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IMPROVEMENT OF THE CHEMICAL ANALYSIS OF ANTIBIOTICS

I. SIMPLE METHOD FOR THE ANALYSIS OF TETRACYCLINES ON SILICA GEL HIGH-PERFORMANCE THIN-LAYER PLATES

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SUMMARY

A technique for determination of tetracyclines using silica gel high-performance thin-layer chromatography followed by densitometry is established. A predevelopment with saturated disodium ethylenediaminetetraacetate aqueous solution and the complementary use of three solvent systems enable a reliable identification. Direct determination using densitometry without further treatment with any reagents is of high sensitivity and reproducibility. A change of measurement wavelength enables the determination of overlapping spots.

INTRODUCTION

Pharmaceutical preparations of tetracycline (TC) contain small quantities of related compounds as impurities, the most of which are 4-epianhydrotetracycline (EATC), anhydrotetracycline (ATC), 4-epitetracycline (ETC) and chlortetracycline (CTC). The permitted concentrations of these impurities are listed in the Federal Register[1] for TC dosage forms marketed in the United States and in the British Pharmacopoeia[2] and European Pharmacopoeia[3] for European markets. EATC has been identified as a cause of Fanconi-type syndrome[4–7]. CTC is a more active antibiotic than TC, and therefore reliance only on tests for microbiological potency for TC may give rise to misleading results if significant amounts of CTC are present. ETC is virtually inactive as an antibiotic. ATC is also inactive, but may undergo epimerization, under storage conditions, leading to the formation of EATC. However, no limits for these compounds are laid down in the Japanese Pharmacopoeia.

Antibiotics are also used in modern agricultural practice, being added to feed and water to increase weight gain and feed conversion efficiency and to prevent disease, and are sprayed on agricultural products as bactericides. Such usage may lead to problems with residues in agricultural products and environmental pollution.

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The regulation of these areas is insufficient in Japan, particularly for fish breeding. Therefore, in this study we have borne in mind the possible occurrence of such problems with regard to tetracyclines (TCs) which play an important rôle in human and veterinary medicine and animal nutrition.

The separation and determination of TCs have been achieved with spectrophotometry[8,9], gas chromatography (GC)[10], high-performance liquid chromatography (HPLC)[11–15] and thin-layer chromatography (TLC)[16–23]. Spectrophotometric methods are insensitive, and interference from other materials cannot always be excluded. A GC method requires prior formation of the trimethylsilyl derivative under carefully controlled conditions. Although many HPLC methods have been reported, most use solvent gradient systems, with consequent difficulties in reproducibility. TLC methods have been used by a number of workers to separate and characterize certain TCs using adsorbent layers of Kieselguhr[16,17], silica gel[18–20] and microcrystalline cellulose[21–23]. In general, TLC is simple and does not require special equipment, but most of the published methods suffer from the excessive time needed for preparation and development. Nishimoto's method[18] using silica gel adsorbents is not suitable for the separation of TC and ETC. Kapadia and Rao[19] could not determine impurities in TCs. Finally, we obtained poor resolution among TC, oxytetracycline (OTC) and CTC under the conditions of Joshi *et al.*[20].

This paper describes a simple and rapid method for analysis of TCs using high-performance thin-layer chromatography (HPTLC) on precoated silica gel followed by densitometry without further treatment with any reagents.

EXPERIMENTAL

Materials

Methanol, chloroform, isopropanol, ethyl acetate, acetone and disodium ethylenediaminetetraacetate (Na_2EDTA) were analytical grade reagents.

Tetracycline, oxytetracycline, chlortetracycline and doxycycline (DC), as their hydrochlorides, were supplied by Pfizer Taito Co., Ltd. 4-Epitetracycline, anhydrotetracycline and 4-epitetracycline, as their hydrochlorides, were prepared according to the methods of Simmon *et al.*[24] and McCormick *et al.*[25].

Treatment of HPTLC plates

A precoated silica gel plate (E. Merck 5641) was predeveloped with saturated Na_2EDTA aqueous solution, dried in air at room temperature for 1 h and then activated at 130°C for 2 h.

