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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

X*. DETERMINATION OF EIGHT TETRACYCLINES USING THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HISAO OKA*, YOSHITOMO IKAI, NORIHISA KAWAMURA, KEIICHI UNO and MASUO YAMADA
Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462 (Japan)
and
KEN-ICHI HARADA, MACHIKO UCHIYAMA, HIROHIKO ASUKABE and MAKOTO SUZUKI
Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468 (Japan)
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SUMMARY

Analytical methods for eight tetracyclines (TCs) were established using silica gel high-performance thin-layer chromatography (HPTLC), reversed-phase thin-layer chromatography (RP-TLC) and high-performance liquid chromatography (HPLC). Good separations of eight TCs were obtained using chloroform–methanol–5% disodium ethylenediaminetetraacetate solution (65:20:5) (lower layer) and methanol–acetonitrile–0.5 *M* oxalic acid solution (1:1:4) (pH 3.0) on silica gel HPTLC and C_8 TLC plates, respectively. A combination of HPTLC and RP-TLC made possible the identification of the eight TCs. Each calibration graph was linear between 0.1 and 1.0 μg using UV densitometry except for rolitetracycline. For detection reagents, the diazonium salts including Fast Violet B gave variously coloured spots with the eight TCs and good sensitivities were obtained except with minocycline. In HPLC, the simultaneous analysis of the eight TCs on a C_8 column was possible using methanol–acetonitrile 0.01 *M* oxalic acid solution (1:1.5:7) adjusted to pH 3.0 as the mobile phase. A linear relationship was obtained between 1.0 and 10 ng using the usual sample preparation except for rolitetracycline. The direct determination of rolitetracycline was possible using tetrahydrofuran, dimethyl sulphoxide and the mobile phase as solvents for preparation of the sample. For the determination of residual rolitetracycline, it was effective to measure the amount of rolitetracycline as tetracycline by HPLC, HPTLC and RP-TLC after conversion of rolitetracycline to tetracycline by incubating for 5 min in methanol at 50°C.

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INTRODUCTION

The tetracycline antibiotics (TCs, Fig. 1) are widely used as veterinary medicines and feed additives. Their widespread utilization leads to increasing resistance factors, so that the usage of TCs should be regulated. For public health agencies it is important to monitor accurately TCs in foods. In Japan, eight TCs are commercially available, of which oxytetracycline, tetracycline, chlortetracycline and doxycycline are permitted to be administered to animals. However, it is a distinct possibility that other TCs may also be used for animals for the following reasons[1]: minocycline has the highest biological activity among TCs and is active against tetracycline-resistant pathogens, methacycline and demeclocycline are more stable than tetracycline and give higher and more sustained blood levels with smaller doses at less frequent intervals and rolitetracycline is readily soluble in water so that it is more suitable than other TCs for injections.

Although many simultaneous analytical methods for TCs have been reported, including thin-layer chromatography (TLC)[2–4] and high-performance liquid chromatography (HPLC)[5–11], most of them have not included rolitetracycline and minocycline as an analyte. Only a few HPLC studies have been published that include rolitetracycline and minocycline[9–11]. However, Nelis and De Leenheer's method is not suitable for the separation of oxytetracycline and minocycline[9]. Reeuwijk and Tjaden's method requires a complicated column preparation for the analysis of TCs[10]. We were not able to obtain a satisfactory separation of the eight TCs under the conditions of Mack and Ashworth[11]. Therefore, it was desirable to establish a simple and rapid analytical method for the eight TCs.

In previous papers we have established chemical methods for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline using HPLC[12], silica gel high-performance TLC (HPTLC) and reversed-phase TLC (RP-TLC) followed by densitometry[13,14] and the use of detection reagents[15].

Because these methods are simple, rapid and reliable and gave good results for the simultaneous determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline, we considered that with slight modifications they would be suitable for the present purpose. Therefore, we applied these techniques to the analysis of the eight TCs and various experiments were carried out in order to establish the optimal conditions. This paper describes the optimal conditions and techniques utilized for the identification and determination of the eight TCs using HPLC, RP-TLC and HPTLC.

EXPERIMENTAL

Chemicals

Chloroform, methanol, acetonitrile, tetrahydrofuran, oxalic acid, disodium ethylenediaminetetraacetate (Na_2EDTA), pyridine and dimethyl sulphoxide were of analytical-reagent grade.

