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Note

Simultaneous determination of fenbendazole and its two metabolites and two triclabendazole metabolites in plasma by high-performance liquid chromatography

K.H. LEHR* and P. DAMM

Hoechst AG, Postfach 80 03 02, D-6230 Frankfurt/M 80 (F.R.G.)

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Fenbendazole and triclabendazole (Fig. 1) belong to the same family of benzimidazole anthelmintics. Fenbendazole has excellent activity against gastrointestinal nematodes and against lungworms in sheep and cattle and triclabendazole has excellent activity against liver flukes. The concomitant use of these two compounds, therefore, provides excellent activity against all the major endoparasites of sheep and cattle [1—5].

Several methods for determining fenbendazole and its metabolites have been published [6, 7], but none for determining the triclabendazole metabolites. Very recently, a method was published for determining triclabendazole and its metabolites, but using a normal-phase high-performance liquid chromatographic (HPLC) column [8].

After administration of fenbendazole to sheep, the sulphoxide II and the sulphone III were detected in the plasma in addition to the parent compound I (Fig. 1) [9, 10]. After administration of triclabendazole, however, the parent compound could not be detected at all or else only in trace amounts whereas high levels of sulphoxide V and sulphone VI were found (Fig. 1) [8, 11].

This paper describes a method that allows all five relevant compounds to be extracted in one step and determined in one HPLC run.

EXPERIMENTAL

Reagents

The following solvents and reagents were used without special purification: dimethylformamide (Spectranal grade), ethyl acetate (analytical-reagent grade),

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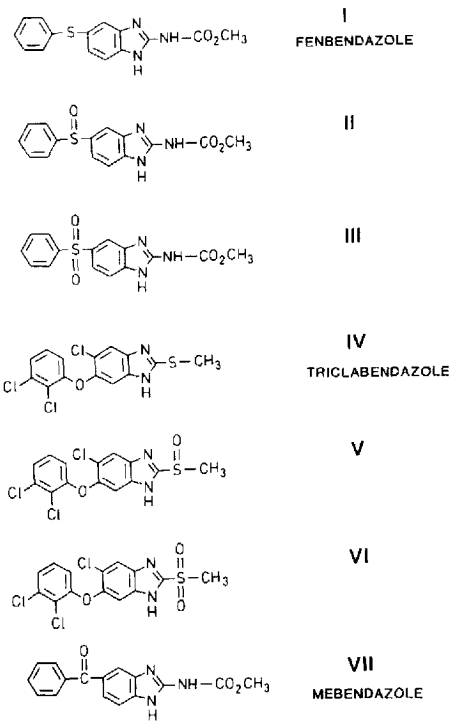

Fig. 1. Structural formulae of the compounds studied.

methanol (analytical-reagent grade), 0.05 *M* borate buffer solution (pH 8), tetraethylammonium hydroxide solution (20%) (Riedel-de Haën, Seelze-Hannover, F.R.G.) and acetonitrile (HPLC grade S) (Rathburn, Walkerburn, U.K.).

Standards

Fenbendazole and its metabolites were supplied by Hoechst (Frankfurt, F.R.G.), the metabolites of triclabendazole by Ciba-Geigy (Basle, Switzerland) and mebendazole (internal standard) by Janssen (Neuss, F.R.G.).

Stock solutions (1 mg/ml) of fenbendazole and its metabolites and of mebendazole were prepared by dissolving 10 mg of the compounds in 10 ml of dimethylformamide. Stock solutions (1 mg/ml) of triclabendazole metabolites were prepared by dissolution in methanol. The solutions were stable in the dark at 4°C for at least six months. Plasma standards were prepared by adding 10 μl of the stock solution of fenbendazole and each of its two metabolites and 100 μl of the stock solutions of the triclabendazole metabolites to drug-free plasma to obtain concentrations of 1 and 10 μg/ml, respectively. The standards were stored frozen at —20°C in 1-ml portions.

A working internal standard solution was prepared by 100-fold dilution of the stock solution with methanol.

Sample preparation

A 1-ml volume of plasma is mixed with 40 μl of the working internal standard solution and with 1 ml of buffer (pH 8) and shaken with 5 ml of ethyl

acetate for 20 s on a vortex mixer. The mixture is then centrifuged (2500 *g*) for 5 min, approximately 4 ml of the organic phase are transferred into a conical centrifuge tube and the ethyl acetate is evaporated for approximately 25 min at 50°C under nitrogen. Care must be taken that the samples do not remain at this temperature for an extended period in a dry condition (there is a danger that fenbendazole will oxidize to sulphoxide II!).

