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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

VI*. DETECTION REAGENTS FOR TETRACYCLINES IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Semi-quantitative screening methods for tetracyclines using detection on silica gel high-performance thin-layer chromatographic (HPTLC) and reversed-phase (RP) TLC plates are described. Good results with respect to the background and detection limits were obtained using detection with 1% Fast Violet B Salt solution followed by heating on the silica gel HPTLC plate and with 0.5% Fast Violet B Salt solution and pyridine without heating on the RP-TLC plate. The above detection method and UV densitometry using the silica gel HPTLC plate were compared with respect to recovery from spiked samples after Sep-Pak C_{18} extraction. For the measurement of impurities in tetracycline drugs, both methods were compared using the RP-TLC plate and similar results were obtained.

INTRODUCTION

Tetracycline antibiotics (TCs) (Table I) are the most widely applied antibiotics for the treatment of fishes in Japan[1]. For measuring residue of TCs in foods, microbiological assays are most often used as they are reliable and sensitive, but they are non-specific and can be time consuming. Therefore, a fluorimetric[2] and thin-layer chromatographic (TLC)[3] methods have been reported for the determination of TCs in foods, but most of them are very complicated. Hence the development of a simple and rapid method for the determination of TCs in foods is desirable.

In previous papers we described chemical methods for the determination of antibiotics and established techniques for determination of TCs and polyether antibiotics[4]. For TCs we used high-performance liquid chromatography (HPLC)[5], silica gel high-performance thin-layer chromatography (HPTLC)[6] and reversed-phase

* For Part V, see ref. 5.

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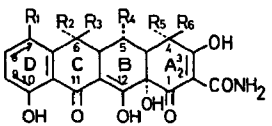
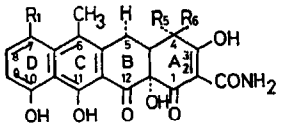
TABLE I

STRUCTURES OF TETRACYCLINES



I



II

Compound	*Structure*	R_1	R_2	R_3	R_4	R_5	R_6
Tetracycline (TC)	I	H	CH_3	OH	H	$N(CH_3)_2$	H
Oxytetracycline (OTC)	I	H	CH_3	OH	OH	$N(CH_3)_2$	H
Chlortetracycline (CTC)	I	Cl	CH_3	OH	H	$N(CH_3)_2$	H
Doxycycline (DC)	I	H	CH_3	H	OH	$N(CH_3)_2$	H
4-Epitetracycline (ETC)	I	H	CH_3	OH	H	H	$N(CH_3)_2$
Anhydrotetracycline (ATC)	II	H	–	–	–	$N(CH_3)_2$	H
4-Epianhydrotetracycline (EATC)	II	H	–	–	–	H	$N(CH_3)_2$

(RP) TLC[7] followed by densitometry, and further we succeeded in measuring the concentration of TCs in fish tissues by a combination of a Sep-Pak C_{18} extraction and HPTLC–densitometry[8]. However, these methods required the use of a densitometer or a high-performance liquid chromatograph to determine TCs. Therefore, we wished to establish a semi-quantitative method for TCs as a screening method without the need for special instrumentation.

In this paper we report a technique for the semi-quantitative analysis of TCs using spray reagents on silica gel HPTLC and RP-TLC plates.

EXPERIMENTAL

Materials

Methanol (CH_3OH), acetonitrile (CH_3CN), chloroform ($CHCl_3$), ethanol (C_2H_5OH), oxalic acid, citric acid, disodium ethylenediaminetetraacetate (Na_2EDTA), sodium hydroxide (NaOH), disodium hydrogen phosphate (Na_2HPO_4), pyridine, 1,5-diazabicyclo[5.4.0]undecene-5 (DBU) and triethanolamine were analytical-reagent grade materials.

Tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC), as their hydrochlorides, were supplied by Pfizer Taito. 4-Epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC), as their hydrochlorides, were prepared according to the methods of Simmon *et al.*[9] and McCormick *et al.*[10].

Preparation of standard tetracycline solutions

Each TC (100 mg) was weighed accurately into a 10-ml volumetric flask and diluted to volume with CH_3OH or water. Dilution was sometimes necessary.