Preparation of tetracycline solutions

Each tetracycline (about 100 mg) was weighed accurately into a 10-ml volumetric flask, and diluted to volume in methanol.

One hundred nanolitres of the reference solution were applied on the silica gel HPTLC plate using a microsyringe. After air drying, the spots can be detected by exposure to UV light (365 nm).

Solvent systems

The organic phases of the following mixtures were used: A, chloroform–methanol–5% Na_2EDTA aqueous solution (65:20:5), lower layer; B, isopropanol–

ethyl acetate–5% Na_2EDTA aqueous solution (3:4:7), upper layer; C, acetone–5% Na_2EDTA aqueous solution (10:1).

Densitometry

The developed HPTLC plate was placed under a Shimadzu CS-910 chromatogram scanner, and the spots of the components determined by UV absorption spectrophotometry. Operating conditions: instrument in dual-wavelength mode; wavelengths λ_{sample} = 360 nm, $\lambda_{reference}$ = 600 nm (TC, OTC, CTC, DC, ETC), λ_{sample} = 450 nm, $\lambda_{reference}$ = 650 nm (ATC, 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

TCs show tailing on the usual silica gel layers and this is considered to be due to chelation of TCs with trace metals in the silica gel. In order to avoid this a plate is generally sprayed with Na_2EDTA aqueous solution and is then activated[26]. We attempted to apply this method in our study, however, we obtained poor resolution, especially for EATC. An improved method was therefore designed: the plate is predeveloped with a saturated Na_2EDTA aqueous solution and is activated before applying sample. Although the development is somewhat time-consuming, satisfactory resolution was achieved with this method.

n-Butanol has often been applied as a developing solvent to separate TCs on silica gel. Therefore, various solvent systems containing *n*-butanol or other organic solvents were investigated but were found to be unsatisfactory. As shown in Fig. 1, a good separation among TCs was achieved using solvent systems A–C. The development time of the chromatograms is about 30 min, which is shorter compared with other systems[23]. Solvent system A yields a better resolution for seven TCs than that of B and C, especially among TC, OTC, CTC and DC. In the case of solvent system B, although the spots of CTC and DC are overlapping, there is a good separation between CTC and EATC which weakly interact in solvent system A. With solvent system C a good separation of TC, CTC, ETC, ATC and EATC is obtained, but DC and TC are overlapping. However, it is not alway necessary to differentiate between all seven TCs. For example, when an analytical method is applied to residues 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.

Densitometry

After developing and drying, the absorbance value for each spot is recorded by a densitometer under the conditions described under Experimental. With this instrument we can choose a linear or zigzag scanning mode. As linear scanning is more sensitive by about two times and faster than zigzag scanning, we opted for the former. The curve linearizer is programmed according to the Kubelka–Munk equation. The absorbance maxima for TC, OTC, CTC, DC and ETC are near 360 nm, for ATC and

