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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

VII*. COMPARISON OF ANALYTICAL METHODS FOR DETERMINATION OF IMPURITIES IN TETRACYCLINE PHARMACEUTICAL PREPARATIONS

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SUMMARY

In order to examine the suitability of our four established methods for the analysis of tetracyclines (high-performance liquid chromatography, reversed-phase thin-layer chromatographic densitometry, reversed-phase thin-layer chromatographic spray reagents and silica gel high-performance thin-layer chromatographic densitometry) for the determination of impurities in tetracycline pharmaceutical preparations, sixteen samples available on the Japanese market were analysed. Because these methods did not always give satisfactory results, their analytical conditions were modified. The comparative studies indicated these methods were accurate and reliable. Therefore, we recommend a combination of these methods for the analysis of impurities, in which the spray reagents are used first as a screening method, and then if the results exceed the limits, a precise determination is carried out using either high-performance liquid chromatography or reversed-phase thin-layer chromatographic densitometry.

INTRODUCTION

Tetracycline (TC) is extensively used in humans and animals for therapeutic and prophylactic purposes. Small amounts of impurities are contained in TC pharmaceutical preparations: most commonly, these are 4-epitetracycline (ETC), chlortetracycline (CTC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). EATC in particular possesses renal toxicity[1–3], so the permitted concentration of EATC is fixed by the Code of Federal Regulation (CFR)[4]. The British Pharmacopoeia (BP) sets limits for ETC, CTC, ATC and EATC in TC pharmaceutical

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preparations[5], but, the Japanese government does not. The CFR prescribes a spectrophotometric screening test for total anhydrotetracyclines in TC pharmaceutical preparations, and if the results exceed the limits, a determination of EATC using column chromatography followed by spectrophotometry is required. In the BP, these impurities are determined by thin-layer chromatography (TLC). Because column chromatography and the preparation of the necessary TLC plates are time-consuming and complicated, a simpler and more precise analytical method is described.

In previous reports, we have established four methods for the determination of TCs: (A) high-performance liquid chromatography (HPLC)[6]; (B) reversed-phase thin-layer chromatography (RP-TLC) followed by densitometry[7]; (C) RP-TLC followed by detection with spray reagents[8]; (D) silica gel high-performance thin-layer chromatography (HPTLC) followed by densitometry[9].

Because these methods gave good results for the determination of TCs, we consider them suitable for the above purpose. Therefore, we used them for a comparative analysis of TC pharmaceutical preparations available on the Japanese market. This paper describes the optimal conditions and analytical results for the determination of impurities in TC pharmaceutical preparations.

EXPERIMENTAL

Chemicals

Methanol, acetonitrile, acetone, oxalic acid, disodium ethylenediaminetetraacetate (Na_2EDTA) and pyridine were analytical grade reagents.

TC and CTC, as their hydrochlorides, were supplied by Pfizer Taito. ETC, ATC and EATC, as their hydrochlorides, were prepared according to the methods of Simmon *et al.*[10] and McCormick *et al.*[11].

Preparation of standard tetracycline solutions

Each TC (100 mg) was weighed accurately into a 10 ml volumetric flask, and diluted to volume in methanol. Further dilution was sometimes necessary before application.

Preparation of sample solutions

For a TC in tablet or capsule form, 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 syrup was dissolved and made up to volume with methanol in a 10-ml volumetric flask.

Thin-layer chromatography

A silica gel HPTLC (E. Merck, 5641) was predeveloped with saturated Na_2EDTA aqueous solution and then dried in air at room temperature for 1 h and activated at 130°C for 2 h. Following the application of 1–5 μl of standard and sample solutions, the plate was developed with acetone–5% Na_2EDTA aqueous solution (10:1). For RP-TLC, following the application of sample and standard solutions a plate (C_{18}-modified silica gel, E. Merck, 15423) was developed with methanol–acetonitrile–0.5 *M* aqueous oxalic acid solution, pH 2.0 in the ratios 1:1:5 and 1:1:2 for the determination of ETC and the others, respectively.

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

Detection with spray reagents

As described previously[8], the developed RP-TLC plate was sprayed evenly with 0.5% Fast Violet B aqueous solution and pyridine, and then heated at 120°C to evaporate pyridine. Amounts of TCs on the plates were estimated immediately by visual comparision of the colour intensity with that of standards after heating TLC plates.

High-performance liquid chromatography

A high-performance liquid chromatograph equipped with a constant-flow pump (Shimadzu LC-5A, Kyoto, Japan) was used, with variable-wavelength detector (UVIDEC-100-IV, Tokyo, Japan) operated at 400 nm. The separation was performed on Nucleosil $5C_{18}$ (5 μm, 150 × 4.6 mm I.D., M. Nagel) with methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution, pH 2.0 (1:1:5) at a flow-rate of 2 ml/min as the mobile phase at room temperature. For determination of the impurities, 2 μl each of sample and standard solution were injected.

