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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

V*. A SIMPLE METHOD FOR THE ANALYSIS OF TETRACYCLINES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic high-performance liquid chromatographic method for the determination of tetracyclines is described using a mobile phase containing oxalic acid and C_8- and C_{18}-modified silica gel columns. For good separations of tetracyclines, oxalic acid concentrations of above 0.01 and 0.2 *M* respectively for parent tetracyclines (group I) and impurities in tetracycline (group II) are required. The optimum pH of the aqueous oxalic acid solution in the mobile phase is 2.0. The combinations of the C_8-modified silica gel column with methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5) and the C_{18}-modified silica gel column with methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3.5) gave satisfactory results for groups I and II, respectively.

INTRODUCTION

Tetracycline antibiotics (TCs) are a group of clinically and agriculturally important compounds, active against a wide range of human and animal pathogens. Especially in Japan, where fish is a major source of protein, TCs are used in great quantity for fish breeding[1]. Such usage may lead to problems with residues in fish and environmental pollution, so a simple, rapid and reliable method for determination of TCs is required. The conventional microbiological procedure provides a good sensitivity but cannot identify certain TCs. Therefore, a number of chemical determinations of TCs were reported[2–20].

In previous studies we have established techniques for determination of TCs[21–23] and polyether antibiotics[24], using thin-layer chromatography (TLC). Al-

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though these TLC methods are simple and available, they require a long development time and are not always sensitive enough to allow detection of small amounts of TCs with good precision. As high-performance liquid chromatography (HPLC) is fast, reliable and has high sensitivity, we have now investigated its use for the sensitive determination of TCs.

TCs form chelate complexes with metal ions[25,26] capable of adsorption on reversed-phase (RP) columns bonded to alkyl chains[8], so that TCs are apt to appear as tailing peaks. Thus, the quantification is less precise because the peak height is more likely to be non-linearly related to the concentration, and measurement of the peak area is more difficult. In order to avoid forming such chelate complexes and their adsorption on RP columns, RP column chromatography using mobile phases containing various acids (phosphoric[9,10], citric[11,12], perchloric[13], Tartaric[12], ethylenediaminetetraacetic acid[14,15]) and ion-pair chromatography[8,16–18] have been used successfully. However, we have found that TCs show extreme tailing on RP-TLC plates even when using mobile phases containing these acids, and that only a mobile phase containing oxalic acid enabled the precise determination of TCs[23].

In the present paper, we report in detail the RP-HPLC separation and determination of TCs using mobile phases containing oxalic acid.

EXPERIMENTAL

Chemicals

Methanol, acetonitrile, aqueous ammonia and oxalic acid were analytical grade reagents.

The HPLC packing material were Cosmosil $5C_8$ and $5C_{18}$ (Nakarai, Kyoto, Japan) which had particle sizes of 5 μm.

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.*[27] and McCormick *et al.*[28].

Apparatus

A chromatograph equipped with a constant-flow pump (Shimadzu LC-5A, Kyoto, Japan) was used together with a variable wavelength detector (JASCO UVIDEC-100-IV, Tokyo, Japan) operated at 360 nm for TC, OTC, CTC and DC, and 400 nm for TC, ETC, CTC, ATC and EATC.

Mobile phase

The mobile phases were made from methanol, acetonitrile and aqueous oxalic acid solution the pH of which was adjusted with 28% aqueous ammonia. The flow-rate was 1 ml/min.

Preparation of tetracycline solutions

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

RESULTS AND DISCUSSION

Chromatographic conditions

We consider that it is not always necessary to differentiate all seven TCs. For example, when an analytical method is applied to residues or biological samples, 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, ETC, CTC, ATC and EATC (group II) is needed. Therefore, groups I and II were treated separately in the subsequent experiments.

In a previous study[23] we reported that the combinations of a C_8 TLC plate and methanol–acetonitrile–0.5 *M* aqueous oxalic acid solution pH 2.0 (1:1:4) and a C_{18} TLC plate and methanol–acetonitrile–0.5 *M* aqueous oxalic acid solution pH 2.0 (1:1:2) showed satisfactory results for groups I and II, respectively. Although we attempted to separate TCs using HPLC under the same conditions as RP-TLC (mobile phase and chemically bonded materials), the results were unsatisfactory.

