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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

III*. SIMPLE METHOD FOR THE ANALYSIS OF TETRACYCLINES ON REVERSED-PHASE THIN-LAYER PLATES

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SUMMARY

A technique for the determination of tetracyclines using reversed-phase thin-layer chromatographic (TLC) plates is described. A solvent system containing 0.5 *M* aqueous oxalic acid solution makes possible the determination of tetracyclines on an untreated reversed-phase TLC plate. The separation is dependent on the pH of the aqueous acidic solution in the solvent system, the optimum pH being 2.0. The combinations of a C_8 TLC plate with methanol–acetonitrile–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:4) as the solvent system, and a C_{18} TLC plate with methanol–acetonitrile–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:2) as the solvent system gave satisfactory results for parent tetracyclines and impurities in tetracycline, respectively. Depending on the purpose, good separations among tetracyclines can be obtained by changing the TLC system.

INTRODUCTION

Tetracycline antibiotics (TCs) continue to play an important role in human and veterinary medicine and in animal nutrition. Because of their great practical importance, TCs have been the subject of many biological, biochemical and analytical studies, and a simple, rapid and reliable method for the determination of TCs has been particularly required. However, the property of TCs to form chelate complexes with metallic ions[1,2] complicates their analysis. The separation and determination of TCs have been achieved using spectrophotometry[3,4], gas chromatography (GC)[5], high-performance liquid chromatography (HPLC)[6–11] and thin-layer chromatography (TLC)[12–18]. As TLC is simple and does not require special equipment, it has been used by a number of workers to separate and characterize certain TCs using an

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adsorbent layer of Kieselguhr[12,13], silica gel[14–16] and microcrystalline cellulose[17,18], but most of the published methods are very complicated.

In previous work[19,20], we established a technique for the determination of TCs using silica gel high-performance thin-layer chromatography (HPTLC) followed by densitometry. We succeeded in measuring the concentration of TCs in fish tissues by this method. However, in order to avoid the formation of chelate complexes of TCs, pre-development with saturated aqueous disodium ethylenediaminetetraacetate (Na_2EDTA) solution and activation before applying samples on to the silica gel HPTLC plate was necessary. Therefore, we wished to achieve the precise separation and determination of TCs without the pre-development step.

Because octyl (C_8) and octadecyl (C_{18}) reversed-phase (RP) systems have been applied successfully to determine TCs by HPLC[6–11], we decided to use pre-coated RP-TLC plates. In this paper, we report a technique for the determination of TCs using RP-TLC plate.

EXPERIMENTAL

Materials

Methanol (CH_3OH), acetonitrile (CH_3CN), isopropanol (i-PrOH), tetrahydrofuran (THF), ethanol (C_2H_5OH), dioxane, aqueous ammonia, phosphoric acid, citric acid, tartaric acid, oxalic acid, ethylenediaminetetraacetic acid and malonic acid were analytical-reagent grade chemicals.

TLC plates pre-coated with C_8- and C_{18}-modified silica gel (E. Merck 15424 and 15423, respectively) were used.

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 method of Simmon *et al.*[21] and McCormick *et al.*[22].

Preparation of tetracycline solutions

Each tetracycline (about 100 mg) was weighed accurately into a 10-ml volumetric flask and diluted to volume with methanol. Further dilution was sometimes necessary prior to application.

A 1-μl volume of the refference solution was applied on the TLC plate using a microsyringe.

Solvent systems

The developing solvents were made from CH_3OH, CH_3CN and various aqueous acidic solutions (phosphoric, citric, ethylenediaminetetraacetic, tartaric, oxalic and malonic acids). The pH of the solutions was adjusted with 28% aqueous ammonia.

Densitometry

The developed TLC plate was placed under a Shimadzu CS-910 chromatogram scanner, and the spots of the components were determined by UV absorption spectrophotometry. The operating conditions were as follows: instrument in dual-wave-

length mode; λ_{sample} = 360 nm and $\lambda_{reference}$ = 600 nm for TC, OTC, CTC, DC and ETC; λ_{sample} = 425 nm and $\lambda_{reference}$ = 650 nm for ATC and EATC; linear scanning in reflection mode, size of scanning beam 0.25 × 9.0 mm; working curve linearizer, LIN SX = 3 program; background correction, on.

