by semipreparative high-pressure liquid chromatography (Metabolite Identification).

Animal Studies. All studies were conducted in accordance with federal guidelines for the care and use of laboratory animals and were approved by the Merck and Co., Inc. Institutional Animal Care and Use Committee. Dosing solutions were prepared by dissolving PHQ in propylene glycol/glycerol formal (60:40). Male Mongolian gerbils were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and weighed approximately 80 g. Male beagle dogs were purchased from Marshals Farms (North Rose, NY) and weighed ~10 kg. Castrated lambs weighing roughly 40 kg were from Branchburg Farm (Merck Research Laboratories) flock. Animals were fasted overnight and were provided access to food 4 h after dosing. Dogs, sheep, and gerbils were housed in metabolism cages throughout the studies.

In Vitro Activity against H. contortus. Activity against H. contortus was performed using a larval development assay based on a previously published technique (Hubert and Kerboeuf, 1992). In the larval development assay, embryonation, hatching, feeding, and molting to the larval stage 3 developmental stage occurred in constant contact with the test compounds during a 5-day period. Larval motility was assessed using a digital camera and a computerized pixel reader.

Pharmacokinetic and Excretion Studies. Pharmacokinetics and excretion of PHQ in sheep and dogs were performed after a single 0.5-mg/kg oral dose of [3H]PHQ. Each animal received ~200 μCi. Blood samples were collected in heparinized tubes via the jugular vein at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12 (sheep only), 24, 48, and 72 h, and plasma was obtained by centrifugation at 3000 rpm for 10 min at 4°C. Urine and feces were collected at 24-h intervals. For sheep, four animals in total were used, and one sheep was euthanized every 24 h. The entire GI tract was divided by sections, and the contents were collected and homogenized. A sample of bile was also collected at each time point. Excretion and metabolism studies in gerbils were performed after a single oral dose of [3H]PHQ (20 mg/kg; ~20 μCi/subject). The total radioactivity content in plasma, urine, and bile was determined by mixing an aliquot (0.02–0.1 ml) with 7 ml of Econo 2 Scintillant (Thermo Fisher Scientific, Waltham, MA) before analysis in a Tri-Carb 1900TR liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Radioactivity in intestinal contents and fecal homogenates was determined after combustion in a sample oxidizer (model 307, PerkinElmer Life and Analytical Sciences) and scintillation counting.

Plasma Pharmacokinetics. Plasma concentrations of PHQ were determined by liquid chromatography-tandem mass spectrometry on a TSQ7000 mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray source operated in the positive mode and interfaced with two PerkinElmer series 200 micropumps and a PerkinElmer series 200 autosampler (PerkinElmer Life and Analytical Sciences). Plasma samples (0.1 ml) were precipitated with 2 volumes of acetonitrile containing an internal standard. Samples were kept at 4°C for 1 h before centrifugation at 3000 rpm for 30 min. The supernatants were analyzed using a ThermoHypersil Betabasic C18 column (20 × 2 mm; 5 μm) (Thermo Fisher Scientific) with a gradient of solvent B (water/acetonitrile/acetic acid; 5:95:0.1) into solvent A (water/acetonitrile/acetic acid; 95:5:0.1). A sample of bile was also collected at each time point. Excretion and fecal homogenates was determined after combustion in a sample oxidizer and scintillation counting.

Results

In Vitro Metabolism. PHQ was rapidly metabolized by sheep (20% remaining after 1 h), dog (10% remaining after 30 min), and gerbil (7% remaining after 15 min) liver microsomes fortified with NADPH. Representative high-pressure liquid chromatography-radiochromatograms of the incubations after 60 min are shown in Fig. 2. The structures of the observed metabolites were elucidated by electrospray ionization multiple-step tandem mass spectrometry (Table 1), and the proposed structures are shown in Fig. 4. The major metabolites detected in sheep liver microsomes were also analyzed by 1H NMR (Table 2).

In positive ionization mode, PHQ gave a protonated molecular ion at m/z 494, which upon CID (MS²) gave a base ion at m/z 476 that further fragmented (MS³) to give a base ion at m/z 419, indicating successive neutral losses of H₂O and CH₃NCO (Table 1; Fig. 3). Subsequent fragmentation of the ion at m/z 419 gave ions at m/z 134...
and 176 (base), which resulted from neutral losses that included the oxindole moiety. The oxindole group itself gave a signature fragment ion at m/z 244. The 1H NMR spectrum of PHQ has been published previously (Liesch and Wichmann, 1990; Blanchflower et al., 1991), and only the chemical shifts of the H12 to 17 protons are given here (Table 2).

