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Development and analytical characterization of a new antiparasitic fenbendazole compound tablet and pharmacokinetic investigations after its oral administration to dogs

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The objective of this study was to prepare a new compound fenbendazole tablet containing 29.7 % fenbendazole, 1.50 % praziquantel and 0.059 % ivermectin for oral administration. The tablets were successfully prepared using mannitol as filler agent, polyvinyl polypyrrolidone as disintegrant, 5 % povidone (PVA_{K30}) as a binder agent and magnesium stearate as lubricant. The appearance, hardness, fragility, time limit of disintegration and fenbendazole dissolution at 45 min all met the technical standards of the Ministry of Agriculture for the People's Republic of China. We used high performance liquid chromatography and electrospray-mass spectrometry for drug detection. Oral administration of 100 mg/kg fenbendazole, 5 mg/kg praziquantel and 0.2 mg/kg ivermectin using a non-compartmental model defined peak plasma concentrations (C_{max}) of 495, 826, 73 ng/mL, and 218 ng/mL for the metabolite oxfendazole, respectively. The area under the curve (AUC_{last}) values for these drugs were 4653, 1045, 1971 and 5525 h×ng/mL, respectively. This study enriches the pharmacokinetic data of compound fenbendazole tablets using dogs as a model system. The new tablet formulation was assimilated quickly and systemically and this study will be beneficial for the clinical application of parasite treatments in dogs.

1. Introduction

Animal parasitic infections with trematodes, nematodes and cestodes are commonly treated with two or more antiparasitic drugs. The macrolides, ivermectin (IVM), praziquantel (PZQ) and benzimidazole derivatives are all effective means of controlling these infections in livestock (Boes et al. 2000; Flisser et al. 2006; Capucchio et al. 2009; Marley et al. 2004; Mehlhorn et al. 2005; Scorza et al. 2006; Grandemange et al. 2007; Borgsteede et al. 2008). Each drug has a unique antiparasitic activity so that the simultaneous administration of multiple drugs is more efficient than a single drug (Bonneau et al. 2009). For example, IVM has a broad spectrum activity against nematodes and external parasites in poultry (Campbell 1984; Goa et al. 1991; Petersen et al. 1996). In contrast, PZQ is highly potent against flukes and tapeworms (Leopold et al. 1978; Pawowski 1990). Fenbendazole (FEN) and its metabolite oxfendazole (OXF) are used for controlling gastrointestinal parasitic diseases as well (Heggem 2008). Therefore, the combination of FEN, PZQ and IVM has become an ideal treatment because it covers a broader spectrum of action with reduced frequency of administration.

Studies of the action of FEN, PZQ, IVM and OXF in dogs are lacking, although they have been carried out in pigs, sheep, horses, kittens and cattle (Borgsteede et al. 2008). There are several products on the market containing two or more drugs that can be used in dogs. However, no tablets containing all three drugs are currently available. Oral PZQ has a drug half-life of 1-3 h so it needs to be given multiple times to maintain effective blood concentrations (Hong et al. 2003, 2016). However, FEN and IVM have long oral half-lives and can maintain effective plasma concentrations. These three drugs also work synergistically, which is an added benefit (McKellar et al. 1990; Tang et al. 2016).

In order to increase drug effectiveness, we developed a new compound FEN tablet and studied its pharmacokinetics in dogs. We used to study the veterinary anthelmintic drugs FEN, OXF, PZQ and IVM in dogs after oral administration of a single compound tablet by high performance liquid chromatography (HPLC) and mass spectroscopy (MS). This study will provide a basis for clinical medication.

2. Investigations and results

2.1. Analytical methods

The compound fenbendazole chewable tablets tested in this study contained FEN (454 mg), PZQ (23 mg), IVM (0.9 mg), mannitol (908 mg), 5 % povidone PVA $_{K30}$, polyvinylpolypyrrolidone (90 mg) and magnesium stearate (15 mg). The appearance of the product was white and its disintegration time limit was in the range of 4.5-5.3 min with a hardness of 8.2-8.7 kg. The friability range was 0.30-0.33 % with a FEN dissolution rate at 45 min of 75-81 %. We determined the actual drug content of the tables using HPLC by comparison with standard solutions. Calibration standards were tested and found to be linear for FEN (0.04-2500 ng/mL), PZQ (100-5000 ng/mL), IVM (2-200 ng/mL) and OXF (0.3-1000 ng/mL) (r > 0.997).

