ELSEVIER

Journal of Chromatography B, 692 (1997) 351–360

JOURNAL OF CHROMATOGRAPHY B

Confirmatory assay for the determination of tetracycline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography–mass spectrometry

W. John Blanchflower*, Robert J. McCracken, Audrey S. Haggan, D. Glenn Kennedy

Department of Agriculture, Veterinary Sciences Division, Stormont, Belfast BT4 3SD, N. Ireland, UK

Received 17 September 1996; revised 11 November 1996; accepted 18 November 1996

Abstract

A confirmatory method is described for the determination of tetracycline, oxytetracycline and chlortetracycline in muscle and kidney using liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry. The tetracyclines were extracted from tissue using glycine–HCl buffer and concentrated using solid-phase extraction. HPLC separation was carried out using a gradient and the tetracyclines were detected using a bench-top LC–MS system. Several ions could be monitored for each tetracycline, allowing ion ratio measurements to be made. The detection limits for the assay were in the region of 10 ng/g in muscle and 20 ng/g in kidney. Validation was carried out at half the maximum residue limit, the maximum residue limit and two times the maximum residue limit. The formation of epimers and tautomers of the tetracyclines, their presence in incurred tissues and difficulties in their accurate quantitation is discussed.

Keywords: Tetracycline; Oxytetracycline; Chlortetracycline

1. Introduction

The tetracyclines are broad-spectrum antibacterials active against a range of organisms such as *Mycoplasma*, *Chlamydia* as well as a number of Gram-positive and Gram-negative bacteria and are widely used in veterinary medicine. Both tetracycline and chlortetracycline are licensed in the UK for use in pigs and poultry and are normally administered orally. Oxytetracycline is licensed for a wide range of species and can be administered either orally or by subcutaneous, intramuscular, or intravenous injection. Withdrawal periods of 5–20 days before slaughter are recommended for food producing animals depending on the species and nature of the food product.

The European Union has set maximum residue limits (MRLs) for tetracyclines in various food products. Member States are therefore required to carry out testing to reduce the likelihood of food containing tetracycline levels above the MRLs from entering the food supply. The levels set for muscle and kidney are 100 and 600 ng/g, respectively and recent EU legislation [1] stipulates that this should comprise the sum of the parent drug and its 4-epimer.

Many methods for the detection of tetracyclines in food products have been published. Screening assays are mainly based on the four plate test [2] and confirmatory assays have mainly involved HPLC

*Corresponding author.

PII S0378-4347(96)00524-5

with a variety of clean-up and detection techniques. Sample preparation steps include solid-phase extraction [3,4], matrix solid-phase dispersion [5], and chelate affinity chromatography [6,7]. Detection techniques used include UV detection [8] and fluorescence detection using derivatisation with metal salts [9] or conversion to the iso derivatives under alkaline conditions [10,11]. Fluorescence detection is generally more specific than UV detection and is less prone to interference from other compounds in the sample matrix.

For confirmatory assays, however, the EU have recommended that where possible, some type of mass spectrometry should be used in order to increase specificity [12]. The tetracyclines are not suitable for GC–MS due to their low volatility but a method has been published [13] using TLC with FAB mass spectrometry. This technique, however, is not suitable for routine use. LC–MS has offered a viable alternative and suitable instruments are now becoming available in many laboratories. A method has been published [14] for the determination of tetracyclines in milk using particle beam LC–MS and it has also been shown that atmospheric pressure chemical ionisation (APCI) and electrospray (ES) interfaces are suitable for the routine determination of a range of veterinary drug residues [15].

In general, the detection and quantitation of tetracycline residues in biological material can present a number of problems. Firstly, they can be difficult to extract into organic solvents and most extraction techniques from tissue use an acidic buffer at pH 2–4. This is usually followed by a solid-phase clean-up using reverse phase material. The tetracyclines readily chelate to metal ions, however, and we have found that the use of some manufacturers SPE cartridges can lead to low and variable recoveries, probably due to the presence of trace metal impurities. This has also been reported by others [16]. It is therefore necessary to use cartridges containing a high purity silica based material in order to prevent this. This can also be a problem with the HPLC column used for separation and the use of a column containing a high purity silica based material is also recommended. It may also be necessary to add a metal blocking agent such as oxalic acid or ethylenediamine tetraacetic acid to the mobile phase to reduce peak tailing and increase sensitivity.

