Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 5, pp. 501–508, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00
Pergamon Journals Ltd.

Simultaneous determination of fenbendazole, its sulphoxide and sulphone metabolites with the corresponding metabolites of triclabendazole in the plasma of sheep and cattle by high-performance liquid chromatography

M. S. BULL* and G. R. E. SHUME

CIBA-GEIGY Australia Limited, Western Road, Kemps Creek, N.S.W. 2171, Australia

Abstract: Pharmacokinetic studies following the simultaneous treatment of sheep or cattle with the anthelmintics triclabendazole and fenbendazole required an assay for determining the analytes in the plasma. An extraction and clean-up procedure is described which uses a C_{18} Sep Pak cartridge, involves no evaporation steps and produces a clean extract from sheep and cattle plasma in which triclabendazole, fenbendazole and their respective oxidation products may be determined by reversed-phase high-performance liquid chromatography. Results from application of the method to a pharmacokinetic study of sheep are presented.

Keywords: *Reversed-phase high-performance liquid chromatography; C_{18} Sep Pak; triclabendazole; fenbendazole; sheep; cattle.*

Introduction

The new benzimidazole anthelmintic, triclabendazole (CGA-89317), is effective against all stages of liver fluke infections in sheep and cattle [1, 2] but, unlike other benzimidazoles, has no activity against gastro-intestinal nematodes. For broad-spectrum control of nematodes and trematodes in sheep and cattle it is necessary to combine triclabendazole with a compound with a wide range of activity against intestinal parasites. Levamisole, pyrantel pamoate and benzimidazole anthelmintics are widely used for nematode control and would appear to be suitable for use in combination with triclabendazole.

The concomitant use of triclabendazole and fenbendazole was examined first. Both are sulphur-containing benzimidazoles which undergo oxidation to their respective sulphoxide and sulphone metabolites (Fig. 1) which have been found in the bloodstream of sheep and cattle following oral administration. However, although the pharmacology displayed by both compounds is similar, at equivalent dosage the concentrations of

*To whom correspondence should be addressed.

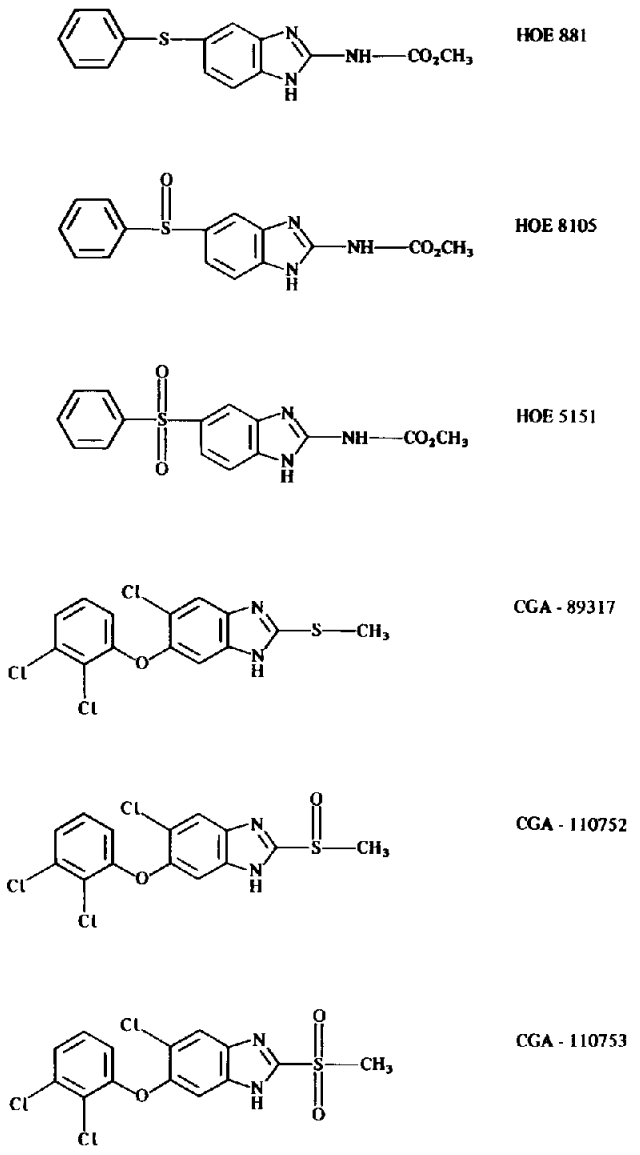

Figure 1
Chemical structure of fenbendazole (HOE 881), its sulphoxide (HOE 8105) and sulphone (HOE 5151), and triclabendazole (CGA-89317) and its sulphoxide (CGA-110752) and sulphone (CGA-110753) metabolites.*

triclabendazole metabolites found in the bloodstream are considerably higher than those of fenbendazole or its metabolites [3–5].

