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Simultaneous determination of thiabendazole and its major metabolite, 5-hydroxythiabendazole, in bovine tissues using gradient liquid chromatography with thermospray and atmospheric pressure chemical ionisation mass spectrometry

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Abstract

A novel method is presented for the determination of thiabendazole and 5-hydroxythiabendazole in animal tissues. Samples are homogenised in buffer at pH=7.0, extracted with ethyl acetate and cleaned up using CN solid-phase extraction columns. Thiabendazole and 5-hydroxythiabendazole are separated chromatographically using gradient elution and analysed by liquid chromatography–mass spectrometry. Deuterated thiabendazole is employed as an internal standard for thiabendazole determination; 5-hydroxythiabendazole is quantified via external standards. Samples are screened by monitoring the protonated molecular ions at m/z=202 for thiabendazole, 206 for deuterated thiabendazole and 218 for 5-hydroxythiabendazole using thermospray LC–MS. Positives are confirmed by multiple ion monitoring using APCI LC–MS. Validation of the method was carried out at 50, 100 and 200 μg kg^{-1}. Recoveries for thiabendazole in bovine muscle, liver and kidney ranged from 96–103% with C.V.s between 0.7 and 4.8% and for 5-hydroxythiabendazole recoveries ranged from 70–85% with C.V.s between 3.1 and 11.5%.

Keywords: Thiabendazole; Hydroxythiabendazole

1. Introduction

Thiabendazole (TBZ; 2-(4-thiazolyl)-1*H*-benzimidazole) is a broad-spectrum anthelmintic effective against gastrointestinal nematodes in ruminants and lungworms in sheep [1]. It is also a versatile fungicide and is widely used both pre- and post harvest to control a range of pathogenic fungi affecting field crops and stored fruits and vegetables. Since its introduction over 30 years ago, other benzimidazole anthelmintics with improved efficacy, such as fenbendazole, albendazole and triclabendazole, have been developed, but TBZ remains in use because it is relatively inexpensive in comparison with the newer compounds. TBZ is metabolised in farm animals via hydroxylation of the benzimidazole ring at the 5-position to form 5-hydroxythiabendazole (5-OHTBZ) with subsequent glucuronide or sulfate conjugation [2,3]. A maximum residue limit (MRL) of 100 μg kg^{-1} for the sum of TBZ and 5-OHTBZ in muscle, liver, kidney, fat and milk has been set by the European Union (EU) [4].

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To prevent the occurrence of residues of TBZ and/or 5-OHTBZ at concentrations greater than the MRL, set withdrawal periods must be observed before treated animals are presented for slaughter. Failure to observe the withdrawal period may lead to elevated TBZ residues entering the human food chain. The use of TBZ treated feedingstuffs for livestock may also contribute to tissue residues of the drug. Methods are therefore required to monitor tissues for residues of TBZ and 5-OHTBZ.

Very few methods have been published for the determination of both TBZ and 5-OHTBZ in biological matrices. TBZ has been determined alone, or in combination with other anthelmintics, by various high-performance liquid chromatography (HPLC) methods with detection by UV, photodiode array or fluorescence spectrophotometry [5–8]. HPLC–UV has been used for the detection of eight benzimidazoles, including TBZ, with subsequent confirmation by derivatisation and gas chromatography–mass spectrometry (GC–MS) [9]. A monoclonal antibody-based ELISA method for TBZ in liver has been reported [10]. However, since the MRL applies to the sum of TBZ and 5-OHTBZ these methods do not meet the requirements of EU legislation. HPLC methods for the determination of both analytes in milk have been described [11,12]. A collaborative study on a multiresidue LC method has been published [13]. However, although results were good from the laboratory of origin of the method, the study data were not satisfactory for TBZ and 5-OHTBZ. A GC–MS confirmatory assay for both analytes at 100 $\mu g\ kg^{-1}$ in animal tissues has also been reported [14]. Mass spectrometry is recommended by the EU [15] as the method of choice for confirmatory residue analysis because of the specificity inherent to the technique. We have previously described simple and rapid liquid chromatography–mass spectrometry (LC–MS) methods for anthelmintic and antimicrobial substances [16–18]. This paper describes a method for the simultaneous determination of TBZ and 5-OHTBZ in liver, kidney and muscle tissues by gradient LC–MS after extraction with ethyl acetate and solid-phase cyanopropyl (CN) clean-up. Deuterated TBZ (D_4-TBZ) is employed as an internal standard for TBZ determination; 5-OHTBZ is determined using external standards. The molecular ions for each analyte and the internal standard are monitored using thermospray (TS) LC–MS, and further confirmation of positives is provided by multiple ion monitoring using atmospheric pressure chemical ionisation (APCI) LC–MS. The limit of determination of the method, defined as the lowest level at which it has been validated, is 50 $\mu g\ kg^{-1}$, or half the MRL for each analyte, as suggested by the EU [15]. The limits of detection, based on a signal to noise ratio of 3:1, for both TBZ and 5-OHTBZ in each tissue are $<10\ \mu g\ kg^{-1}$.

