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Short Communication

Determination of tetracycline antibiotics by reversed-phase high-performance liquid chromatography with fluorescence detection

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ABSTRACT

A highly sensitive method for the determination of tetracycline antibiotics (TCs) using reversed-phase high-performance liquid chromatography with fluorescence detection is presented This method was based on the use of disodium ethylenediaminetetraacetate (EDTA) and calcium chloride as fluorescence-increasing reagents in the mobile phase The concentrations of each reagent in the mobile phase greatly influenced the fluorescence intensity of TCs When the concentration of EDTA and calcium chloride were 25 and 35 m*M*, respectively, and the pH of the mobile phase was 6 5, the maximum fluorescence intensity was obtained The column temperature hardly influenced the fluorescence intensity At 3 75 ng of TCs injected, the precision (relative standard deviation) ranged from 1 12 to 2 20% In the range 0 075–37 5 ng for tetracycline and oxytetracycline and 0 225–37 5 ng for chlortetracycline, a linear response was observed The detection limits of this method were 49–190 pg for three different TCs The proposed method was applied to the determination of one of the TCs in pharmaceuticals by the internal standard method using other TCs as internal standards and was also applied to determination of TCs added to fish tissue

INTRODUCTION

Tetracycline antibiotics (TCs) are widely used in the stockbreeding and fishery industries and a selective and sensitive analytical method is still required for pharmacokinetic studies of TCs Recently, various high-performance liquid chromatographic (HPLC) methods for the determination of TCs have been reported [1–9] These methods use spectrophotometric detection of TCs, which limits the sensitivity that can be achieved More recently, Blanchflower *et al* [10] discussed this and other disadvantages of these methods, and developed an HPLC method for chlorotetracycline (CTC) at residual levels with fluorescence detection This method allowed the detection of CTC at picomole levels, but it is restricted to CTC An HPLC method for TCs with electrochemical detection was reported by Hou and Wang [11] This allowed highly selective detection, but electrochemical detectors are not widely used compared with spectrophotometric and fluorimetric detectors

It is well known that TCs combine with divalent metal ions to give fluorescent chelate compounds Although only TCs show fluorescence under basic conditions, the fluorescence intensity of the chelate under neutral or alkaline conditions is stronger

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than that of free TCs This suggests the possibility of their highly sensitive detection Indeed, this has applied to the determination of TCs by the manual methods [12,13] However, application to an HPLC–fluorescence detection method for TCs using this property has not been reported, because TCs experience problems in reversed-phase HPLC In order to resolve these problems, an acidic mobile phase must be used, but this results in quenching of the fluorescence The problems are a decrease in recovery from the column owing to chelation between the TCs and trace metal ions in the packing and on the surface of the column tube, and asymmetric peaks arising from interactions between TCs and residual silanols present in the packing, resulting in poor sensitivity

In order to overcome the above problems, in this work calcium chloride (CC) and disodium ethylenediaminetetraacetate (EDTA) were added to the neutral mobile phase and a metal ion-free packing was used, resulting in a highly sensitive HPLC method for the determination of tetracycline (TC), oxytetracycline (OTC) and CTC with fluorimetric detection The method was applied successfully to the determination of TCs present in pharmaceutical preparations and added to fish tissue

EXPERIMENTAL

Apparatus

The HPLC system consisted of an L-6200 delivery system (Hitachi, Tokyo, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, USA), a Capcell C_{18} type SG-120 (5-μm) prepacked column (250 × 4 6 mm I D) (Shiseido, Tokyo, Japan) and an L-1200 spectrofluorimeter (Hitachi) The column was thermostated at 30 ± 0 2°C The detector excitation and emission wavelengths were set at 390 and 512 nm, respectively Results were recorded on a D-2500 chromato-integrator (Hitachi)