After various experiments, we succeeded in the separation of TCs using RP-HPLC. Typical separations are illustrated in Fig. 1 under the optimum conditions. For group I, a satisfactory result is obtained using a C_8-modified silica gel column and methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5) as a mobile phase. A good separation among group II is obtained by elution with methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3.5) on a C_{18}-modified silica gel column.

Various experimental results are described below. In interpreting the chromatograms the following three parameters are used: (1) capacity factor, $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the sample peak and t_0 is the retention time for a non-retained peak; (2) resolution factor, $R_s = 2(t_1 - t_2)/(w_1 + w_2)$, where

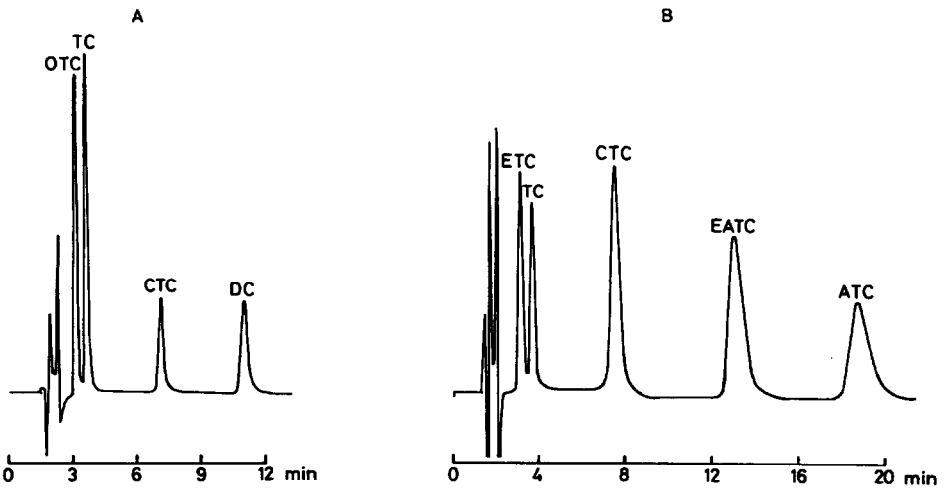

Fig. 1. HPLC separation of tetracyclines. A, Column: Cosmosil $5C_8$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5). Flow-rate: 1 ml/min. Detection: 360 nm. B, Column: Cosmosil $5C_{18}$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3.5). Flow-rate; 1 ml/min. Detection: 400 nm.

t_2 and t_1 are retention times and w_1 and w_2 the corresponding peak widths at the baseline and (3) asymmetry factor, A_s, which is the ratio of the rear (tailing edge) to the front (leading edge) lengths of the peak along a line parallel to and 10% (of its height) distant from its base.

(a) Comparison of C_8- and C_{18}-modified silica gel columns. In a previous study[23], although the R_F values of OTC and TC, and of ETC and TC, were similar, the usage of a C_8 TLC plate showed better resolution between OTC and TC than did a C_{18} TLC plate, whereas a good resolution was achieved between ETC and TC using the C_{18} plate. In this study the k' values of OTC and TC, and of ETC and TC, were also similar. Therefore, in order to compare C_8- and C_{18}-modified silica gel columns for their suitability in TC analysis using methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5) as a mobile phase, we estimated the R_s values between OTC and TC and between ETC and TC for groups I and II, respectively. The R_s values between OTC and TC are 1.46 and 1.18 using C_8- and C_{18}-modified silica gel columns, where the corresponding R_s values between ETC and TC are 1.20 and 1.35 respectively. Therefore, C_8- and C_{18}-modified silica gel columns were used for groups I and II, respectively, in all subsequent experiments.