2. Experimental

2.1. Materials

All solvents were of HPLC grade and other chemicals were of analytical reagent grade. Distilled or deionized water was used throughout. TBZ was obtained from Sigma (Poole, Dorset, UK). D_4-TBZ was synthesised by Quchem (The Queen's University of Belfast, UK). 5-OHTBZ was obtained from Merck (Whitehouse Station, NJ, USA). Stock standard solutions (1 $mg\ ml^{-1}$) of TBZ, D_4-TBZ and 5-OHTBZ were prepared in methanol and were stable for at least 6 months when stored at 4°C. Dilute standard solutions (10 $\mu g\ ml^{-1}$) of each compound were prepared by dilution of the stock standards with methanol and were stable for at least 3 months when stored at 4°C. A mixed working standard containing TBZ, D_4-TBZ and 5-OHTBZ at 1 $\mu g\ ml^{-1}$ was prepared with every batch of samples by mixing aliquots (100 μl) of each dilute standard solution (10 $\mu g\ ml^{-1}$), evaporating the mixture to dryness under nitrogen and redissolving in methanol–acetonitrile–water (2:1:7, v/v/v, 1 ml). Phosphate buffer (0.5 *M,* pH=7.0) was prepared by dissolving sodium dihydrogen orthophosphate 1-hydrate (69 g) in water, adjusting to pH=7.0 with sodium hydroxide (10% w/v) and adjusting the volume to 500 ml with water.

2.2. Equipment

Bakerbond SPE Cyano (CN) 3 ml disposable extraction columns (J.T. Baker, Phillipsburg, NJ, USA) were conditioned immediately before use with hexane (4 ml) and were not allowed to dry out

before sample application. The columns were used with a Vac–Elut vacuum manifold fitted with stainless steel Luer stopcocks (Varian, Harbour City, CA, USA).

2.3. TS LC–MS system

The HPLC system comprised a Hewlett Packard (Stockport, Cheshire, UK) series 1100 binary pump, autosampler and solvent degasser and a Phenomenex (Macclesfield, Cheshire, UK) Prodigy 5 ODS 3, 150×4.6 mm I.D. column with 2 μm column prefilter (Alltech Associates/Applied Science, Carnforth, Lancashire, UK). Mobile phase A was ammonium acetate (0.1 *M*) and mobile phase B was acetonitrile. The LC was linked via a thermospray interface to a Hewlett–Packard 5989A MS Engine controlled by an HP MS ChemStation. The MS was tuned daily according to the manufacturer's instructions. The instrument was operated in positive-ion mode with thermospray ionisation. The ion source and analyser were set to 250 and 100°C, respectively. The fragmenter electrode was set to 170 V and the electron multiplier to approximately 2000 V. Spectra were obtained over the range m/z=50–350 in scan mode using direct flow injection (no column connected) with a mobile phase composition of 50% A:50% B. The vaporiser stem temperature was set to 95% of the temperature required for complete vaporisation. For sample analysis, selected ion monitoring (SIM) mode and gradient chromatography were employed. The LC gradient programme had a total run time of 10 mm, and ramped from an initial composition of 25% B from 0–2 min to 80% B at 6 min, returning to 25% B at 8 min and maintaining this composition for 2 min to permit re-equilibration. The vaporiser stem temperature was ramped to follow the mobile phase gradient, maintaining the stem temperature at approximately 95% of the take-off temperature throughout the programme. The vaporiser probe stem temperature ramp lagged behind the mobile phase composition ramp by 1 min to allow for the delay as any given composition of mobile phase passed through the LC column before reaching the probe. Peak area data were obtained for the ions at m/z=202 (TBZ), 206 (D_4-TBZ) and 218 (5-OHTBZ). The dwell time for each ion was 500 ms and low mass resolution was enabled.

2.4. APCI-LC–MS system

The binary gradient HPLC equipment consisted of an L6200A intelligent pump, an L6000 pump and an A52000 autosampler (Merck, Poole, Dorset, UK). The HPLC column, mobile phases and gradient programme were as described above. The LC was coupled to the APCI probe of a Platform LC–MS system (Micromass, Altrincham, Cheshire, UK). The source was maintained at 150°C and the APCI probe at 450°C. The flow-rates of the sheath and drying gases (nitrogen) were 50 and 200 l h^{-1}, respectively. Spectra were obtained in positive mode over the range m/z=50–350 using a range of cone voltages between 10 and 60 V. SIM was performed for four ions from each analyte with a dwell time of 200 ms for each ion and with the cone set to 55 V. The ions for 5-OHTBZ were monitored from 0–5 min and those for TBZ from 5–10 min.

2.5. Sample extraction

Aliquots (3.0 g) of minced bovine liver, kidney or muscle, which had been obtained from animals held on a government-owned farm, were weighed into 110×25 mm I.D. centrifuge tubes. Fortified samples were prepared by adding an appropriate amount of TBZ and 5-OHTBZ to known negative samples. Internal standard (D_4-TBZ, 10 μg ml^{-1}, 30 μl) was added to unknowns, negatives and fortified negatives and the samples allowed to stand for 10 min. Phosphate buffer (0.5 *M,* pH=7.0, 7 ml) was added and the samples homogenised for 1 min using a Silverson homogenizer. Ethyl acetate (15 ml) was added and the tubes were capped and shaken for 40 sec. The tubes were centrifuged (600 *g*, 4°C, 10 min) and aliquots (10 ml) of the supernatants transferred into 100×12 mm tubes. The volume was reduced to approximately 1 ml at 70°C under a stream of nitrogen. Hexane (5 ml) was added and the tubes agitated to mix the contents.

