*Journal of Chromatography*, 576 (1992) 135–141
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6267

# Reversed-phase ion-pair high-performance liquid chromatographic determination of triclabendazole metabolites in serum and urine

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(First received October 29th, 1991; revised manuscript received January 3rd, 1992)

ABSTRACT

An ion-pair high-performance liquid chromatographic method was developed for measuring the concentrations of triclabendazole metabolites (sulphoxide and sulphone) in plasma and urine samples. The diluted biological fluids are ultrafiltered before chromatography through a 30 000 relative molecular mass cut-off filter and then injected into a $C_{18}$ column. They are then isocratically eluted with a mobile phase consisting of 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (55:45, v/v) with addition of 1.0 mmol/l sodium decanesulphonate and monitored by ultraviolet–visible spectrophotometry at 312 nm. Recoveries over the range 0.01–9.0 μg/ml for triclabendazole sulphoxide and sulphone are, respectively, 91.7% and 91.6% in serum and 90.3% and 90.2% in urine. For both metabolites, the limit of detection is 10 ng/ml in both urine and serum.

## INTRODUCTION

Triclabendazole [4-chloro-5-(2,3-dichlorophenoxy)-2-methylthiobenzimidazole] (Fig. 1) is a benzimidazole anthelmintic shown to exhibit high activity against immature and mature stages of *Fasciola hepatica* and *Fasciola gigantica* in sheep and cattle [1,2]. After oral administration to these species [3,4] it undergoes rapid and extensive metabolism, first to its sulphoxide and sulphone (Fig. 1), with the parent compound remaining undetectable in the plasma of treated animals.

The purpose of this study was to develop a reversed-phase ion-pair high-performance liquid chromatographic (HPLC) method for determining triclabendazole metabolites in serum and urine samples. To this end we studied the influ-

0378-4347/92/$05.00 

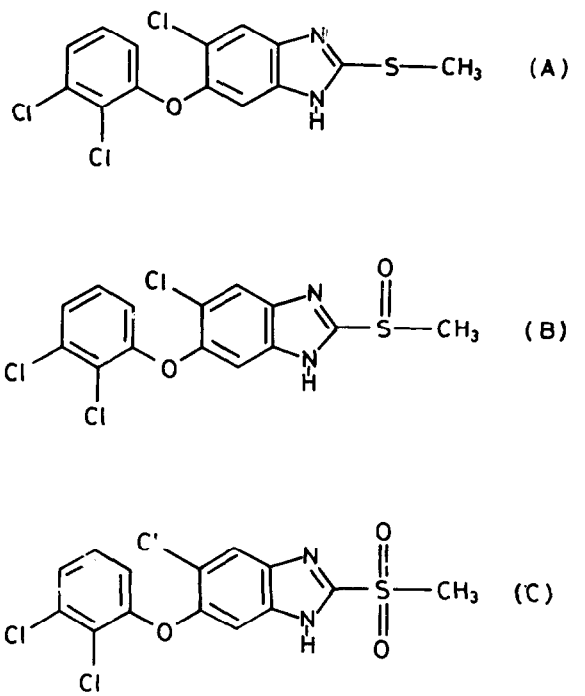


Fig. 1. Structures of (A) triclabendazole, (B) triclabendazole sulphoxide and (C) triclabendazole sulphone.

ence on their retention time of pH, mobile phase composition and the nature of sodium alkyl sulphonate added to the mobile phase [5,6].

## EXPERIMENTAL

### *Reagents and materials*

Triclabendazole and its metabolites (sulphoxide and sulphone) were supplied by Ciba-Geigy Agricultural Division (Barcelona, Spain). Fasinex (which contains 10% triclabendazole) was supplied by Ciba-Geigy. The sodium salts of propane-, pentane- and decanesulphonic acids were purchased from Fluka (Buchs, Switzerland) and HPLC-grade acetonitrile form Carlo Erba (Milan, Italy). The water used was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical-reagent grade. The ultrafiltration system, with a relative molecular mass cut-off of 30 000 (Ultrafree-MC UFC 3 LTK) was purchased from Millipore. New Zealand white rabbits weighing 2.5–3 kg were used.