Oxytetracycline, tetracycline, chlortetracycline, doxycycline and methacycline were supplied by Pfizer Taito, minocycline and demeclocycline by Lederle (Japan) and rolitetracycline by Banyu.

Preparation of detection reagents

Aqueous solutions of the following diazonium salts 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) and Fast Red B salt (Sigma).

Preparation of standard tetracycline solutions

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

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 chloroform–methanol–5% Na_2EDTA solution (65:20:5) (lower layer). For RP-TLC, a sample was applied to a C_8-modified silica gel TLC plate (E. Merck, 15424) and developed with methanol–acetonitrile–0.5 *M* oxalic acid solution (1:1:4) (pH 3.0).

Determination of tetracyclines

UV densitometry. The developed TLC plate was placed under a chromatogram scanner (Shimadzu CS-910, Kyoto, Japan) and the components were determined by UV absorption spectrophotometry. The operating conditions were as follows; dual-wavelength mode, $\lambda_{sample} = 360$ nm and $\lambda_{reference} = 600$ nm; linear scanning in the reflection mode, size of beam 0.25 × 9.0 mm; working curve linearizer, LINSX = 3 program; background correction, on.

Detection reagents. The developed silica gel HPTLC plate was sprayed evenly with each diazonium salt solution and then heated at 120°C to produce coloured spots. The developed RP-TLC plate was sprayed evenly with each diazonium 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.

High-performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant-flow pump (Shimadzu LC-5A) was used, together with a variable-wavelength UV detector (Shimadzu SPD-2A) operated at 350 nm. The separation was performed on Chemcosorb 3C8 (3 μm, 75 × 4.6 I.D.) (Chemco, Osaka, Japan) with methanol–acetonitrile–0.01 *M* oxalic acid solution (1:1.5:7) (pH 3.0) as the mobile phase at a flow-rate of 1 ml/min at room temperature.

RESULTS AND DISCUSSION

In previous papers[12–15] we established five analytical methods for oxytetracycline, tetracycline, chlortetracycline and doxycycline and the conditions are listed in Table I. Although these methods were efficient and reproducible for the simultaneous analysis of four TCs, they did not always give satisfactory results for the analysis of all eight TCs. After carrying out various experiments, good results were obtained for

TABLE I

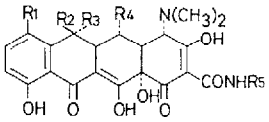
ESTABLISHED ANALYTICAL METHODS FOR OXYTETRACYCLINE, TETRACYCLINE, CHLORTETRACYCLINE AND DOXYCYCLINE AND THEIR OPTIMAL CONDITIONS

Method	*Stationary phase*	*Solvent system*	*Flow-rate*	*Detection*
HPLC	Cosmosil $5C_8$ (Nakarai, Japan)	Methanol–acetonitrile–0.01 *M* oxalic acid (pH 2.0) (1:1.5:5)	1 ml/min	350 nm
HPTLC–densitometry	Silica gel HPTLC (E. Merck, 5641)	Chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer)	–	360 nm
HPTLC–spray reagents	Silica gel HPTLC (E. Merck, 5641)	Chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer)	–	1% Fast Violet B
RP-TLC–densitometry	C_8 TLC (E. Merck, 15424)	Methanol–acetonitrile–0.5 *M* oxalic acid (pH 2.0) (1:1:4)	–	360 nm
RP-TLC–spray reagents	C_8 TLC (E. Merck, 15424)	Methanol–acetonitrile–0.5 *M* oxalic acid (pH 2.0) (1:1:4)	–	0.5% Fast Violet B and pyridine

TABLE II

OPTIMAL CONDITIONS FOR ANALYSIS OF EIGHT TETRACYCLINES

Method	*Stationary phase*	*Solvent system*	*Flow-rate*	*Detection*
HPLC	Chemcosorb 3C8 (Chemco, Japan)	Methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:8) (pH 3.0)	1 ml/min	350 nm
HPTLC–densitometry[13]	Silica gel HPTLC (E. Merck, 5641)	Chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer)	–	360 nm
HPTLC–spray reagents[15]	Silica gel HPTLC (E. Merck, 5641)	Chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer)	–	1% Fast Violet B
RP-TLC–densitometry	C_8 TLC (E. Merck, 15424)	Methanol–acetonitrile–0.5 *M* oxalic acid (1:1:4) (pH 3.0)	–	360 nm
RP-TLC–spray reagents	C_8 TLC (E. Merck, 15424)	Methanol–acetonitrile–0.5 *M* oxalic acid (1:1:4) (pH 3.0)	–	0.5% Fast Violet B and pyridine



