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Short Communication

Determination of luxabendazole in biological fluids by high-performance liquid chromatography

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ABSTRACT

Luxabendazole, a new benzimidazole, is a highly potent broad-spectrum anthelmintic. A high-performance liquid chromatographic method has been developed for its determination in serum and urine samples. In order to optimize the clean-up of samples we compared two procedures: C_{18} Sep-Pak cartridges and ultrafiltration through a cellulose membrane with a 30 000 relative molecular mass cut-off. In order to obtain the most suitable mobile phase, we studied the influence of pH and acetonitrile content on the capacity factor (k'). Chromatographic separation and quantification were performed on a reversed-phase column packed with 5-μm Nucleosil C_{18}. The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 7.0), (40:60, v/v). The column effluent was monitored by ultraviolet–visible spectrophotometry at 290 nm. The method shows good recovery, precision and accuracy. The lower limit of detection for luxabendazole is 15 ng/ml in serum samples and 25 ng/ml in urine samples.

INTRODUCTION

The benzimidazoles are broad-spectrum anthelmintics first recognized over 25 years ago [1] and now available in many different forms. Recent reports by several researchers [2–5] have shown that luxabendazole, methyl-5-(4-fluorophenylsulphonyloxy)benzimidazole-2-carbamate (Fig. 1), is a highly effective anthelmintic against trematodes (*Fasciola, Dicrocroelium*), cestodes (*Moniezia*) and nematodes (*Haemonchus, Ostertagia, Trichostrongylus, Trichinella*). In sheep, the efficacy of a single dose of 10 mg of luxabendazole/kg body weight against immature and mature liver flukes of *Fasciola hepatica* was 95.2 and 95.9%, respectively [2]. The performance of this drug compared favourably with that of Dipling Kombi (oxiclozanide and levamisole), which was used as a reference drug [5].

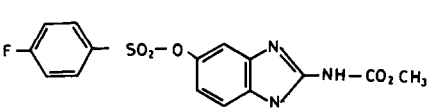

Fig. 1. Structure of luxabendazole.

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This paper describes the development of a high-performance liquid chromatographic (HPLC) method for the determination of luxabendazole in the plasma and urine of treated animals.

EXPERIMENTAL

Reagents and materials

Luxabendazole was kindly supplied by Hoechst (Frankfurt, Germany). HPLC-grade acetonitrile was supplied by Farmitalia Carlo Erba (Milan, Italy). N,N-Dimethylformamide, for UV spectroscopy, was supplied by Fluka Chemie (Buchs, Switzerland). The water was purified with a Milli-Q water purification system purchased from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade. The ultrafiltration system, with a 30 000 relative molecular mass cut-off (Ultrafree-M.C. UFC3-LTK), and the Sep-Pak cartridges, each packed with 360 mg of C_{18} sorbent, were also from Millipore. New Zealand White rabbits weighing 2.5–3 kg were used.

Equipment

The HPLC system comprised a Shimadzu LC-6A solvent-delivery pump, a Shimadzu C-12-6A detector and a Rheodyne 7125 20-μl loop injector. Analyses were performed on a reversed-phase Nucleosil C_{18} column (5 μm particle size, 20 cm × 0.2 cm I.D.) purchased from Tecnocroma (Barcelona, Spain). A guard column (2 cm × 2 mm I.D.) packed with Perisorb RP-18 (30–40 μm pellicular), supplied by Upchurch Scientific (Oak Harbor, WA, USA), was used when biological samples were injected.

Preparation of standard solutions

Standard solutions of luxabendazole were prepared by dissolving it in N,N-dimethylformamide to obtain solutions of 1.0 mg/ml, 10 μg/ml and 1.0 μg/ml, which were stored at 4°C.

Preparation of serum and urine samples

Procedure I. To 1 ml of serum or urine containing known amounts of standard solutions of luxabendazole, we added 1 ml of N,N-dimethylformamide. The mixture was shaken vigorously and centrifuged for 15 min at 10 000 *g*, after which an aliquot of 1 ml of the supernatant was passed through a C_{18} Sep-Pak cartridge conditioned previously with 5 ml of each of methanol and water. Then 2 ml of water and 2 ml of N,N-dimethylformamide were run through the cartridge to elute the luxabendazole, and the eluate was evaporated to dryness using nitrogen at 40°C. The sample was then reconstituted with 0.5 ml of N,N-dimethylformamide and injected into the HPLC column.