### *Equipment*

The HPLC system consisted of a Shimadzu LC-6A solvent-delivery pump, a Shimadzu C-12-6A spectrophotometric detector and a Rheodyne Model 7125 injector with a 20-$\mu$l loop. Analyses were performed on a $C_{18}$ Spherisorb ODS-2 (5 $\mu$m particle size) reversed-phase column (25 cm × 0.4 cm I.D.) purchased from Tecknocroma (Barcelona, Spain). A guard column (2 cm × 2 mm I.D.) packed with Perisorb RP-18 (30–40 $\mu$m, pellicular), supplied by Upchurch Scientific (Oak Harbor, WA, USA), was utilized when biological samples were injected.

### *Preparation of serum and urine samples*

Standard solutions of triclabendazole and of the sulphoxide and sulphone (1 mg/ml) were prepared by dissolution in methanol and were stored under nitrogen.

Samples were prepared by adding 0.2 ml of ethanol–acetonitrile–water (40:10:50, v/v/v) to 0.2 ml of serum or urine [7] containing known amounts of standard solutions of triclabendazole and each of its two metabolties in order to obtain concentrations in the range 10–0.01 $\mu$g/ml. These were then vortex mixed for 20 s and fed into an Ultrafree-MC ultrafiltration system with a 30 000 relative molecular mass cut-off cellulose membrane and centrifuged for 10 min at 5000 *g*. The clear filtrate was used directly for chromatographic analysis. For concentrations of less than 1.0 $\mu$g/ml the sample volume was reduced by half using nitrogen at room temperature.

### *HPLC method*

The different mobile phases used were prepared with 0.05 *M* phosphate buffer, obtained by adding a saturated solution of sodium hydroxide to a 0.05 *M* solution of phosphoric acid until the required pH was reached, varying the pH, percentage of acetonitrile and the amount and nature of the sodium alkylsulphonate added. For the final analysis, it consisted of 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (55:45, v/v) containing 1.0 mmol/l sodium decanesulphonate, added in solid form. The flow-rate was 1.4 ml/min for serum and 1.0 ml/min for plasma sam-

ples. The detection wavelength was 312 nm. The system was kept at room temperature (22 ± 3°C).

## RESULTS AND DISCUSSION

Interference from endogenous serum and urine constituents was minimized by protein precipitation and ultrafiltration through 30 000 relative molecular mass cut-off cellulose membranes. The use of ethanol–acetonitrile–water (40:10:50, v/v/v) to precipitate proteins also apparently prevented the binding of triclabendazole and its metabolites to blood components, as borne out by the recoveries obtained.

This method of sample preparation simplified the analysis, as it is intrinsically even faster than the methods using Sep-Pak cartridges [2,3,9] while allowing several samples to be prepared simultaneously. This simplification does not impair the recovery or accuracy, however, nor does it affect the detection limit. On the other hand, the existence of ion-pair-forming agents in the mobile phase allows the capacity factor, $k'$, to be easily altered while making it adaptable for use with other biological systems.

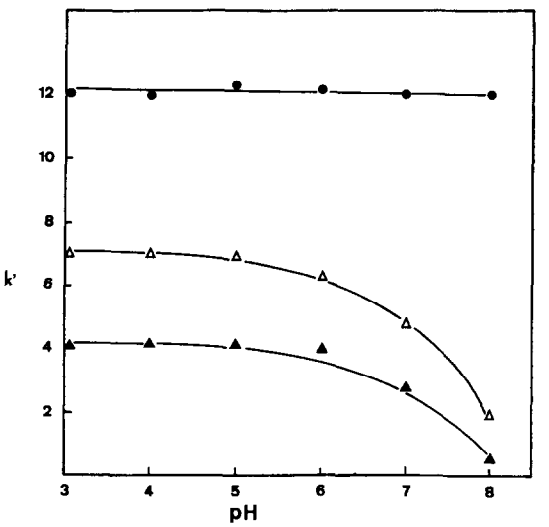


Fig. 2. Effect of variation of the capacity factor, $k'$, for (●) triclabendazole, (△) triclabendazole sulphoxide and (▲) triclabendazole sulphone in a mobile phase containing 0.05 *M* phosphate buffer of different pH (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0)–acetonitrile (60:40, v/v) and 1.0 mmol/l sodium decanesulphonate.