The chromatographic systems that gave three times peak height above baseline and 10 times above baseline represented the limit of detection (LOD) and limit of quantitation (LOQ) in a plasma matrix, respectively. These values were 0.02 and 0.04 for FEN, 40 and 100 for PZQ, 1 and 2 for IVM and 0.1 and 0.3 ng/mL for OXF, respectively. Recoveries of the four drugs ranged from 86 to 108 % (Table 1). The interday accuracy and stability of the extraction and chromatographic procedures showed a variation between 0.05 % and 11.30 % (Table 2). We found no major changes in the sample stability of any of the four drug stock solutions kept for 24 h either at room temperature or at -20 °C (Table 3). This indicated that our processing and depositing methods were suitable for routine analyses.

2.2. Pharmacokinetics

The present study first determined pharmacokinetic parameters of the four test drugs after a single 225 mg/kg oral dose to dogs. We

found PZQ, IVM, FEN and OXF at every sampling point for each dog. Drug concentrations in plasma ranged from 6-704 ng/mL for FEN, 118-1562 ng/mL for PZQ, 4-125 ng/mL for IVM and 1-788 ng/mL for OXF. The plasma concentrations for all four drugs were under the LOD for all dogs at 48 h, 16 h, 336 h and 48 h, respectively. Plasma FEN concentrations achieved the C_{max} (495.4 ng/mL) within 2.0 h and the $t_{1/2}$ was 16.3 h (Fig. 1 and Table 4). PZQ reached C_{max} (826.7 ng/mL) in plasma within 1.5-3 h after treatment. These data indicated that the drugs were absorbed quickly from the gastrointestinal tract (Fig. 2 and Table 4). IVM was also absorbed and the C_{max} in plasma was 73 ng/mL (Fig. 3 and Table 4). OXF is FEN metabolite and was cleared with a C_{max} of 218.5 ng/mL, a $t_{1/2}$ of 20.6 h and a t_{max} of 2.1 h (Fig. 4 and Table 4).

3. Discussion

3.1. *Optimization of sample processing conditions*

The aim of this initial study was to define the proper conditions for pre-processing of the three drugs, each with different solubilities. Solid-phase extraction (SPE) had been used previously to extract PZQ from plasma but the recoveries were generally less than 45 % (Morovján et al. 1998). The present study therefore chose a liquid-liquid extraction method to correct this situation. A previous study for PZQ plasma extraction used methyl-tert. butyl ether/dichloromethane and reached recoveries of 75 % (Tang et al. 2016). The present study showed that used this procedure and increased the number of extractions and applied ultrasonication and vortexing to reach recoveries greater than 90 %.

Table 1: Drug Recoveries from dog plasma (n=6)

	Nominal concentration (ng/ml)	Accuracy (%)	RSD (%)
Fenbendazole	0.04	108.00±11.12	10.30
	100	104.00±4.98	4.79
	2500	107.00±4.38	4.09
Praziquantel	100	91.39±9.15	10.00
	500	92.27±4.89	5.30
	5000	94.07±2.35	2.50
Ivermectin	2	104.50±8.64	8.27
	20	95.50±9.17	9.60
	200	92.60±4.17	5.38
Oxfendazole	0.3	94.80±4.68	4.90
	100	105.00±4.32	4.10
	1000	86.00±8.00	9.39

Table 2: Precision and accuracy of drug recovery from dog plasma (n=15)

	Nominal concentration (ng/ml)	N	Mean measured concentration (ng/ml)	RSD (%)
Fenbendazole	0.04	15	0.04±0.01	11.30
	200	15	213.30±9.92	4.65
	2500	15	2809.00±114.60	4.08
Praziquantel	100	15	107.00±10.70	10.00
	500	15	417.00±4.59	1.10
	5000	15	4616.00±2.31	5.01
Ivermectin	2	15	2.03±0.17	8.27
	20	15	21.03±2.02	9.60
	200	15	156.33±8.41	5.38
Oxfendazole	0.3	15	0.28±0.01	4.93
	100	15	107.00±4.41	4.12
	1000	15	992.00±91.15	9.39

Extraction procedures for FEN and OXF relied on chloroform as an extracting agent and gave only 56 % recoveries from plasma (Klausz et al. 2015). The present study applied methyl-tert. butyl ether extraction and achieved recoveries greater than 92 %. This solvent also has the advantage of lower toxicity and boiling point such that it could be quickly evaporated under a stream of nitrogen at 45 ºC. This reduced utilization of nitrogen and time minimized oxidation by decreasing air exposure time.