Reagents

Methanol was of HPLC grade, all other reagents were of analytical-reagent grade Water was purified by distillation, followed by final clean-up through a Milli-Q Labo system (Nihon Millipore, Tokyo, Japan) TC HCl and OTC HCl were obtained from Wako (Osaka, Japan) and CTC HCl from Sigma (St Louis, MO, USA) A 1 mg/ml stock solution of each TC was prepared with 50 m*M* HCl The mobile phase consisted of 0 1 *M* acetate buffer (pH 6 5) containing 35 m*M* CC and 25 m*M* EDTA (buffer A) and methanol

Procedure for standard samples

Standard solutions of each TC were prepared by diluting the stock solution to appropriate concentrations with 50 m*M* HCl To 150 μl of the standard solution were added 250 μl of 24% trichloroacetic acid (TCA) solution in methanol An aliquot (10 μl) of the resulting mixture was injected on to the HPLC column

Procedure for determining OTC and TC in pharmaceutical formulations by the internal standard method

For Terramycin capsules (Pfizer) One capsule was dissolved and diluted with 50 m*M* HCl in a 250-ml calibrated flask, and this solution was centrifuged for 15 min at 2000 *g* Further, 100 μl of the supernatant were diluted with 50 m*M* HCl in a 50-ml calibrated flask To 150 μl of this solution and the standard solution of OTC (2 μg/ml) were added 250 μl of internal standard solution (prepared by dissolving TC in 24% methanolic TCA solution concentrated to 2 μg/ml), respectively An aliquot (10 μl) of the each resulting mixture was injected on to the HPLC column successively The content of OTC in the capsule was calculated by using the ratio of the peak area of OTC to that of the internal standard (TC) for each chromatogram

For Achromycin V syrup (Lederle) A 1-ml volume of syrup was diluted with 50 m*M* HCl in a 25-ml calibrated flask and this solution was centrifuged for 15 min at 2000 *g* Further, 100 μl of the supernatant were diluted with 50 m*M* HCl in a 50-ml calibrated flask To 150 μl of this solution and the standard solution of TC (2 μg/ml) were added 250 μl of internal standard solution (prepared by dissolving OTC in 24% methanolic TCA solution concentrated to 2 μg/ml), respectively An aliquot (10 μl) of the each resulting mixture was injected on to the HPLC column successively The content of TC in the syrup was calculated by using the ratio of the peak area of TC to that of the internal standard (OTC) for each chromatogram

Procedure for determining three TCs spiked in silver salmon tissue

A 1-g amount of tissue was homogenized with 3 ml of 1 *M* HCl, then further homogenized with 6 ml of 24% methanolic TCA solution This mixture was centrifuged at 2000 *g* for 15 min To 600 μl of the supernatant were added 600 μl of buffer A After the mixture had been vortex mixed and allow to stand for 5 min, it was centrifuged at 2000 *g* for 10 min An aliquot (50 μl) of the resulting supernatant was injected on to the HPLC column

RESULTS AND DISCUSSION

Effects of calcium ion and EDTA on fluorescence intensity and elution from a reversed-phase column

The effects of calcium ion and EDTA on fluorescence intensity were investigated by a flow-injection method The mobile phase containing CC alone gave fluorescence but its intensity was less than half of that when both CC and EDTA were present With EDTA alone or no additive hardly any fluorescence was obtained This suggests that the presence of the appropriate amount of EDTA further increased the fluorescence of the TC–calcium chelates

Fig 1 shows typical chromatograms of three TCs obtained with the above mobile phase systems using a reversed-phase column The mobile phase containing both CC and EDTA gave three symmetrical peaks (Fig 1A), whereas the three TCs could not be detected using mobile phases containing CC alone, EDTA alone or no additive (Fig 1B–D) Undesirable interactions between TC chelates and the packing were diminished by addition of EDTA to a CC-containing mobile phase

In order to compare with fluorescence intensity of TC–magnesium chelates, a mobile phase containing magnesium chloride instead of CC was also studied by the flow-injection method A slightly stronger fluorescence of the three TCs was observed with CC compared with magnesium chloride