A further complication in the determination of tetracyclines, and particularly chlortetracycline, is the formation of isomers, a factor often overlooked in the literature. It has been shown that tetracyclines rapidly isomerise in aqueous solutions at pH 2–6 at the C-4 dimethylamino group to form 4-epitetracyclines [11]. In addition, keto-enol tautomers are also rapidly formed in aqueous solutions [17]. Depending on the conditions and type of column used, up to four peaks consisting of the keto and enol isomers of both CTC and epi-CTC can therefore be obtained using an HPLC assay for CTC.

In this paper, we present a confirmatory assay using LC–MS with APCI for the determination of TC, OTC and CTC in muscle and kidney and attempt to overcome some of the difficulties discussed above. The assay has been validated at half the MRL and above for both muscle and kidney and the determination of the isomers of CTC is also discussed.

2. Experimental

2.1. Chemicals

All solvents were HPLC grade and other chemicals were analytical grade. Tetracycline, oxytetracycline, chlortetracycline and their epimers were obtained from Acros Chimica (Newton Hyde, UK) as the hydrochloride salts. A certificate of analysis was supplied for each standard. Stock standards (1 mg/ml as the dry free base) were prepared in methanol and were stable for up to 1 month stored at 4°C in amber vials. Spiking standards (10 μg/ml) were prepared fresh as required by dilution of the stock standard in acetonitrile–water (25:75, v/v). Working standards (1 μg/ml) were prepared by dilution of the spiking standards in 20 m*M* oxalic acid–acetonitrile (80:20 v/v). These were prepared at the same time as sample extracts and were allowed to sit overnight at 4°C before analysis using LC–MS.

2.2. HPLC system

The HPLC system was a binary gradient system comprising a Merck-Hitachi L6200A intelligent pump, L6000 pump and an AS2000 autosampler

(Merck, Poole, UK). The HPLC column was an endcapped high purity C_{18} silica column, 150×4.6 mm Prodigy ODS2 (Phenomenex, Macclesfield, UK). An Inertsil ODS2 column (GL Sciences, Tokyo, Japan) was found to provide similar results.

Mobile phase A was a mixture of acetonitrile–water (10:90) containing 0.04% (v/v) heptafluorobutyric acid (HFBA), 10 m*M* oxalic acid and 10 μ*M* ethylenediamine tetraacetic acid, ammonium salt (EDTA). Mobile phase B was a mixture of acetonitrile–water (90:10) with the same concentrations of HFBA, oxalic acid and EDTA as in mobile phase A. The flow-rate was 1 ml/min and the gradient profile (where T refers to time in min) was as follows: T_0 %B=10, T_{10} %B=50, T_{11} %B=90, T_{14} %B=90, T_{16} %B=10, T_{20} %B=10. Total run time=20 min.

2.3. LC–MS system

The LC–MS system comprised a VG Platform II (Micromass, Altrincham, UK) fitted with an APCI probe. This was coupled to the outlet of the HPLC column using a length of PEEK tubing. The instrument was operated in the positive ion mode. Full scan data were collected in order to obtain spectra from standards and single ion data (dwell time 0.5 s) were collected when analysing samples. The source of the instrument was maintained at 150°C and the flow-rates of the drying and sheath gases were 300 and 50 l/h, respectively. The temperature of the APCI probe was 500°C and it was operated in an off-centre axis to reduce the volume of vaporised mobile phase entering the source. This had the effect of increasing sensitivity towards the tetracyclines and reducing contamination of the source. The source was cleaned after about 18 h operating time, a simple procedure which took about 0.5 h. The instrument was calibrated weekly using a polyethylene glycol mixture.

2.4. Extraction procedure

The extraction procedure used was identical to that already described [9]. Frozen tissue samples were pulverised using a domestic food blender and were either analysed fresh or stored at −20°C until analysis. Portions (5 g) were weighed into 125 ml polythene centrifuge bottles. Tissue samples for recovery studies were also set up at this stage by spiking with the required volume of standard (10 μg/ml), allowing them to stand for 10 min and then treating them as for normal samples. Glycine–HCl buffer (45 ml, 0.1 *M* glycine in 1 *M* HCl) was added and the mixture was homogenised for 1 min using a Silverson Laboratory Mixer (Silverson Machines, Chesham, UK). Ammonium sulphate (5 g) was added to the homogenate, the bottles were shaken for 30 s and left to stand for 10 min before centrifuging at 2000 *g* for 10 min. The supernatant was filtered through a plug of glass wool into a beaker. A further 50 ml of glycine–HCl buffer was added to each of the tissue precipitates and the extractions were repeated. The extracts were combined and aliquots (approximately 30 ml) were centrifuged at 2000 *g* for 10 min.