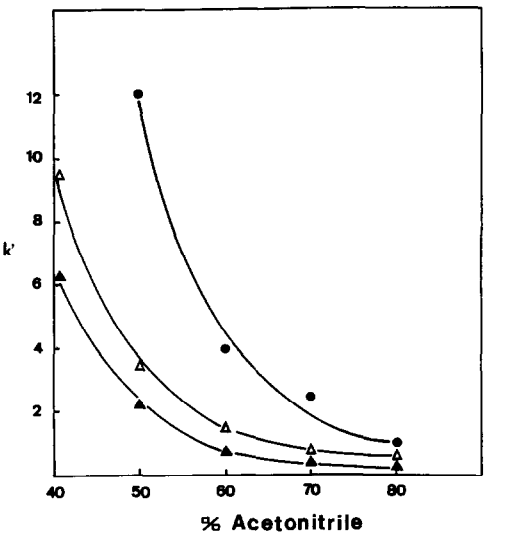


Fig. 3. Effect of varying the percentage of acetonitrile in the mobile phase on the capactiy factor, $k'$, of (●) triclabendazole, (△) triclabendazole sulphoxide and (▲) triclabendazole sulphone. The mobile phase contained 0.05 *M* phosphate buffer (pH 7.0) and 40, 50, 60 70 and 80% of acetonitrile, with 1.0 mmol/l sodium decanesulphonate.

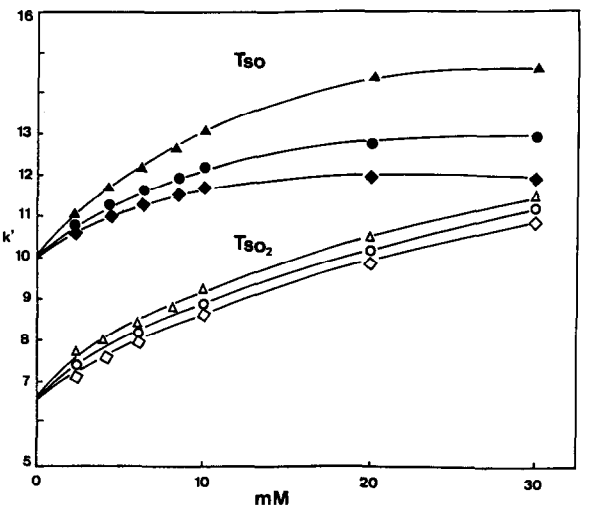


Fig. 4. Effect of concentration and length of side-chain on the capacity factor, $k'$, for triclabendazole sulphoxide ($T_{SO}$) and triclabendazole sulphone ($T_{SO_2}$). The mobile phase was 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (60:40, v/v) containing sodium alkylsulphonates at concentrations of 0, 10, 20 and 30 mmol/l: sodium salts of (◆) propane-, (●) pentane- and (▲) decanesulphonic acid.

In order to obtain an efficient method for the determination of triclabendazole sulphoxide and sulphone, we investigated the effect on the capacity factors ($k'$) of varying some of the chromatographic parameters, pH, percentage of acetonitrile in the mobile phase and the concentration and chain length of the ion-pair-forming agent.

For the variation of the mobile phase pH 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)–acetonitrile (60:40, v/v) and 1.0 mmol/l sodium decanesulphonate. The $k'$ values remain very high and constant for triclabendazole over the whole pH range (Fig. 2). We believe this to be