Optimization of HPLC conditions

In order to optimize the HPLC conditions, the effects of the concentration of each additive in the mobile phase, pH and column temperature were investigated The maximum fluorescence intensity of the TCs was obtained when the CC, EDTA and

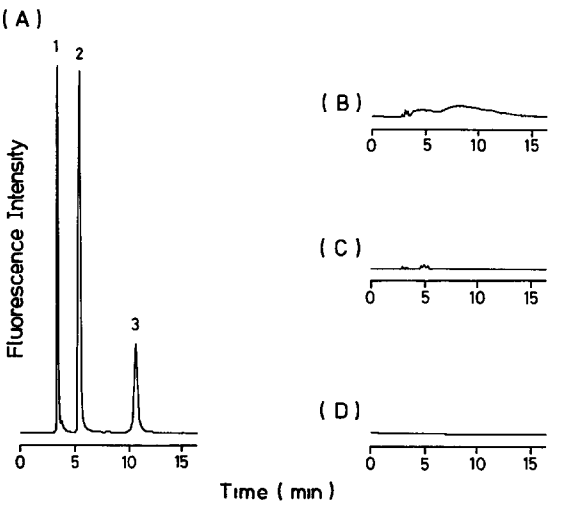

Fig 1 Chromatographic profiles of three TCs obtained on a reversed-phase column using various mobile phases Mobile phase (A) methanol–buffer A (45 55), (B) without EDTA, (C) without CC, (D) without both EDTA and CC Flow-rate, 1 0 ml/min, sample concentration, 37 5 ng per injection Peaks 1 = OTC, 2 = TC, 3 = CTC

sodium acetate concentrations in the mobile phase were ⩾35 m*M*, 25 m*M* and ⩾ 0 01 *M*, respectively

The mobile phases were tested in the pH range 5 0–7 5 Only OTC showed a different pattern from the other two TCs, but the fluorescence intensity of the three TCs was almost constant in the pH range 6 5–7 5, lower pH values decreased the intensity (Fig 2) Figure 3 shows the effect of column temperature on the fluorescence intensity in the range 20–40°C In this range, the fluorescence intensity of TC and CTC remained almost constant, whereas that of OTC decreased slightly with an increase in temperature Consequently, the HPLC conditions given under Experimental were adopted

Under these conditions, column packings, various kinds of conventional ODS (including end-capped types) and a few metal-free type ODS, were also investigated A Capcell C_{18} type SG-120 (metal-free type) gave the most symmetrical peaks, and was adopted The details are omitted As TCA is generally used in bioanalysis as deproteinization reagent for TCs, TCA was added to the standard solution

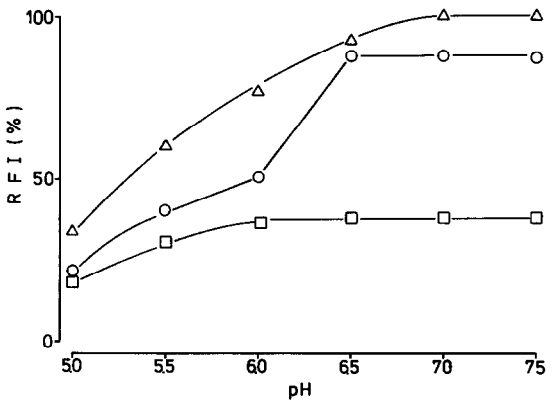

Fig 2 Effect of pH of buffer A on the fluorescence intensity of TCs Mobile phase, methanol–buffer A (45 55), flow-rate, 1 0 ml/min, column temperature, 30°C, sample concentration, 37 5 ng per injection △ = TC, ○ = OTC, □ = CTC R F I = relative fluorescence intensity