Preparation of spray reagents

Aqueous solutions of the following reagents were used for detection: Fast Violet B Salt (Wako), Fast Blue BB Salt (Sigma), Fast Blue B Salt (E. Merck), Fast Red ITR Salt (Sigma), Fast Red B Salt (Sigma), Fast Garnet GBC Salt (Sigma) and Fast Red PDC Salt (Sigma).

Extraction and clean-up procedure for determination of TCs in foods

As described previously[8], a sample (20 g) was blended twice using 50 ml of 0.1 *M* Na_2EDTA–McIlvaine buffer (pH 4.0) and centrifuged. The supernatant was adsorbed on a Sep-Pak C_{18} cartridge pre-treated with 0.2 *M* Na_2EDTA solution, the cartridge was washed with water, TCs were eluted with 10 ml of C_2H_5OH and the concentrated residue was spotted on a silica gel HPTLC plate.

Preparation of test solution to determine impurities in TC drugs

For tablets and capsules, twenty tablets or capsules of TC · HCl selected at random were combined and finely powdered when required. A 100-mg amount of the powder or dry syrup was dissolved in and made up to volume with CH_3OH in a 10-ml volumetric flask.

Thin-layer chromatography

A silica gel HPTLC plate (E. Merck, 5641) was pre-developed with saturated Na_2EDTA solution and then dried in air at room temperature for 1 h and activated at 130°C for 2 h. After applying a sample, the plate was developed with $CHCl_3$–CH_3OH–5% Na_2EDTA solution (65:20:5) (lower layer). For RP-TLC, the plates (E. Merck, 15423 and 15424) were developed with CH_3OH–CH_3CN–0.5 *M* oxalic acid solution (pH 2.0) (1:1:2) and (1:1:4), respectively.

Determination of TCs

UV densitometry. The developed TLC plate was placed under a Shimadzu CS-910 chromatogram scanner and the components were determined by UV absorption spectrophotometry. The operating conditions were as follows: dual-wavelength mode, λ_{sample} = 360 nm and $\lambda_{reference}$ = 600 nm for TC, OTC, DC and ETC; λ_{sample} = 425 nm and $\lambda_{reference}$ = 650 nm for ATC and EATC; linear scanning in the reflection mode, size of beam 0.25 × 9.0 mm; working linearizer, LIN SX = 3 programme; background correction, on.

Detection. The developed silica gel HPTLC plate was sprayed evenly with 1.0% Fast Violet B Salt solution and then heated at 120°C to produce coloured spots. The developed RP-TLC plate was sprayed evenly with 0.5% Fast Violet B Salt solution and pyridine, then heated at 120°C to evaporate the pyridine. The amounts of TCs on both TLC plates were measured immediately by visual comparison of the intensities of the colours with those of standards after heating the TLC plates.

RESULTS AND DISCUSSION

Iron (III) chloride[11], antimony pentachloride[11], sulphuric acid[11], diazotized *p*-nitroaniline[12], modified Sakaguchi reagent[12], diphenylpicrylhydrazyl reagent[12] and Fast Blue B Salt[11–13] have been reported previously as detection reagents for TCs on TLC plates, and has been found that Fast Blue B Salt gives better results than other reagents[12]. Gyanchandani *et al.*[12] and Radecka and Wilson[13] succeeded in determining TC and its degradation products using 0.5% Fast Blue B Salt solution alone or followed by 0.1 *N* NaOH solution and heating on a Kieselguhr TLC plate.

Silica gel HPTLC plate

Spray reagent. We attempted to use 0.5% Fast Blue B Salt solution and 0.1 *N* NaOH solution as spray reagents according to the method of Gyanchandani *et al.*[12] on a silica gel HPTLC plate. However, we obtained unsatisfactory results with respect to the background and detection limits. In an attempt to find a spray reagent that is useful for the semi-quantitative analysis of TCs by TLC, several diazonium salts (Fast Violet B Salt, Fast Blue BB Salt, Fast Blue B Salt, Fast Red ITR Salt, Fast Red B Salt, Fast Garnet GBC Salt and Fast Red PDC Salt) were examined.

