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Determination of tetracyclines in bovine and porcine muscle by high-performance liquid chromatography using solid-phase extraction

John R. Walsh*, Laurence V. Walker and Jonathan J. Webber

Regional Veterinary Laboratory, Department of Agriculture, Ballarat Road, Hamilton, Victoria 3300 (Australia)

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ABSTRACT

A method is presented for the determination of the three tetracyclines oxytetracycline, tetracycline and chlortetracyline in muscle, spiked at 100 ng/g, using high-performance liquid chromatography (HPLC). The concentration and extraction steps are carried out using Waters Environmental Sep-Pak cartridges. The principal steps involve homogenizing the sample in EDTA–McIlvaine buffer followed by centrifugation and precipitation of the supernatant using trichloroacetic acid. After further filtration and concentration on a Sep-Pak cartridge, the sample is eluted and analysed by HPLC with UV detection and confirmation by diode-array. The column used is a Nova-Pak C_{18} (4 μm) cartridge (10 cm × 8 mm I.D.). A phosphate–citrate–acetonitrile buffer, utilizing ion suppression, is the mobile phase. The analytes are detectable at levels down to 10 ng/g. The analyte identity can be confirmed at 20 ng/g by the use of diode-array detection and spectral library comparison.

INTRODUCTION

In Australia, oxytetracycline (OTC) and chlortetracycline (CTC), together with various members of the sulphonamide group, are the most widely used antibacterials in the meat-production industry. A monitor of the use of these antibiotics and their subsequent residues in food is of serious concern to consumers in the export and domestic markets. A reliable, robust and reproducible method was required for the determination of tetracyclines in muscle. The method would form part of a panel of analyses that could be used throughout Australia to monitor both domestic and export meat for the presence of a range of antibacterial residues. To this end, all equipment, chemicals and products had to be available and supported in Australia.

The maximum residue limits (MRL) in Australia are 250 ng/g for OTC and 50 ng/g for CTC. The aim of this study was to produce a method for the determination of tetracyclines that would produce consistent peak areas in tissues spiked at the 100 ng/g level. At the same time, a reasonable laboratory throughput had to be maintained. This method should permit a very favourable signal-to-noise ratio at around the MRL, such that it would be easy to quantify concentrations of analytes in muscle significantly below the stated MRLs.

Many methods have been published for the determination of tetracyclines in a number of matrices [1–8] that can be reproduced with varying degrees of success. These methods utilize a number of purification techniques: partitioning [3], ultrafiltration [7], solid-phase dispersion (SPD) [8] and solid-phase extraction (SPE) involving ion exchange [5], adsorption [4] or reversed phases (C_{18}) [1,2,6,7]. The last group can be subdivided into external, or preconcentration of the sample on a cartridge [1,6], and on-column, where the sample is directly concentrated on the analytical column [2,7]. In most of these reports, samples were spiked at 1000 ng/g, and the sensitivity was extrapolated to much lower concentrations. There are only two reports [6,7] of samples spiked at 20–100 ng/g. We believe that the re-

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coveries are not constant throughout the ranges, and it is necessary to spike samples in the required range of analysis. Other published methods appear to have a less favourable signal-to-noise ratio at these MRL levels. The method of Oka *et al.* [1] was adopted and modified by techniques that allow much higher sample loads in a smaller final sample volume. The chromatographic conditions were modified to permit the use of diode-array detection and to minimize analyte losses in the analytical column.

EXPERIMENTAL

Chemicals and materials

Environmental Sep-Pak cartridges (0.85 g, 55–105-μm C_{18}; end-capped silica; 12% carbon loading) (Waters), water purified in a Milli-Q apparatus, methanol and acetonitrile (Millipore, Milford, MA, USA). Trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA), sodium salt, citric acid, disodium monohydrogenorthophosphate dihydrate, potassium dihydrogenorthophosphate, hydrochloric acid (HCl) and tetramethylammonium chloride were of analytical-reagent grade.

EDTA–McIlvaine buffer (pH 4) was prepared by dissolving 15 g of disodium monohydrogenorthophosphate dihydrate, 13 g of citric acid and 3.72 g of EDTA in water and diluting to 1 l.