Evaluation of present method

Linear responses were observed in the range 0 075–37 5 ng for OTC and TC and 0 225–37 5 ng for CTC The equations of the regression lines for the calibration graphs were $y = 4\ 787x - 0\ 343$ for OTC, $y = 6\ 245x + 0\ 114$ for TC and $y = 2\ 751x + 0\ 070$ for CTC (y = peak area 10^{-5}, x = ng) The correlation coefficient for the three TCs were > 0 9999

Several analyses were performed to determine the reproducibility of the method Table I gives the relative standard deviations of peak area for the three TCs at a concentration of 3 75 ng per injection The detection limits with both fluorimetric and spectrophotometric (350 nm) detection (signal-to-noise ratio = 3) are also given in Table I The results demonstrate that highly sensitive detection for all three TCs was achieved

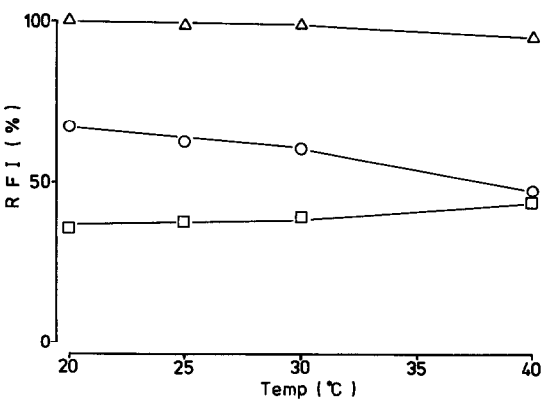

Fig 3 Effect of column temperature on the fluorescence intensity of TCs pH of buffer A, 6 5, other conditions and symbols as in Fig 2

TABLE I

REPRODUCIBILITIES OF PEAK AREA AND DETECTION LIMITS FOR TETRACYCLINES

Sample	R S D (%)		Detection limit[c] (ng)	
	Intra-assay[a]	Inter-assay[b]	Fluorimetric detection	UV detection[d]
OTC	1 46	2 20	0 051	1 9
TC	1 24	1 48	0 049	3 6
CTC	1 18	1 12	0 190	9 6

[a] 3 75 ng per injection, $n = 10$
[b] 3 75 ng per injection, $n = 5$
[c] Signal-to-noise ratio = 3
[d] Wavelength, 350 nm

Application to determination of TCs in pharmaceuticals

The method was applied to the determination of OTC in Terramycin capsules and TC in Achromycin V syrup using the internal standard method as an example TC and OTC were used as internal standards in the former and latter analyses, respectively Fig 4 shows chromatograms for (A) Terramycin capsules and (B) Achromycin V syrup The mean results (± S D , $n = 5$) were 106 7 ± 3 8% OTC in Terramycin capsules and 92 3 ± 1 1% TC in Achromycin V syrup

Application to determination of TCs added to fish tissue

As the conventional HPLC methods for the determination for TCs mainly used spectrophotometric detection, they required concentration steps involving extraction using organic solvents or a precolumn as a pretreatment for the determination of TCs in biological fluids These methods are generally tedious and are responsible for unsystematic errors in the analysis In contrast, the present method allows the highly sensitive detection of TCs, suggesting the possibility of the determination of TCs in biological fluids without the need for tedious pretreatment

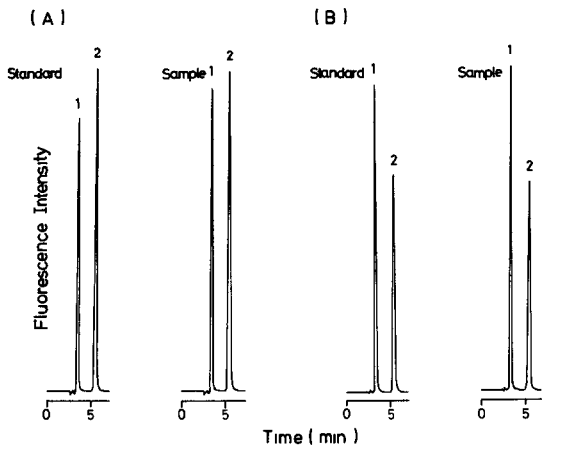

Fig 4 Chromatograms for determining OTC in (A) Terramycin capsules and TC in (B) Achromycin V syrup by the internal standard method Mobile phase, methanol–buffer A (45 55), flow-rate, 1 0 ml/min Peaks 1 = OTC, 2 = TC