In order to investigate the pharmacokinetics of the two products given simultaneously a method of assay was needed which would quantify the parent compounds and their oxidation products in the bloodstream. A further requirement was that the procedure should be simple enough to allow for the processing of a large number of samples without causing any loss of performance in quantitation. The present paper describes such a

*Courtesy of Dr K. H. Lehr, Hoescht, Frankfurt.

method using solid phase extraction with Sep Pak C_{18} cartridges and HPLC for the determination of fenbendazole, its sulphoxide and sulphone metabolites and the metabolites of triclabendazole in plasma of sheep or cattle. Triclabendazole itself was not quantified by the HPLC conditions used because of its long retention time relative to those of the other five compounds. However this was not considered to be disadvantageous since the levels of triclabendazole in the plasma rarely rise above 50 ng/ml.

Materials and Methods

Chemicals and reagents

Analytical standards of fenbendazole (HOE 881), oxfendazole (HOE 8105) and fenbendazole sulphone (HOE 5151) were supplied by Hoechst Laboratories (Frankfurt, Germany). Triclabendazole (CGA-89317), its sulphoxide (CGA-110752) and sulphone (CGA-110753) were obtained from CIBA-GEIGY Limited (Agro Division, Basel, Switzerland). All chemicals used were reagent grade and the acetone and methanol were redistilled in glass. The acetonitrile was HPLC Grade (Millipore Pty. Ltd., Part No. 84935). All HPLC solvents were filtered through a 0.45 μm Sartorius membrane filter.

Liquid chromatography system

The reversed-phase HPLC system (Waters Associates Inc., Milford, USA) comprised a 6000A solvent delivery pump, a 710B Wisp sample processor, a 300 × 3.9 mm i.d. column-packed with 10-μm C_{18}-microBondapak and a Lambda-Max Model 481 LC spectrophotometer detector set at 300 nm. A Hewlett–Packard 3390A integrator was used to quantify the peaks. The mobile phase was acetonitrile–0.04 M diammonium hydrogen orthophosphate (41:59, v/v). The pH of the mixture was adjusted to 7.5–7.6 dropwise with 20% (m/v) orthophosphoric acid solution so that the sulphone peaks of triclabendazole and fenbendazole were resolved. The mixture was degassed under vacuum with the aid of an ultrasonic bath and the flow-rate was 1.5 ml/min. The retention times were 3.2 min for HOE 8105, 4.5 min for HOE 5151, 5.2 min for CGA-110753, 9 min for CGA-110752, 11.3 min for HOE 881 and 40 min for CGA-89317.

Standard curve preparation. Stock solutions of CGA-110752, CGA-110753, HOE 881, HOE 5151 and HOE 8105 were prepared in methanol at concentrations of 1000 μg/ml. The stock solutions were diluted with the mobile phase to provide five-component standards at four different concentrations between 0.05 and 5 μg/ml. 100 μl of each standard was injected on to the column to enable calibration curves to be prepared over the range of 5–500 ng for all five compounds. The concentration of each component in the plasma sample extracts (80-μl injections) was determined by comparing the peak height or area with that from the standard curve for each compound.

Procedure

The cyclic procedure used the Waters Sep Pak cartridge rack vacuum system (Part No. 22030) or appropriate glass syringes to pass the various fluids through the C_{18} Sep Pak (Waters Part No. 51910).