Antibiotics

Oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) (Sigma, St. Louis, MO, USA) were each dissolved in 0.01 *M* HCl at 0.1 mg/ml. The working solution was a mixture, prepared by dilution of 1 ml of each stock-solution 100-fold with 0.1 *M* potassium dihydrogenorthophosphate solution, containing 0.01 *M* EDTA to produce a 1000 ng/ml standard. All solutions were stored in the dark at 4°C.

Apparatus

The following equipment was used: Janke & Kunkel homogenizer using the 18N probe; Sorvall refrigerated centrifuge; 50-ml (nominal) Sorvall centrifuge tubes; 400-ml tall-form beakers; 250-, 25- and 10-ml measuring cylinders; 75-mm glass funnels; Whatman 11-cm No. 541 filter-papers; large ice-bath; magnetic stirrer; stirring bars; 50-ml polypropylene plastic syringe barrels (reservoirs); 10-ml gas-tight syringes; water vacuum pump and a simple manifold for multiple vacuum outlets.

The chromatograph used was a Varian Star system, equipped with a ternary pump (Model 9010), a diode-array UV detector (Model 9065), an autosampler (Model 9095) and a computer for control and data handling (Varian Star Software).

Method

A 35–40-g amount of trimmed meat (muscle) was homogenized with 5 ml/g of EDTA–McIlvaine buffer (4°C) using a Janke & Kunkel homogenizer (18N probe). The weight (w_1) and volume (v_1) used were recorded. The homogenate was centrifuged at 2700 *g* for 15 min at 4°C. The supernatant was collected and its volume (v_2) recorded.

A volume (v_3) of trichloroacetic acid solution (1 g/ml), equal to 10% of the supernatant volume, was slowly added to the supernatant with constant stirring. The mixture was stirred for a further 1 min, then placed in a bed of ice for 15 min. The mixture was filtered through a No. 541 Whatman filter into a 250-ml measuring cylinder and the volume (v_4) recorded.

At this point, the weight of muscle (w_2) to be loaded on to the Environmental Sep-Pak cartridge can be calculated using the equation

$$w_2 = w_1[v_2/(v_2+v_3)][v_4/(w_1+v_1)]$$

The assumption is made that the muscle is virtually all water, *i.e.*, w_1 g contributes w_1 ml, and that it all contributes to the dilution of v_1.

The Sep-Pak cartridge, conditioned with 5 ml each of methanol and then EDTA–McIlvaine buffer, is attached to a reservoir, made from the barrel of a 50-ml polypropylene syringe, supported by a clamp. The outlet of the Sep-Pak cartridge is connected to a water pump, and the sample is aspirated through at no more than 10 ml/min. When the sample has been passed through, the Sep-Pak cartridge is disconnected, flushed with 5 ml of EDTA–McIlvaine buffer and the surplus liquid blown out.

The technique of minimum volume elution is used. Into a 10-ml gas-tight syringe exactly 2 ml of a 50:50 mixture of acetonitrile and EDTA–McIlvaine buffer are aspirated. The syringe is attached to the Sep-Pak cartridge and about 0.6 ml of this eluent, or enough for one drop of liquid of eluate to appear

at the outlet, is injected. After waiting 30 s for equilibration, the remainder of the eluent is injected and the eluate is collected in a 10-ml measuring cylinder. The syringe is removed, refilled with air and reconnected to the Sep-Pak cartridge, and the remaining eluate is expelled into the measuring cylinder. The volume (v_5) (ml) in the measuring cylinder is measured. A 150-μl volume is injected into the liquid chromatograph. The equivalent weight of muscle injected is calculated as 0.15 w_2/v_5 g.

High-performance liquid chromatography (HPLC)

Solution A was 0.01 *M* citric acid–0.01 *M* dipotassiumorthophosphate and solution B was acetonitrile. The mobile phase was A–B (72:28), containing 0.005 *M* tetramethylammonium chloride and 0.1 g/l of EDTA.

The column used was a Nova-Pak C_{18} (4-μm; end-capped) cartridge (10 cm × 8 mm I.D.) with a μBondapak C_{18} guard column (Waters). The flow-rate was 2.4 ml/min. The concentrations of the analytes were quantified using the data from the 365-nm channel.

RESULTS

Muscle from a calf, known not to contain tetracycline residues, was used for the study. A blank and five replicates spiked at 100 ng/g were processed according to the preceding method.