2.6. CN clean-up

The extracts were applied to CN cartridges prepared as described above. The tubes were washed with hexane (4 ml) which was also applied to the CN cartridges, and the cartridges allowed to dry under vacuum for 10 min. The analytes were eluted into

100×12 mm tubes with 2×2 ml portions of methanol containing 0.2% (v/v) triethylamine. The eluates were evaporated to dryness at 70°C under nitrogen and the residues redissolved in methanol–acetonitrile–water (2:1:7, v/v/v, 200 μl) by vortexing. A working standard was also prepared at this stage as described above. The tubes were sonicated in an ultrasonic bath for 10 min and the samples transferred to microvials for analysis.

2.7. LC–MS analysis

Mobile phase at the initial composition was pumped through the column at a flow-rate of 1 ml min^{-1} to equilibrate the system before beginning the analyses. Aliquots (25 μl) of the mixed working standard (TBZ, D_4-TBZ and 5-OHTBZ, 1 μg ml^{-1}) were run on the TS LC–MS system using the gradient programme described above until reproducible peak areas were obtained (usually three injections). Aliquots of sample extracts were then injected, with a standard injection after every 3 samples. Peak area data for the ions at $m/z=202$ (TBZ), 206 (D_4-TBZ) and 218 (5-OHTBZ) were recorded. Results for TBZ were calculated on an internal standard basis by comparing the peak area ratio ($m/z=202/206$) in a sample with the ratio in the standard, whereas for 5-OHTBZ results were calculated by comparison of the sample peak areas at $m/z=218$ with those of the standard peak areas.

For confirmation by APCI, aliquots of standards and samples were analysed using the same chromatographic conditions. Peak area data for the ions at $m/z=202$, 175, 131 and 92 were recorded for TBZ and at $m/z=218$, 191, 147 and 108 for 5-OHTBZ.

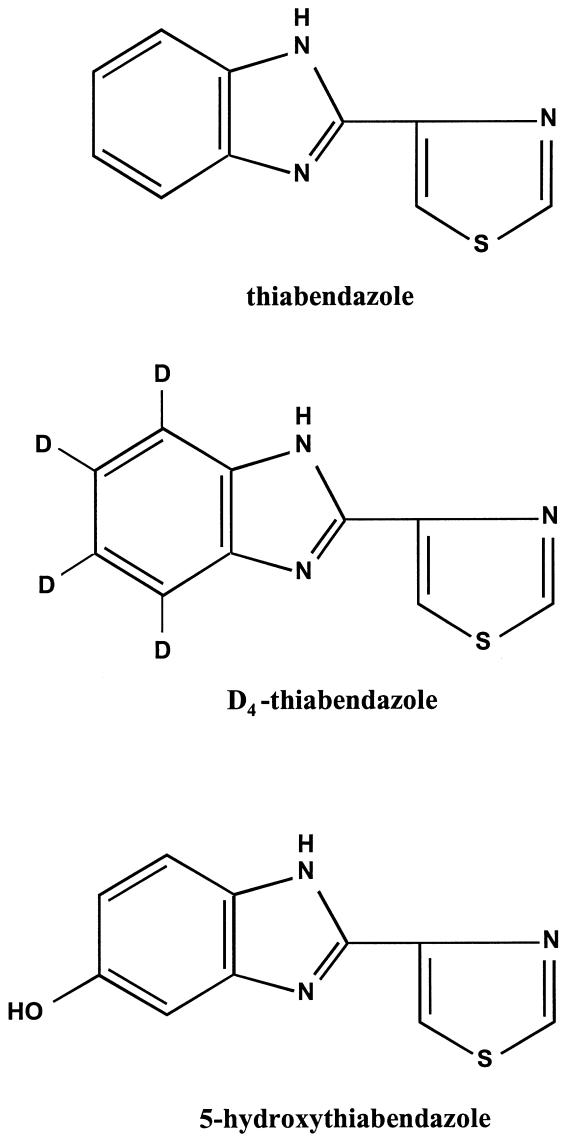

Fig. 1. Structures of thiabendazole, deuterated (D_4) thiabendazole (internal standard) and 5-hydroxythiabendazole.

3. Results and discussion

The structures and positive-ion thermospray mass spectra of TBZ, D_4-TBZ and 5-OHTBZ are shown in Figs. 1 and 2, respectively. Each compound exhibits an ionisation pattern with minimal fragmentation, characteristic of thermospray LC–MS. The base peak for each of the compounds is the protonated molecular ion $[M+H]^+$ at $m/z=202$ for TBZ, $m/z=206$ for D_4-TBZ and $m/z=218$ for 5-OHTBZ. Each spectrum also exhibits a peak corresponding to an acetonitrile adduct, but the formation of these adducts was variable and they were usually not evident at low concentrations of the analytes and therefore not useful for analytical purposes.