(b) Influence of oxalic acid concentration. Using methanol–acetonitrile–aqueous oxalic acid solution (pH 2.0) (1:1.5:5 and 1:1:3.5) as mobile phases for groups I and II, respectively, the influence of oxalic acid concentration was examined. As shown in Fig. 2, the A_s values improved with increasing acid concentration, so that good A_s values are obtained above 0.01 and 0.2 *M* for groups I and II, respectively. The k' values are also related to the acid concentration as shown in Fig. 3. When 0.01 *M* aqueous oxalic acid solution is used for group I, the k' values are sufficient to separate the TCs, and the R_s value between the OTC and TC peaks is 1.46. Using 0.2 *M* aqueous oxalic acid solution, suitable retention times are shown for group II and the R_s value between ETC and TC is 1.29. Although good resolution of TC spots was obtained above 0.3 *M* in RP-TLC[23], we chose 0.01 and 0.2 *M* aqueous oxalic acid solution for groups I and II, respectively, in this study.

(c) pH of the aqueous oxalic acid solution. The influence of the pH of the aqueous oxalic acid solution in the mobile phase on k' and A_s values was investigated,

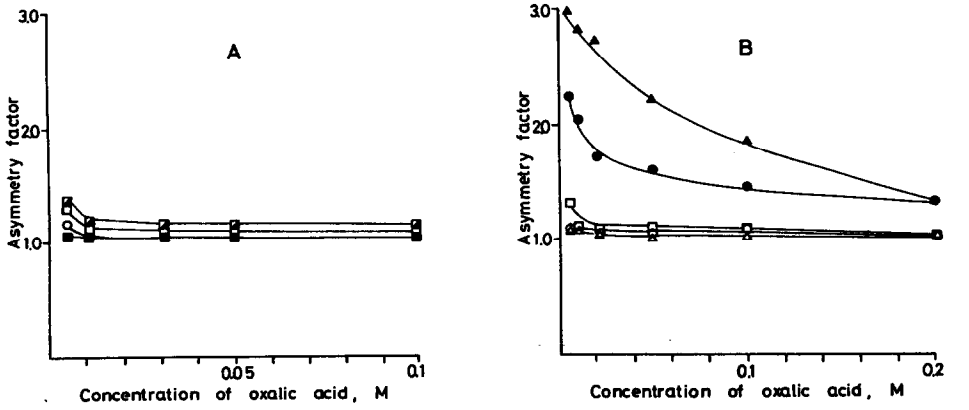

Fig. 2. Influence of oxalic acid concentration on the asymmetry factor. Conditions as in Fig. 1. Curves: ○, TC; ■, OTC; □, CTC; ◪, DC; △, ETC; ●, EATC; ▲, ATC.

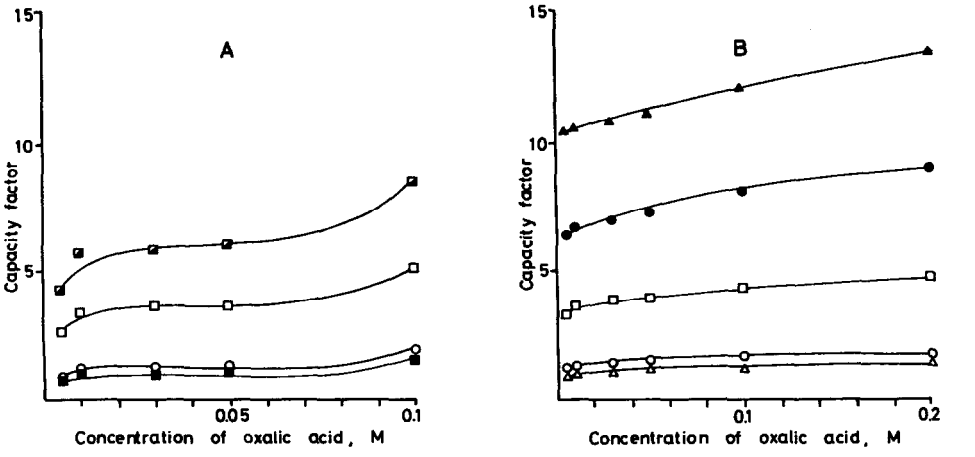

Fig. 3. Influence of oxalic acid concentration on the capacity factor. HPLC conditions and key as in Figs. 1 and 2.

using methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution (1:1.5:5) and methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution (1:1:3.5) for groups I and II, respectively. As shown in Fig. 4, CTC and DC were completely separated below pH 3.0, but they overlapped above pH 4.0. In group II, the k' values of EATC and ATC were too large above pH 2.5, and above pH 3.5 oxalic acid was precipitated in the mobile phase. All the TCs had the best asymmetry values at pH 2.0. Therefore, we conclude that the optimum pH of the aqueous oxalic acid solution is 2.0.

(d) Proportions of methanol and acetonitrile. Although we rested various proportions of methanol or acetonitrile with 0.01 or 0.2 *M* aqueous oxalic acid solution pH 2.0 as the mobile phase, unsatisfactory results were obtained as in the previous

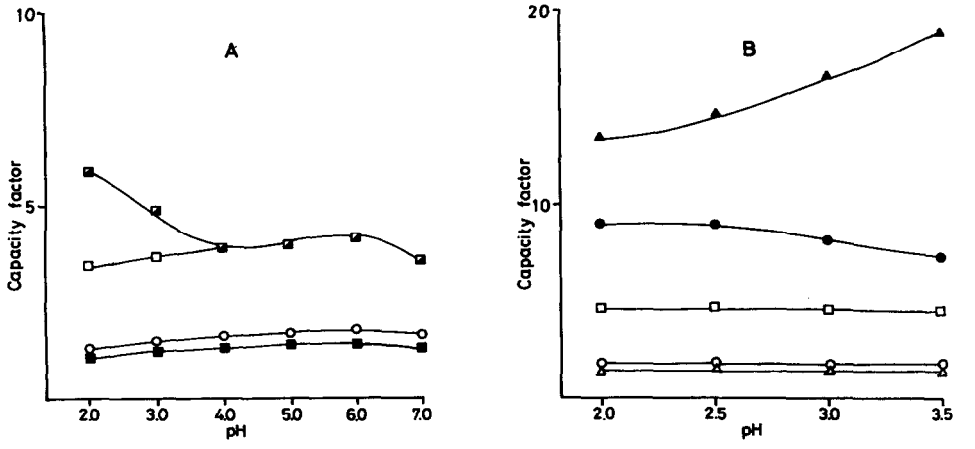

Fig. 4. Influence of pH of the aqueous oxalic acid solution in the mobile phase on the capacity factor. A, Column: Cosmosil $5C_8$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.01 *M* aqueous oxalic acid solutions (1:1.5:5). Flow-rate: 1 ml/min. Detection: 360 nm. B, Column: Cosmosil $5C_{18}$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution (1:1:3.5). Flow-rate: 1 ml/min. Detection: 400 nm. For key see Fig. 2.

RP-TLC study[23], suggesting that a mixture of methanol and acetonitrile is required to separate TCs. So various ratios of methanol and acetonitrile were tested using the organic solvent (methanol–acetonitrile)–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:2) and the organic solvent (methanol–acetonitrile)–0.2 *M* aqueous oxalic acid solution pH 2.0 (2:3.5) for groups I and II, respectively. As shown in Fig. 5, 1:2, 1:1.5 and 1:1 mixtures of methanol and acetonitrile gave good separations of group I, however, with the 1:1 mixture the retention times of TCs are too long, and the 1:1.5 mixture gave a better resolution between OTC and TC than did the 1:2 mixture. If the volume of methanol used was the same as that of acetonitrile, the mobile phase gave good separations among group II. But the 2:1 mixture and methanol alone gave too long retention times. Thus, the 1:1.5 and 1:1 mixtures were employed for groups I and II, respectively, in subsequent work.