RESULTS AND DISCUSSIONS

Chromatographic conditions

Our established analytical methods for TCs and their optimal conditions are summarized in Table I[6–9]. Although the chromatograms exhibited excellent properties, the methods did not always give satisfactory results for the analysis of impurities in TC pharmaceutical preparations. Modified optimal conditions for the latter analysis are listed in Table II.

Using our previously reported method A, we could not accurately determine ETC because a large amount of TC in the sample solution interfered with a small amount of ETC in the chromatograms. Under the modified conditions shown in Table II, a precise determination of ETC was obtained. Typical chromatograms of TC pharmaceutical preparations under these conditions are shown in Fig. 1. The detection limit for impurities in TC pharmaceutical preparations was 0.05%. Although the determination of impurities in TC pharmaceutical preparations was achieved using the conditions of method B listed in Table I, we found that the slightly modified conditions shown in Table II gave better separation of ETC from a large amount of TC. Other impurities (CTC, EATC and ATC) were completely determined under the conditions in Table I. Typical densitometric profiles are shown in Fig. 2. Detection limits were 0.5% and 0.1% for ETC and the other impurities, respectively. The modified method C led to the determination of the impurities in a similar manner;

TABLE I

ESTABLISHED ANALYTICAL METHODS FOR TETRACYCLINES AND THEIR OPTIMAL CONDITIONS

Method	*Stationary phase*	*Solvent system*	*Flow-rate*	*Detection*
HPLC (method A)	Cosmosil $5C_{18}$ column (Nakarai, Japan)	CH_3OH–CH_3CN–0.2 *M* oxalic acid (aq. pH 2.0) 1:1:3.5	1 ml/min	400 nm
RP-TLC densitometry (method B)	C_{18}-TLC plate (E. Merck, 15423)	CH_3OH–CH_3CN–0.5 *M* oxalic acid (aq. pH 2.0) 1:1:4 (for ETC) and 1:1:2 (for the others)	–	360 nm (for ETC and CTC) 425 nm (for EATC and ATC)
RP-TLC-spray reagents (method C)	C_{18}-TLC plate (E. Merck, 15423)	CH_3OH–CH_3CN–0.5 *M* oxalic acid (aq. pH 2.0) 1:1:4 (for ETC) and 1:1:2 (for the others)	–	0.5% Fast Violet B (aq.) and pyridine
HPTLC (method D)	Silica gel HPTLC plate (E. Merck, 5641)	CH_3COCH_3–5% Na_2EDTA (aq.) (10:1)	–	360 nm (for ETC and CTC) 450 nm (for EATC and ATC)

TABLE II

OPTIMAL CONDITIONS FOR ANALYSIS OF IMPURITIES IN TETRACYLINE PHARMACEUTICAL PREPARATIONS

Method	*Stationary phase*	*Solvent system*	*Flow-rate*	*Detection**
HPLC (method A)	Nucleosil $5C_{18}$ (M. Nagel)	CH_3OH–CH_3CN–0.2 *M* oxalic acid (aq. pH 2.0) 1:1:5	2 ml/min	400 nm
RP-TLC densitometry (method B)	C_{18}-TLC plate* (E. Merck, 15423)	CH_3OH–CH_3CN–0.5 *M* oxalic acid (aq. pH 2.0) 1:1:5 (for ETC) and 1:1:2 (for the others)	–	360 nm (for ETC and CTC) 425 nm (for EATC and ATC)
RP-TLC spray reagents (method C)	C_{18}-TLC plate* (E. Merck, 15423)	CH_3OH–CH_3CN–0.5 *M* oxalic acid (aq. pH 2.0) 1:1:5 (for ETC) and 1:1:2 (for the others)	–	0.5% Fast Violet B (aq.) and pyridine
HPTLC (method D)**	–	–	–	–

* These conditions are as in Table I.

** Unsuitable for practical application.

TABLE III

CONTENT OF IMPURITIES IN SOME TETRACYCLINE PHARMACEUTICAL PREPARATIONS

Values are the average of six measurements. A = HPLC; B = densitometry; C = detection with spray reagents; ND = not detected. For analytical methods see Experimental.