Metabolite M1, the predominant metabolite in dog microsomes, had a protonated molecular ion at m/z 428 corresponding to a metabolic loss of 66 Da from the parent PHQ. The characteristic ion at m/z 176 observed in the MS³ product ion spectrum of M1 indicated this loss could arise only from cleavage of the dioxepene moiety. The structure of M1 was confirmed by comparison with an authentic standard prepared by incubation of PHQ with 0.1 N HCl (data not shown), which selectively cleaves the isoprenoid group.

Metabolite M2 was the second most predominant metabolite in dog liver microsomes and had a protonated molecular ion at m/z 408. Subsequent CID analysis resulted in product ions at m/z 351 (loss of 57 Da or CH₃NCO), 174, and 132. Its structure (Fig. 4) was proposed by analogy with that of M3 (see below).

Metabolite M3 was the major metabolite formed when PHQ was incubated with sheep liver microsomes. It had a protonated molecular ion at m/z 474, which upon CID yielded a base ion at m/z 417 (loss of 57 Da). Further fragmentation of the ion at m/z 417 gave product ions at m/z 244, 174, and 132 (Table 1). The first of these ions indicated that the oxindole portion of the molecule was unchanged. The latter two ions, along with the absence of water loss in the product ion scan of the molecular ion of M3, suggested that M3 was the product of dehydration and a net dehydrogenation. The 1H NMR spectrum of M3 was characterized by absence of the methylene groups at positions 15 and 16 and of the methyl group at position 17 and appearance of new vinylic signals at 6.90, 6.34, and 6.25 ppm (Table 2), consistent with the formation of a conjugated system at the pyrrolidine ring of PHQ (Fig. 4).

Coeluting metabolites M4 and M5 were formed in a 3:1 mixture (by 1H NMR; data not shown) and were the major metabolites identified in gerbil liver microsomes. M4 had a protonated molecular ions at m/z 506 and product ions at m/z 488, 431, and 403, corresponding to the subsequent neutral losses of H₂O, CH₃NCO, and CO (Table 1). The loss of CO suggested the presence of a carbonyl group. The 1H NMR spectrum of M4 was characterized by the absence of the methylene signals at position 15 and 16 that were replaced by a single vinylic signal at δ = 6.08 ppm. The original methyl group at position

![Fig. 2. Representative radiochromatograms of incubations of PHQ (10 μM) with sheep, dog, and gerbil liver microsomes (2 mg/ml).](image-url)
17 was replaced with a methylene group that was shifted ~3 ppm downfield (Table 2). Together, these data indicated that M4 arose from successive oxidation at C14 to a carbonyl group, dehydration, and hydroxylation at C17 (Fig. 4). Formation of M4 was nearly quantitative when PHQ was incubated with NADPH-fortified sheep liver microsomes (data not shown). Metabolite M5 had a protonated molecular ion at m/z 508, which upon CID (MS²) gave fragment ions at m/z 480 and 449, indicating neutral losses of CO and CH₃NCO. Further fragmentation of m/z 449 resulted in loss of water (m/z 431), suggesting the presence of a primary or secondary hydroxyl group. The ¹H NMR spectrum of M5 was characterized by loss of the methylene signal at position 14 and only minimal changes in the methyl signal at position 17. The structure of M5 was consistent with oxidation at position 16 to the corresponding pyridone.

Metabolites M6 and M8 had protonated molecular ions at m/z 519 corresponding to the addition of 25 Da. CID of M6 resulted in a base ion at m/z 492 (~ HCN) and an ion at m/z 501 (~ H₂O). The MS³ spectra of M6 and M8 were very nearly identical, with a base peak at m/z 444 and ions at m/z 417, 244, and 174. The ¹H NMR spectra of M6 and M8 were characterized by disappearance of a single proton signal at position 16. The only possible structures consistent with the spectroscopic data of M6 and M8 are two diastereomeric cyano derivatives at position 16 (Fig. 4). High-resolution accurate mass analysis of M6 and M8 afforded molecular ions at m/z 519.2584 (Δ = −3.5 ppm) and 519.2587 (Δ = −2.9 ppm), respectively, in good agreement with the expected mass of 519.2602 (C₂₉H₃₅N₄O₅). This finding suggested a contamination of the microsomal incubation systems with cyanide, possibly arising from metabolism of acetonitrile, the solvent used to dispense PHQ (Freeman and Hayes, 1988). Replacement of acetonitrile with methanol as carrier and reaction-quenching solvent resulted in complete disappearance of M6 and M8. Furthermore, the molecular ion peak of M6 and M8 was increased by 1 mass unit, to m/z 520, when the incubation was run in the presence of CH₃CN as carrier solvent.