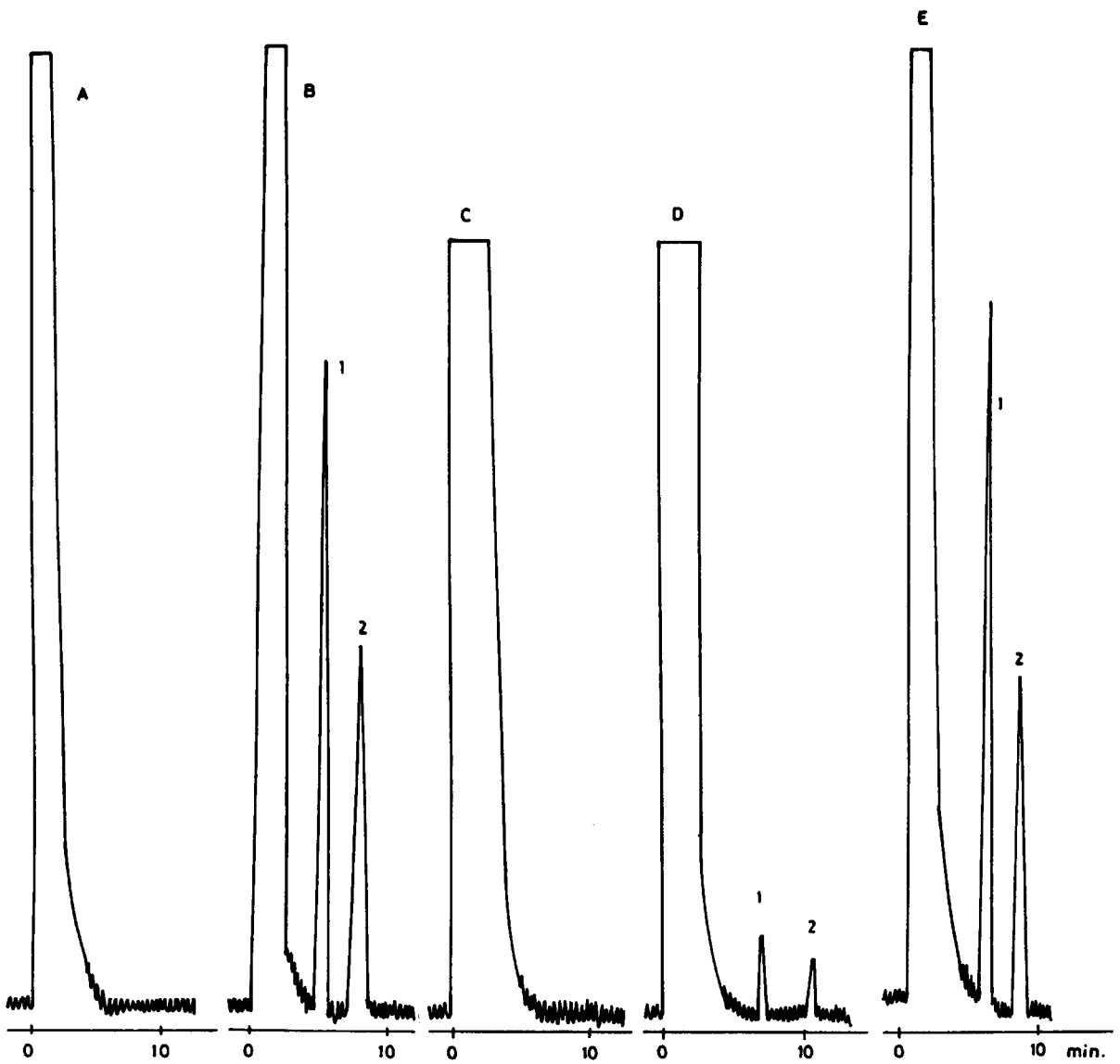


Fig. 5. Chromatograms of (A) blank serum; (B) serum containing 500 ng/ml triclabendazole sulphone (1) and triclabendazole sulphoxide (2); (C) blank urine; (D) urine containing 50 ng/ml triclabendazole sulphone (1) and triclabendazole sulphoxide (2); and (E) rabbit serum 24 h after oral administration of Faxinex (10 mg/kg). The mobile phase was 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (55:45, v/v) containing 1.0 mmol/l sodium decanesulphonate. The flow-rate was 1.4 ml/min for serum and 1.0 ml/min for urine samples, the wavelength was 312 nm and the column was $C_{18}$ Sperisorb ODS-2.

due to the high hydrophobicity of this molecule, which outweighs the retarding effect of the retention times produced by ion-pair formation.

Major differences in $k'$ are found when the pH is altered for triclabendazole sulphoxide and sulphone, as the hydrophobicity of these molecules is lower, revealing the effect of the formation of ion pairs with alkyl sulphonates. The $pK_a$ and $pK_b$ values for these products are 10.5 and 2.5, respectively. The base group is responsible for the formation of ion pairs, so in the pH range 3.0–6.0 the amino group is highly protonated, which is why there is a predominant formation of ion pairs and the $k'$ values are high. For pH > 6.0, the concentration of protonated amine decreases with a consequent drastic reduction in the formation of ion pairs and in the $k'$ values.

As can be seen in Fig. 3, an increase in the percentage of acetonitrile in the mobile phase led to an expectedly large decrease in the $k'$ values for triclabendazole and its metabolites. This effect was determined by using mobile phases consisting of 0.05 *M* phosphate buffer (pH 7.0) and 40, 50, 60, 70 and 80% of acetonitrile and containing 1 mmol/l sodium decanesulphonate.