Procedure II. To 1 ml of serum or urine samples containing known amounts of standard solutions of luxabendazole, we added 1 ml of N,N-dimethyformamide. The mixture was shaken vigorously and centrifuged for 15 min at 10 000 *g*. An aliquot of 0.4 ml was transferred to an Ultrafree-MC ultrafiltration system containing a cellulose membrane with a 30 000 relative molecular mass cut-off, and centrifuged for 10 min at 5000 *g*. The clear filtrate was used directly for chromatographic analysis. For concentrations of less than 100 ng/ml the filtrate volume was halved by evaporation with nitrogen at 40°C.

HPLC method

The different mobile phases used were prepared with 0.05 *M* phosphate buffer at different pH values and with different percentages of acetonitrile. For the final analysis, we used acetonitrile–0.05 *M* phosphate buffer (pH 7.0) (40:60, v/v). The flow-rate was 0.5 ml/min, and the detection wavelength was 290 nm. All injection volumes for HPLC analysis were 40 μl, a 20-μl loop being used. The whole system was kept at room temperature (22 ± 3°C).

RESULTS AND DISCUSSION

Luxabendazole is practically insoluble in water and common solvents but dissolves well in N,N-dimethylformamide, dimethyl sulphoxide and hot glacial acetic acid [3]. For this reason we used N,N-dimethylformamide for extracting luxabendazole from serum and urine samples, where it

also served as a protein-precipitating agent. Two procedures for removing precipitated proteins were assayed: (I) a Sep-Pak cartridge packed with C_{18} and (II) ultrafiltration through an Ultrafree-MC cellulose membrane with a 30 000 relative molecular mass cut-off. The results from the two procedures are shown in Tables I and II, where it may be seen that the accuracy and recovery values are not significantly different, results being good in both cases. Both methods are therefore equally suitable, although ultrafiltration is faster because it involves fewer operations per sample and it is possible to process several samples simultaneously. However, the Sep-Pak method is to be preferred for urine samples because the blank urine obtained by ultrafiltration interferes with the luxabendazole peak (Fig. 2) under the chromatographic conditions we consider most suitable for the analysis of this compound. Changing the flow-rate to 0.3 ml/min solves this problem, but more time is needed.

The percentage recovery of luxabendazole from serum and urine samples was determined by injection of cleaned samples at each concentration studied. The area obtained in each case was divided by the area obtained on injecting a sample of luxabendazole at the same concentration in N,N-dimethylformamide. Assays were repeated ten times for each concentration.

The coefficient of variation (C.V.) was determined from the equation C.V. = S.D./$\bar{x}$. Ten assays were made for each concentration.

In order to achieve a good separation of luxabendazole from endogenous compounds of serum and urine samples, we investigated the effectiveness of varying the chromatographic parameters to increase the retention time of the drug with respect to those of the endogenous compounds.

An increase in the proportion of acetonitrile in the mobile phase caused a big drop in the value of k', when mobile phases of 0.05 M phosphate buffer (pH 7.0)–acetonitrile in proportions of 30, 40, 50, 60, 70 and 80% were used.

We also studied the variation in k' when the pH of the mobile phase was altered (Fig. 3). A difference of five units in the pH gave rise to an increase of nearly one unit of the k' value. We used mobile phases consisting of 0.05 M phosphate buffer of different pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) and acetonitrile (60:40, v/v).

The results of this study allow us to select the best conditions for the determination of luxabendazole from serum and urine samples by HPLC in a mobile phase comprising acetonitrile–0.05 M phosphate buffer (pH 7.0) (40:60, v/v). The flow-rate was 0.5 ml/min.

The chromatograms obtained from serum and urine samples using Sep-Pak and ultrafiltration for sample clean-up are shown in Fig. 2.

The limit of detection of luxabendazole is 15 ng/ml in serum using Sep-Pak or ultrafiltration.

TABLE I

RECOVERY OF LUXABENDAZOLE FROM SERUM AND URINE

Samples were treated with a Sep-Pak cartridge or ultrafiltration; n = 10.