IVM sample processing procedures used an initial acetonitrile step to remove serum proteins followed by SPE prior to derivatization (Montigny et al. 1990; Tang et al. 2012). However, the present study found that the SPE column was easily blocked using this method so we substituted an ethyl acetate extraction. We also modified the IVM derivatization step because our attempts after drying under nitrogen at 45 ºC failed. We corrected this by adding an additional 4 min drying time in a 50 ºC oven. IVM derivatization does not occur in the presence of water and placement in the oven should be optimized for steam removal. These procedures allowed recoveries for IVM of more than 94.3 %.

3.2. *Method validation*

A first step in our analyses was to determine the existence of potential interfering substances in plasma samples (Morovján et al. 1998). The LOD values in plasma were 0.02 for FEN, 40 for PZQ, 1 for IVM and 0.1 ng/mL for OXF. Overall, our results for extraction recoveries at three spiked concentration levels were all greater than 90 % (Table 1). The interday accuracy was 107 % for FEN, 92 % for PZQ, 77 % for IVM and 95 % for OXF. The RSD values for all samples were within acceptable limits indicating high levels of precision and accuracy. Our processing and storage methods were therefore applicable for routine analyses (Table 2).

3.3. *Preparation of the tablet process*

There are commercial sources of oral compounds that include FEN, PZQ and IVM for parasitic infections in animals except dogs. Our compounding procedure was successful in delivering acceptable dosages in dogs orally. However, oral PZQ preparations must be administered frequently to cure infections such as schistosomiasis and clonorchiasis. This is due to an extensive and rapid conversion of the drug to an inactive complex through first-pass liver metabolism (Dayan 2003; Hong et al. 2003; King 1989). Therefore, we developed a new compound FEN tablet to prolong the effect of PZQ. After inspecting and optimizing the filler, binder, disintegrant and lubricant concentrations, we showed that a product was obtained with the appearance, hardness, fragility, time limit of disintegration and FEN dissolution at 45 min that conformed to the Technical Standards of the Ministry of Agriculture of People's Republic of China.

3.4. *Pharmacokinetic parameters*

When delivering a combination drug preparation, the pharmacokinetic features of one active drug can be influenced by the others in the composition. For example, PZQ effects are reduced by simultaneous administration of antiepileptic drugs or corticosteroids. IVM plasma concentrations were doubled when administered with verapamil (Pérez et al. 2010). The effective plasma concentration of FEN was prolonged when given together with methimazole (Gascon 1995). In order to assess the interactions between drugs, pharmacokinetic parameters were compared between single and compound drug doses. For example, FEN_1 and OXF_1 concentrations increased quickly after oral administration and achieved the C_{max} (495 and 218 ng/mL) within 2 h with half times of 16 and 20.6 h, respectively. The AUC for FEN_1 was 4654 and 5525 ng×h/ml for OXF_1 (Table 4). Previous reports in dogs after oral administration with the same dose gave maximum mean concentrations for FEN_2 and OXF_2 of 470 and 490 ng/ml with T_{max} values of 3.2 and 10 h, respectively (McKellar et al. 1990). Our T_{max} values were 1.56 and 2.13 h for FEN_1 and OXF_1, respectively (Table 4). Our compound tablets were orally active and rapidly assimilated into the circulatory system compared with the