The peaks observed were confirmed by comparison with the retention times of known standards and with the spectra in a spectral library of tetracyclines. As seen in Table I, the recoveries were 70% [relative standard deviation (R.S.D.) = 8%] for OTC, 58% (R.S.D. = 8%) for TC and 50% (R.S.D. = 10%) for CTC. Although the recovery of CTC is much lower than that of the other tetracyclines, it is easier to detect and confirm because it appears in a part of the chromatogram relatively free from other peaks. Results of spiking studies in routine laboratory analyses have been included in Table I to show the inter-assay variability.

The chromatograms for the standards, a representative spiked sample and the blank sample (negative) are shown in Fig. 1. Figs. 2 and 3 show the spectra for OTC and CTC from a representative spiked sample, together with their software-selected overlays from the library.

Two pigs were injected with therapeutic doses of either OTC or CTC. The animals were killed 24 h later and their muscle tissue was collected, in small samples, and frozen at −20°C. Samples from these pigs were later thawed and tested using the same procedure as for the spiked samples. This experiment was used to demonstrate the applicability of the method using genuine incurred residues. The chromatograms and spectra with the overlaid library matches from these tests are shown in Figs. 4–7. In fig. 5, there is a peak that corresponds to TC. It has been observed in this laboratory that in samples containing a very high concentration of

TABLE I

ANALYSIS OF SPIKED MEAT SAMPLES

A 40-g amount of each sample was processed according to the preceding method. The extracts were chromatographed isocratically [A–B (72:28)] at a flow-rate of 2.4 ml/min and quantified at 365 nm.

Parameter	Oxytetracycline	Tetracycline	Chlortetracycline
Spike level (ng/g)	100	120	100
Intra-assay (n=5)			
Average recovery (ng/g)	70	70	50
Average recovery (%)	70	58	50
S.D. (ng/g)	10	10	0
R.S.D. (%)	8	7	10
Inter-assay (n=5)			
Average recovery (%)	61	60	47
S.D. (ng/g)	12	6	10
R.S.D. (%)	19	10	21

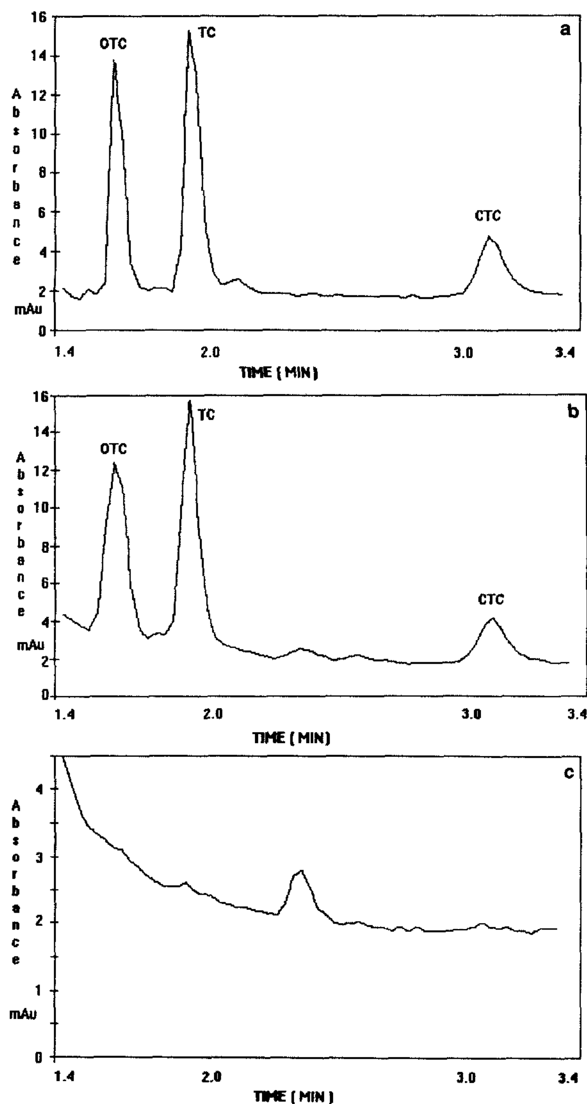

Fig. 1. (a) Chromatogram of three tetracycline standards (150 ng). (b) Chromatogram of blank meat sample spiked with three tetracyclines at 100 ng/g (150 μl). (c) Chromatogram of blank meat-sample (150 μl). HPLC conditions: Nova-Pak C_{18} column (10 cm × 8 mm I.D.); mobile phase, A–B (72:28); flow-rate, 2.4 ml/min; detection at 365 nm.