The influence of the length of the side-chain of the ion-pair-forming agent is shown in Fig. 4 for triclabendazole sulphoxide and sulphone. Here, the mobile phase consisted of 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile (60:40, v/v) containing and sodium propane-, pentane- and decanesulphonate at concentrations of 0, 2, 4, 6, 8, 10, 20 and 30 mmol/l. High values found for $k'$ are not shown, which were of the order of 30 for triclabendazole. Triclabendazole sulphoxide and sulphone have $k'$ values of about 10 in the absence of alkylsulphonate, increasing as the concentration of alkylsulphonate increases. No major variation in $k'$ was observed when the length of the side-chain of the alkylsulphonate is increased by 3–10 carbons, however. The behaviour of triclabendazole sulphone is analogous to that of the sulphoxide, but starting form a lower $k'$ value (7.0), and with a smaller difference in the increase in $k'$ as the length of the alkylsulphonate side-chain is altered. This behaviour may be explained by the increase in the degree of oxidation of the molecule and the consequent increase in its polarity hindering the increase in $k'$, owing to the formation of ion pairs with alkylsulphonates with different side-chain lengths.

The results of the study allow us to select as the optimum conditions for the determination of triclabendazole metabolites in serum and urine samples by HPLC a mobile phase consisting of 0.05 *M* phosphate buffer (pH 7.0)–acetonitrile

TABLE I

RECOVERY OF TRICLABENDAZOLE SULPHOXIDE ($T_{SO}$) AND TRICLABENDAZOLE SULPHONE ($T_{SO_2}$) IN SERUM AND URINE SAMPLES

| Added concentration (μg/ml) | Recovery (mean ± S.D., $n$ = 10) (%) | | | |
|---|---|---|---|---|
| | Serum | | Urine | |
| | $T_{SO}$ | $T_{SO_2}$ | $T_{SO}$ | $T_{SO_2}$ |
| 0.01 | 88.8 ± 5 | 85.4 ± 3 | 82.3 ± 4 | 85.3 ± 4 |
| 0.10 | 89.1 ± 2 | 97.8 ± 2 | 92.7 ± 2 | 86.6 ± 3 |
| 0.25 | 94.7 ± 4 | 94.7 ± 2 | 86.4 ± 5 | 90.0 ± 3 |
| 0.50 | 101.5 ± 2 | 104.5 ± 2 | 92.0 ± 2 | 92.3 ± 1 |
| 1.0 | 92.3 ± 3 | 90.0 ± 3 | 89.2 ± 3 | 85.4 ± 2 |
| 3.0 | 88.9 ± 5 | 84.2 ± 5 | 82.7 ± 4 | 83.2 ± 3 |
| 6.0 | 92.7 ± 1 | 89.3 ± 4 | 96.2 ± 2 | 101.0 ± 2 |
| 9.0 | 86.5 ± 6 | 86.9 ± 4 | 101.0 ± 2 | 98.2 ± 3 |

TABLE II

ACCURACY AND PRECISION OF THE DETERMINATION OF TRICLABENDAZOLE SULPHOXIDE ($T_{SO}$) AND TRICLABENDAZOLE SULPHONE ($T_{SO_2}$) IN SERUM AND URINE SAMPLES