(e) Proportions of the organic solvent and aqueous oxalic acid solution. Various ratios of the organic solvent (methanol–acetonitrile, 1:1.5 and 1:1) and aqueous oxalic acid solution (0.01 and 0.2 *M*, pH 2.0) were tested as mobile phases. As shown in Fig. 6, for group I, a poor separation between OTC and TC was obtained using methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:2.5). When methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:7.5) was used, the best separation between OTC and TC was achieved, but the retention times of DC and CTC were too long. A good separation among group I was obtained on a C_8-modified silica gel column using methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5). For group II, the *k'* values of EATC and ATC were too large with methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:4) as a mobile phase, and using methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3) the resolution between ETC and TC was poor. So we recommend methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3.5) as a mobile phase to separate group II.

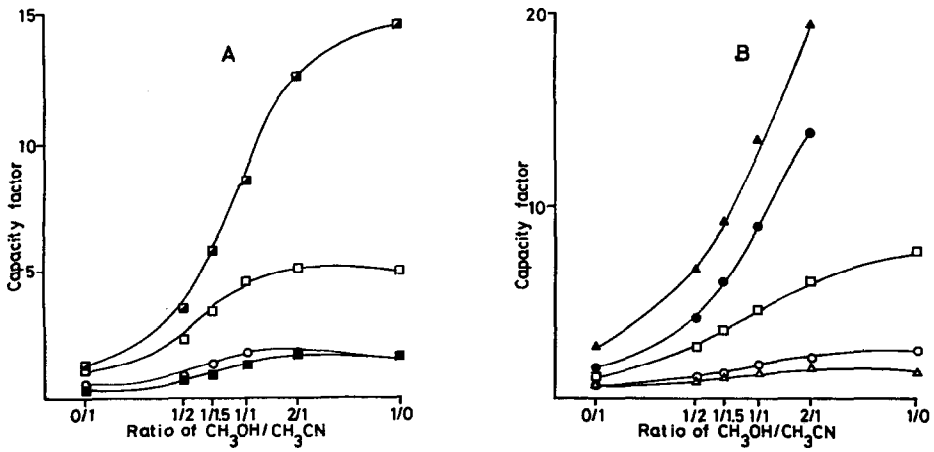

Fig. 5. Effect of ratio of methanol to acetonitrile on the capacity factor. A, Column: Cosmosil $5C_8$, 150 × 4.6 mm I.D. Mobile phase: (methanol–acetonitrile)–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:2) Flow-rate: 1 ml/min. Detection: 360 nm, B. Column: Cosmosil $5C_{18}$, 150 × 4.6 mm I.D. Mobile phase: (methanol–acetonitrile)–0.2 *M* aqueous oxalic acid solution (pH 2.0) (2:3.5). Flow-rate: 1 ml/min. Detection: 400 nm. For key see Fig. 2.

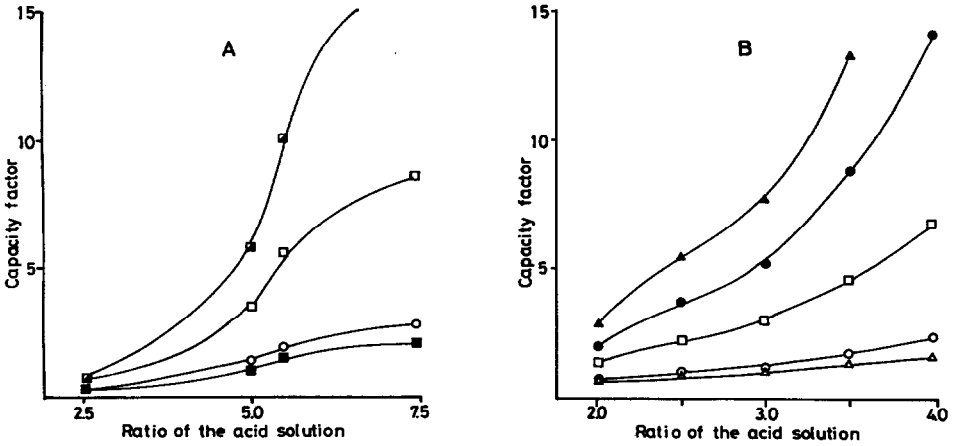

Fig. 6. Effect of ratio of organic solvent to aqueous oxalic acid solution on the capacity factor. A, Column: Cosmosil $5C_8$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:X). Flow-rate: 1 ml/min. Detection; 360 nm. B, Column: Cosmosil $5C_{18}$, 150 × 4.6 mm I.D. Mobile phase: methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:X). Flow-rate: 1 ml/min. Detection: 400 nm. For key see Fig. 2.