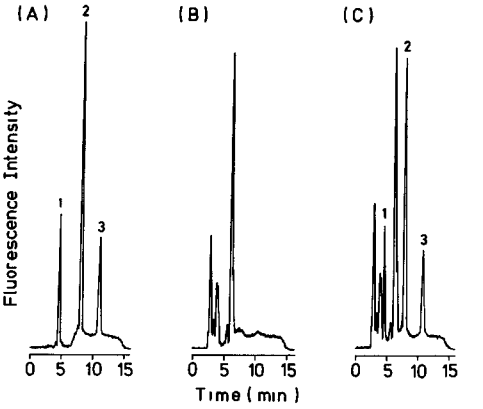

Fig 5 Chromatograms of (A) standard TCs, (B) tissue blank and (C) TCs added to tissue Mobile phase, mixture of 90% methanol and buffer A (using 0 3 *M* instead of 0 1 *M* sodium acetate) Gradient programme 0–1 min, 67% buffer A, 1–8 5 min, 45% buffer A, 8 5 min onwards, 67% buffer A Flow-rate, 1 0 ml/min, sample concentration, 1 ppm Peaks 1 = OTC, 2 = TC, 3 = CTC

The effect of the extraction reagent on the recovery from silver salmon tissue is shown in Table II The extraction conditions specified under Experimental gave maximum recoveries Fig 5 shows chromatograms of standard TCs, tissue blank and the three TCs added to tissue Isocratic elution as in Fig 1 suffered interference from endogenous materials in the detection of OTC This problem was resolved by a combination of gradient elution as indicated in the caption of Fig 5 and the use of 0 3 *M* instead of 0 1 *M* sodium acetate in buffer A Under these conditions, the calibration graphs for OTC, TC and CTC were linear in the ranges of 0 08–10, 0 06–10 and 0 1–10 ppm (all with r >0 999), respectively The recovery and reproducibility [relative standard deviation (R S D)] for OTC at 0 1, 0 5 and 1 0 ppm were 73 6–91 9% and R S D 2 77–4 44% (n = 7), for TC 90 6–95 5% and R S D 2 62–4 63% (n = 7) and for CTC 85 6–93 4% and R S D 3 48–4 70% (n = 7) The reproducibility of the retention time of each TCs was R S D = 0 77–1 78% (n = 7)

TABLE II

RECOVERY OF TETRACYCLINES FROM SILVER SALMON FISH TISSUE BY THE USE OF VARIOUS EXTRACTION REAGENT SYSTEMS

Method[a]	Recovery (%)		
	OTC	TC	CTC
24% TCA in methanol, 3 ml	76 8	75 9	79 4
1 *M* HCl, 3 ml	72 6	73 4	70 9
24% TCA in methanol, 3 ml			
1 *M* HCl, 3 ml	91 0	93 5	93 4
24% TCA in methanol, 6 ml			
24% TCA in methanol, 9 ml	87 8	86 0	93 1

[a] For 1 g of muscle tissue, sample concentration, 1 ppm

The detection limits for the three TCs were 32–90 ppb (signal-to-noise ratio = 3) The three TCs were stable for at least 24 h in the resulting mixture at room temperature

Although the tedious pretreatment step discussed above was omitted, the detection limits were as good as those for the conventional methods [1,3,4] This demonstrates the possibility of the simple determination of TCs in biological fluids and also of a more sensitive determination by a combination of the present method and concentration steps Concentration steps that are as simple as possible and suitable for the present method are currently being studied

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