Metabolite M7 had a protonated molecular ion at m/z 490, which upon CID gave a base ion at m/z 433 (~ CH₃NCO) that in turn fragmented to yield an ion at m/z 405 (~ CO). The ¹H NMR spectrum of M7 was characterized by loss of the proton signal at position 16 and disappearance of the methylene signal at position 15. These signals were replaced by a single vinylc proton at δ = 5.92 ppm.
Taken together, these data indicated that M7 was likely formed by oxidation at position 16 to a carbonyl group followed by elimination of water to form an α,β unsaturated lactam (Fig. 4).

The incubation of PHQ with dog liver microsomes was characterized by several unique metabolites. Metabolite M9 had a protonated molecular ion at m/z 510 consistent with monoxygenation. The fragmentation pattern did not provide sufficient information to assign a site of metabolism. Metabolite M10 had a protonated molecular ion at m/z 608. Its product ion spectrum included ions at m/z 590 (loss of H₂O), which further fragmented to yield a base ion at m/z 510 (neutral loss of 80 Da) and an ion at m/z 492 (neutral loss of 98 Da). These two ions strongly suggest the presence of either an aliphatic sulfate group or an aliphatic phosphate group. High-resolution accurate mass analysis of M10 yielded a molecular ion at m/z 608.2352, in agreement with the molecular formula of a phosphate conjugate (C₂₈H₃₉N₃O₁₀P, calc. m/z 608.2368, Δ = 2.6 ppm). A possible structure is given in Fig. 4. Metabolite M11 had a protonated molecular ion at m/z 588 which upon CID gave a base ion at m/z 531 (loss of CH₂NCO), which subsequently lost 80 (m/z 451) and 96 Da (m/z 433). A tentative structure was proposed by analogy with that of M10 (Fig. 4). Metabolites M12 and M13 had a protonated molecular ions at m/z 453 consistent with cyanide adducts of M1, the main metabolite in dog liver microsomes.

In Vitro Activity of PHQ Metabolites against H. contortus. The major metabolites of PHQ identified in sheep liver microsomes were isolated and tested for nematodicidal activity against H. contortus at 1 μg/ml. At that concentration, PHQ causes an 80% decrease in the motility of H. contortus larvae. None of the metabolites tested were significantly more active than PHQ (Fig. 5). However, M4 showed a significantly reduced activity. Prior testing in our laboratories indicated that M1, the major metabolite identified in dog liver microsomes was...
that in dogs (Table 3). In sheep, the PHQ AUC accounted for roughly
shorter (1.5 h), so that at 10 h after dose, the plasma concentration of
8.5 h, whereas the apparent
\[ C_{\text{max}} \] (ng/ml) 38 46 73 167
\[ T_{\text{max}} \] (h) 0.5 8 0.5 0.5
\[ \text{AUC}_{\text{0-last}} \] (ng · h/ml) 547 1192 136 1387
\[ t_{1/2} \] (h) 8.5 13 1.5 23

TABLE 3
Pharmacokinetic of PHQ and total DRM in sheep [n = 4 (0–24 h); n = 3 (24–48 h); n = 2 (48–72 h)] and beagle dogs (n = 2) following a single 0.5-mg/kg oral dose of [\(^3\)H]PHQ

TABLE 4
Excretion of radioactivity following a single oral dose of [\(^3\)H]PHQ to sheep, dogs, and gerbils

TABLE 5
Distribution of radioactivity in the gastrointestinal tract of sheep following a single 0.5-mg/kg oral dose of [\(^3\)H]PHQ

somines, was pharmacologically inactive against nematodes. M5 could
not be isolated in sufficient amounts, and its activity against H. contortus was not tested; however, it was inactive against T. colubriformis in vivo (Liesch and Wichmann, 1990; Blanchflower et al., 1991).