Sample	*Content (C.V.) (%)*											
	ETC			*CTC*			*EATC*			*ATC*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Capsule A	0.78 (0.6)	0.85 (2.3)	1.0	ND	ND	ND	0.11 (6.9)	ND	0.1	0.25 (3.2)	0.32 (4.6)	0.3
Capsule B	0.53 (0.8)	0.61 (3.4)	1.0	ND	ND	ND	0.08 (5.7)	ND	0.1	0.33 (1.5)	0.34 (1.4)	0.3
Capsule C	0.90 (0.9)	0.94 (0.8)	1.0	ND	ND	ND	0.08 (6.2)	ND	ND	0.15 (7.2)	0.18 (8.7)	0.2
Capsule D	0.50 (2.0)	0.54 (4.2)	0.8	ND	ND	ND	ND	ND	ND	0.12 (6.3)	0.16 (8.7)	0.1
Capsule E	2.51 (1.9)	2.48 (3.5)	2.8	ND	ND	ND	ND	ND	ND	0.12 (6.8)	0.16 (5.9)	0.1
Capsule F	0.58 (1.3)	0.52 (3.3)	0.8	ND	ND	ND	ND	ND	ND	0.12 (6.0)	0.20 (8.7)	0.3
Capsule G	1.07 (0.6)	1.30 (6.3)	1.0	ND	ND	ND	ND	ND	ND	0.16 (4.6)	0.15 (5.5)	0.2
Powder A	1.25 (1.0)	1.41 (5.8)	1.6	ND	ND	ND	ND	ND	ND	0.30 (3.9)	0.38 (2.3)	0.5
Powder B	1.69 (1.2)	1.78 (2.5)	1.8	ND	ND	ND	ND	ND	ND	0.25 (4.1)	0.30 (3.1)	0.4
Powder C	2.65 (1.2)	2.57 (2.3)	3.0	0.59 (0.8)	0.54 (5.5)	0.5	ND	ND	ND	0.40 (1.2)	0.46 (1.7)	0.5
Syrup A	0.15 (2.4)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Syrup B	0.13 (3.7)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dry syrup	1.32 (2.3)	1.67 (4.6)	2.0	ND	ND	ND	ND	ND	ND	0.12 (4.0)	0.16 (5.8)	0.1
Tablet	2.10 (0.5)	2.19 (2.3)	2.0	ND	ND	ND	0.12 (3.9)	0.10 (9.8)	0.1	0.27 (2.9)	0.30 (7.0)	0.4
For animal A	0.11 (2.3)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
For animal B	0.07 (5.5)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

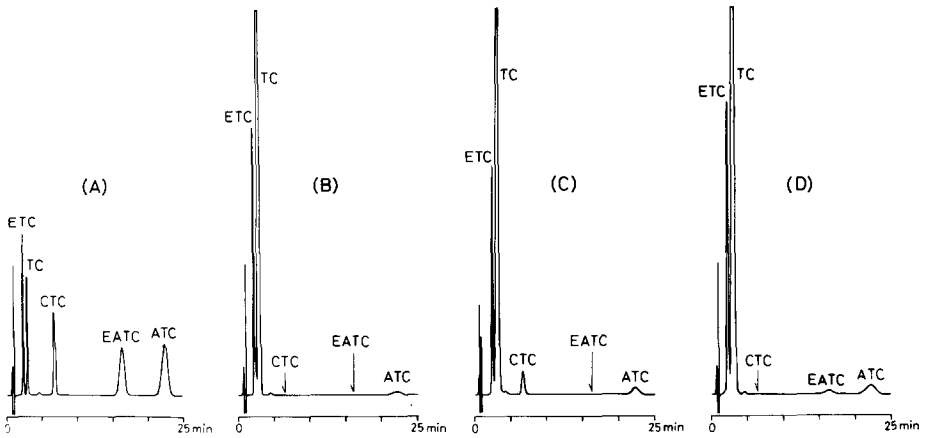

Fig. 1. Typical high-performance liquid chromatograms of tetracycline pharmaceutical preparations. (A) Standard of TCs (300 ng); (B) capsule E; (C) powder C; (D) tablet. For analytical conditions see Experimental.

detection limits on RP-TLC were 0.3% and 0.06% for ETC and other impurities, respectively.

The modified method D showed good separation of ETC from a large amount of TC, but the analytical values of ETC were uncommonly large. We suppose that these results are due to contamination by an unknown substance which shows the same R_F value as ETC on HPTLC plates, and we are investigating further. Therefore, method D would seem at present to be unsuitable for practical application.