CTC in muscle or urine, some TC is also observed at varying concentrations. TC is not used in animals in Australia and its source has not been investigated.

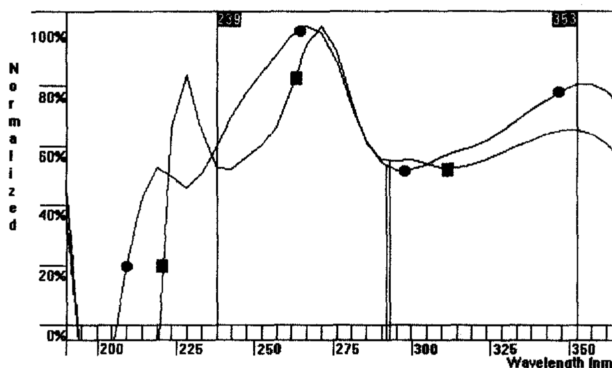

Fig. 2. (■) Diode-array spectra of spiked oxytetracycline sample and (●) oxytetracycline spectrum from the library. For experimental conditions, see Fig. 1.

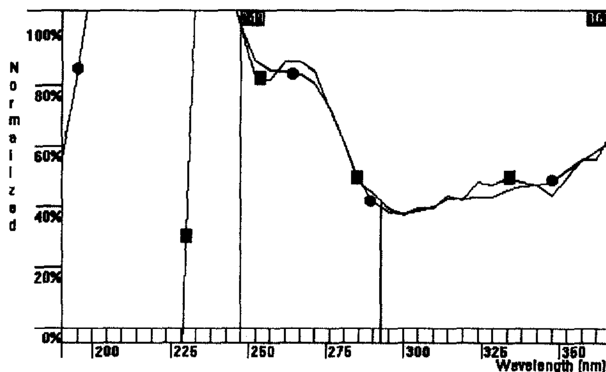

Fig. 3. (■) Diode-array spectra of spiked chlortetracycline sample and (●) chlortetracycline spectrum from the library. For experimental conditions, see Fig. 1.

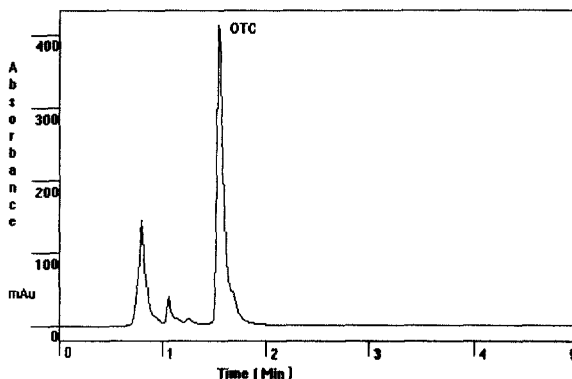

Fig. 4. Chromatogram of incurred oxytetracycline residue sample. For experimental conditions, see Fig. 1.

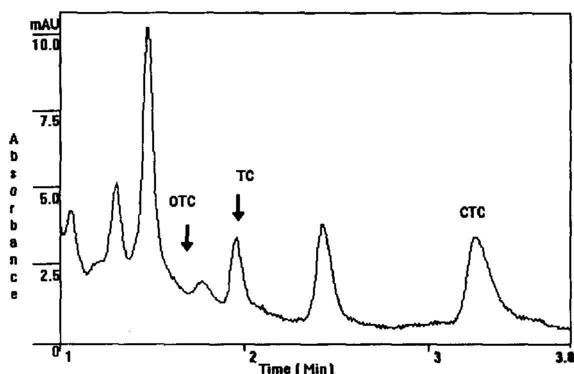

Fig. 5. Chromatogram of incurred chlortetracycline residue sample. The retention-times of OTC and TC are marked. Note the TC peak. For experimental conditions, see Fig. 1.

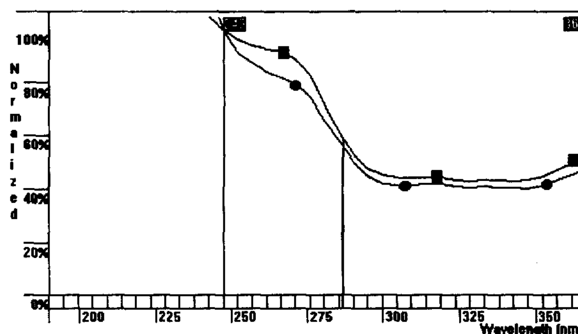

Fig. 7. (■) Diode-array spectra of incurred chlortetracycline residue and (●) chlortetracyline spectrum from the library. For experimental conditions, see Fig. 1.