Table 3: Drug stability in dog plasma under different storage conditions

Status		Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	RSD (%)
Room temperature (24 h)	Fenbendazole	0.04	0.040±0.01	111.00±10.29	9.09
		200	213.30±10.48	99.00±4.86	4.91
		2500	2709.00±110.70	121.00±4.95	4.09
	Praziquantel	100	91.39±9.26	94.50±9.57	10.13
		500	461.50±24.52	95.50±5.07	5.31
		5000	4703.00±117.4	97.60±2.43	2.49
	Ivermectin	2	2.09±0.17	104.50±8.64	8.27
		20	19.10±1.83	95.50±9.17	9.60
		200	185.20±8.34	92.60±4.98	5.38
	Oxfendazole	0.3	0.28±0.01	105.00±8.82	8.40
		100	104.90±8.81	85.00±5.14	6.05
		1000	854.60±51.70	107.00±11.77	11.00
-20°C (24 h)	Fenbendazole	0.04	0.04±0.01	112.00±14.90	13.30
		200	203.41±11.73	111.00±6.40	5.77
		2500	2516.00±98.56	116.00±4.55	3.92
	Praziquantel	100	90.97±3.70	92.50±3.76	4.07
		500	461.70±25.53	94.50±5.06	5.35
		5000	4540.00±184.50	84.60±3.43	4.06
	Ivermectin	2	2.05±0.14	101.50±8.13	8.01
		20	18.10±1.73	94.50±8.98	9.50
		200	157.20±8.56	79.60±4.22	5.30
	Oxfendazole	0.3	0.27±0.014	90.20±7.23	8.01
		100	101.10±7.73	94.50±8.98	9.50
		1000	875.20±58.56	95.60±5.07	5.30

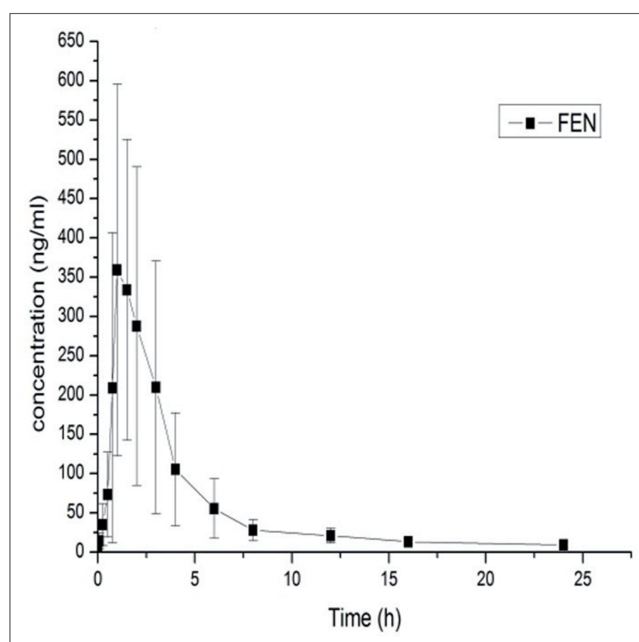

Fig. 1: Mean plasma concentration profiles of FEN in Beagle dogs (mean ± SD).

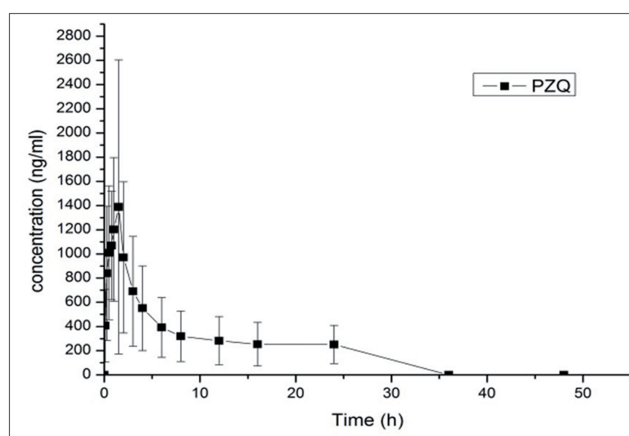

Fig. 2: Mean plasma concentration profiles of PZQ in Beagle dogs (mean ± SD).

single administration of the drugs. The reason for the differences in the C_{max} and AUC with FEN_2 (McKellar et al. 1990) may be the result of differences in plasma protein binding, distribution of metabolites as well as the detection method.