For APCI, with cone voltages less than 30 V, the mass spectra produced were similar to the thermospray spectra, the only difference being the absence of acetonitrile adducts. As the voltage was increased above 30 V fragmentation was initiated. Spectra for

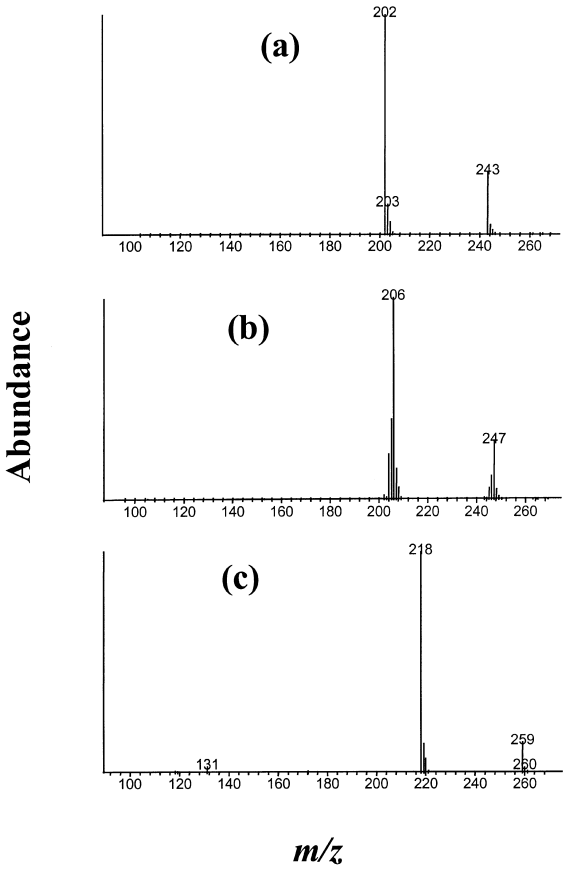

Fig. 2. Positive-ion thermospray mass spectra of (a) thiabendazole, (b) deuterated (D_4) thiabendazole and (c) 5-hydroxythiabendazole.

TBZ and 5-OHTBZ with the cone set to 55 V are shown in Fig. 3. Four major ions were produced for TBZ at m/z=202 $[M+H]^+$, m/z=175 and 131, probably due to the loss of HCN and CH–S–CHN, respectively, from the thiazolyl moiety, and m/z=92, probably corresponding to cleavage of the imidazole ring. The same pattern was observed for D_4-TBZ, ions being present at m/z=206, 179, 135 and 96, with corresponding isotope clusters. The ions produced for D_4-TBZ were discrete from those for TBZ and therefore the use of the internal standard caused no interference in the confirmatory analysis for TBZ. 5-OHTBZ also fragmented in the same way, giving ions at m/z=218, 191, 147 and 108. For the confirmatory analysis of tissue extracts, it was found that the ion at m/z=108 was not sufficiently resolved from chromatographic interferences to permit its use

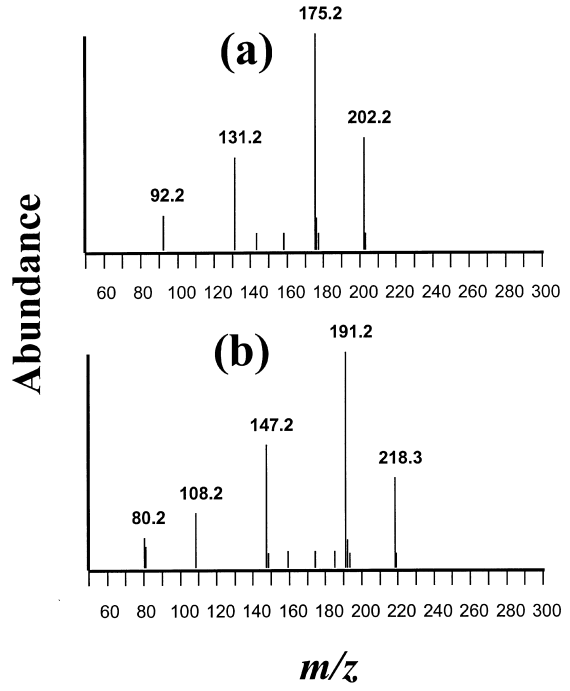

Fig. 3. Positive-ion APCI spectra of (a) thiabendazole and (b) 5-hydroxythiabendazole. The skimmer cone was set to 55 V.

as a diagnostic ion, so the ratios of three rather than four ions were used to confirm 5-OHTBZ.

Fig. 4 shows SIM TS chromatograms for a mixed standard containing TBZ, D_4-TBZ and 5-OHTBZ equivalent to 100 μg kg^{-1} in tissue, a negative liver and a liver sample fortified with TBZ and 5-OHTBZ at 50 μg kg^{-1}. The retention times for TBZ and 5-OHTBZ are approximately 6 min and 3.2 min, respectively. The chromatograms are free from any significant interfering peaks. Chromatograms for kidney and muscle (not shown) were similar. The application of gradient LC enabled both compounds to be analysed in a single chromatographic run with a short run-time. The best signal-to-noise ratios were obtained using thermospray ionisation without filament or discharge electrode assistance. A slight increase in sensitivity was observed when the fragmenter electrode was maintained at 170 V.