Sep Pak Cycle No. 1. The plasma sample (3 ml) in a 5-ml polypropylene disposable centrifuge tube was buffered with 1 ml of 0.075 M diammonium hydrogen phosphate

(adjusted to pH 5.3 with orthophosphoric acid). A 5-ml luerlock glass syringe was connected to the Sep Pak. The Sep Pak was pre-conditioned by passing, under vacuum through to waste, 10 ml of methanol followed by 10 ml of water. Then 3 ml of the buffered plasma sample was transferred by pipette into the syringe and passed dropwise through the Sep Pak; and the eluate was collected in a 10-ml polypropylene disposable centrifuge tube. The syringe was rinsed with 2 ml of water and the washings passed through the Sep Pak into the centrifuge tube. This aqueous fraction, which contained triclabendazole and its metabolites, with some unretained fenbendazole, was taken for removal of plasma protein. The Sep Pak was further washed with 10 ml of water and the washings discarded. The syringe was removed and a dry 2-ml luerlock glass syringe reconnected to the Sep Pak. Acetonitrile (2 ml) was transferred by pipette into the barrel of the syringe and the solvent passed dropwise through the Sep Pak; the eluate, which contained the fenbendazole group of compounds, was collected in a 50-ml graduate measuring cylinder.

Protein precipitation and extraction

To the aqueous sample in the centrifuge tube was added by pipette 5 ml of acetone down the inside wall. The capped tube was immediately inverted, shaken vigorously for 30 s and then centrifuged at 1500 g for 15 min.

Sep Pak Cycle No. 2. The Sep Pak under vacuum was washed with 10 ml of methanol followed by 10 ml of water. The supernatant solution from the centrifuge tube was decanted into a 50-ml graduated measuring cylinder containing 20 ml of water. The mixture was drawn dropwise through the Sep Pak under vacuum and the eluate discarded. The Sep Pak and measuring cylinder were washed with 10 ml of water and the eluate discarded. The 2-ml glass syringe was attached to the Sep Pak and with 2 ml of acetonitrile the absorbed compounds were slowly eluted from the Sep Pak into the acetonitrile retained from Cycle 1. The combined acetonitrile eluates were diluted with 40 ml of water.

Sep Pak Cycle No. 3. The Sep Pak under vacuum was washed with 10 ml of methanol followed by 10 ml of water. The aqueous acetonitrile extract was passed through the Sep Pak under vacuum and the eluate discarded. The 2 ml glass syringe was re-attached to the Sep Pak and the compounds eluted dropwise from the cartridge into a 4-ml septum capped Wheaton bottle with 2.5 ml of a solution of 0.2% trifluoroacetic acid (m/v) in acetonitrile. To this was added 1.5 ml of 0.06 M diammonium hydrogen phosphate and the contents mixed and retained for injection on to the HPLC. The Sep Pak was prepared for the next sample by washing under vacuum first with 10 ml of methanol and then with 10 ml water.

Kinetic study

The suitability of this method for the analysis of plasma from sheep treated with the two compounds was examined. Four sheep were treated by oral administration of a 5% (m/v) suspension of fenbendazole and triclabendazole at a dose of 5 mg/kg body weight for each compound. Blood (20 ml) was withdrawn from the jugular vein at 2, 4, 6, 8, 16, 24, 36, 48, 72 and 96 h after dosage and transferred to tubes containing EDTA as an anticoagulant. After centrifuging at 1500 g for 15 min the plasma was separated immediately and stored at −18°C until analysed.

Results

Control values, limit of determination and recoveries

Control samples of sheep plasma were analysed according to the procedure described and no significant peaks were found to interfere with the analysis (Fig. 2). Since the maximum concentrations of fenbendazole and its metabolites in sheep plasma following an oral dose were considerably lower than those found for triclabendazole metabolites [4], a lower limit of determination (10 ng/ml) for fenbendazole and its metabolites was set than that for the metabolites of triclabendazole (100 ng/ml). The calibration curves for the five compounds were linear throughout the range 5–500 ng.

The percentage recovery of each compound from control cattle plasma is summarised in Table 1. Six determinations were carried out at each concentration of added compound. Adulteration of the plasma was minimised by addition to the plasma of up to 30 μl of a 1000 μg/ml three-way standard of fenbendazole with its metabolites and a 1000 μg/ml two-way standard of triclabendazole metabolites by using a micro-syringe. The standards were prepared in dimethyl sulphoxide.