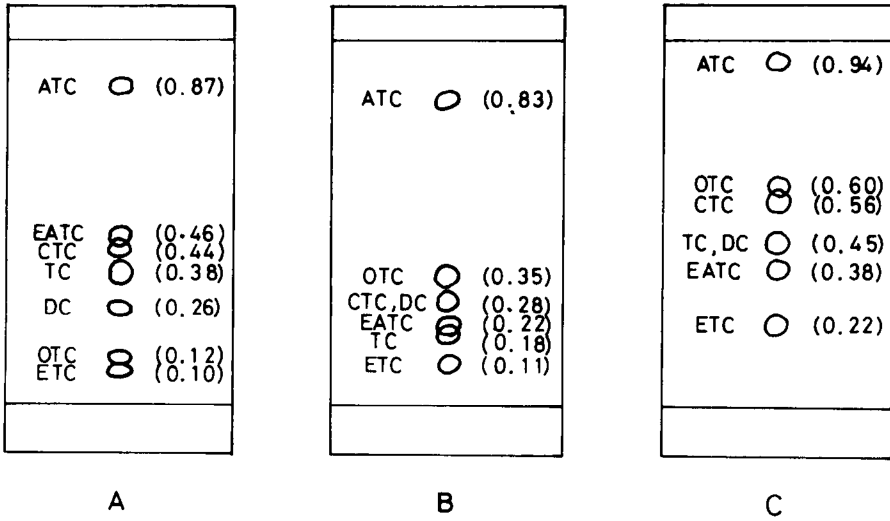

Fig. 1. Separation of tetracyclines on silica gel HPTLC plate predeveloped with saturated. Na_2EDTA aqueous solution and then activated for 2 h at 130°C. For solvent system, A–C see Experimental.

EATC, near 425 nm. Since CTC causes slight interference with EATC at 425 nm, the measurement wavelength is set at 450 nm for ATC and EATC.

Fig. 2 shows densitometric profiles for TCs (each 1 μg) on silica gel HPTLC using solvent system A. The chromatograms of group I show good separation. In group II, there is slight overlapping between CTC and EATC using this solvent

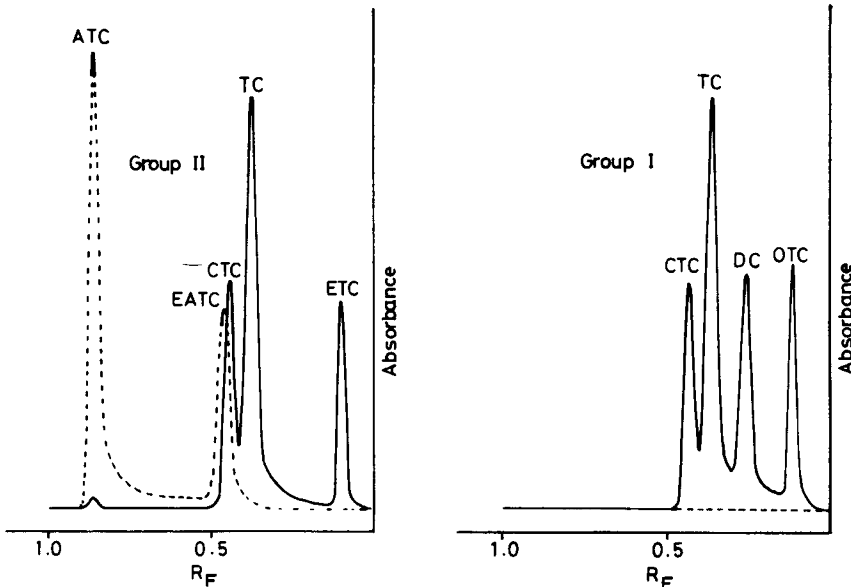

Fig. 2. Densitometric profiles of tetracyclines on silica gel HPTLC using solvent system A. Measurement wavelengths: ——— λ_s = 360 nm; ---, λ_s = 450 nm.