**Pharmacokinetics.** The oral pharmacokinetics of PHQ and total DRM were evaluated in sheep and dogs after a single 0.5-mg/kg dose of [\(^3\)H]PHQ. The absorption of PHQ was rapid in both species, and maximum plasma concentration was reached at a \( T_{\text{max}} \) of 0.5 h, with a \( C_{\text{max}} \) of 38 and 73 ng/ml in sheep and dogs, respectively (Fig. 6). In sheep, the plasma concentration of PHQ reached a second concentration maximum plasma concentration was reached at a

\[ C_{\text{max}} \] (ng/ml) 38 46 73 167
\[ T_{\text{max}} \] (h) 0.5 8 0.5 0.5
\[ \text{AUC}_{\text{0-last}} \] (ng · h/ml) 547 1192 136 1387
\[ t_{1/2} \] (h) 8.5 13 1.5 23

**Excretion.** After oral administration of [\(^3\)H]PHQ to sheep, dogs, and gerbils (0.5, 0.5, and 20 mg/kg, respectively), >80% of the radioactivity was recovered in feces and urine after a 72- to 96-h

**In Vivo Metabolism.** *Feces.* Representative radiochromatograms of feces extracts of sheep, dogs, and gerbils after oral dosing of [\(^3\)H]PHQ are shown in Fig. 7. Unchanged PHQ accounted for <10% of total radioactivity in sheep and gerbil feces. No PHQ could be detected in dog feces. M14, the major metabolite detected in sheep and gerbil feces, had a protonated molecular ion at \( m/z \) 476, consistent with dehydration of PHQ. The exact position of the double bond could not be determined, but it was tentatively assigned as endocyclic (Fig. 4). Previous unpublished studies in our laboratories have shown that dehydration at the pyrrolidine moiety resulted in a loss in activity in vitro. Other metabolites in sheep and gerbil feces included M3, M4, and M5. Interestingly, the cyanide adducts M6 and M8 identified in microsomal incubations were also observed in gerbil feces. Similar to that observed in liver microsomes incubations, the metabolite profile in dog feces was markedly different from those obtained from sheep and gerbils, and M1 and M2 were the main metabolites excreted in dog feces. M15, the third most abundant metabolite in dog feces, had a protonated molecular ion at \( m/z \) 530 that yielded product ions at \( m/z \) 512, 410 [neutral loss of 102 Da (C\(_3\)H\(_5\)NCO) from \( m/z \) 512], and 353 (loss of CH\(_3\)NCO from \( m/z \) 410). This fragmentation pattern is consistent with a ring opening of the dioxepene moiety followed by reduction of the resulting aldehyde. M16 had a protonated molecular ion at \( m/z \) 410, and it was assigned to dehydrated M1. M17 had a protonated molec-
ular ion at $m/z$ 544, which gave fragment ions at $m/z$ 526, 469, 410, and 353. The latter two fragments indicated that the structure of M17 was related to that of M15. The additional 14 Da in its molecular ions suggested oxidation of the terminal hydroxyl group to a carboxylic acid.

**Urine.** The major metabolites in sheep and gerbil urine were M3, M4, and M5 (Fig. 8). In contrast, M21 was the major drug-related component in dog urine. It had a protonated molecular ion at $m/z$ 508, which gave fragment ions at $m/z$ 490 (loss of H$_2$O) and 410 (loss of 80 Da from $m/z$ 490). Taken together, these data suggested that M21 is a sulfate conjugate of M1. Other metabolites unique to dog urine were M18 ($m/z$ 618), a glucuronic acid conjugate of M1, and M19 ($m/z$ 442), a pyridinol derivative of M1. Interestingly, M20 was detected in dog and gerbil urine. It had a protonated molecular ion at $m/z$ 631, with characteristic fragment ions at $m/z$ 469, 410, and 353 that were also observed in M17 (Table 1), indicating opening of the dioxepene ring. An additional fragment ion at $m/z$ 526 corresponded to a neutral loss of 105 Da (C$_3$H$_7$O$_3$N), suggesting that M20 is a serine conjugate of M17.

**Plasma.** PHQ was the major radioactive component identified in sheep plasma and gerbil blood (Fig. 9). Other circulating metabolites in sheep plasma included M3, M4, M5, M7, and M14. The two cyanide adducts (M6 and M8) detected in microsomal incubations were also found in significant amounts in gerbil blood. In dog plasma, the main circulating components were PHQ (<30%), M15, and M21.