2.5. Solid-phase clean-up

Isolute cyclohexyl (CH-endcapped) cartridges (500 mg, 3 ml, Jones Chromatography, Hengoed, UK) were prepared by washing with methanol (10 ml) followed by water (10 ml). Aliquots (20 ml) of the tissue extracts were passed through the cartridges using a syringe, and after washing with water (10 ml), any tetracyclines present were eluted with methanol (7 ml). The methanol was evaporated to dryness at 65°C under nitrogen and the residues were dissolved in 20 m*M* oxalic acid–acetonitrile (80:20, v/v, 0.5 ml for muscle extracts and 1 ml for kidney) using vortex mixing. The extracts were transferred to Eppendorf tubes and centrifuged at 5000 *g* for 10 min using a microcentrifuge. Aliquots of the supernatants (250 μl) were transferred to conical autosampler vials and were allowed to sit overnight at 4°C before analyses using LC–MS.

2.6. LC–MS analyses

The HPLC and LC–MS systems were operated for 30 min to allow for equilibration prior to sample analyses. The LC–MS was set to collect multiple single ion data for the M+1 ions at m/z 445, 461 and 479 for tetracycline, oxytetracycline and chlortetracycline, respectively. The autosampler was programmed to inject 50 μl aliquots of sample extracts and a mixed standard containing 1 μg/ml of each of

Table 1
Some of the fragment ions of the tetracyclines and their proposed structures produced using positive ion LC–MS with APCI

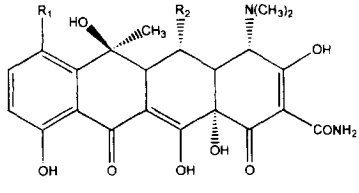
	Tetracycline	Oxytetracycline	Chlortetracycline
$[M+H]^+$	445	461	479, 481[a]
$[M+H-NH_3]^+$	428	444	462
$[M+H-H_2O]^+$	427	443	461
$[M+H-NH_3-H_2O]^+$	410	426	–
$[M+H-2H_2O]^+$	409	425	443

[a] Chlorine isotopic ion.

the tetracyclines. The standard was re-injected after every three sample extracts and results were calculated with reference to peak area data of the standards (equivalent to 500 ng/g muscle or 1000 ng/g kidney). Where ion ratio data were required for confirmatory purposes, the appropriate extracts and standards were re-injected, collecting data for 3 ions for each tetracycline. Useful diagnostic ions which can be used for ion ratio measurements are shown in Table 1.

3. Results and discussion

The structures of the three tetracyclines are shown in Fig. 1 and their full scan LC–MS APCI spectra at a cone voltage of 25 V are shown in Fig. 2. The ratio of the ions can be changed by varying the cone voltage and 25 V was chosen as giving a good selection of ions for each tetracycline. A summary of the fragment ions and their proposed structures are shown in Table 1 and these can be used for ion ratio measurements. The spectra of the isomers and tautomers (see later) were similar to the parent compounds.



Compound	R_1	R_2
Oxytetracycline	H	OH
Tetracycline	H	H
Chlortetracycline	Cl	H

Fig. 1. Molecular structures of the three tetracyclines.

Single ion chromatograms of the M+1 ions from a mixed standard (1 μg/ml), a negative muscle extract and an extract of muscle spiked with each of the tetracyclines at the MRL (100 ng/g) are shown in Fig. 3. The chromatograms for oxytetracycline and chlortetracycline were free of interfering peaks. The chromatograms for tetracycline in the negative and spiked muscle extracts showed two small peaks in close proximity to the tetracycline peak, but were clearly separated from it. These peaks were not present when additional ions were monitored for tetracycline at *m/z* 410 and 427 and were also not present in kidney extracts. The small peaks eluting