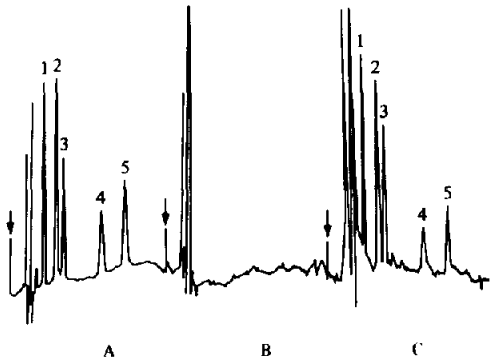

Figure 2
HPLC chromatogram of fenbendazole, its sulphoxide and sulphone metabolites and the corresponding metabolites of triclabendazole in sheep plasma. (A) Standard solution in mobile phase (5 ng of each): 1 = fenbendazole sulphoxide, 2 = fenbendazole sulphone, 3 = triclabendazole sulphone, 4 = triclabendazole sulphoxide, and 5 = fenbendazole; (B) Untreated sheep extract from plasma (45 mg injected); (C) Sheep extract from plasma (45 mg injected) to which had been added 0.1 μg/ml each of 1 = fenbendazole sulphoxide, 2 = fenbendazole sulphone, 3 = triclabendazole sulphone, 4 = triclabendazole sulphoxide, 5 = fenbendazole.

Table 1
The recovery of fenbendazole (HOE 881), its metabolites (HOE 8105) and (HOE 5151), triclabendazole (CGA-110752) and (CGA-110753) from cattle plasma

Added concentration (μg/ml)	% Recovery ± RSD*				
	HOE 881	HOE 8105	HOE 5151	CGA-110752	CGA-110753
0.1	99 ± 4	74 ± 3	76 ± 2	80 ± 5	76 ± 4
1.0	88 ± 3	77 ± 5	77 ± 2	83 ± 2	75 ± 2
10	87 ± 1	83 ± 10	86 ± 3	82 ± 1	83 ± 2

*RSD = Relative Standard Deviation (n = 6).

Discussion

Methodology

The major difference between the procedure described in the present paper and that reported by Lehr and Damn [6] is that due attention has been given to the clean-up of the extracts prior to evaluation by HPLC. This has ensured that the analysis of hundreds of plasma samples from sheep and cattle did not induce any deterioration in the performance of the HPLC column. The procedure uses minimal glassware and occupies very little bench space in the laboratory. Furthermore only 30 ml of methanol, 6.5 ml of acetonitrile and 5 ml of acetone are used in the extraction and clean-up of each sample. The Sep Pak can be used for at least 4 samples without any loss of performance. No concentration steps are required. This is advantageous if no precautions are taken to expel air since fenbendazole has the tendency to oxidise to its sulphone metabolite during evaporation.

During the development of the procedure it was found necessary to remove the metabolites of fenbendazole from the plasma by passing the plasma through the Sep Pak prior to precipitation of the plasma protein with acetone. This step prevented occlusion of the metabolites on to the precipitate and thus ensured their availability for extraction. Fenbendazole and triclabendazole and its metabolites are not tightly bound to the precipitate after the denaturing process.

The rate of elution from the Sep Pak was found to be important. The sample and extracts of the sample should be applied to the Sep Pak in such a manner that individual droplets are seen to come from the cartridge. The flow rate of washings through the Sep Paks was found not to be as critical.

This procedure enables a technician to analyse easily 12 samples in a day and also enables all but triclabendazole to be analysed in the one sample injection on the HPLC. If the virtual absence of triclabendazole in the plasma needs to be confirmed an increase in the acetonitrile content of the mobile phase to about 60% and re-injection of the sample will provide sufficient sensitivity for quantitation. Alternatively triclabendazole can be determined by the Sep Pak procedure with acetone precipitation [5] without any interference from the other compounds present.

Kinetic study

In contrast to triclabendazole, which does not appear in the bloodstream above the detectable limit, fenbendazole was detected for up to 72 h (Fig. 3). Maximum concentrations of oxfendazole, the sulphoxide metabolite of fenbendazole, were only 1–3.5% of those of the corresponding sulphoxide of triclabendazole (Figs 3 and 4). The times for maximum values to be reached were similar for the sulphoxides of both parent compounds. Residues of the sulphone metabolite of fenbendazole were correspondingly reduced and were only just detectable. The levels in the plasma for the combination of fenbendazole and triclabendazole are similar to that reported by Lehr and Damm [6] and for the individual compounds at equivalent doses are proportional to the results reported by Marriner and Bogen [3], Alvinerie and Galtier [4] and Bull and Shume [5]. These results indicate that the oral administration to sheep of the combination of fenbendazole and triclabendazole appears to be additive, thus resulting in the same pharmacokinetics as those of the individual anthelmintics given alone.