RESULTS AND DISCUSSION

Separation of tetracyclines

As described previously[19], it is not always necessary to differentiate among all seven TCs. For example, when an analytical method is applied in residue or biological work it is necessary to separate completely TC, OTC, CTC and DC (group I), and for the determination of impurities in TC drugs a good separation of TC, CTC, ETC, ATC and EATC (group II) is needed. Therefore, groups I and II were treated separately in subsequent experiments.

As a result of various experiments, we achieved the precise separation of TCs on RP-TLC plates without any pre-development. Typical separations of TCs obtained using optimal conditions are illustrated in Fig. 1. For group I, a satisfactory result is obtained on a pre-coated C_8 TLC plate using CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:4) (solvent system A). A good differentiation among group II is obtained by development with CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:2) (solvent system B) on a pre-coated C_{18} TLC plate. When a precise separation between ETC and TC is needed, it can be improved using solvent system A on a pre-coated C_{18} TLC plate.

Various parameters were examined in order to obtain optimal conditions for the separation of TCs, and the results are reported below.

Addition of acid. TCs showed extreme tailing on RP-TLC plates using aqueous solutions of CH_3OH, CH_3CN, THF, i-PrOH, C_2H_5OH or dioxane as solvent systems. In several HPLC studies, the use of mobile phases containing various acids

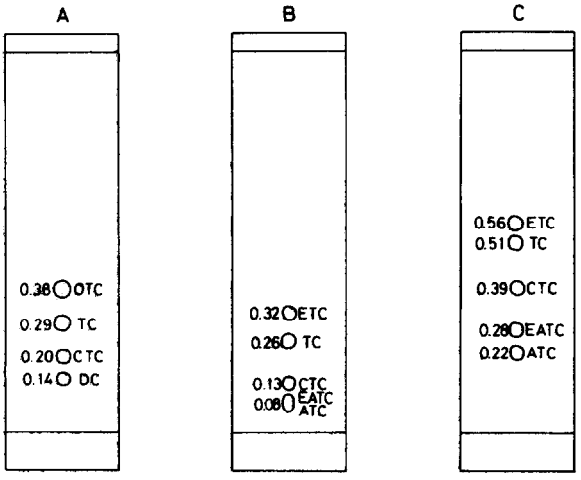

Fig. 1. Separation of tetracyclines on RP-TLC plates. (A) Group I on a C_8 TLC plate using solvent system A; (B) group II on a C_{18} TLC plate using solvent system A; (C) group II on a C_{18} TLC plate using solvent system B.

(phosphoric[6,7], citric[8,9], tartaric[9], ethylenediaminetetraacetic[10,11] and malonic acids[9]) gave good results for the determination of TCs. However, when we attempted to apply these acids in our study, we obtained tailing spots with all TCs. When TCs are purified from culture broth, oxalate is usually added to prevent the formation of chelate complexes of TCs[2,3]. Therefore, we used CH_3OH–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1) as a solvent system on C_{18} TLC plates. In spite of giving imperfect separations among the TCs, no tailing was exhibited. Consequently, we concluded that only aqueous oxalic acid solution is effective in preventing tailing on RP-TLC plates.

Using CH_3OH–aqueous oxalic acid solution (pH 2.0) (1:1) as the solvent, the influence of the acid concentration was examined. The resolution of the spots increased on increasing the acid concentration, so that good resolution was obtained above 0.3 *M*. Because oxalic acid was deposited on drying the plate when acid concentrations above 0.6 *M* were used we chose 0.5 *M* aqueous oxalic acid solution for subsequent work.

Comparison of C_8 and C_{18} TLC plates. We tested C_8 and C_{18} TLC plates using CH_3OH–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1) as a solvent system. Although poor resolution was obtained with group II using C_8 TLC plates, good resolution was obtained with group I. On the other hand, good resolution was achieved with group II using C_{18} TLC plates, but it was unsatisfactory for group I. Therefore, in subsequent experiments, C_8 and C_{18} TLC plates were used for groups I and II, respectively.