**PHQ metabolite distribution in the GI tract of sheep.** After a single oral dose of 0.5 mg/kg [3H]PHQ to sheep, the concentration of DRM in the GI tract 24 h after dose varied from ~350 ng Eq/g in the reticulum to 8150 ng Eq/g in the colon (Table 6). At this time point, the total concentration of DRM in plasma was only ~20 ng Eq/ml (Fig. 6). At 48 h after dose, the concentration of DRM in the GI tract varied from 69 ng Eq/g in the rumen to 1220 ng Eq/g in the colon, whereas the DRM in plasma decayed to ~7 ng Eq/ml. Metabolite profiling of the GI tract content at 24 h after dosing indicated that PHQ was the main component of DRM in the four chambers of the sheep stomach. A small amount (~8%) of the cyano derivative, M6, was detected in the rumen. The major components of sheep bile at 24 h after dose were M3 and M7, whereas PHQ constituted only 17%
of the bile. The overall composition of intestinal content undergoes changes as the DRM travels down the intestinal tract, and M14 and M4/M5 were the major components identified in sheep colon.

Discussion

The metabolism of paraherquamidine in sheep, dogs, and gerbils in vivo and in vitro is extensive, but it is almost exclusively limited to the pyrrolidine and dioxepene groups. Metabolism and ultimate loss of the dioxepene moiety are most effective in dog liver microsome preparations and also constituted the major route of metabolism in that species in vivo. A likely mechanism for M1 formation is presented in Fig. 10. Epoxidation of the isoprenoid double bond followed by hydrolysis leads to formation of a cyclic hemiacetal in equilibrium with its ring-opened isomeric α-hydroxy aldehyde. The oxindolyl catechol M1, being a good leaving group, can be displaced by a retro-Michael reaction. Interestingly, in vivo in the dog, the α-hydroxy aldehyde intermediate is oxidized to the acid M17 that is further conjugated with serine to yield M20. Conjugation with serine is, apparently, a relatively rare event (Reif and Sinsheimer, 1975), probably because serine is a poor substrate for acyl CoA:amino acid N-acyl transferases (van der Westhuizen et al., 2000). Metabolism of the pyrrolidone moiety of PHQ is most likely initiated by oxidation of the methylene α to the nitrogen (Fig. 11). The intermediate carbinolamine undergoes further oxidation to M5 or two successive dehydration steps, leading to the formation of M3. Alternatively, it seems that it is also converted to an iminium ion, which is trapped by cyanide to form the stereoisomeric cyano conjugates M6 and M8 (Fig. 11). The identification of these two metabolites in microsomal incubations from all species was intriguing since cyanide was not added to the incubation mixtures. Incorporation of C-13 cyanide into M6 and M8 when the reaction was carried out in the presence of 1% CH313CN indicated that cyanide was produced from microsomal metabolism of acetonitrile (Freeman and Hayes, 1988). From the kinetics of this reaction, it is expected that the levels of cyanide could reach up to 250 μM at 60 min and therefore reach sufficient levels to trap any iminium ions formed from PHQ. Surprisingly, M6 and M8 were also observed
TABLE 6

Gastrointestinal content composition following a single 0.5-mg/kg oral dose of [3H]PHQ to sheep

Values in parentheses for sheep 1 to 4 represent time of necropsy.

<table>
<thead>
<tr>
<th></th>
<th>Sheep 1 (24 h)</th>
<th>Sheep 2 (48 h)</th>
<th>Sheep 3 (72 h)</th>
<th>Sheep 4 (96 h)</th>
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<tr>
<td></td>
<td>Total PHQ M3 M14 M4/M5 M7 M6</td>
<td>Sheep 2 (48 h)</td>
<td>Sheep 3 (72 h)</td>
<td>Sheep 4 (96 h)</td>
</tr>
<tr>
<td>Rumen</td>
<td>766 81 4 N.D. N.D. N.D. 8</td>
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<tr>
<td>Reticulum</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasum</td>
<td>672 99 N.D. N.D. N.D.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile</td>
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<td></td>
<td></td>
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<tr>
<td>Duodenum</td>
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<td></td>
</tr>
<tr>
<td>Jejunum</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
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<td></td>
<td></td>
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<tr>
<td>Cecum</td>
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<td></td>
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<tr>
<td>Large intestine</td>
<td>1890 17 9 39 23 8 N.D.</td>
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<tr>
<td>Colon</td>
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<td>Feces (0–24 h)</td>
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<tr>
<td></td>
<td>total ng Eq/g</td>
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<td></td>
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N.D., not detected.
in vivo in gerbil excreta and blood but not in sheep or dogs. This species difference might due to differences in the cyanogenic glycosides content of their respective diets. In vivo, PHQ undergoes significant dehydration to M14 in the gastrointestinal tract of sheep, which seems to occur primarily in cecum, likely due to the activity of the resident microflora (Table 6). The same metabolic fate of PHQ is also observed in gerbils in which M14 was the major constituent in feces. In both species, M14 was also observed in plasma, suggesting that it might also be absorbed, albeit to a small extent.