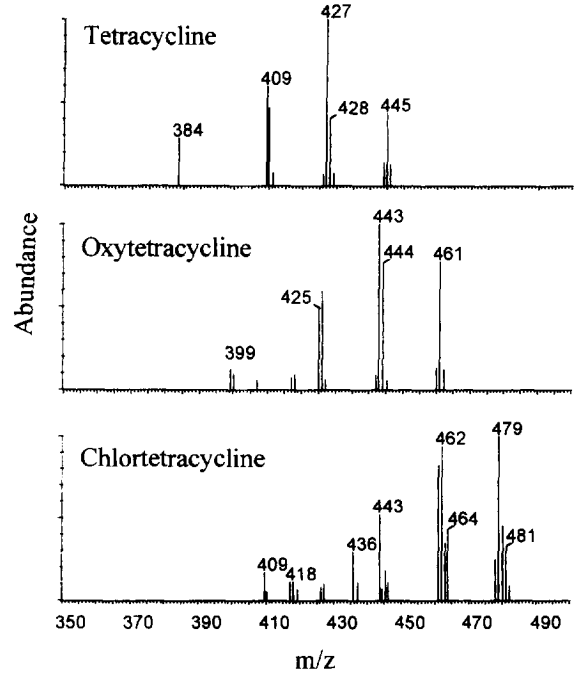


Fig. 2. Full scan APCI LC–MS spectra of the tetracyclines at a cone voltage of 25 V.

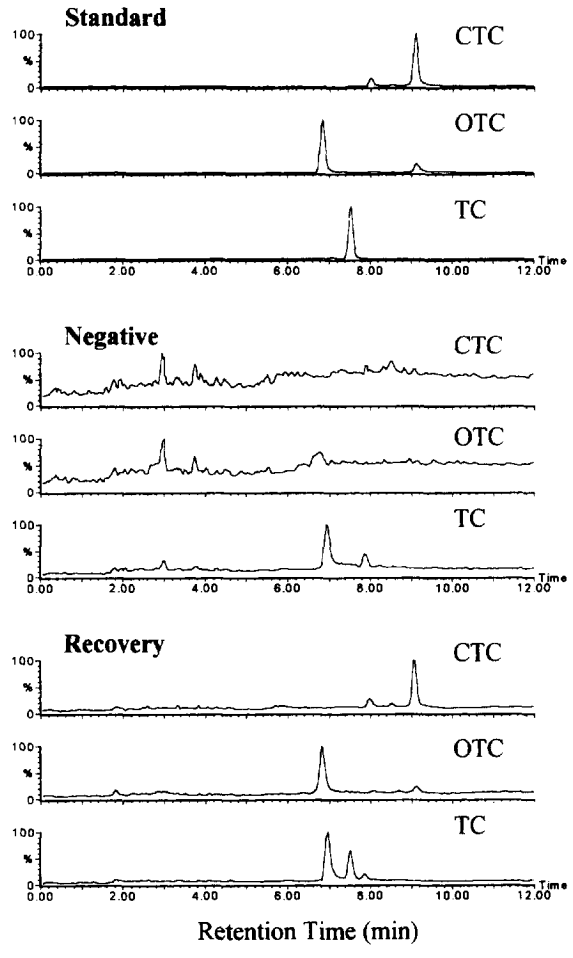


Fig. 3. Single ion chromatograms of the M+1 ions at m/z 445, 461 and 479 for tetracycline, oxytetracycline and chlortetracycline, respectively in a mixed standard (1 μg/ml), a negative muscle extract and an extract of muscle which had been spiked with each tetracycline at the MRL (100 ng/g).

just before chlortetracycline in the standard and recovery were due to isomers of chlortetracycline and will be discussed later.

The method has been validated at one half the MRL, the MRL and two times the MRL for both muscle and kidney, according to EU guidelines. The results are shown in Tables 2 and 3. The overall recoveries for muscle ranged from 59 to 82% and for kidney were from 67 to 83%. The overall coefficients of variation (C.V.s) ranged from 12.8 to 22.9% for muscle and from 11.7 to 28.1% for kidney. These values are higher than would normally be expected from an analytical method and would be improved by the use of deuterated internal standards should they become available. The high values also reflect the difficulty in measuring compounds which can form a number of isomers and are relatively unstable in solution (see later).

For confirmatory analysis using MS, it is recommended that ion ratio measurements should be made in order to increase specificity. The reproducibility of ion ratio measurements using this assay is shown in Table 4. Five replicates of negative muscle and kidney samples were spiked at the MRL and taken through the assay. Three ions were monitored for each tetracycline. The C.V.s of the ion ratios ranged from 8.6 to 14.7% for tetracycline, 10.2 to 25.7% for oxytetracycline and 4.0 to 13.5% for chlortetracycline.

The linearity of the assay was checked by injecting a series of standards from 1 to 20 μg/ml into the LC–MS system. The assay was found to be linear up to the highest value measured. The linear regression coefficients (r) were 0.9984, 0.9997 and 0.9998 for tetracycline, oxytetracycline and chlortetracycline, respectively.