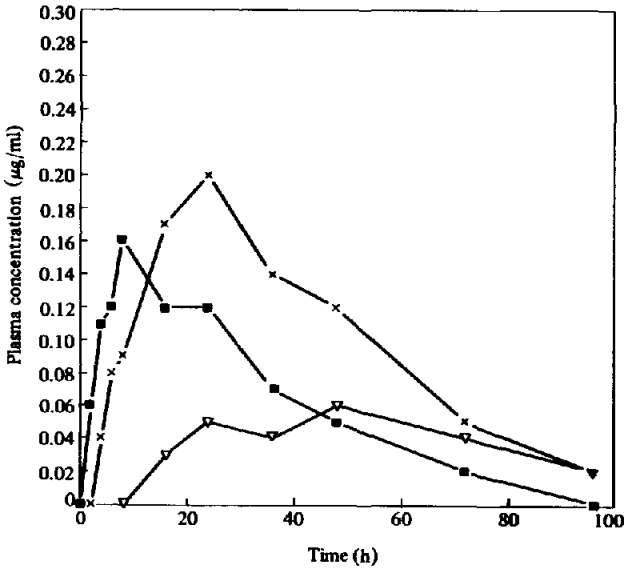

Figure 3
Plasma levels of fenbendazole and its sulphoxide and sulphone metabolites in sheep after oral administration of fenbendazole and triclabendazole at 5 mg/kg. ■ = fenbendazole, X = fenbendazole sulphoxide, ▽ = fenbendazole sulphone.

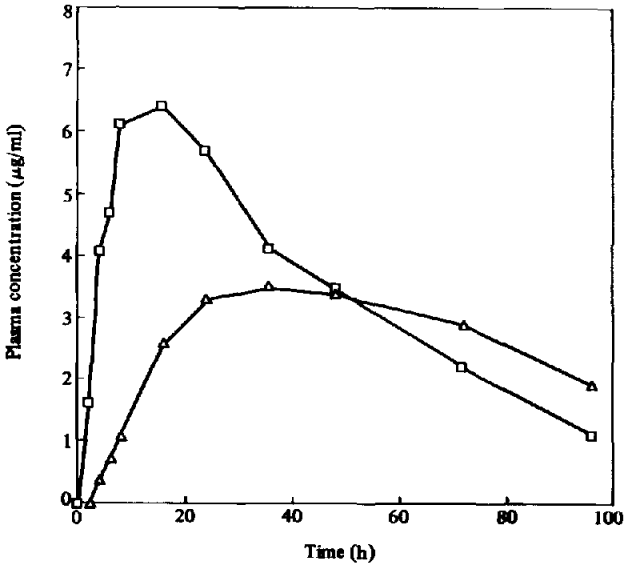

Figure 4
Plasma levels of triclabendazole sulphoxide and sulphone metabolites in sheep after oral administration of fenbendazole and triclabendazole at 5 mg/kg. □ = triclabendazole sulphoxide, △ = triclabendazole sulphone.

References

[1] J. C. Boray, P. D. Crowfoot, M. B. Strong, M. Schellenbaum, M. von Orelli and G. Sarasin, *Vet. Rec.* **113**, 315–317 (1983).
[2] J. Eckert, G. Schneiter and K. Wolff, *Berl. Münch. Tierärztl. Wschr.* **91**, 349–356 (1984).
[3] S. E. Marriner and J. A. Bogan, *Am. J. Vet. Res.* **42**, 1146–1148 (1981).
[4] M. Alvinerie and P. Galtier, *J. Chromatogr.* **374**, 409–414 (1986).
[5] M. S. Bull and G. R. E. Shume, *J. Pharm. Biomed. Anal.*, submitted for publication.
[6] K. H. Lehr and P. Damm, *J. Chromatogr.* **382**, 355–360 (1986).

[*First received for review 10 March 1986; revised manuscript received 10 December 1986*]