Compound	R_1	R_2	R_3	R_4	R_5
Tetracycline (TC)	H	OH	CH_3	H	H
Oxytetracycline (OTC)	H	OH	CH_3	OH	H
Chlortetracycline (CTC)	Cl	OH	CH_3	H	H
Doxycycline (DC)	H	H	CH_3	OH	H
Minocycline (MINO)	$N(CH_3)_2$	H	H	H	H
Methacycline (MTC)	H	$=CH_2$		OH	H
Demeclocycline (DMCTC)	Cl	OH	H	H	H
Rolitetracycline (PRMTC)	H	OH	CH_3	H	*

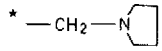


Fig. 1. Structure of tetracyclines.

the analysis of the eight TCs using the modified optimal conditions given in Table II. Various experimental results are detailed below.

Separation of tetracyclines

Thin-layer chromatography. As shown in Fig. 2, good separations among the eight TCs on HPTLC and RP-TLC plates were achieved using the modified optimal conditions listed in Table II. For RP-TLC, although we attempted to apply the con-

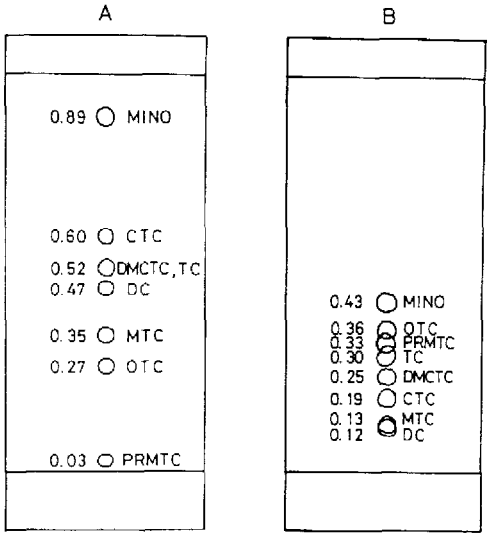


Fig. 2. Separation of tetracyclines by TLC. (A) Pre-developed silica gel HPTLC plate with saturated Na_2EDTA and then activated for 2 h at 130°C. Solvent system: chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer). (B) C_8 TLC plate. Solvent system: methanol–acetonitrile–0.5 *M* oxalic acid (1:1:4) (pH 3.0).

ditions shown in Table I in the present study, the separations between doxycycline and methacycline and between tetracycline and demeclocycline were not sufficient for their identification. Therefore, various combinations of organic solvent (methanol–acetonitrile, 1:1) and 0.5 *M* oxalic acid solution (pH 2.0) were investigated, however, we could not obtain satisfactory results. As the separation of TCs in RP-TLC depends on the pH of the solvent system[14], the effect of pH (adjusted with 28% aqueous ammonia) on the separation of the eight TCs was examined using methanol–acetonitrile–0.5 *M* oxalic acid solution (1:1:4). The separation between tetracycline and demeclocycline improved with increasing pH of the solvent system, but no particular change was observed in the separation between doxycycline and methacycline. The best resolution among most of the TCs was obtained at pH 3.0. Therefore, we conclude that the optimal solvent system in RP-TLC is methanol–acetonitrile–0.5 *M* oxalic acid solution (1:1:4) (pH 3.0) and that good separations of the eight TCs, except for doxycycline and methacycline, are possible using RP-TLC. The previously reported HPTLC method[13] gave good results except for the separation between tetracycline and demeclocycline (Fig. 2). Consequently, we used the same conditions as in Table I for the present HPTLC work.

As described above, when employing the conditions listed in Table II, good separations among minocycline, oxytetracycline, rolitetracycline, tetracycline, demeclocycline and chlortetracycline and among minocycline, chlortetracycline, doxycycline, methacycline, oxytetracycline and rolitetracycline were obtained using RP-TLC and HPTLC, respectively. Therefore, combination of RP-TLC and HPTLC permits the identification of the eight TCs.