While Fast Garnet GBC and Fast Red PDC gave a dirty background, five remaining spray reagents gave various coloured spots with the parent TCs and their degradation products as shown in Table II. Spraying with diazonium salt solution followed by heating gave good sensitivities (30–100 ng) as in our previous work[6], and the colour intensities of the spots were related to the amounts of TCs present. We

consider that these spray reagents are suitable for the semi-quantitative analysis of TCs. 1.0% Fast Violet B Salt solution gave the best results with respect to the background and detection limits, and is the most suitable for the detection of TCs. It was therefore employed in subsequent work.

Determination of TCs in foods. To test the detection with 1.0% Fast Violet B Salt solution on a silica gel HPTLC plate, we determined TCs in spiked samples at the 1.0 ppm level using Sep-Pak C_{18} extraction, and compared the results with those obtained by UV densitometry. The results for rainbow trout, eel, milk, egg and chicken, beef and pork muscle samples are given in Table III. Both methods gave similar average recoveries, but the standard deviations were 1.4–6.6% and 3.4–17.6% for UV densitometry and detection with 1.0% Fast Violet B Salt solution, respectively. Although the latter method showed a higher standard deviation, no spots were revealed except TCs and the detection limits for foods were as good as those of UV densitometry (0.1 ppm). The combination of Sep-Pak C_{18} extraction and detection with 1.0% Fast Violet B Salt solution dose not require special instrumentation and its operation can be achieved simply in any laboratory. Therefore, we consider this combination is of great practical use as a screening method.

Reversed-phase TLC plate

Spray reagent. As described under Experimental, the RP-TLC plate was developed with CH_3OH–CH_3CN–0.5 *M* oxalic acid solution (pH 2.0) (1:1:4). Although solutions of spray reagents at the same concentration as for the silica gel HPTLC method were sprayed on the RP-TLC plate, we were not able to obtain satisfactory coloured spots. We consider that this reaction involves coupling of diazonium salts with TCs to produce coloured spots, and in this coupling an aromatic ring which contains a powerful electron-donating group, generally –OH, $-NR_2$, –NHR or $-NH_2$, undergoes attack by the diazonium ion, and its substitution usually occurs *para* to the activating group. Applying similar reasoning to TCs, it would be expected that the substitution would occur *para* to the hydroxy group on the D-ring. As the electron-donating ability of the hydroxy group in mildly alkaline or neutral media is higher than that in acidic media, coupling of diazonium ions with TCs occurs rapidly in mildly alkaline or neutral media. In general, this is accomplished by addition of a suitable amount of sodium hydroxide[14].

The results reported above suggest that the residual oxalic acid on the TLC plate interfered with the coupling of the diazonium salts, as the RP-TLC plate is developed with organic solvents containing oxalic acid. When 0.1 *N* NaOH solution was sprayed as a basic reagent after spraying with 0.5% Fast Violet B Salt solution, coloured spots were obtained. However, the treatment did not always give reproducible results, so the use of 28% ammonia and 0.2 *M* Na_2HPO_4 solution (aqueous) as inorganic bases and 5% methanolic DBU solution, 10% methanolic triethanolamine solution and pyridine as organic bases to give basic conditions was examined. Weakly coloured spots were produced by use of 0.2 *M* Na_2HPO_4 solution and 10% methanolic triethanolamine solution. Although 28% ammonia solution produced clearly coloured spots, it gave poor reproducibility, and 5% methanolic DBU solution yielded a dirty background on the TLC plate. In contrast to the other basic reagents, pyridine gave clearly coloured spots and good reproducibility without heating for all TCs. The detection limits and colours of the spots obtained with various

TABLE II

DETECTION LIMITS OF TETRACYCLINES BY HPTLC WITH DIFFERENT SPRAY REAGENTS

Plate: silica gel HPTLC pre-developed with saturated Na_2EDTA, activated at 130°C for 2 h. Solvent system: $CHCl_3$–CH_3OH–5% Na_2EDTA (65:20:5) (lower layer).