PZQ is rapidly converted into an inactive complex with first-pass liver metabolism after oral administration (King 1989). In our study, the pharmacokinetics of the compound FEN tablet was different from those of the PZQ_2 (Morovján et al. 1998) preparation and reached a maximum concentration of 826.67 ng/mL in plasma within 2-3 h after treatment. The MRT (6 h) was higher than previous reports indicating C_{max} and $t_{1/2}$ values in dogs after an oral single dose of 5 mg/kg were 300-700 ng/mL and 2-3 h, respectively (Morovján et al. 1998). After a single PZQ_3 dose of 30 mg/kg, the C_{max} was 270 ng/mL with a $t_{1/2}$ of 2.3 h and an MRT of 4.1 h in pigs (Tang et al. 2012). In sheep, the maximum concentration of PZQ_4 achieved after a single oral dose at 100 mg/kg was 889 ng/mL with $t_{1/2}$ and MRT values of 3.6 h and 0.87 h, respectively (Tang et al. 2016). The effectiveness of PZQ relies on the time which parasites are exposed to a valid drug concentration (Jung et al. 1997). A longer $t_{1/2}$ time indicated a slower release of PZQ from the compound preparation. A 100 ng/mL level of PZQ had been regarded as the minimal effective concentration to control flukes and tapeworms (Pica-Mattoccia 2004). PZQ_1 releases from the compound tablet (12 h) was longer than the PZQ_3 (4.16 h) (Tang et al. 2012). Thus, our new compound FEN

Table 4: Pharmacokinetic parameters of FEN, PZQ, IVM and OXF in dogs after oral administration of compound fenbendazole tablets

Pharmacokinetic parameters	FEN_1 (100mg/kg)	FEN_2 (100mg/kg)	OXF_1	OXF_2
$t_{1/2}$(h)	16.26±12.38	-	20.60±16.70	-
T_{max}(h)	1.56±0.62	3.17±0.54	2.13±1.00	10.00±2.87
C_{max}(ng/ml)	495.37±351.21	470.00±30.00	218.54±216.00	490.00±40.00
AUC_{INF} (ng.h/ml)	4653.81±1021.47	7200.00±1050.00	5525.00±1339.00	8430.00±940.00
Pharmacokinetic parameters	PZQ_1 (5mg/kg)	PZQ_2 (5mg/kg)	PZQ_3 (30mg/kg)	PZQ_4 (100mg/kg)
C_{max}(ng/ml)	826.67±250.83	300-700	270.00±140.00	889.47±459.28
$T_{1/2}$(h)	2.67±0.60	2-3	2.36±0.93	3.62±0.99
MRT(h)	6.01.00±2.45		4.10	0.87
Pharmacokinetic parameters	IVM_1 (0.2mg/kg)	IVM_2 (0.2mg/kg)	IVM_3 (0.3mg/kgIM)	IVM_4 (0.3mg/kgSC)
C_{max}(ng/ml)	73.00±58.40	51.32	9.43	14.26
$T_{1/2}$(h)	95.10±53.10	25.40	113.15	141.15
AUC_{INF} (ng.h/ml)	1971.90±526.80	137.1	1425.04	1864.68
MRT(h)	169.80±77.40	-	157.68	152.95

1 represents the parameters of this experiment. 2,3,4 represents the parameters in the literature.

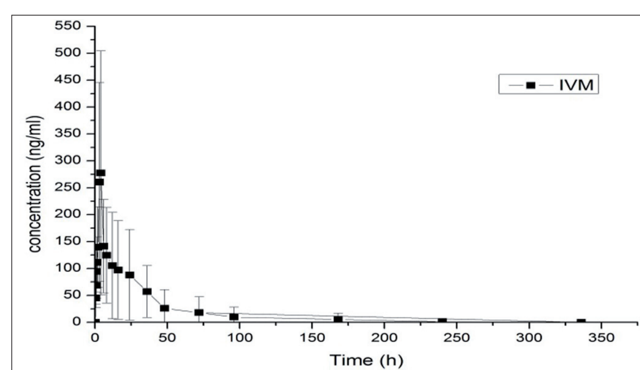

Fig. 3: Mean plasma concentration profiles of IVM in Beagle dogs (mean ± SD).