Combination of methanol and acetonitrile. As imperfect separations were obtained on development with CH_3OH–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1) and CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1), various combinations of CH_3OH and CH_3CN were examined in the system (CH_3OH–CH_3CN)–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1). As shown in Fig. 2, 2:1 and 1:1 mixtures of CH_3OH and CH_3CN gave good separations among group I. For group II, satisfactory results were obtained using 1:1 and 1:2 mixtures of CH_3OH

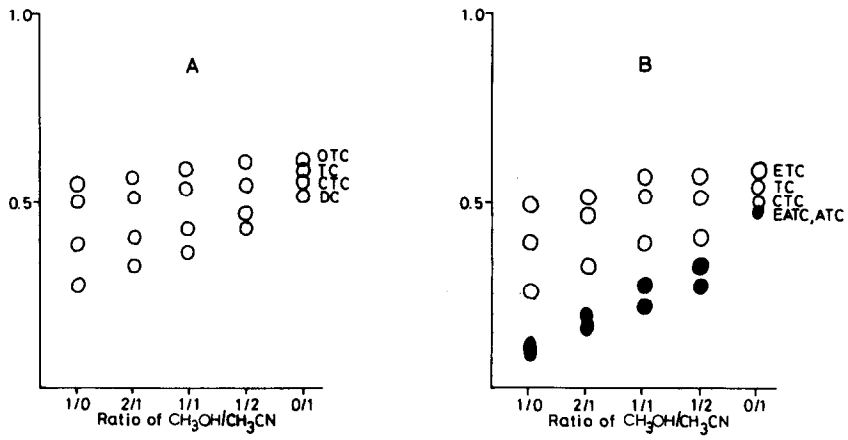

Fig. 2. Effect of ratio of CH_3OH and CH_3CN on R_F values. (A) Group I on a C_8 TLC plate; (B) group II on a C_{18} TLC plate. Solvent system: (CH_3OH–CH_3CN)–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1).

and CH_3CN. Because it is more convenient to use the same solvent composition for both groups I and II, the 1:1 mixture was employed in subsequent work.

Adjustment of pH of aqueous oxalic acid solution. The influence of the pH of the aqueous oxalic acid solution (adjusted with 28% aqueous ammonia) in the solvent systems on the R_F values was investigated. When CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (1:1:2) was used all of the TCs showed tailing at pH 1.2, as shown in Fig. 3. above pH 3.0, EATC and ATC showed considerable tailing and the separations between CTC and DC and between ETC and TC were unsatisfactory. CTC and DC were overlapping at pH 5.0. As a precise separation and good resolution among groups I and II were obtained at pH 2.0, we conclude that the optimum pH of 0.5 *M* aqueous oxalic acid solution is 2.0.

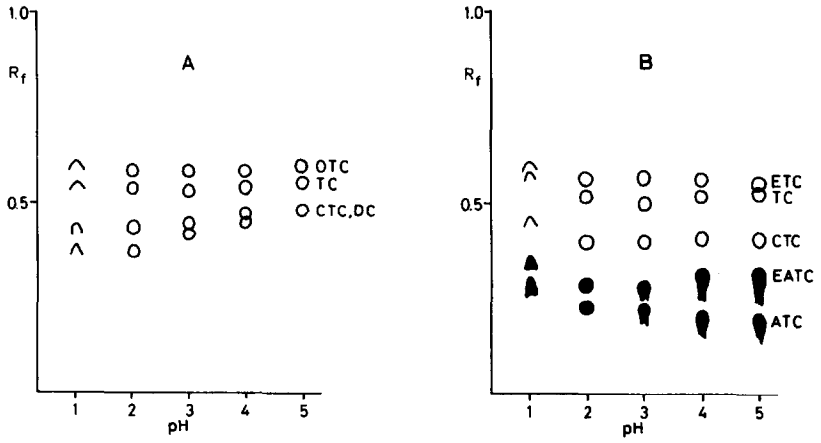

Fig. 3. Influence of pH of 0.5 *M* aqueous oxalic acid solution on R_F values. (A) Group I on a C_8 TLC plate; (B) group II on a C_{18} TLC plate. Solvent system: CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (1:1:2).