DISCUSSION

The method of Oka *et al.* [1] was adapted to suit our requirements, as follows: (a) a larger sample size (40 g), with connective tissue and fat carefully trimmed off; (b) larger capacity SPE cartridges (Environmental Sep-Pak); (c) removal of most extraneous compounds (proteins) that otherwise would saturate the Sep-Pak cartridge and the chromatogram and, ultimately, block the column irreversibly, no matter what house-keeping techniques are used [7]; (d) buffers that have minimum UV absorbance above 220 nm; (e) high-resolution liquid chromatographic columns [Nova-Pak C_{18} (10 cm × 8 mm I.D.)] and (f) application of the concept of minimum volume elution.

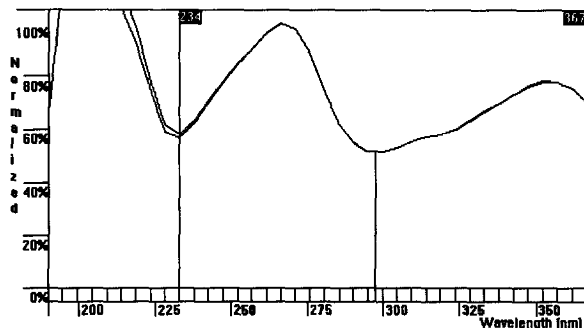

Fig. 6. Diode-array spectra of incurred oxytetracycline residue and oxytetracycline spectrum from the library. For experimental conditions, see Fig. 1.

Measuring the volume at each step was employed for a faster throughput. This enables supernatants to be poured off, not aspirated and re-washed a number of times. The same applies to filtrates. The time per analysis would be doubled or tripled if exhaustive extraction was used. Analyses must be carried out on the same day, as the analytes deteriorate overnight, even if frozen. The R.S.D. of inter-assay spikes (Table I) was double that of the intra-assay spikes. The larger scatter was associated with a lower mean recovery. This has been shown to be due to individual samples not being analysed within the ideal time-frame used when the method was developed. When a large number of samples (8–20) are batched, *i.e.*, the whole group goes through each step together, some samples spend a longer time in each step than is desirable. The spiked sample used in each batch was always placed last in the queue, which caused any delays to affect the spiked sample the most. Results have indicated that samples should be analysed in batches of five or less to maximize recoveries.

Elution of the analytes from the Sep-Pak cartridge in the smallest possible volume is critical to the production of useful peak areas at low concentrations of TCs.

Originally, oxalic acid [1] and methanol [2] were used in the aqueous phases, but were discarded in favour of the present mixtures in order to make diode-array library matches possible at these levels. The use of oxalic acid in the mobile phase inhibits losses of the TCs in the analytical column and connecting tubing, possibly by scavenging divalent ions. EDTA [9] was substituted when oxalic acid in

the mobile phase proved unsuitable for diode-array use. Tetramethylammonium chloride was used to improve the peak shapes via the mechanism of ion suppression [2].

At low wavelengths, the contribution of the buffer and residual matrix to the UV spectrum distorts the subject spectrum. This is very apparent below 240 nm. It can seen by the comparison of Figs. 2 and 6 for OTC and Figs. 3 and 7 for CTC that the library matches for the compounds deteriorates as the level of analyte decreases. This is a function of the greater contribution of the background to the whole spectrum relative to the analyte. The contribution of the buffer and residual matrix to the spectrum is very large compared with that of the analyte and is not constant throughout the chromatogram. It is for this reason that the library of spectra contains a number of spectra of each species created using different levels of spiking in samples. The retention time of the analyte is still the primary means of identification, with the diode-array system important in supplying a further source of confirmation. Tetracycline (TC) was originally included in the study to test its usefulness as an internal standard as it is not used in the meat-production industry. Its appearance with high concentrations of CTC has precluded its use.

In conclusion, this method has proved to be reliable and robust, and possesses the required sensitivity to detect the improper use of OTC and CTC in the meat-production industry in Australia, and it has been used for surveillance (compliance) and monitoring of tetracycline residues in over 300 animals.

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