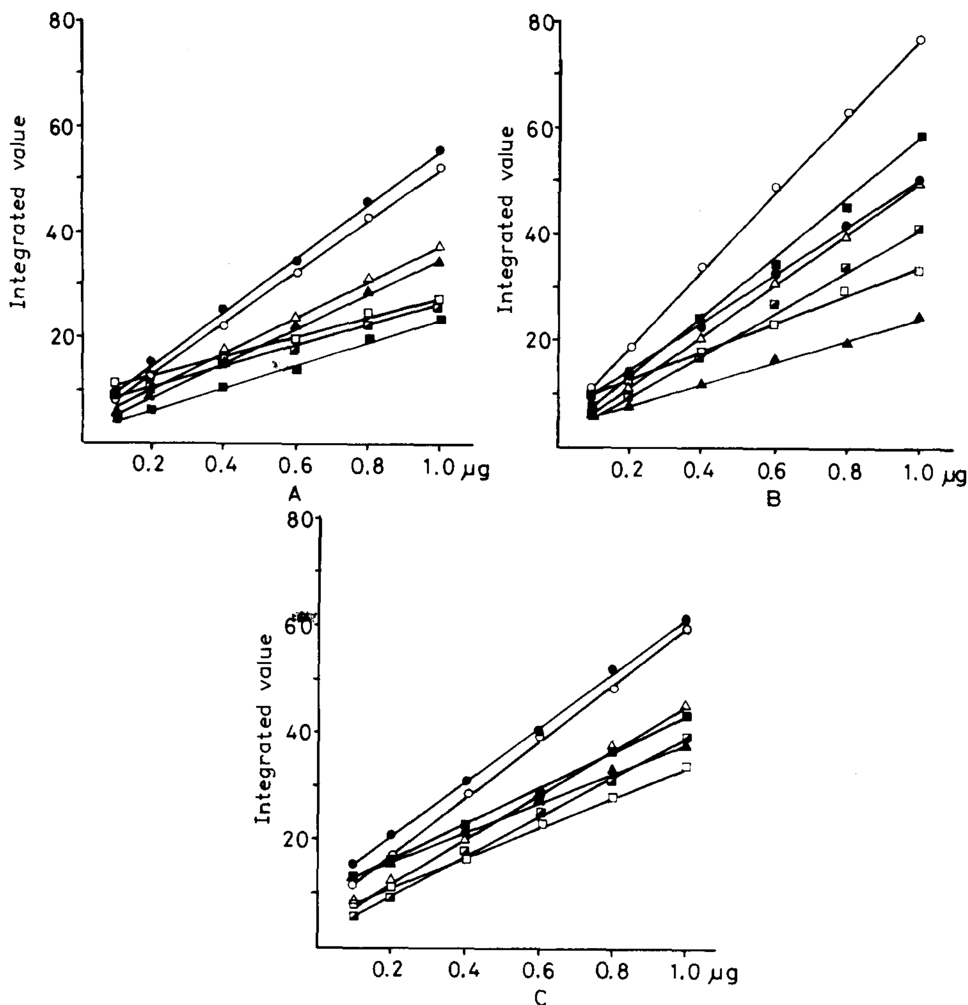

Fig. 3. Calibration curves for TC (○), OTC (■), CTC (□), DC (◪), ETC (△), ATC (●) and EATC (▲) using solvent systems A–C.

system, however, they can be determined by changing the measurement wavelength as described above.

In group I, CTC and DC cannot be distinguished with solvent system B, nor can OTC and CTC, TC and DC with solvent system C. In group II, CTC and EATC overlap in solvent system B, but can be determined by changing the wavelength. As shown in Fig. 1, solvent system C gives a good separation among group II.

Consequently, we recommend the following solvent systems for determination of TCs: group I, solvent system A; group II, solvent system C. In both cases, solvent system B can be subsidiarily used. The complementary use of the three solvent systems enables an identification for most purposes.

Calibration curves

As a general rule, UV-light scanning in densitometry yields a non-linear plot of absorbance against concentration[27]. Therefore, we employed a linearizer programmed according to the Kubelka–Munk equation. After various amounts of TCs are spotted on a plate and developed, the integrated absorbance value for each spot is recorded by a densitometer. Fig. 3 shows a typical result, with a linear relationship between 0.1 and 1.0 μg. This indicates that the present method is about two times more sensitive than other reported methods[23].

In conclusion, a technique for determination of TCs using HPTLC is established

(1) A predevelopment with a saturated Na_2EDTA aqueous solution makes possible the determination of TCs on precoated silica gel HPTLC plates.

(2) Appropriate developing solvents can be selected, solvent system A being very effective in separating TCs.

(3) Direct determination using densitometry without further treatment with any reagents is of high sensitivity and reproducibility.

(4) In the case of overlapping spots between TC and ATC determination can be achieved by a change in the wavelength of measurement.

(5) Each calibration curve shows a linear relationship between 0.1 and 1.0 μg.

Finally, we consider that HPTLC–densitometry can be applied to determine impurities in TC drugs, TCs in foods and biological samples. Further work will be conducted along these lines.

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