Combination of organic solvents and aqueous oxalic acid solution. Various combinations of the organic solvents (CH_3OH:CH_3CN = 1:1) and the acidic solution (0.5 *M* aqueous oxalic acid solution, pH 2.0) were tested to choose suitable solvent systems for use with RP-TLC plates. As shown in Fig. 4, for group I an unsatisfactory separation between OTC and TC was obtained using CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:1). When CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:6) was used, the separation between CTC and DC was unsatisfactory. A precise separation among group I was obtained on C_8 TLC plates using CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:4) (solvent system A). For group II, EATC and ATC overlapped when solvent system A was used, but a precise separation between ETC and TC was obtained. Using CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:2) (solvent system B), all of group II were well separated on C_{18} TLC plates. Therefore, group I can be separated with solvent system A on C_8 TLC plates and group II can be separated with solvent system B on C_{18} TLC plates.

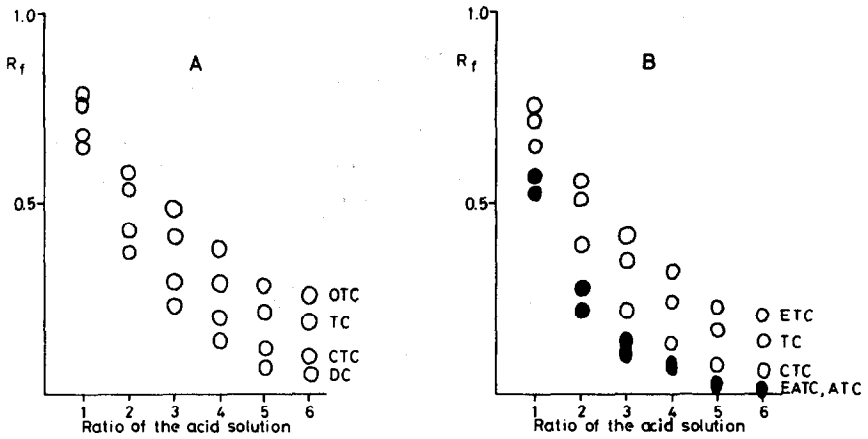

Fig. 4. Effect of ratio of organic solvents and 0.5 *M* aqueous oxalic acid solution. (A) Group I on a C_8 TLC plate; (B) group II on a C_{18} TLC plate. Solvent system: CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:*x*).

Densitometry

After development and drying, the absorbance value for each spot is recorded with a densitometer under the conditions described under Experimental. These conditions are same as those used previously[19] except for the measurement wavelength for EATC and ATC. As CTC interfered slightly with EATC at 425 nm, which is its absorption maximum, the measurement wavelength was set at 450 nm for EATC and ATC on silica gel HPTLC plates[19]. As we can separate CTC and EATC completely, the measurement wavelength for EATC and ATC was set at 425 nm.

Fig. 5 shows densitometric profiles for TCs (1 μg each) on pre-coated C_8 and C_{18} TLC plates using solvent systems A and B. The chromatogram of group I shows