High-performance liquid chromatography. When the modified HPLC conditions summarized in Table II were used, a good separation among the eight TCs was achieved, as shown in Fig. 3. However, it was not possible to identify rolitetracycline on the chromatogram using the usual solvents for sample preparation such as meth-

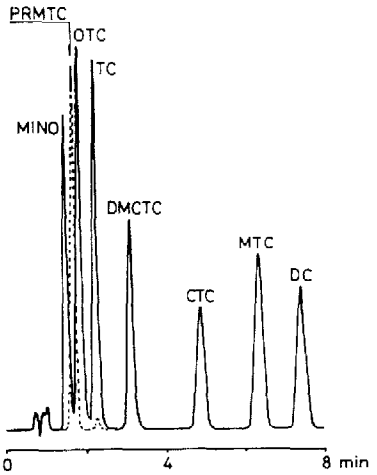


Fig. 3. Separation of tetracyclines by HPLC. Column: Chemcosorb 3C8 (3 μm, 75 × 4.6 mm I.D.). Mobile phase: methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:8) (pH 3.0). Flow-rate, 1 ml/min; detection, 350 nm. ——, Chromatogram of seven tetracyclines; – – –, chromatogram of rolitetracycline freshly prepared using the mobile phase as solvent.

anol and water, because rolitetracycline decomposed rapidly to give tetracycline. The identification of rolitetracycline itself is described under *Determination of rolitetracycline*. Hence the chromatogram of rolitetracycline is shown as a simple approach by a dotted line.

In previous work[12] we found that variation of the ratio of organic solvents and oxalic acid solution (pH 2.0) in the mobile phase permits good separations among oxytetracycline, tetracycline, chlortetracycline and doxycycline on C_8 packing materials from different suppliers. In this work, the optimal ratio was examined on a short C_8 column (Chemcosorb 3C8, 3 μm, 75 × 4.6 mm I.D.) to obtain a high sensitivity and to shorten the analytical time, and the use of methanol–acetonitrile–0.01 *M* oxalic acid solution (1:1.5:7) gave suitable capacity and resolution factors for most TCs except for minocycline. However, minocycline was not retained on the column using this mobile phase.

De Leenheer and Nelis[16] reported that minocycline is one of the most lipophilic TCs at neutral pH, but its capacity factor (k') at lower pH is too small. We considered that this fact was caused by the R_1 (dimethylamino) group in minocycline and that k' was a function of the pH of the mobile phase. Therefore, the influence of the pH of mobile phase on k' was investigated using methanol–acetonitrile–0.01 *M* oxalic acid solution (1:1.5:7). As shown in Fig. 4, the k' value of minocycline increased with increasing pH of the mobile phase and was suitable for its analysis above pH 3.0. As good separations among the eight TCs were obtained at pH 3.0, we chose methanol–acetonitrile–0.01 *M* oxalic acid solution (1:1.5:7) (pH 3.0) as the mobile phase.

As mentioned above, the simultaneous identification of the eight TCs by HPLC using an oxalic acid-containing mobile phase adjusted to pH 3.0 on Chemcosorb 3C8 is possible.

Determination of tetracyclines

UV detection. The determination of TCs was achieved using a densitometer and UV detector for TLC and HPLC, respectively. For TLC, the densitometric profiles of the TCs on HPTLC and RP-TLC plates which were measured under the conditions listed in Table I are shown in Fig. 5. Each spot on the TLC plate was

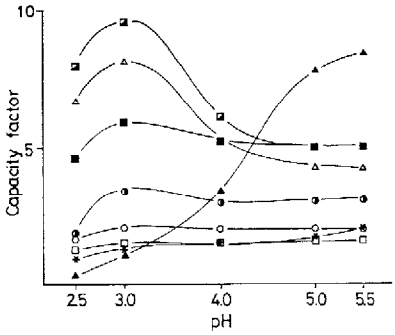


Fig. 4. Influence of the pH of the mobile phase on the capacity factor. Column: Chemcosorb 3C8 (3 μm,. 75 × 4.6 mm I.D.). Mobile phase: methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:8). ○, Tetracycline; □, oxytetracycline; ■, chlortetracycline; ◪, doxycycline; ▲, minocycline; △, methacycline; ◑, demeclocycline; ∗, rolitetracycline.