Typical limits of detection (three times signal-to-noise) for the assay are in the region of 10 ng/g for each of the tetracyclines in muscle and 20 ng/g in kidney. As with any MS method, however, this can vary depending on the cleanliness of the source, tuning parameters, and alignment of the LC–MS interface etc. The limits of quantitation, defined as the lowest levels at which the assay was validated, are 50 ng/g for muscle and 300 ng/g for kidney. It was not thought necessary to go lower than these values, which represent half the MRL in each case.

Recent EU legislation on MRLs for the tetracyclines stipulates that for each tetracycline, the reported values should comprise the sum of the parent compound and its 4-epimer. Validation was therefore also carried out by spiking negative tissue samples with the epimers themselves. Five replicates of muscle and kidney were spiked at the MRLs (100 ng/g for muscle and 600 ng/g for kidney) and carried through the assay. The results (not shown) were found to be similar to those from the parent compounds. Recoveries ranged from 56 to 74% and C.V.s ranged from 6.2 to 24%.

When running standards of the epimers, using the

Table 2
Inter- and intra-assay validation for tetracycline, oxytetracycline and chlortetracycline in muscle using the proposed method

Added	50 ng/g			100 ng/g			200 ng/g		
	TC	OTC	CTC	TC	OTC	CTC	TC	OTC	CTC
Day 1									
Mean (ng/g)	35.1	33.0	35.6	68.4	60.2	66.6	103.0	117.0	119.5
S.D.	4.8	4.4	2.2	6.1	3.0	4.3	17.1	11.7	14.3
C.V. (%)	13.7	13.3	6.1	9.0	5.1	6.4	16.6	10.0	12.0
Recovery (%)	70	66	71	69	60	67	52	58	60
Day 2									
Mean (ng/g)	25.0	43.8	38.7	69.3	68.5	76.2	112.6	149.2	163.4
S.D.	4.4	5.6	4.4	12.6	7.0	10.6	20.7	19.2	8.9
C.V. (%)	17.6	12.9	11.3	18.1	10.2	13.9	18.4	12.8	5.4
Recovery (%)	50	87	77	69	68	76	56	75	82
Day 3									
Mean (ng/g)	36.5	47.1	49.7	77.2	95.7	81.7	136.8	165.0	152.4
S.D.	7.3	3.9	7.8	9.6	9.9	6.8	30.5	17.8	20.0
C.V. (%)	20.0	8.3	15.7	12.4	10.3	8.3	22.3	10.8	13.1
Recovery (%)	73	94	99	77	96	82	68	82	76
Overall									
Mean (ng/g)	31.9	40.9	40.7	71.6	74.8	74.8	117.5	143.7	145.0
S.D.	7.3	7.6	7.6	10.0	17.0	9.6	26.2	25.7	23.9
C.V. (%)	22.9	18.6	18.7	14.0	22.7	12.8	22.2	17.9	16.5
Recovery (%)	64	82	81	72	75	75	59	72	72

Negative samples were spiked with 50, 100 and 200 ng/g of each of the tetracyclines and carried through the assay. The M+1 ions were monitored for each. Five replicates were measured at each level on three different days.

HPLC conditions described, epitetracycline eluted from the column approximately 0.5 min before the parent compound. Epioxytetracycline and the parent compound were not separated and eluted at the same retention times. Epichlortetracycline eluted approximately 0.6 min before chlortetracycline, but a second relatively large peak (approximately half the area of the epi-CTC peak) was apparent in the chromatograms with a retention time 1 min earlier than epi-CTC. A small peak was also apparent in the same position as the parent CTC due to reversion of the epimer to the parent compound. This picture was further complicated when samples from pigs which had been treated with CTC were analysed. These samples were found to produce four peaks in the single ion chromatograms for CTC at m/z 462, 479 and 481. A typical chromatogram of the M+1 ion at m/z 479 from an incurred pig muscle sample is shown in Fig. 4. Two of the peaks (retention times 8.6 min and 9.2 min) were identified from their retention times as epi-CTC and CTC, respectively. The other two peaks have been tentatively identified as the keto tautomers of epi-CTC and CTC with reference to papers from Bryan et al. [11] and Naidong et al. [17]. These authors showed that isomerisation occurred in aqueous standards of CTC, particularly at low pH. They state that as a solid, epi-CTC and CTC exist as enols but in aqueous solutions an equilibrium is formed between the keto and enol tautomers (Fig. 5). We have found that the keto tautomer of epi-CTC is formed rapidly in fresh acidic aqueous solutions of epi-CTC (enol) and reaches an equilibrium with the enol in under one hour. The peak area of the keto tautomer equilibrates to about 50% of that of the enol. With time, small amounts of keto-CTC and enol-CTC are also formed from epi-CTC. Fresh aqueous solutions of CTC are more stable than epi-CTC and the keto tautomer forms more slowly (over a period of days) and equilibrates at less than 10% of the enol (from peak area measurements).