Figs. 5 and 6 show APCI SIM chromatograms for a mixed TBZ/5-OHTBZ standard and a liver sample fortified with TBZ and 5-OHTBZ at 50 μg kg^{-1}. It was observed that the ion ratios for standard injections drifted slightly over a number of hours,

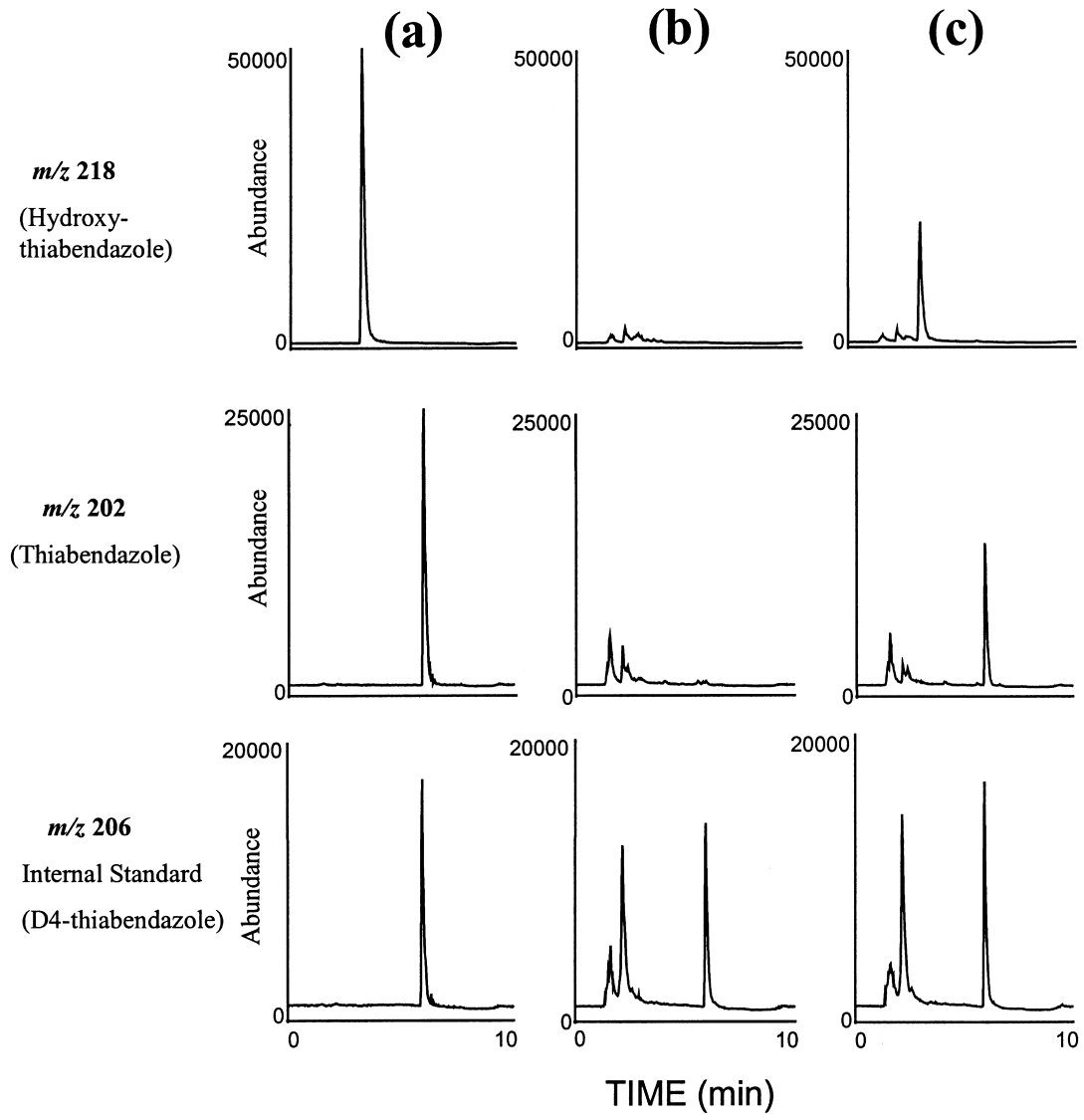

Fig. 4. SIM thermospray LC–MS chromatograms for (a) a mixed standard containing 5-hydroxythiabendazole, thiabendazole and D_4 thiabendazole, (b) a known negative liver and (c) a known negative liver fortified with 5-hydroxythiabendazole and thiabendazole at 50 μg kg^{-1}.

possibly due to the ion source or skimmer cone becoming contaminated. To overcome this, a standard was injected after every three sample injections and the ion ratios in any sample were compared with the means of the standards injected before and after that sample.

During the development of the method, samples and working standards were initially dissolved in acetonitrile–water (25:75, v/v). This caused some peak broadening for the early-eluting 5-OHTBZ peak in standards, whereas a reasonable peak shape was obtained for tissue extracts, probably due to the effect of the matrix. Injecting the standard dissolved in methanol or methanol–water (25:75, v/v) gave a good peak shape for 5-OHTBZ but caused broadening of the later-eluting TBZ peak. Symmetrical peaks