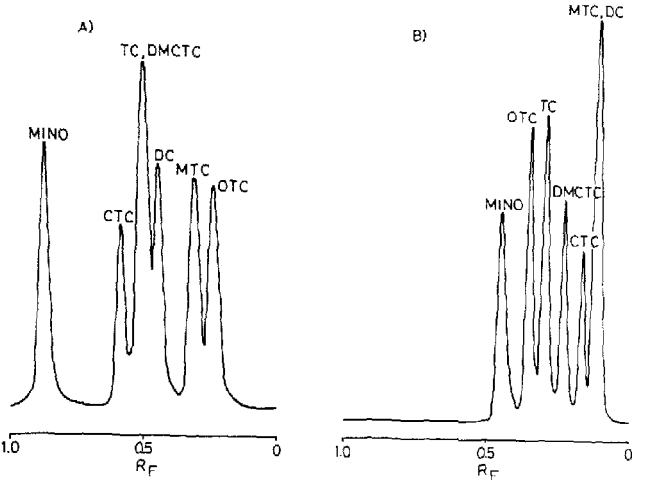


Fig. 5. Typical densitometric profiles of tetracyclines on (A) HPTLC and (B) RP-TLC plates. Measurement wavelength: 350 nm. For chromatographic conditions, see Fig. 2.

detected as a sharp peak and the calibration graphs prepared by the peak-height method showed linear relationships between 0.1 and 1.0 μg, except for rolitetracycline (HPTLC: minocycline, $y = 4.587x+0.412$, $r = 0.9998$; methacycline, $y = 4.250x+0.300$, $r = 0.9996$; oxytetracycline, $y = 4.134x+0.409$, $r = 0.9996$; tetracycline, $y = 3.991x+0.268$, $r = 0.9998$; chlortetracycline, $y = 3.025x+0.325$, $r = 0.9998$; doxycycline, $y = 2.787x+0.325$, $r = 0.9997$; demeclocycline, $y=2.663x+0.337$, $r = 0.9998$. RP-TLC: tetracycline, $y = 8.750x+0.250$, $r = 0.9998$, oxytetracycline, $y = 8.375x+0.225$, $r = 0.9997$; methacycline, $y = 7.500x+0.200$, $r = 0.9999$, doxycycline, $y = 6.875x+0.025$, $r = 0.9999$; demeclocycline, $y = 6.625x-0.125$, $r = 0.9999$; minocycline, $y = 6.125x-0.125$, $r = 0.9996$; chlortetracycline, $y = 5.250x-0.250$, $r = 0.9998$). It is well known that rolitetracycline is rapidly hydrolysed in aqueous solution to give TC and that the hydrolysis occurs even during TLC development[17–19]. The degradation of rolitetracycline was also found in this TLC work, and therefore it is difficult to determine rolitetracycline directly on TLC plates. However, as described in the next section, it is probably possible to determine rolitetracycline as tetracycline after complete conversion.

For HPLC, the calibration graphs prepared by the peak-height method showed linear relationships between 1 and 10 ng, except for rolitetracycline (tetracycline, $y = 1.087x+1.425$, $r = 0.9999$; oxytetracycline, $y = 1.0687x+1.163$, $r = 0.9998$; minocycline, $y = 1.031x+0.037$, $r = 0.9998$; methacycline, $y = 0.506x+0.937$, $r = 0.9999$; demeclocycline, $y = 0.468x+0.863$, $r = 0.9999$; doxycycline, $y = 0.375x+0.600$, $r = 0.9999$; chlortetracycline, $y = 0.356x+0.337$, $r = 0.9999$). Although it is difficult to determine rolitetracycline directly using the usual solvents for sample preparation such as methanol and water for the same reasons as in TLC, we consider that it is possible to achieve a successful analysis by using suitable solvents for sample preparation and by converting rolitetracycline into tetracycline as described in the next section. The proposed method is about five times more sensitive

than the previous method[12], indicating that the use of a short column is effective for improving the sensitivity, as we expected.