The pharmacodynamic action of anthelmintic agents is a complex phenomenon due in large part to the broad range of compartments in which these parasites reside and to a lack of understanding of how parasites acquire the active drug (Geary et al., 1999). It is nevertheless accepted that to achieve their effects, these agents must reach high enough concentrations in all compartments, and plasma concentrations, by themselves, are not necessarily sufficient to assess bioavailability of drug to parasitic nematodes. With the exception of O. columbianum, PHQ is effective against most common sheep GI parasites after a single oral dose of 0.5 mg/kg. At this dose, plasma levels of PHQ rapidly reached a maximum plasma concentration of ~40 ng/ml. The elimination rate was such that by 24 h the PHQ concentration was ~8 ng/ml and below 1 ng/ml at 72 h (Fig. 6). Although circulating metabolites of PHQ that retained some in vitro activity were observed in sheep plasma, by 48 h their combined levels were already below 10 ng/ml. These concentrations are well below those required to effectively reduce larval motility of H. contortus, T. colubriformis, and O. circumcinta in vitro (Gill and Lacey, 1993), suggesting that exposure to active components in the GI tract compartment is responsible for the activity of PHQ in sheep. At 24 h after dose, PHQ was the major DRM in the abomasum, the site of residence of H. contortus and O. circumcinta, and its concentration was roughly 700 ng/ml (Table 6). By 42 h, the concentration of PHQ had fallen to

Fig. 10. Proposed mechanism of the oxidative cleavage of the benzodioxepene moiety of PHQ in the dog.

Fig. 11. Proposed mechanism of formation of the major metabolites in sheep and gerbil.
~100 ng/ml. It is anticipated that the concentrations of PHQ in the abomasum at earlier time points were significantly above 700 ng/ml; therefore, the concentrations of PHQ until 48 h after dose would have been sufficient to reduce the motility of these nematodes and cause them to be flushed out of the stomach. PHQ was somewhat less effective against *O. columbianum*, a parasite resident in the large intestine of sheep (Shoop et al., 1990). In agreement with this observation, we have found that PHQ undergoes metabolism in the cecum to a dehydrated product that was shown to have much less activity in vitro so that the concentration of active components in the large intestine is much reduced, necessitating higher effective dose of PHQ to eliminate *O. columbianum* from the large intestine.

The absorption of PHQ in dogs was rapid, and plasma levels reached a maximum concentration at 30 min (Table 3; Fig. 6). Contrary to what was observed in sheep, PHQ is subsequently rapidly eliminated from plasma. The absence of PHQ in feces and urine indicated that, in dogs, PHQ is eliminated exclusively by metabolism. A sulfate conjugate of M1, M21, was identified as the major circulating metabolite in dog plasma. M21 was also the major PHQ-related component in dog urine, indicating that its major route of clearance was renal extraction. Although M21 was not tested for activity against *H. contortus*, it is most likely inactive since the benzodioxepine group is required for activity. PHQ would therefore be the major pharmacologically active component in dog plasma. The rapid rise and drop in systemic exposure to PHQ was accompanied with transient protrusion of the nictitating membrane and ataxia in both dogs dosed. These signs, which abated rapidly (by 24 h after dose, the animals had fully recovered), are in agreement with the previously reported toxicological effects of PHQ in dogs (Shoop et al., 1991, 1992a), and they are consistent with ineffective cholinergic muscular transmission. The mode of action of PHQ and related analogs is believed to occur via nicotinic cholinergic antagonism (Zinser et al., 2002; Qian et al., 2006), and, taken together, these observations strongly suggest that the adverse effects observed in dogs are mechanism-based and largely due to the parent PHQ. The majority of the administered dose in dogs was eliminated in the feces as metabolites lacking an intact benzo-dioxepine group, and none of these metabolites would be expected to have significant nematocidal activity. The lack of efficacy of PHQ in dogs can therefore be rationalized by its rapid and extensive absorption and by an equally rapid conversion to inactive metabolites so that gastrointestinal parasites are only in short and insufficient contact with active drug.

In summary, the data presented herein indicate that the wide differences observed in the nematocidal efficacy of PHQ in sheep, gerbils, and dogs is likely due to differences in metabolism in these species.