The formation of isomers makes the accurate

Table 3
Inter- and intra-assay validation for tetracycline, oxytetracycline and chlortetracycline in kidney using the proposed method

Added	300 ng/g			600 ng/g			1200 ng/g		
	TC	OTC	CTC	TC	OTC	CTC	TC	OTC	CTC
Day 1									
Mean (ng/g)	241	230	249	459	436	467	885	1033	985
S.D.	33.1	38.1	21.9	60.3	59.4	65.6	97.6	74.1	71.1
C.V. (%)	13.7	16.6	8.8	13.1	13.6	14.0	11.0	7.2	7.2
Recovery (%)	80	77	83	76	73	78	74	86	82
Day 2									
Mean (ng/g)	205	183	205	364	340	386	769	712	729
S.D.	36.0	16.0	17.4	38.8	35.4	23.9	23.5	51.0	35.3
C.V. (%)	17.6	8.7	8.5	10.7	10.4	6.2	3.1	7.2	4.8
Recovery (%)	68	61	68	61	57	64	64	59	61
Day 3									
Mean (ng/g)	304	336	249	416	440	504	981	937	1092
S.D.	38.0	11.1	15.0	78.5	38.4	35.0	119	83.2	99.7
C.V. (%)	12.5	3.3	6.0	18.9	8.7	6.9	12.1	8.9	9.1
Recovery (%)	101	112	83	69	73	84	82	78	91
Overall									
Mean (ng/g)	250	250	234	413	404	450	878	894	932
S.D.	53.9	70.2	27.3	68.9	64.4	66.1	122.6	153.7	166
C.V. (%)	21.6	28.1	11.7	16.7	15.9	14.7	14.0	17.2	17.9
Recovery (%)	83	83	78	69	67	75	73	74	78

Negative samples were spiked with 300, 600 and 1200 ng/g of each of the tetracyclines and carried through the assay. The M+1 ions were monitored for each. Five replicates were measured at each level on three different days.

quantitation of tetracyclines more difficult. With the LC–MS assay we have found that the response of a fresh standard of epi-CTC is only 86% of that of CTC, using peak area measurements of the M+1 ion. With the fluorescence method [9] using the aluminium complex, the response of epi-CTC was 94% of that of CTC and with the iso fluorescence method [10] for CTC, the response was 93%. This

Table 4
Reproducibility of ion ratio measurements

	Tetracycline		Oxytetracycline		Chlortetracycline	
	410/445	426/445	426/461	443/461	462/481	479/481
Muscle						
Mean	6.61	2.24	1.91	2.24	3.59	2.23
S.D.	0.57	0.29	0.49	0.23	0.43	0.30
C.V. (%)	8.6	12.9	25.7	10.3	12.0	13.5
Kidney						
Mean	4.49	4.47	1.24	1.96	2.45	2.47
S.D.	0.66	0.52	0.24	0.20	0.10	0.10
C.V. (%)	14.7	11.6	19.3	10.2	4.1	4.0

Five replicates of negative muscle and kidney were spiked at the MRL (100 and 600 ng/g, respectively) and taken through the assay. Three standards (1 μg/ml) were run with each set. The ions at m/z 410, 426 and 445 were monitored for tetracycline, 426, 443 and 461 for oxytetracycline and 462, 479 and 481 for chlortetracycline. The results show the reproducibility of ion ratios for each set which includes standards. Muscle and kidney were run on different days.

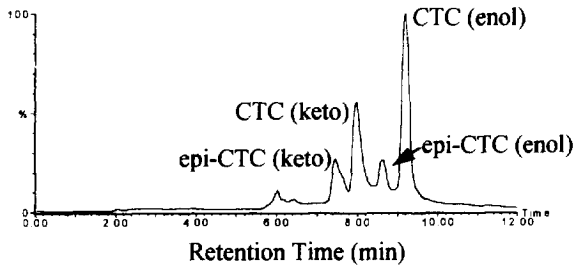


Fig. 4. Single ion chromatogram at *m/z* 479 for a muscle extract from a pig which had been treated with chlortetracycline.