Determination of rolitetracycline. In order to determine rolitetracycline by the proposed HPTLC, RP-TLC and HPLC methods, the stability of rolitetracycline in various solutions (methanol, acetonitrile, tetrahydrofuran, dimethyl sulphoxide, dioxane and the mobile phase) was examined using the HPLC method. It was more stable in dimethyl sulphoxide, tetrahydrofuran and the mobile phase than in methanol, acetonitrile and dioxane. Therefore, the linearity between peak-height and amount of freshly prepared solutions in dimethyl sulphoxide, tetrahydrofuran and the mobile phase was investigated. A linear relationship between 1 and 10 ng ($y = 1.009x + 1.165$, $r = 0.9998$) was obtained. Therefore, we considered that the simultaneous determination of rolitetracycline and tetracycline is possible using tetrahydrofuran, dimethyl sulphoxide and the mobile phase as sample solvents and that it is effective for the analysis of rolitetracycline in pharmaceutical preparations. However, if the proposed HPLC and TLC methods are applied to residue analysis, it is expected that rolitetracycline will be rapidly converted into tetracycline *in vivo* or during the analytical procedure[18]. Therefore, in practical applications it is useful to determine tetracycline as rolitetracycline after complete conversion. As the conversion of rolitetracycline to tetracycline is temperature dependent[18,19], the influence of temperature on the conversion in methanol was examined. After incubation of rolitetracycline in methanol for 0, 2, 5, 10 and 15 min at 25, 30, 40 and 50°C, these samples were injected into the HPLC system and then the peak-height ratios of rolitetracycline to tetracycline were calculated. As shown in Fig. 6, the conversion of rolitetracycline is affected significantly by temperature and rolitetracycline was completely converted to tetracycline after incubation for more than 5 min at 50°C. However, when the sample solution was incubated for more than 15 min at 50°C, 4-epitetracycline appeared on the chromatograms. Therefore, we prepared calibration graphs for rolitetracycline after conversion to tetracycline by incubating for 5 min in methanol at 50°C, and obtained linear relationships between 1 and 10 ng and between 0.1 and 1.0 μg using HPLC and both TLC methods, respectively (HPLC, $y =$

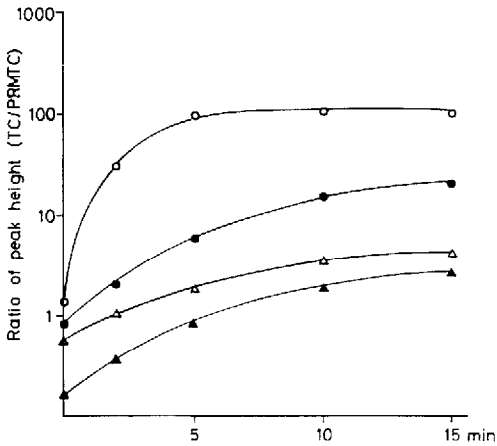


Fig. 6. Influence of temperature on conversion of rolitetracycline to tetracycline. ○, 50°C; ●, 40°C; △, 30°C; ▲, 25°C. For chromatographic conditions, see Fig. 3.

TABLE III

DETECTION LIMITS OF TETRACYCLINES USING HPTLC WITH DIFFERENT SPRAY REAGENTS

Plate: pre-developed silica gel HPTLC with saturated Na_2EDTA and then activated at 130°C for 2 h. Solvent system: chloroform–methanol–5% Na_2EDTA (65:20:5) (lower layer).

Spray reagent (aq.)	*Detection limit (μg)**								
	Oxytetracycline	*Tetracycline*	*Chlortetracycline*	*Doxycycline*	*Minocycline*	*Demeclocycline*	*Methacycline*	*Rolitetracycline*	*Background*
2.0% Fast Red ITR	0.1 (Y)	0.05(Or)	0.1 (Or)	0.1 (Y)	0.1(Y)	0.05(Y)	0.05(Or)	−(Y)	−(Y-Or)
1.0% Fast Red B	0.03(Or)	0.03(Or)	0.03(Or)	0.03(Y)	0.1(Or-Y)	0.05(Or)	0.05(Br)	−(Or-Y)	−(Y-Or)
0.5% Fast Blue BB	0.03(R)	0.05(R)	0.05(R)	0.03(R)	0.1(Br)	0.03(R-V)	0.03(R-V)	−(R-V)	−(Y-Or)
1.0% Fast Violet B	0.03(R)	0.03(R)	0.03(R)	0.03(R)	0.1(Or)	0.03(R)	0.03(R)	−(R-V)	−(W)
0.2% Fast Blue B	0.03(R)	0.05(R)	0.05(R)	0.05(R)	0.1(R)	0.03(R-V)	0.03(R-V)	−(R-V)	−(Y-Or)

* Colours in parentheses: Y = yellow; Or = orange; R = red; V = violet; Br = brown; W = white.