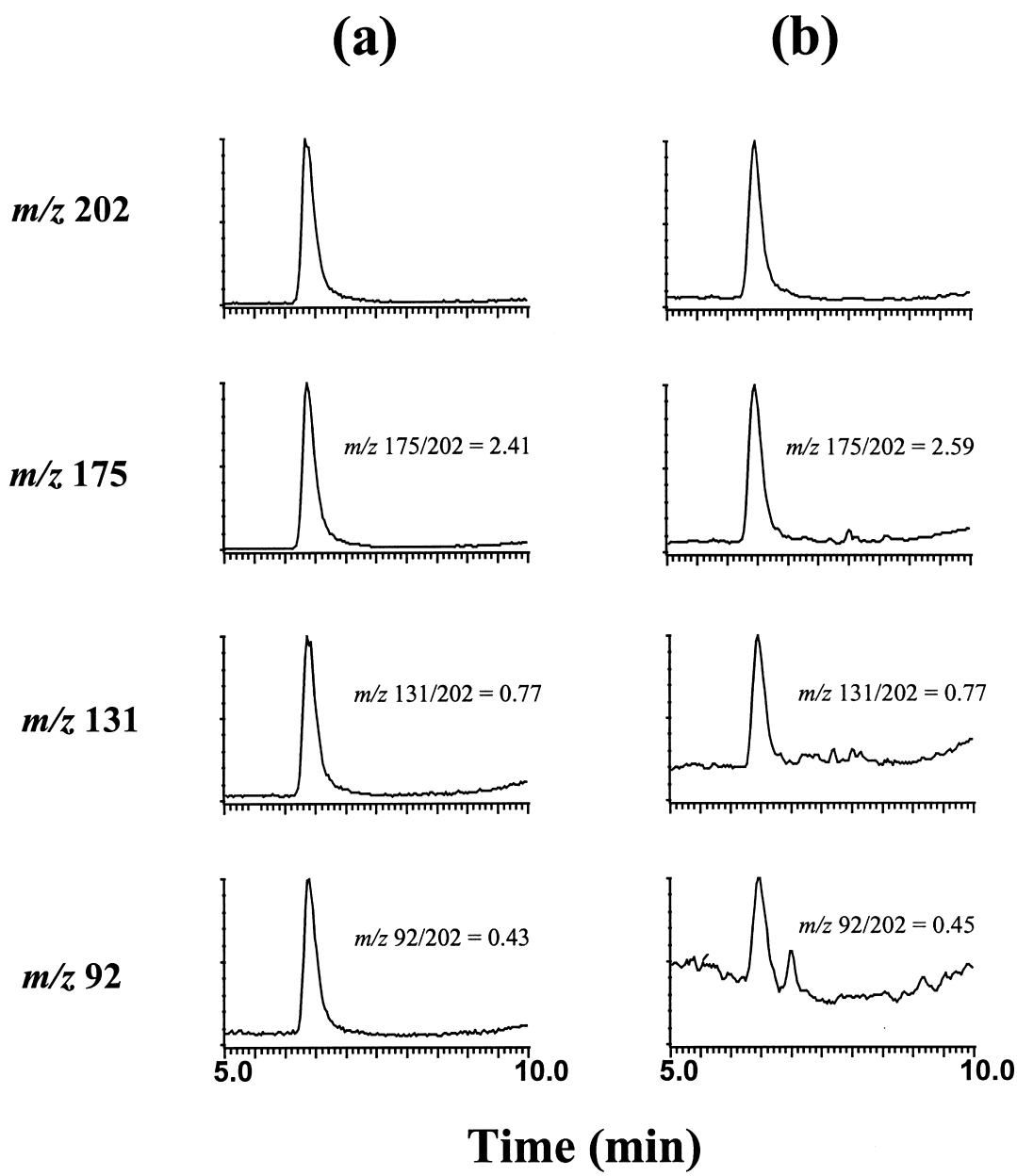

Fig. 5. Normalised APCI chromatograms for (a) a thiabendazole standard equivalent to 100 μg kg^{-1} and (b) a negative liver sample fortified at 50 μg kg^{-1}. Figures on chromatograms indicate peak area ratios.

and baseline resolution for both analytes could be obtained by modifying the mobile phase by the addition of trifluoroacetic acid (0.1%, v/v), but this caused ionisation suppression for 5-OHTBZ, thus affecting the sensitivity of the method. Optimum chromatography for both peaks was obtained by dissolving working standards and tissue extracts in methanol–acetonitrile–water (2:1:7, v/v/v).

D_4-TBZ was used as an internal standard for TBZ, but no suitable labelled compound was available for