obviously makes accurate quantitation difficult since it is not possible to obtain single isomer standards and in any case conversion will take place in solutions. One approach is to allow standards and samples to equilibrate overnight before measurement and to sum the peak areas. The picture is further complicated with incurred samples, as the isomers will also be formed in vivo and will be present in tissues. As already shown in Fig. 4, both the keto and enol tautomers of epi-CTC and CTC were present in an incurred pig muscle extract. Using the enol peak only, the level of CTC present was estimated to be 1.2 μg/g, and using the sum of the peaks, the level was estimated at 1.8 μg/g. A comparison of the levels of CTC found in 10 incurred samples using the LC–MS assay and a fluorescence method [9] is shown in Table 5. When the results for enol-CTC in both methods were

Table 5
Comparison of levels of CTC-enol and total isomers (sum of -keto and -enol tautomers of epi-CTC and CTC) found in 10 incurred samples carried through 2 different methods

	Fluorescence		LC–MS	
	-Enol	Total	-Enol	Total
1	1.33	1.75	0.72	1.10
2	1.93	2.60	1.42	2.21
3	0.81	1.07	0.49	0.84
4	0.18	0.26	0.14	0.30
5	1.18	1.83	0.82	1.29
6	0.17	0.21	0.15	0.31
7	0.10	0.18	0.06	0.18
8	0.74	0.93	0.60	0.86
9	0.69	0.97	0.55	0.78
10	1.11	1.61	0.90	1.58

Fluorescence method=Ref. [2]., LC–MS=present method. Results are in μg/g tissue and are corrected for recoveries. The values were calculated with reference to a CTC enol standard.

compared using Student's *t*-test, the LC–MS method gave significantly lower values than the fluorescence method. In contrast, there were no significant differences in the values for the total isomers between the two methods. This emphasises the importance of measuring the sum of the isomers rather than only the CTC enol itself, which is the case in most methods.

With oxytetracycline, it is not possible to separate epi-OTC from OTC using any of the described assays and it is therefore not possible to determine if

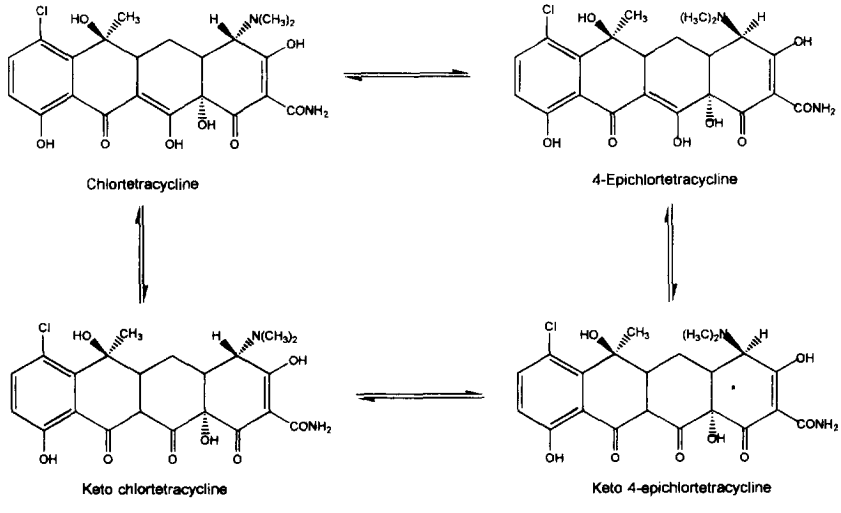


Fig. 5. Structures and transformation of the epimers and tautomers of chlortetracycline.

any isomers are present in incurred samples or are indeed formed in aqueous standards. A comparison of the levels of OTC found in seven incurred tissues from a ring test, however, using the aluminium-fluorescence method [10] and the LC–MS assay, showed good agreement. The mean value for the fluorescence method was 0.22 μg/g and for the LC–MS assay was 0.23 μg/g (both corrected for recoveries).

Tetracycline and epitetracycline can be separated using HPLC and the standards appear to be more stable and less prone to isomer formation than CTC. Tetracycline is also less widely used than OTC or CTC and no incurred samples were available to us for testing or comparing methods.