TABLE IV

DETECTION LIMITS OF TETRACYCLINES USING RP-TLC WITH DIFFERENT SPRAY REAGENTS

Plate: C_8 TLC. Solvent system: methanol–acetonitrile–0.5 *M* oxalic acid solution (pH 3.0) (1:1:4).

Spray reagent (aq.)	*Detection limit (μg)**								
	Oxytetracycline	*Tetracycline*	*Chlortetracycline*	*Doxycycline*	*Minocycline*	*Demeclocycline*	*Methacycline*	*Rolitetracycline*	*Background*
0.5% Fast Red ITR	0.05(Y)	0.05(Or)	0.05(Or-Y)	0.05(Y)	0.1(Y)	0.05(Y)	0.05(Y)	−(Or)	−(Y-Or)
0.5% Fast Red B	0.05(Or-Y)	0.05(Or-Y)	0.05(Y)	0.05(Y)	0.1(Or-Y)	0.05(Or-Y)	0.03(Br)	−(Or-Y)	−(Y-Or)
0.5% Fast Blue BB	0.03(R-V)	0.03(B-V)	0.03(R-V)	0.03(R)	0.1(Br)	0.03(V)	0.03(V-R)	−(V)	−(Y-Or)
0.5% Fast Violet B	0.03(R)	0.03(R-V)	0.03(R)	0.03(R-V)	0.1(Or)	0.03(R-V)	0.03(R-V)	−(R)	−(W)
0.5% Fast Blue B	0.03(R)	0.03(R-V)	0.03(R-V)	0.03(R)	0.1(R-V)	0.03(R-V)	0.03(V)	−(R-V)	−(Y-Or)

* Colours in parentheses: Y = yellow; Or = orange; R = red; V = violet; Br = brown; B = blue, W = white.

$0.887x+0.225$, $r = 0.9999$; HPTLC, $y = 2.678x+0.716$, $r = 0.9999$; RP-TLC, $y = 6.713x+0.638$, $r = 0.9999$). We consider that this conversion method is very effective for residue analysis of rolitetracycline.

Detection reagents. We have previously established semi-quantitative methods on TLC plates using diazonium detection reagents for oxytetracycline, tetracycline, chlortetracycline and doxycycline[15]. These methods are very useful for screening purposes without special instrumentation, so we attempted to apply them to the eight TCs. As shown in Tables III and IV, five diazonium detection reagents gave variously coloured spots with the eight TCs and most of the TCs gave good sensitivities (30–50 ng), but the detection limit of minocycline was not so good (100 ng).

Because the colour intensities of the spots were related to the amount of TCs present, we conclude that these detection reagents are suitable for the semi-quantitative analysis of the eight TCs.

CONCLUSION

Analytical methods for eight TCs were established using silica gel HPTLC, RP-TLC and HPLC. The optimal conditions obtained are summarized in Table II. Good separations among minocycline, oxytetracycline, rolitetracycline, tetracycline, demeclocycline and chlortetracycline and among minocycline, chlortetracycline, doxycycline, methacycline, oxytetracycline and rolitetracycline were obtained on silica gel HPTLC and RP-TLC plates, respectively. A combination of HPTLC and RP-TLC made possible the identification of the eight TCs. Each calibration graph was linear between 0.1 and 1.0 μg using UV densitometry, except for rolitetracycline. As detection reagents, diazonium salts including Fast Violet B gave variously coloured spots with the eight TCs and good sensitivities were obtained except for minocycline. With respect to HPLC, the simultaneous analysis of the eight TCs was possible on a C_8 column using methanol–acetonitrile–0.01 *M* oxalic acid solution (1:1.5:7) at pH 3.0 as the mobile phase. A linear relationship was obtained between 1.0 and 10 ng using the usual sample preparation, except for rolitetracycline. The direct determination of rolitetracycline was possible using tetrahydrofuran, dimethyl sulphoxide and the mobile phase as sample solvents. For the determination of residual rolitetracycline, it was effective to measure the amount of rolitetracycline as tetracycline by HPLC, HPTLC and RP-TLC after conversion of rolitetracycline to tetracycline by incubating for 5 min in methanol at 50°C.

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