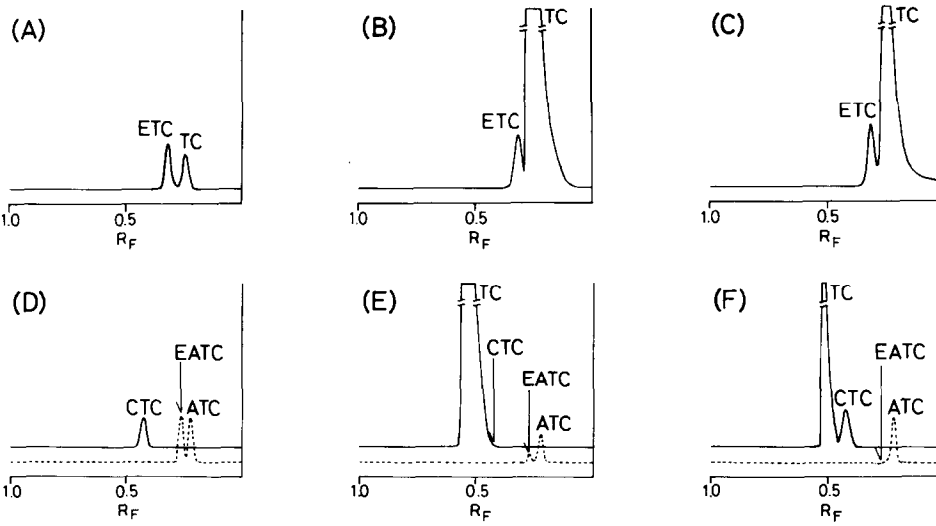

Fig. 2. Typical densitometric profiles of tetracycline pharmaceutical preparations. (A) Standard of TCs (0.2 μg); (B) tablet; (C) capsule E; (D) standard of TCs (0.25 μg); (E) tablet; (F) powder C. For analytical conditions see Experimental.

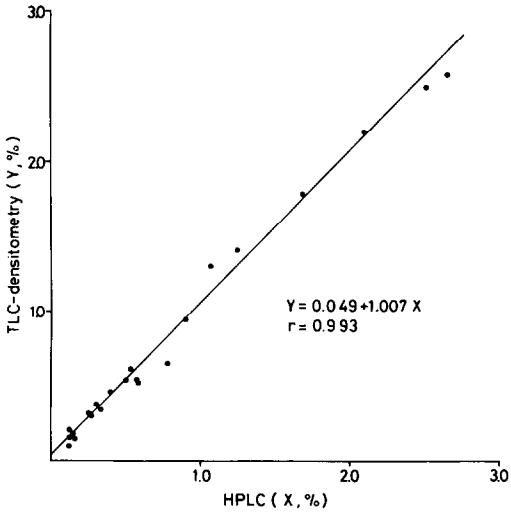

Fig. 3. Correlation of quantitative data for HPLC and RP-TLC densitometry. Points represent the values listed in Table III.

Comparison of analytical results

The TC pharmaceutical preparations analysed were capsules (7), tablets (1), syrups (3), powders (3) and for animals (2) available on the Japanese market (Table III). The results from method A show better a coefficient of variation than those from method B. When the analytical values are close to 0.1%, even the coefficient of variation for method A is not so good. However, as the coefficients of variation for both methods are within 10%, it is considered that both are suitable in practice.

Fig. 3 shows a correlation of the quantitative data from methods A and B, using the least-squares method. The corresponding parameters, method B value = a (method A value) + b, are $a = 1.007$, $b = 0.049$ and $r = 0.993$. Points represent the values listed in Table III. If the values from either method A or method B were not determined, this point is omitted. If such values were assigned at half the detection limit, the parameters were 1.006, 0.051 and 0.992 for a, b and r, respectively. The values of both parameters a and b indicate that the accuracies of the two methods are almost identical, so we conclude that these analytical results are very reliable.

Method C is not as accurate as other methods but it has some advantages, namely that it does not require special instruments, it shows better detection limits than method B and it is the quickest of these methods (the analytical times per five samples are 2.5 h, 2.5 h and 2.0 h by methods A, B and C, respectively). As method C shows analytical values close to the others (Table III), it is concluded to be very effective as a screening method.

Therefore, we recommend a combination of method C and either method A or method B as a simple and rapid analytical system for impurities in TC pharmaceutical preparations. A screening test of the impurities using method C is first carried out and, if the results exceed the limits, a precise determination using method A or B should follow.

Because no limits for the impurities in TC pharmaceutical preparations are

laid down in the Japanese Pharmacopoeia, we cannot directly evaluate these results. However, our results do not exceed the BP limits. In the near future, we hope to set the limits for the impurities in TC pharmaceutical preparations in Japan.

CONCLUSION

The comparative studies using these methods indicate that methods A and B showed good accuracy and their analytical values were very reliable, and that method C gave also analytical values close to the others and required the shortest analysis time. Some of the advantages are as follows: the usage of an isocratic solvent system for method A and the usage of commercially available precoated TLC plates for methods B and C. Further, method C does not require special instruments.

We recommend a combination of method C and either method A or method B for analysis of the impurities. Method C is used as an initial screening method; if the results exceed the limits, a precise determination should be carried out using method A or B. Because this system can be readily achieved everywhere, we consider that it is suitable for a replacement of the official methods.

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