Spray reagent (aq.)	*Detection limit (μg) (colour)**							
	OTC	*TC*	*CTC*	*DC*	*ETC*	*ATC*	*EATC*	*Background*
2.0% Fast Red ITR	0.1 (Y)	0.05(Or)	0.1 (Or)	0.1 (Y)	0.05(Or)	0.1(R-V)	0.1(R-V)	–(Y-Or)
1.0% Fast Red B	0.03(Or)	0.03(Or)	0.03(Or)	0.03(Y)	0.03(Or)	0.1(R-V)	0.1(R-V)	–(Y-Or)
0.5% Fast Blue BB	0.03(R)	0.05(R)	0.05(R)	0.03(R)	0.03(R)	0.1(B-V)	0.1(B-V)	–(Y-Or)
1.0% Fast Violet B	0.03(R)	0.03(R)	0.03(R)	0.03(R)	0.03(R)	0.1(B-V)	0.1(B-V)	–(W)
0.2% Fast Blue B	0.03(R)	0.05(R)	0.05(R)	0.05(R)	0.03(R)	0.1(R-V)	0.1(R-V)	–(Y-Or)

* Y = Yellow; Or = orange; R = red; V = violet; B = blue; W = white.

TABLE III

COMPARISON OF UV DENSITOMETRY AND DETECTION WITH 1% FAST VIOLET B ON HPTLC

Plate: silica gel HPTLC pre-developed with saturated Na_2EDTA, activated at 130°C for 2 h. Solvent system: $CHCl_3$–CH_3OH–5% Na_2EDTA (65:20:5) (lower layer). A = UV densitometry; B = detection with 1.0% Fast Violet B.

Sample	*No. of experiments*	*Recovery (av. ± S.D.) (%)*							
		OTC		*TC*		*CTC*		*DC*	
		A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Rainbow trout	6	74.8±3.4	76.6±16.9	67.0±4.5	61.6±17.6	61.8±4.5	59.7±10.3	59.3±3.4	43.3± 8.9
Eel	6	78.3±1.4	67.5±14.2	67.5±1.5	59.3± 6.0	65.3±1.4	50.8± 7.3	65.2±1.8	50.8±11.0
Milk	6	85.6±3.6	75.4± 4.8	73.0±3.5	66.6± 9.4	79.0±4.4	73.3±12.5	80.6±4.9	73.3±12.5
Egg	6	76.6±6.6	65.8±15.3	72.3±4.5	73.3±13.4	75.2±5.2	83.3± 4.9	61.0±3.2	59.2± 7.2
Chicken muscle	6	65.7±3.1	75.0±11.0	70.5±3.9	65.8± 3.4	73.0±4.8	65.8± 8.3	72.2±3.5	65.0± 4.1
Beef muscle	6	75.7±1.9	61.7±10.3	62.4±2.7	60.8± 5.3	65.5±3.7	66.7± 9.9	61.0±2.3	50.0±10.2
Pork muscle	6	78.0±2.3	70.0±10.1	70.3±2.7	83.3± 9.8	66.7±2.5	81.1± 8.6	68.2±2.1	56.6±13.5

TABLE IV

DETECTION LIMITS OF TETRACYCLINES BY RP-TLC WITH DIFFERENT SPRAY REAGENTS FOLLOWED BY PYRIDINE

Group I: on C_8 TLC plate using CH_3OH–CH_3CN–0.5 *M* oxalic acid (pH 2.0) (1:1:4). Group II: on C_{18} TLC plate using CH_3OH–CH_3CN–0.5 *M* oxalic acid (pH 2.0) (1:1:2). Group I = TC, OTC, CTC and DC; group II = ETC, TC, CTC, ATC and EATC.