| Added concentration ($\mu$g/ml) | Serum | | | | Urine | | | |
|---|---|---|---|---|---|---|---|---|
| | $T_{SO}$ | | $T_{SO_2}$ | | $T_{SO}$ | | $T_{SO_2}$ | |
| | Concentration found[a] ($\mu$g/ml) | C.V.[b] (%) | Concentration found[a] ($\mu$g/ml) | C.V.[b] (%) | Concentration found[a] ($\mu$g/ml) | C.V.[b] (%) | Concentration found[a] ($\mu$g/ml) | C.V.[b] (%) |
| 0.01 | $8.88 \cdot 10^{-3} \pm 4 \cdot 10^{-4}$ | 4.5 | $8.50 \cdot 10^{-3} \pm 2 \cdot 10^{-4}$ | 2.3 | $8.23 \cdot 10^{-3} \pm 3 \cdot 10^{-4}$ | 3.6 | $8.50 \cdot 10^{-3} \pm 3 \cdot 10^{-4}$ | 3.5 |
| 0.1 | $0.09 \pm 2 \cdot 10^{-3}$ | 2.2 | $0.09 \pm 2 \cdot 10^{-3}$ | 2.2 | $0.09 \pm 2 \cdot 10^{-3}$ | 2.2 | $0.08 \pm 2 \cdot 10^{-3}$ | 2.5 |
| 0.25 | $0.23 \pm 9 \cdot 10^{-3}$ | 3.9 | $0.23 \pm 5 \cdot 10^{-3}$ | 2.1 | $0.21 \pm 0.01$ | 4.7 | $0.22 \pm 6 \cdot 10^{-3}$ | 2.7 |
| 0.50 | $0.51 \pm 0.01$ | 1.9 | $0.53 \pm 0.01$ | 0.1 | $0.46 \pm 0.01$ | 2.1 | $0.46 \pm 5 \cdot 10^{-3}$ | 1.1 |
| 1.0 | $0.92 \pm 0.03$ | 3.2 | $0.90 \pm 0.03$ | 3.3 | $0.89 \pm 0.02$ | 2.2 | $0.85 \pm 0.01$ | 1.2 |
| 3.0 | $2.66 \pm 0.13$ | 4.9 | $2.52 \pm 0.13$ | 5.1 | $2.48 \pm 0.09$ | 3.6 | $2.49 \pm 0.07$ | 2.8 |
| 6.0 | $5.56 \pm 0.05$ | 1.0 | $5.36 \pm 0.21$ | 3.9 | $5.77 \pm 0.11$ | 1.9 | $6.06 \pm 0.12$ | 1.9 |
| 9.0 | $7.80 \pm 0.47$ | 6.0 | $7.70 \pm 0.31$ | 4.0 | $9.09 \pm 0.18$ | 1.9 | $8.80 \pm 0.26$ | 2.9 |

[a] Mean ± S.D. ($n = 6$).

[b] Coefficient of variation.

(55:45, v/v) containing 1.0 mmol/l sodium decanesulphonate. The chromatograms obtained for serum and urine samples are shown in Fig. 5. The results for the recovery of triclabendazole sulphoxide and sulphone are given in Table I. To determine the recovery, we injected, after processing, ten samples each of serum and urine, and divided the peak area by that of one of ten control samples containing a known amount of drug dissolved in methanol. Table II gives accuracy and precision data, obtained by analysing six samples of each concentration of serum and urine. The limit of detection for both metabolites is 10 ng/ml in serum and urine samples, determined as the lowest detectable concentration of the compounds giving a response at least three times the baseline noise.

## CONCLUSIONS

A rapid and sensitive method for determining triclabendazole metabolites in biological fluids using a simple extraction technique and isocratic elution has been developed. The recoveries and detection limits are similar to those of previously reported procedures. The proposed technique has advantages of simple sample preparation, thus making the total analysis time much shorter.

## ACKNOWLEDGEMENT

We are grateful to Ciba-Geigy for the supply of triclabendazole and its metabolites.

## REFERENCES

1 J. C. Boray, P. D. Crowfoot, M. B. Strong, J. R. Allison, M. Schellenbaum, M. Von Orelli and G. Sarasin, *Vet. Rec.*, 113 (1983) 315.
2 K. Wolf, J. Ecker, G. Schneiter and H. Lute, *Vet. Parasitol.*, 13 (1983) 145.
3 D. R. Henessy, E. Lacey, J. W. Steel and R. K. Pritchard, *J. Vet. Pharmacol. Ther.*, 10 (1987) 64.
4 L. D. B. Kinabo and J. A. Bogan, *J. Vet. Parmacol. Ther.*, 11 (1988) 254.
5 A. P. Goldberg, E. Nowanowska and P. E. Antle, *J. Chromatogr.*, 316 (1984) 241.
6 R. Gloor, and E. L. Johnson, *J. Chromatogr. Sci.*, 15 (1977) 413.
7 K. Tyczkowska, D. P. Aucoin, D. C. Richardson and A. L. Aronson, *J. Liq. Chromatogr.*, 10 (1987) 2631.
8 K. H. Lehr and P. Damm, *J. Chromatogr.*, 382 (1986) 355.
9 M. S. Bull and G. R. E. Shome, *J. Pharm. Biomed. Anal.*, 5 (1987) 527.