The residue is dissolved in 200 μl of mobile phase [acetonitrile—tetraethyl ammonium phosphate (TAAP) buffer (0.005 *M*), pH 5.9, 30:70] and injected.

Chromatographic conditions

The chromatographic system consisted of a Waters M-45 solvent pump with a Rheodyne 7100 injection port (100-μl sample loop) and a 125 mm × 4.6 mm I.D. column (Bischoff, Leonberg, F.R.G.) packed with Hypersil SAS (a mixed C_2-, C_4-, C_6-silica), particle size 5 μm.

The mobile phase was TAAP buffer consisting of 0.005 *M* phosphoric acid (adjusted to pH 5.9 with tetraethylammonium hydroxide solution) and acetonitrile (70:30). The absorbance was monitored with a Kratos Spectroflow 757 spectrophotometer at 300 nm. The chromatograph was operated at ambient temperature (18—22°C) with a flow-rate of 2.0 ml/min. Under these conditions, the retention times were approximately 1.5 min for II, 2.5 min for III, 3.2 min for mebendazole, 8.6 min for fenbendazole, 10.4 min for VI and 13.0 min for V.

Chromatograms of a plasma blank, a spiked sample and a real sample are shown in Fig. 2.

Quantification was based on the peak-area ratio of the substance of interest to that of the internal standard.

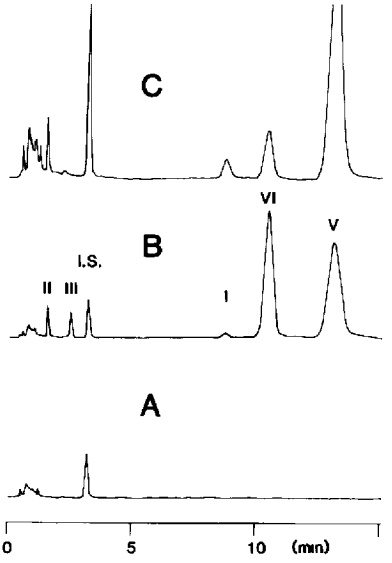

Fig. 2. Chromatograms of plasma extracts. (A) Drug-free plasma. (B) Plasma spiked with 200 ng/ml each of I, II and III and 5000 ng/ml each of V and VI. (C) Plasma 5 h after oral administration of fenbendazole and triclabendazole (5 mg/kg each) to a sheep. In C only 0.5 ml plasma were used and the detector signal was amplified by factor of 4.

RESULTS

The compounds were mixed in various concentrations with a blank plasma. Each mixture was divided into six portions and extractions and chromatography were carried out in one series. Other mixtures were determined as control standards between assays (Table I).

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF SPIKED HORSE PLASMA SAMPLES

In each instance, the three values given are (A) amount measured (mean within-assay, ng/ml), (B) within-assay coefficient of variation (%) (n=6), (C) between-assay coefficient of variation (%) (n=6)

Amount added (ng/ml)	I (fenbendazole)			II			III			V			VI		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
20	21.3	11 0	—	23 8	16 6	—	19 0	3 3	—						
50	51 2	9 9	—	56 0	4 2	—	51 5	1 6	—	50 2	8 1	—	51 2	8 5	—
100	99 5	2 6	—	109.0	3 5	—	103 2	1 0	—	101 2	9 7	—	99 2	6 4	—
200	200 7	4 0	15 4	211 0	3 0	6 1	205 2	1 2	4 3						
500	473 2	6 2	9 7	509.2	1 8	2 9	497 0	0 5	4 2	520 8	1 1	—	515 2	1 1	—
1000	953 0	1 8	10 4	1001 5	0 7	4 2	996 8	0 3	3 2	1046 2	3 7	—	1019 7	3 7	—
5000										4907 5	4 3	14.4	4860 8	5 1	7 3
10000										9514 0	5 7	13 0	9978 8	0 8	5 9

Linearity, accuracy and precision

The linearity, accuracy and precision of the method were assessed over the concentration range 20—1000 ng/ml in plasma for fenbendazole and its metabolites and 50—10 000 ng/ml for triclabendazole metabolites (Table I).