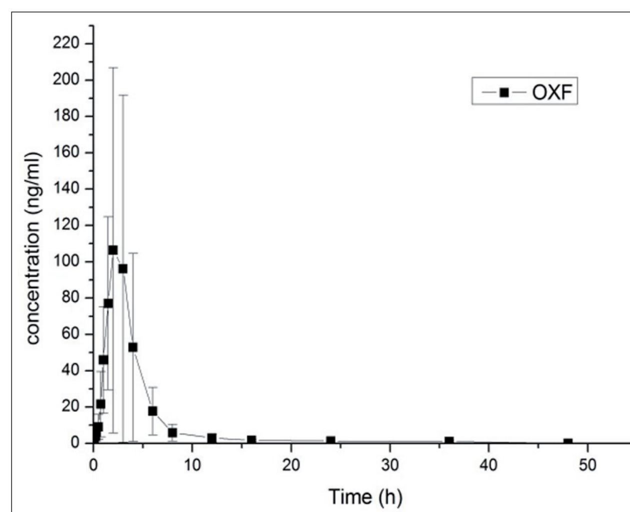

Fig. 4: Mean plasma concentration profiles of OXF in Beagle dogs (mean ± SD).

tablets enable continued effective plasma concentrations equal to a curative effect. The MRT increase the half-life of the drug which indicated the effective concentration of PZQ in dogs was longer than of unilateral preparations.

For IVM_1, its peak concentration, T_{max}, $t_{1/2}$, AUC_{INF} and MRT in plasma were 73.0 ng/mL, 3.25 h, 95.00 h, 1971 ng×h/mL and 169.80 h, respectively. In horses after an oral single dose at 0.2 mg/kg these values were 51.3 ng/mL, 3.60 h, 25.40 h, 137 ng×day/ml and 100.80 h, respectively (Péreza et al. 2002). In pigs, the C_{max} was 9.4 ng/mL and the $t_{1/2}$ was 113.00 h with an AUC_{INF} of 1425 ng×h/mL and a MRT of 157.68 h after intramuscular injection of 0.3 mg/kg (Tang et al. 2012). Subcutaneous injections of 0.3 mg/kg IVM_3 altered these values to 14 ng/mL, 141 h, 1864 ng× h/mL and 152 h, respectively (Tang et al. 2012).

Our results indicated that the C_{max} of IVM from the compound tablet was higher than IVM given alone, but had a slower $t_{1/2}$ compared with the injection methods. This may be the result of first-pass liver metabolism. However, compared with IVM_2 (0.2 mg/kg), the half-life of IVM_1 was significantly higher although the AUC and C_{max} were lower than IVM_2. Moreover, on the basis of *in vitro* pharmacological assays, the concentrations of 1 ng/mL plasma IVM is the minimal drug level essential for antiparasitic activity against many endoparasites (Lifschitz et al. 2000, 2004). In the present study, IVM_1 in the compound tablet and IVM_2 remained under 1 ng/mL at 336 h and 328 h, respectively. According to this standard, the effect levels of the two processes were equal against endoparasites. For ticks, it is necessary to maintain a concentration of 5 ng/mL (Davey et al. 2010). Therefore, the time duration of IVM_1 in a compound tablet under 5 ng/ml can reach 146 h whereas IVM_2 administered by either injection type was only 91.80 h; a significant difference. The C_{max} (73 ng/mL) and AUC_{INF} (1971 ng×h/mL) of IVM_1 in the compound tablet was significantly higher ($P < 0.05$) than those of IVM_2. This means that the IVM in this compound product was more fully absorbed (Tang et al. 2012).

These pharmacokinetic parameters indicated that FEN, PZQ, IVM and OXF in the dogs were rapidly absorbed and there was a synergistic effect between the four drugs.

In conclusion, a new compound FEN tablet for livestock to control parasitic co-infections was developed in this study for the first time. The analytical method compared favorably with the previously reported methods but with the advantages of high sensitivity, recovery and accuracy. These results indicated that these tablets meet pharmacokinetic study standards for use in dogs. In addition, we found no pharmacokinetic interplay between FEN, PZQ, IVM and OXF in this associated product. The pharmacokinetic conduct of these four drugs, especially PZQ, were significantly improved. The combined efficacy of IVM and PZQ towards sensitive parasites can be achieved with a single oral dose. This trial provides a scientific basis for the clinical study of these new fenbendazole tablets in dogs.