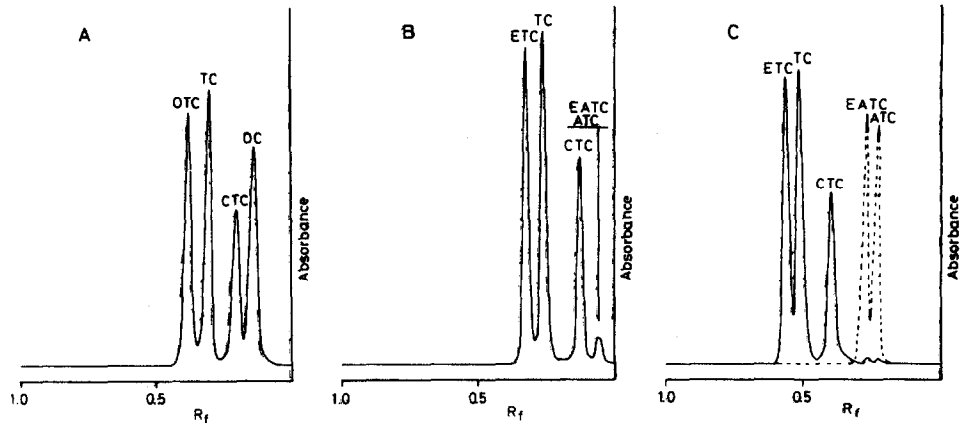

Fig. 5. Densitometric profiles of tetracyclines on RP-TLC plates. (A) Group I on a C_8 TLC plate using solvent system A; (B) group II on a C_{18} TLC plate using solvent system A; (C) group II on a C_{18} TLC plate using solvent system B; Measurement wavelengths: ——, λ_s = 360 nm; – – –, λ_s = 425 nm.

a good separation on C_8 TLC plates using solvent system A. The resolution, R_s, between the OTC and TC and the CTC and DC peaks are 1.29 and 1.14, respectively [$R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_2 and t_1 are R_F values and w_1 and w_2 are the corresponding peak widths at the baseline].

In group II, they can be determined on C_{18} TLC plates using solvent system B and then the R_s between ETC and TC and between ATC and EATC are 1.03 and 1.06, respectively. Further, as the R_s between ETC and TC is 1.27 on C_{18} TLC plates using solvent system A, the use of this solvent system is effective for the determination of ETC and TC. We consider this resolution allows an acceptable level of precision in these analyses, and we recommend the following TLC systems for the separation of TCs: group I, solvent system A on C_{18} TLC plates; group II, solvent system B on C_{18} TLC plates. When it is necessary to separate completely TC and ETC, solvent system A on C_{18} TLC plates should be used. Depending on the purpose, good results can be obtained by changing the TLC system.

Calibration graphs

In previous work[19], we employed a linearizer programmed according to the Kubelka–Munk equation. After various amounts of TCs have been spotted on a plate and developed, the absorbance value of each spot is recorded with a densitometer. Fig. 6 shows typical results, with a linear relationship between 0.1 and 1.0 μg.

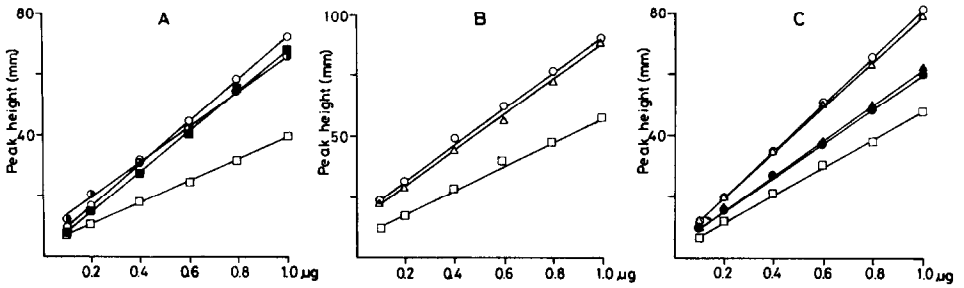

Fig. 6. Calibration graphs for tetracyclines. (A) C_8 TLC plate using solvent system A; (B) C_{18} TLC plate using solvent system A; (C) C_{18} TLC plate using solvent system B; ○, TC; ■, OTC; □, CTC; ◪, DC; △, ETC; ●, ATC; ▲, EATC.

CONCLUSION

A technique for the determination TCs using reversed-phase TLC plates has been established, with the following characteristics.

A solvent system containing oxalic acid makes possible the determination of TCs on untreated RP-TLC plates. The separation of TCs is dependent on the pH of the aqueous oxalic acid solution in the solvent system and the optimal pH is 2.0. The resolution of the spots increases with increasing oxalic acid concentration and good resolution is obtained above 0.3 *M*. The combinations of a C_8 TLC plate with CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:4) (solvent system A) and a C_{18} TLC plate with CH_3OH–CH_3CN–0.5 *M* aqueous oxalic acid solution (pH 2.0) (1:1:2) (solvent system B) give satisfactory results for groups I and II, re-

spectively. Each calibration graph shows a linear relationship between 0.1 and 1.0 μg.

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