Added concentration (μg/ml)	Recovery (mean ± S.D.) (%)			
	Sep-Pak		Ultrafiltration	
	Serum	Urine	Serum	Urine
10	89.3 ± 3	92.3 ± 3	89.6 ± 2	96.1 ± 1
5	100.6 ± 4	84.4 ± 3	102.1 ± 5	85.1 ± 2
1	102.0 ± 3	101.8 ± 4	103.7 ± 5	104.5 ± 6
0.5	88.5 ± 6	101.4 ± 4	102.3 ± 5	102.0 ± 6
0.1	97.7 ± 4	93.2 ± 5	60.3 ± 4	101.0 ± 2
0.05	108.0 ± 1	102.0 ± 4	76.0 ± 2	116.0 ± 4

TABLE II

ACCURACY AND PRECISION OF DETERMINATION OF LUXABENDAZOLE IN SERUM AND URINE

Samples were treated with a Sep-Pak cartridge or ultrafiltration; O.C. = observed concentration.

Added concentration (μg/ml)	Sep-Pak				Ultrafiltration			
	Urine		Serum		Urine		Serum	
	O.C. (μg/ml)	C.V. (%)	O.C. (μg/ml)	C.V. (%)	O.C. (μg/ml)	C.V. (%)	O.C. (μg/ml)	C.V. (%)
10	9.20 ± 0.35	3.80	8.03 ± 0.53	3.60	9.60 ± 0.32	3.33	8.96 ± 0.22	2.45
5	4.22 ± 0.16	3.70	5.03 ± 0.30	3.90	4.10 ± 0.20	3.80	4.50 ± 0.28	3.20
1	1.05 ± 0.05	4.73	1.02 ± 0.05	4.80	1.02 ± 0.07	4.38	1.05 ± 0.06	3.70
0.5	0.50 ± 0.03	5.00	0.44 ± 0.02	4.54	0.60 ± 0.03	4.00	0.52 ± 0.03	3.76
0.1	$0.09 \pm 5 \cdot 10^{-3}$	4.55	$0.01 \pm 5 \cdot 10^{-3}$	5.00	$0.09 \pm 3 \cdot 10^{-3}$	3.03	$0.05 \pm 3 \cdot 10^{-3}$	4.20
0.05	$0.05 \pm 3 \cdot 10^{-3}$	4.35	$0.05 \pm 2 \cdot 10^{-3}$	3.50	$0.05 \pm 1 \cdot 10^{-3}$	1.75	$0.04 \pm 2 \cdot 10^{-3}$	3.80