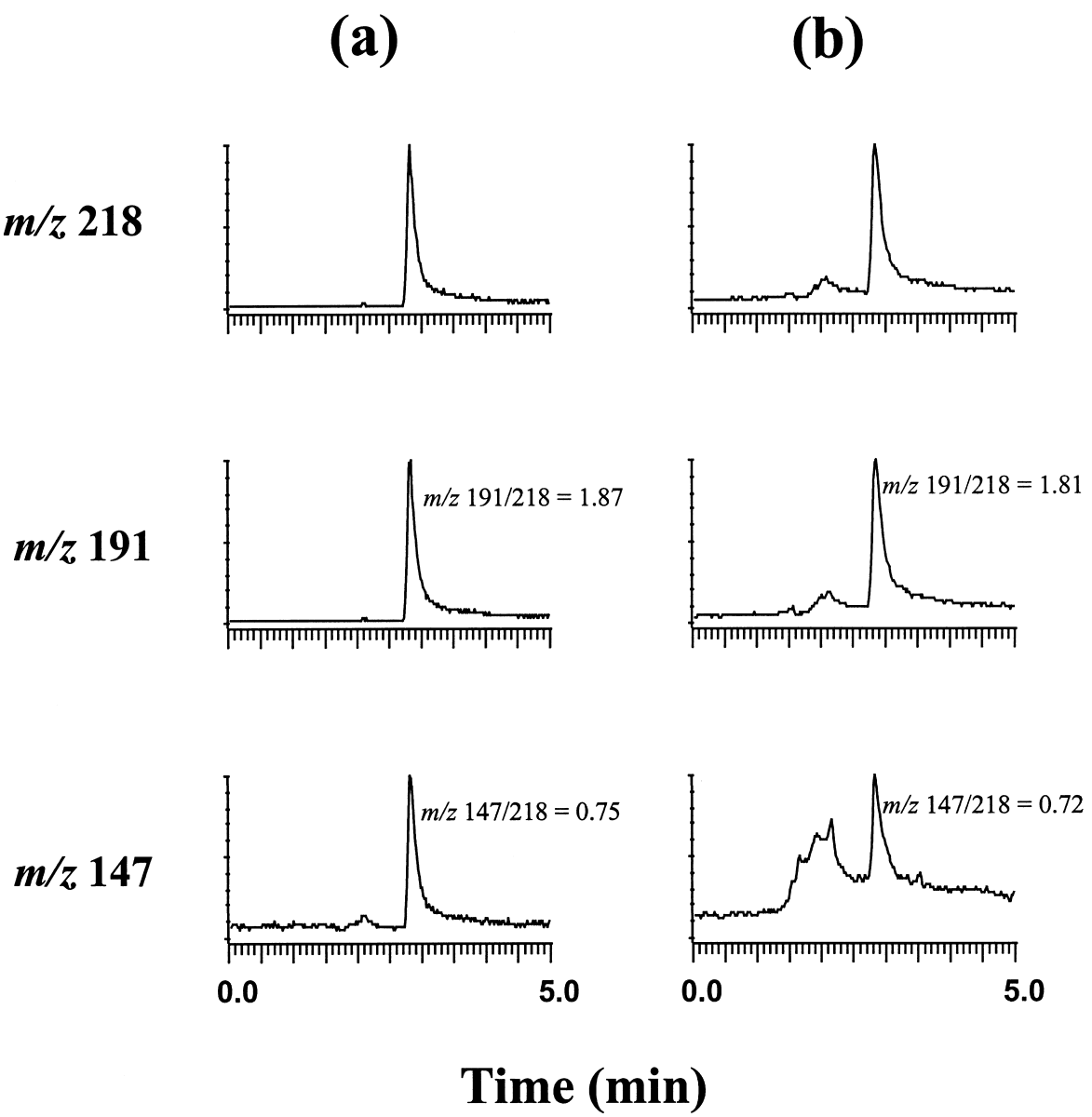

Fig. 6. Normalised APCI chromatograms for (a) a 5-hydroxythiabendazole standard equivalent to 100 μg kg^{-1} and (b) a negative liver sample fortified at 50 μg kg^{-1}. Figures on chromatograms indicate peak area ratios.

use as an internal standard for 5-OHTBZ. The extraction and clean-up were therefore optimised for 5-OHTBZ, since minor shortcomings in the procedure for TBZ would be corrected for by the internal standard. Typical absolute recoveries for TBZ ranged from approximately 60% to 80%, whereas corrected recoveries were between 96% and 103%, indicating the efficacy of the internal standard. D_4-TBZ was not used as internal standard for 5-OHTBZ since the recovery of 5-OHTBZ was approximately 70–85% and the internal standard would therefore have over-corrected. The robustness

of the method for 5-OHTBZ would be improved by the inclusion of an internal standard such as deuterated 5-OHTBZ.

The response of the TS LC–MS was shown to be linear for both TBZ and 5-OHTBZ by analysing a series of standard solutions over the range 0–800 μg ml^{-1} (equivalent to 0–800 μg kg^{-1}), with correlation coefficients (R) of 0.9977 for TBZ and 0.9984 for 5-OHTBZ. A standard curve was also prepared for TBZ over the range 0–8000 ng ml^{-1} with the internal standard included at 1000 ng ml^{-1}. The peak area ratio (m/z=202/206) was plotted against TBZ concentration, giving a correlation coefficient of 0.9997.

The TS LC–MS method was validated for liver, kidney and muscle. Inter- and intra-assay precision and recovery were assessed by extracting and analysing five replicates of negative bovine liver, spiked at 50, 100 and 200 μg kg^{-1} with TBZ and 5-OHTBZ, on three separate occasions. The results are presented in Tables 1 and 2. Intra-assay validation was also carried out for muscle and kidney spiked at 50 μg kg^{-1} (Table 3). Six replicates of negative bovine liver spiked at 50 μg kg^{-1} were also analysed by APCI LC–MS. The ratios of the four ions monitored for TBZ and those of the three ions monitored for 5-OHTBZ were within ±10% of the ratios found in standards.