Spray reagent (aq.)	*Detection limit (μg) (colour)*							
	TC	*ETC*	*ATC*	*EATC*	*OTC*	*CTC*	*DC*	*Background*
0.5% Fast Red ITR	0.05(Or)	0.05(Or)	0.03(R-V)	0.03(R-V)	0.05(Y)	0.05(Or-Y)	0.05(Y)	–(Y-Or)
0.5% Fast Red B	0.05(Or-Y)	0.05(Or-Y)	0.03(R-V)	0.03(R-V)	0.05(Or-Y)	0.05(Y)	0.05(Y)	–(Y-Or)
0.5% Fast Blue BB	0.03(B-V)	0.03(B-V)	0.03(G-B)	0.03(G-B)	0.03(R-V)	0.03(R-V)	0.03(R)	–(Y-Or)
0.5% Fast Violet B	0.03(R-V)	0.03(R-V)	0.03(G-B)	0.03(G-B)	0.03(R)	0.03(R)	0.03(R-V)	–(W)
0.5% Fast Blue B	0.03(R-V)	0.03(R-V)	0.03(G-B)	0.03(G-B)	0.03(R)	0.03(R-V)	0.03(R)	–(Y-Or)

* Y = Yellow; Or = orange; R = red; V = violet; B = blue; W = white; G = green.

TABLE V

COMPARISON OF UV DENSITOMETRY AND DETECTION WITH 0.5% FAST VIOLET B ON RP-TLC

Plate: C_{18} TLC. Solvent system: CH_3OH–CH_3CN–0.5 *M* oxalic acid (pH 2.0) [(1:1:2) for CTC, ATC and EATC; (1:1:4) for ETC]. A = UV densitometry; B = detection with 0.5% Fast Violet B.

Sample	*Content (%)*							
	ETC		*CTC*		*EATC*		*ATC*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Capsule A	3.3	2.5	<0.1	<0.1	0.2	0.1	0.4	0.2
Capsule B	1.8	1.6	<0.1	<0.1	<0.1	<0.1	0.1	0.2
Capsule C	1.3	1.5	<0.1	<0.1	0.2	0.2	0.4	0.3
Capsule D	3.1	2.5	<0.1	<0.1	<0.1	<0.1	0.2	0.2
Capsule E	1.9	1.8	<0.1	<0.1	<0.1	<0.1	0.2	0.2
Capsule F	2.6	1.6	<0.1	<0.1	<0.1	<0.1	0.1	0.1
Capsule G	1.4	1.6	<0.1	<0.1	<0.1	<0.1	0.1	0.2
Dry syrup	0.3	0.8	<0.1	<0.1	<0.1	<0.1	0.2	0.1
Tablet	3.6	3.2	<0.1	<0.1	0.3	0.3	0.4	0.4

diazonium salts followed by spraying with pyridine are shown in Table IV. These diazonium salts showed good sensitivity (30 ng), similar to the results obtained on the silica gel HPTLC plate; 0.5% Fast Violet B Salt solution gave the best results with respect to the background and the colour intensities of the spots relative to the amounts of TCs present. We consider that a combination of 0.5% Fast Violet B Salt solution and pyridine is the most suitable for the identification and determination of TCs. Further, for ATC and EATC, which has renal toxicity, this method gives a better sensitivity than that on the silica gel HPTLC plate, and is suitable for their analysis in TC drugs.

Analysis of impurities in TC drugs. As described above, a combination of 0.5% Fast Violet B Salt solution and pyridine is the most suitable for the identification and determination of TCs on RP-TLC plates, and we consider this method is effective for the analysis of impurities in TC drugs, and a comparison of this method with UV densitometry is shown in Table V. The samples of TC drugs were found by both method to contain small amounts of impurities, below the permitted limits specified in the British Pharmacopoeia[15], and both methods gave similar results. Therefore, we conclude that the proposed method is effective as a screening technique.

CONCLUSION

Techniques involving detection on silica gel HPTLC and reversed-phase TLC plates for the semi-quantitative screening of tetracyclines were established. The optimal conditions obtained are summarized in Table VI. The detection and UV densitometric techniques using silica gel HPTLC plates were compared with respect to recoveries from spiked samples after Sep-Pak C_{18} extraction. For the measurement of impurities in TC drugs, both methods were also compared on RP-TLC plates and similar results were obtained.

TABLE VI

OPTIMAL CONDITIONS FOR DETECTION OF TETRACYCLINES

Plate	*Spray reagent*	*Base*	*Heating*
Silica gel HPTLC	1.0% Fast Violet B Salt solution	None	120°C
RP-TLC	0.5% Fast Violet B Salt solution	Pyridine	None

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