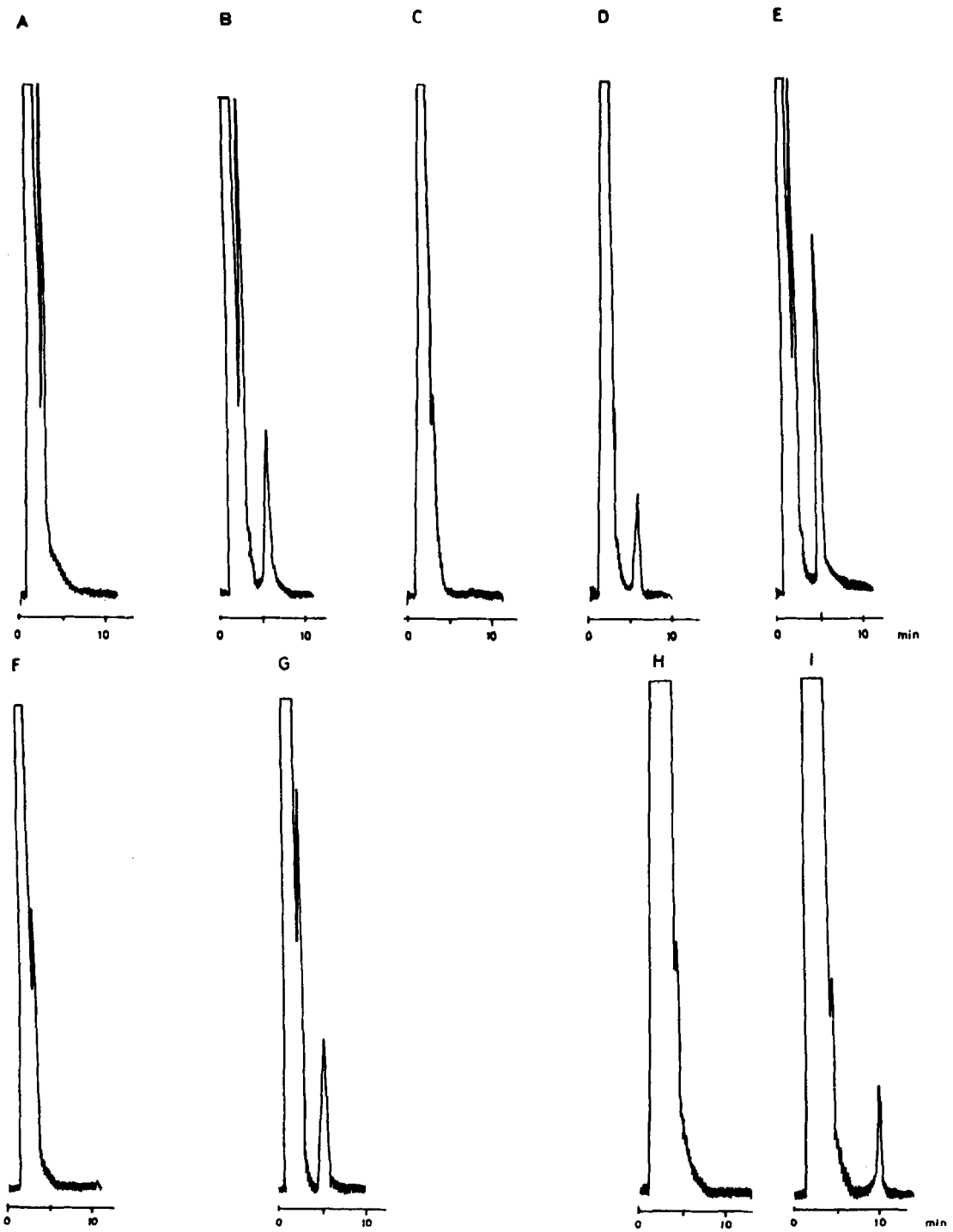

Fig. 2. Chromatograms of samples treated by procedure I (Sep-Pak C_{18} cartridges): (A) blank serum; (B) serum containing 100 ng/ml luxabendazole; (C) blank urine; (D) urine containing 100 ng/ml luxabendazole; (E) rabbit serum 8 h after intravenous administration of 10 mg/kg luxabendazole. Chromatograms of samples treated by procedure II (ultrafiltration): (F) blank serum; (G) serum containing 100 ng/ml luxabendazole; (H) blank urine; (I) urine containing 100 ng/ml luxabendazole (flow-rate 0.3 ml/min). The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 7.0) (40:60, v/v). The flow-rate was 0.5 ml/min except for chromatogram I. The detection wavelength was 290 nm, and the column packing was 5-μm Nucleosil C_{18}.

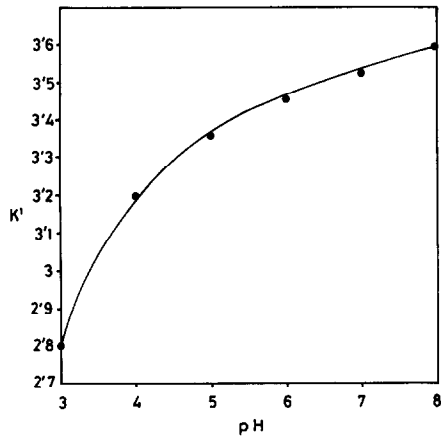

Fig. 3. Variation of k' of luxabendazole when the mobile phase pH is altered. The mobile phase contains acetonitrile and 0.05 M phosphate buffer with different pH: 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 (40:60, v/v).

For urine the limit of detection is 25 ng/ml with Sep-Pak. The limit of detection was determined as the smallest detectable value for the concentration of the compounds giving a response of at least three times that of baseline noise.

CONCLUSION

An isocratic HPLC method was established for monitoring the new benzimidazole, luxabendazole, in serum and urine samples in both therapeutic and pharmacokinetic studies. To develop this method, Sep-Pak and ultrafiltration were used for clean-up, the two procedures being found to be similar in precision and accuracy, although ultrafiltration was not used for the treatment of urine samples as it would have implied working with a lower flow-rate and a consequent increase in analysis time.

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