A limitation of TS-LC–MS is the characteristic soft ionisation, typically producing a single ion for each analyte. APCI-LC–MS has the advantage that, by increasing the skimmer cone voltage, collision induced dissociation may be initiated, thereby producing further diagnostic ions for the compounds of interest. Techniques that produce more than one diagnostic ion provide more structural information about the analytes and therefore offer improved specificity. The GC–MS method described by VandenHeuvel et al. [14] employed on-column methylation to form the methyl and the dimethyl derivatives of TBZ and 5-OHTBZ, respectively. Identification and quantification were achieved by SIM of the M−1, M and M+1 ions from *N*-methylthiabendazole and the M and M−15 ions from *N*,*O*-di-

Table 1
Inter- and intra-assay reproducibility and recovery for thiabendazole in bovine liver spiked at 50, 100 and 200 μg kg^{-1} [a]

	Day 1	Day 2	Day 3	Overall
50 μg kg^{-1}				
Mean (μg kg^{-1})	50.6	50.4	48.0	49.7
S.D. [b] (μg kg^{-1})	2.02	1.90	3.00	2.50
C.V. [c] (%)	4.0	3.8	6.3	5.0
Mean recovery (%)	101.2	100.8	96.0	99.4
n [d]	5	5	5	15
100 μg kg^{-1}				
Mean (μg kg^{-1})	103.0	97.4	102.9	101.1
S.D. (μg kg^{-1})	2.10	3.61	2.05	3.68
C.V. (%)	2.0	3.7	2.0	3.6
Mean recovery (%)	103.0	97.4	103.0	101.1
n	5	5	5	15
200 μg kg^{-1}				
Mean (μg kg^{-1})	194.1	201.8	197.3	197.7
S.D. (μg kg^{-1})	6.33	1.43	4.67	5.38
C.V. (%)	3.3	0.7	2.4	2.7
Mean recovery (%)	97.1	100.9	98.7	98.9
n	5	5	5	15

[a] Results are calculated using internal standard.
[b] *S.D.*=Standard deviation
[c] *C.V.*=Coefficient of variation
[d] *n*=number of replicates

Table 2
Inter- and intra-assay reproducibility and recovery for 5-hydroxythiabendazole in bovine liver spiked at 50, 100 and 200 μg kg^{-1}

	Day 1	Day 2	Day 3	Overall
50 μg kg^{-1}				
Mean (μg kg^{-1})	37.8	42.4	41.7	40.6
S.D. (μg kg^{-1})	1.15	4.86	3.46	3.88
C.V. (%)	3.1	11.4	8.3	9.5
Mean recovery (%)	75.6	84.9	83.5	81.3
n	5	5	5	15
100 μg kg^{-1}				
Mean (μg kg^{-1})	73.3	73.0	69.6	72.0
S.D. (μg kg^{-1})	5.16	4.20	3.99	4.50
C.V. (%)	7.0	5.8	5.7	6.2
Mean recovery (%)	73.3	73.0	69.6	72.0
n	5	5	5	15
200 μg kg^{-1}				
Mean (μg kg^{-1})	147.5	153.0	143.6	148.0
S.D. (μg kg^{-1})	7.66	12.70	8.05	9.87
C.V. (%)	5.2	8.3	5.6	6.7
Mean recovery (%)	73.8	76.5	71.8	74.0
n	5	5	5	15

methyl-5-hydroxy thiabendazole. Marti et al. [9] also used GC–MS to confirm the identity of a number of anthelmintics, including TBZ but not 5-OHTBZ. Multiple ions were produced from the methyl derivative of TBZ in electron-impact mode, and from the pentafluorobenzyl derivative in chemical-ionisation mode. However, both of these GC–MS methods employed relatively lengthy and laborious clean-up procedures and also required the derivatisation of the analytes. The LC–MS method described in this paper is rapid and requires no derivatisation, and also provides multiple ions for confirmation. The initial screening of samples by thermospray, with APCI used for confirmation only, facilitates better time-management of analytical equipment which is already under heavy demand. However, APCI-LC–MS may be the choice for the future, given that it can achieve a much greater degree of fragmentation than TS-LC–MS.

4. Conclusions

The method described employs a simple extraction and rapid solid-phase clean-up, enabling 12 samples to be extracted and analysed in one working day. Both TBZ and 5-OHTBZ are determined in contrast to most of the published methods for residues in meat, which measure only TBZ. The method is reproducible and robust, although it would be further

Table 3
Intra-assay reproducibility and recovery for thiabendazole and 5-hydroxy-thiabendazole in bovine muscle and kidney spiked at 50 μg kg^{-1}

	Thiabendazole		5-Hydroxythiabendazole	
	Muscle	Kidney	Muscle	Kidney
Mean (μg kg^{-1})	50.0	49.4	35.9	40.05
S.D. (μg kg^{-1})	2.41	1.08	1.97	4.62
C.V. (%)	4.8	2.2	5.5	11.5
Mean recovery (%)	100.0	98.7	71.7	80.1
n	5	5	5	5

improved by the inclusion of a suitable internal standard for 5-OHTBZ. The sensitivity is comparable to that of other published methods and the use of MS rather than UV or fluorescence detection provides enhanced specificity for the analytes. In accordance with European Union Commission Decision 93/256/EEC [15] which proposes that, for compounds with an established MRL, methods should be validated at half of the MRL, this method has been validated at 50 μg kg^{-1} for both TBZ and 5-OHTBZ.

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