The relatively complex mobile phases used in this assay reflect the difficulties in obtaining good chromatographic resolution and recoveries of the tetracyclines from the HPLC system. An important requirement for LC–MS is that all components of the mobile phase should be volatile. HFBA was therefore used in place of the more common buffer systems in order to obtain the acidic conditions required for good chromatography of the tetracyclines. It also had an ion pairing effect and increased retention times, particularly for tetracycline and oxytetracycline, which are poorly retained by most HPLC columns. Oxalic acid was included to improve resolution and improve peak shapes and a low concentration of EDTA (10 μ*M*) was found to be essential to obtain good recovery of tetracyclines from the column. If this was omitted, the peak areas of standards or sample extracts were often lower than expected and on some occasions the tetracyclines appeared to be completely retained on the column. The reason for this is not clear as other workers found that the addition of oxalic acid to the mobile phase was sufficient, but is probably due to metal chelation somewhere in the system. Although EDTA is not volatile, the inclusion of the very low concentration required did not appear to be detrimental to the MS interface or source.

The HPLC gradient conditions chosen for separation of the tetracyclines were from 10% mobile phase B to 50% mobile phase B over 10 min. It was found necessary, however, to include a column cleaning step after each injection of sample extract by increasing the concentration of B up to 90% for 2 min. Without this step, the sensitivity of the assay fell significantly throughout a sample run due to a build-up of material which appeared to retain the tetracyclines on the column.

4. Conclusions

The recent change in EU legislation relating to MRLs for tetracyclines may have to be further modified to take account of all isomers and not only epimers. In the past, it may be that the isomers of CTC were not separated and were in fact included unknowingly in the determination of CTC. With the advent of improved HPLC columns and improvements to methods in general, the isomers are now separated and CTC should be determined as the sum of the peaks, provided the responses of the detector system are similar. It is also important to include incurred as well as spiked samples as part of any tetracycline method development, otherwise important information can be overlooked. Incurred samples will contain a mixture of isomers, whereas spiked samples may not. It would be useful to carry out a ring test, using CTC incurred tissue, to determine the differences in reported values for CTC from different laboratories using their own methods.

The development of modern, dedicated LC–MS instruments allow methods such that described in this paper to be performed routinely and with little more difficulty than LC methods with pre or post column derivatisation. In addition they provide mass related data which is to be preferred for confirmatory analyses.

References

[1] Commission Regulation No. 281/96 of 14 February 1996, Off. J. Eur. Commun., L 37/9–L 37/11.
[2] R. Bogaerts and F. Wolf, Fleischwirtschaft, 60 (1980) 672.
[3] J.R. Walsh, L.V. Walker and J.J. Webber, J. Chromatogr., 596 (1992) 211.
[4] S. Horii, J. Liq. Chromatogr., 17 (1994) 213.
[5] A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, J. Assoc. Off. Anal. Chem., 73 (1990) 864.
[6] W.H.H. Farrington, J. Tarbin, J. Bygrave and G. Shearer, Food Addit. Contam., 6 (1991) 55.

[7] J.M. Degroot, B. Wyhowski De Bukanski and S. Srebnik, J. Liq. Chromatogr., 16 (1993) 3515.
[8] H. Oka, H. Matsumoto, K. Uno, K. Harada, S. Kadowaki and M. Suzuki, J. Chromatogr., 325 (1985) 265.
[9] R.J. McCracken, W.J. Blanchflower, S.A. Haggan and D.G. Kennedy, Analyst, 120 (1995) 1763.
[10] W.J. Blanchflower, R.J. McCracken and D.A. Rice, Analyst, 114 (1989) 421.
[11] P.D. Bryan, K.R. Hawkins, J.T. Stewart and A.C. Capomacchia, Biomed. Chromatogr., 6 (1992) 305.
[12] Commission Regulation No. 93/257 of 15 April 1993, Off. J. Eur. Commun., L 118/75.
[13] H. Oka, I. Yoshitomo and J. Havakawa, J. AOAC Int., 77 (1994) 891.
[14] P.J. Kijak, M.G. Leadbetter, M.H. Thomas and E.A. Thompson, Biol. Mass Spectrom., 20 (1991) 789.
[15] W.J. Blanchflower, A. Cannavan, R.J. McCracken, S.A. Hewitt, P.J. Hughes, S.A. Haggan and D.G. Kennedy, in N. Haagsma and A. Ruiter (Editors), Proceedings of the EuroResidue III Conference, University of Utrecht, Netherlands, 6–8 May 1996.
[16] J.D. MacNeil, V.K. Martz, G.O. Korsrud, C.D.C. Salisbury, H. Oka, R.L. Epstein and C.J. Barnes, J. AOAC Int., 79 (1996) 405.
[17] W. Naidong, E. Roets, R. Busson and J. Hoogmartens, J. Pharm. Biomed. Anal. 8 (1990) 881.