4. Experimental

4.1. Reagents

Fenbendazole tablets were obtained from Nanjing Golden Shield Animal Pharmaceutical. (Nanjing, China). Ivermectin (H_2B_{1a} purity ≧ 96.2 %), fenbendazole (100 %), praziquantel (99.7 %), ivermectin (92.0 %) and oxfendazole (98.0 %) standards were supplied by The China Institute of Food and Drug Control (Beijing, China). Dichloromethane, ethyl acetate, methanol and acetonitrile were obtained from Tianjin Fuyu Fine Chemicals (Tianjin, China). Ammonia and phosphoric acid were purchased from Laiyang City Kant Chemicals (Laiyang, China). Methyl *tert*-butyl ether was provided by Shanghai Runjie Chemical Reagent (Shanghai, China). Anhydrous ethanol was from Yantai Sanhe Chemical Reagent (Yantai, China). N-methyl imidazole and trifluoroacetic anhydride were purchased from Sigma-Aldrich (Germany); PVPP and PVA_{K30} were purchased from Boai New Open Source Pharmaceutical (Henan, China). Mannitol was obtained from Shandong Tianli Pharmaceutical (Weifang, China) and magnesium stearate from Tianjin Bodi Chemical (Tianjin, China).

4.2. Preparation of a new compound fenbendazole tablet

A compound tablet was developed using a wet granulation method. Granulation of FEN, PZQ and IVM was accomplished by mixing the drugs with the binder and other vehicles in a tableting machine (TDP-5, Shanghai Tianfeng Pharmaceutical Equipment, Shanghai, China). The optimized formulation was obtained by evaluating the appearance, hardness, fragility, time limit of disintegration and FEN dissolution at 45 min as the indices. An orthogonal design test ranged was used to survey the amount of filler, binder, disintegrants and lubricant (magnesium stearate), in which each element had three levels.

In brief, FEN and mannitol were mixed in a 1:2 ratio using a 200-mesh sieve and PZQ and IVM were forced through 100-mesh sieves. IVM (9 g) was dissolved in 5 % PVP_{K30} in 20 % ethanol. PZQ was mixed with the FEN - mannitol mixture (1:2) in equal amounts in a homogenizer (CH-50, Shanghai Tianfeng Pharmaceutical Equipment Shanghai, China). The material was blended for 5 min and the disintegrant and flavoring agent were added and mixed for an additional 3 min. The soft material was prepared by adding the IVM-soluble binder under humidity control. The kneaded mixture was then screened through a 24-mesh sieve. The wet granulations were dried in a box-type dryer (DHG-9146A, Shanghai Jinghong Experimental Equipment, Shanghai, China) at 60 ºC until the water content reached 3 % to 5 %, and the mixture was then sieved through a 24-mesh sieve. For production, formulation amounts of disintegrating agent and the lubricant magnesium stearate were mixed with the above granulations. The finished product was packed using aluminum blister.

4.3. In vivo studies

Five male and five female dogs each weighing about 10 kg were treated simultaneously with 100 mg/kg FEN, 5 mg/kg PZQ and 0.2 mg/kg IVM orally with commercial tablets. The dogs received no medication for one month prior to the experiment. Water was provided *ad libitum* throughout and the dogs were fasted the day before treatments. Blood samples (6 mL) were collected in heparinized tubes at 0.083, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48 ,72, 96, 168, 240, 336, 504 h after dosing. Blank blood samples (5 mL each) were gathered before the main experiment from the ten animals. Plasma from each sample was separated immediately after collection by centrifuging at 3000 g for 10 min (4 ºC), and stored at -20 ºC until sample testing (maximum two weeks). Blood samples in similar groups were pooled for analysis. This study followed the Qingdao Agricultural University Animal Experiment Committee. All animal experiments conformed to the care and use policies for laboratory animals.

4.4. Sample preparation

4.4.1. Standard solutions

Stock solutions of FEN, PZQ, IVM and OXF were prepared in methanol at concentrations of 1 mg/ml. All standard reagents were preserved from light and were stored at 4 ºC. They were diluted in methanol for the working standard solutions: FEN (0.04–2500 ng/ml), PZQ (40-5000 ng/ml), IVM (2-200 ng/ml) and OXF (0.3-1000 ng/ml).