Extraction yield

Under the processing conditions used, the extraction yields of fenbendazole and the metabolites II, III and V from horse, cattle and sheep plasma were

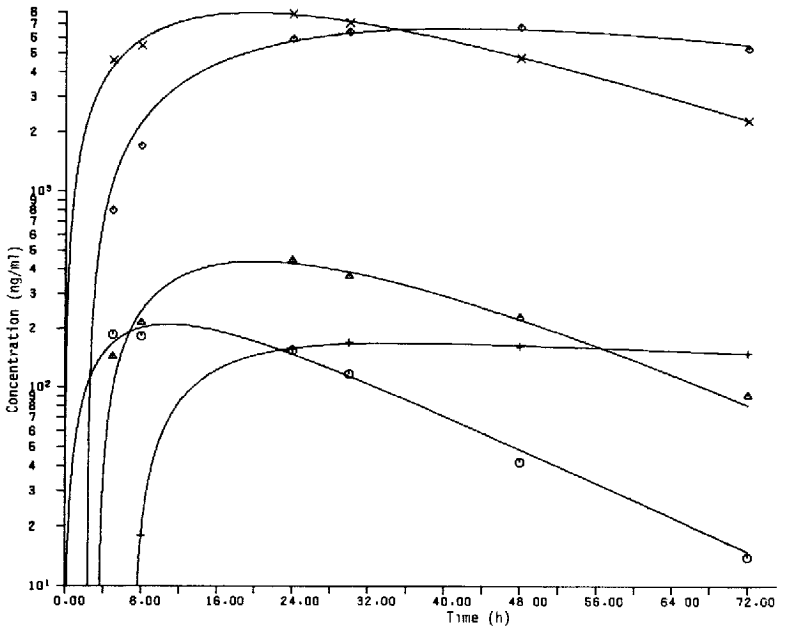

Fig. 3. Plasma concentrations of fenbendazole (○), II (△), III (+), V (×) and VI (◇) for a sheep that received orally 5 mg/kg fenbendazole and 5 mg/kg triclabendazole.

85%, irrespective of the animal species. With metabolite VI, extraction yields of 60 and 70% were obtained for cattle and sheep plasma, respectively. Consequently, the standards should be mixed with plasma of the species whose level has just been determined.

Limit of detection

The limit of detection, corresponding to three times the noise level, was 10 ng/ml for metabolites II and III, 15 ng/ml for fenbendazole and 20 ng/ml for metabolites V and VI.

Plasma concentration profile in sheep

A single dose of 5 mg each of fenbendazole and triclabendazole per kilogram body weight was administered orally to a sheep*. The plasma concentrations of all five compounds of interest were monitored using the assay method described (Fig. 3). The pharmacokinetics show that fenbendazole and, to a greater extent, triclabendazole were metabolized by progressive oxidation at the sulphur atom. It is noteworthy that the plasma concentration of the triclabendazole metabolites was an order of magnitude higher than that of fenbendazole and its metabolites at the same dosage.

DISCUSSION

In the search for a suitable chromatographic system, the following three partially contradictory conditions must be satisfied: (1) all compounds must be eluted within 15 min; (2) the k' value of the least retained compound must not be smaller than 1; (3) fenbendazole, which is only present in low concen-

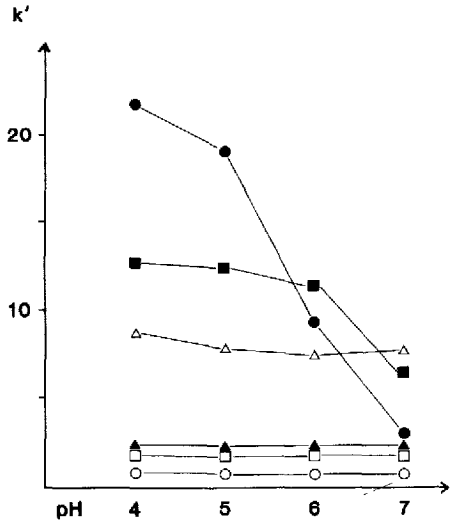

Fig. 4. Effect of mobile phase pH on k' value of fenbendazole (△), II (○), III (□), V (■), VI (●) and mebendazole (▲).

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trations in plasma, must, for reasons of detection sensitivity, appear before the two triclabendazole metabolites in the chromatogram.

With the mobile phase (pH 2) described for fenbendazole and its metabolites [7], the retention times of the triclabendazole metabolites are more than 30 min. Fortunately, as the pH increases, the k' values of the triclabendazole metabolites clearly decrease, whereas those of fenbendazole and its metabolites remain relatively stable (Fig. 4). In order to meet the third condition, however, a mixed C_2, C_4, C_6 reversed phase had to be used instead of a C_{18} or C_8 stationary phase. As the degree of ionization of V and particularly of VI has such a strong influence on the retention times of these compounds (Fig. 4), the pH value, ionic strength of the buffer and temperature must be closely adhered to in this chromatographic system.

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