Calibration curves for tetracyclines

As shown in Fig. 7, we obtained linear relationships between 4 and 65 ng and between 40 and 200 ng for groups I and II, respectively. We have successfully applied this HPLC system to determine TCs in foods and biological samples and to measure impurities in TC drugs. These results will be reported elsewhere, and this HPLC system will be compared with our previously reported TLC techniques[21–23].

In conclusion, a technique for the determination of TCs using reversed-phase HPLC has been established, with the following characteristics.

(1) A mobile phase containing oxalic acid makes possible the determination of TCs on C_8- and C_{18}-modified silica gel columns.

(2) The resolutions and asymmetries of TC peaks are dependent upon the pH of the aqueous oxalic acid solution in the mobile phase and the optimum pH is 2.0.

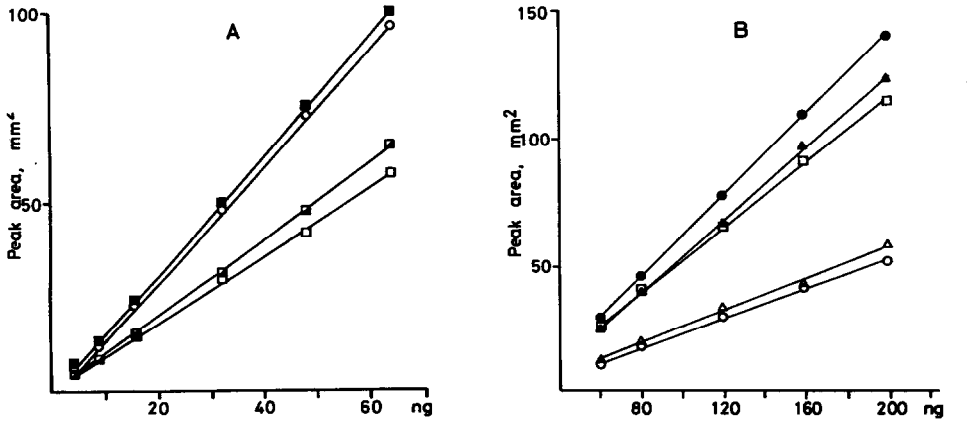

Fig. 7. Calibration curves for tetracyclines. For HPLC conditions and key see Figs. 1 and 2.

(3) The resolutions and asymmetries of TC peaks improve with increasing oxalic acid concentration, good results being obtained above 0.01 and 0.2 *M* for groups I and II, respectively.

(4) The combination of a C_8-modified silica gel column with methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:5) and a C_{18}-modified silica gel column with methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:3.5) give satisfactory results for groups I and II, respectively.

(5) The calibration curves show good linear relationships between 4 and 64 ng and between 40 and 200 ng for groups I and II, respectively.

Finally, although we were able to obtain good results using Cosmosil as packing material, it is well known that large differences in the chromatographic behaviour of TCs have been observed using packing materials from different suppliers[8,19]. For instance, when LiChrosorb RP-8 (E. Merck) and Nucleosil $5C_8$ (M. Nagel) were used as packing materials, we obtained good results for group I using methanol–acetonitrile–0.01 *M* aqueous oxalic acid solution pH 2.0 (1:1.5:2 and 1:1.5:7.5) as mobile phases, respectively. In the case of μBondapak C_{18} (Waters), the solvent system of methanol–acetonitrile–0.2 *M* aqueous oxalic acid solution pH 2.0 (1:1:4.5) gave a satisfactory chromatogram for group II. These results show that even though packing materials from different suppliers are employed, variation of the ratio of organic solvent and aqueous oxalic acid solution in the mobile phase enables good separations. Therefore, we recommend the described HPLC system for analysis of TCs.

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