4.4.2. Extraction of fenbendazole and oxfendazole

After thawing at room temperature, a volume of 0.5 mL of each plasma sample was mixed with 1 mL acetonitrile in 2 mL Eppendorf tubes. The mixture was ultrasonicated for 2 min, vortexed for 60 s then centrifuged at 12,000 rpm for 10 min at 4 ºC. The supernatant was transferred to a new tube and the above experimental steps were repeated three times. Finally, the upper organic layer evaporated to dryness under a stream of nitrogen at 45 ºC. The residue was reconstituted in 0.5 mL of methanol and 5 µL were injected into the HPLC/MS (Agilent, Santa Clara, CA, USA) instrument after filtration through a 0.22 µm filter.

4.4.3. Praziquantel

For the determination of PZQ, plasma was extracted with 1 mL methyl tert-butyl ether/methylene chloride (2/1) and the remainder of the procedure followed that of FEN extraction. 50 µL was used for HPLC analysis after filtration with a 0.22 µm filter.

4.4.4. Ivermectin

Plasma (0.2 mL) was mixed with 1 mL ethyl acetate to precipitate proteins. The mixture was vortexed for 1 min and then centrifuged at 12000 rpm for 10 min. The supernatant was extracted two more times and the extracts were combined. The sample was evaporated to dryness under a stream of nitrogen at 45 ºC, and then dried for 5 min at 50 ºC. The dried residue was derivatized using 100 µL N-methylimidazole/acetonitrile (1:1, v/v) by vortexing for 30 s. The mixture was then added to 150 µL trifluoroacetic anhydride/acetonitrile (1:2, v/v) and vortexed for an additional 30 s. This mixture was added to 250 µL methanol and placed in the dark for 30 min, filtered as above, and 50 µL was used for HPLC analysis.

4.5. Analytical procedures

4.5.1. Equipment and HPLC/MS for fenbendazole and oxfendazole

Determination of FEN and OXF were implemented using a Zorbax RRHD Eclipse Plus C_{18} (2.1 × 50 mm, 1.8 µm) column (Agilent, Santa Clara, CA). The mobile phase consisted of 0.1% formic acid in water: methanol (60:40, v/v). The flow-rate was 0.2 mL/min and FEN and OXF were detected at 292 nm. The MS was operated in positive ionization modes (ESI^+) using nitrogen as the nebulizer gas with a flow rate of 8.0 L/min. The desolvation gas was heated to 350 °C and the temperature of the sheath gas was set at 350 °C. The MS parameters were as follows: capillary voltage 3840V (ESI^+); acceleration voltage 3V and collision energy 25eV. The ion pairs for quantitation were 300/268 (FEN) and 316/159 (OXF) *m/z*.

4.5.2. Analytical conditions of praziquantel

An Xbriage Shield RP18 (5µm, 4.6×250mm) column (Waters Millipore, Milford, MA) was used to the determination of PZQ. The mobile phase was acetonitrile and water (48:52, v/v) and the flow rate was 1 mL/min. Aliquots of 50µLwere used for injection with UV detection at 217 nm.

4.5.3. Assay selectivity of ivermectin

IVM HPLC analysis used fluorescence detection with an excitation of 365 and emission wavelength of 475 nm. The separation column was an Xbriage Shield RP18 (as above) with a mobile phase of methanol, acetonitrile and water a (45:45:10 (v:v:v) and a flow rate of 1 mL/min. The chromatographic analysis was performed at 25 ºC.

4.6. Data analysis

Non-compartmental analysis (NCA) was used to calculate PK parameters using WinNonlin version 5.2 (Pharsight Corporation, USA). The parameters were as follows: time of reaching maximum concentration (T_{max}), the maximum plasma concentration (C_{max}), the area under the plasma concentration time curve from time zero to the last time point (AUC_{last}), volume of distribution (V_z) and mean residence time (MRT) extrapolated to infinity (MRTINF_obs) were predicted. Values are presented as mean± standard deviation (SD). Data were analyzed using SPSS 15.0 and a $P < 0.05$ was considered as statistically significant.

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