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IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

XII*. SIMULTANEOUS ANALYSIS OF SEVEN TETRACYCLINES IN HONEY

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 and MAKOTO SUZUKI
Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468 (Japan)

SUMMARY

An analytical system for the simultaneous determination of residual oxytetracycline, tetracycline, chlortetracycline, doxycycline, methacycline, demethylchlortetracycline and minocycline in honey has been established by a combination of simple thin-layer chromatographic (TLC) and precise high-performance liquid chromatographic (HPLC) methods. In this system, screening by TLC can detect tetracyclines (TCs) at a level of 0.1 ppm in honey without the need for special equipment, and the quantitative method by HPLC can determine TCs with good recovery (83.7–99.6%) and coefficient variation (0.9–4.3%).

INTRODUCTION

In order to prevent foul brood of honey-bees, tetracycline antibiotics (TCs) are widely used all over the world in honey-bee culture[1,2]. Such usage may result in residues of TCs in honey. In Japan, oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) have been administered to honey-bees. Minocycline (MINO), demethylchlortetracycline (DMCTC) and methacycline (MTC) may also be used. Therefore, the establishment of a method for residue analysis of the above seven TCs in honey, which can readily be applied everywhere, is required. Two high-performance liquid chromatographic (HPLC) methods for OTC, TC and CTC in honey have been reported[3,4]. However, Jürgen's method was achieved without any clean up, so many peaks of interfering substances appeared on the chromatogram[3]. Takeba's method did not have enough sensitivity to apply for routine analysis.

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In our previous report[5], we established a simple, rapid and reliable HPLC method in combination with a tandem cartridge clean-up system (Baker 10 C_{18} and COOH cartridges) for the analysis of four TCs (OTC, TC, CTC and DC) in honey. However, this method required the use of a high-performance liquid chromatograph and therefore could not readily be applied everywhere. Therefore, we wished to establish a simple thin-layer chromatographic (TLC) method without the need for special equipment as a screening method and a precise HPLC method for the quantitation of seven TCs in honey by improvement of the previous tandem cartridge–HPLC method. Further, we wished to develop a combination of the TLC and HPLC methods for the simultaneous analysis of TCs in honey.

EXPERIMENTAL

Materials

Methanol, acetonitrile, ethyl acetate, pyridine, triethanolamine (TEA), chloroform, disodium ethylenediaminetetraacetate (Na_2EDTA), citric acid, disodium hydrogenphosphate, oxalic acid, magnesium chloride and Fast Violet B salt were analytical grade materials. OTC, TC, CTC, DC and MTC were supplied by Pfizer (Tokyo, Japan) and MINO and DMCTC by Lederle (Tokyo, Japan). TCs (each 100 mg) were weighed accurately into 10-ml volumetric flasks and made up to volume in methanol and water. Dilution was sometimes necessary with methanol or with water.

Silica gel HPTLC plates (E. Merck, 5641) and RP-8 TLC plates (E. Merck, 15424) were used in normal-phase HPTLC and reversed-phase TLC, respectively.

Baker-10 C_{18} (500 mg, Lot. No. 608125) and Baker-10 COOH (500 mg, Lot No. 427154) cartridges were provided by J. T. Baker (Phillipsburg, NJ, U.S.A.). After activation of a Baker-10 C_{18} cartridge with 10 ml of methanol and with 10 ml of water, the cartridge was conditioned with 10 ml of saturated aqueous Na_2EDTA.

Systematic analytical procedure

A sample of honey (25 g) was dissolved in 50 ml of 0.1 *M* Na_2EDTA–McIlvaine buffer (pH 4.0). After filtration of the sample solution, the filtrate was applied to a Baker-10 C_{18} (500 mg) cartridge pretreated with Na_2EDTA. The C_{18} cartridge containing the sample was washed with 20 ml of water and 0.4 ml of ethyl acetate and then air-dried under vacuum for 5 min. TCs were eluted from the C_{18} cartridge with 50 ml of 10% methanol in ethyl acetate and collected in a 50-ml voluetric flask. A 40-ml volume of the eluate (total volume 50 ml) was concentrated under vacuum at 30°C, and the residue was dissolved in 0.1 ml of methanol. The methanol solutions (each 5 μl) were spotted on normal-phase HPTLC and reversed-phase TLC plates for the identification of MINO, CTC, DC, MTC and OTC and for OTC, TC, DMCTC and CTC, respectively, and were developed and detected according to the procedure in the next section. When the TCs were identified, 5 ml of 10% methanol in ethyl acetate (eluate from C_{18} cartridge) was applied to a Baker-10 COOH cartridge activated with 10% methanol in ethyl acetate. The TCs were eluted from the COOH cartridge with 10 ml of the mobile phase used for HPLC, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (2:3:16), and collected in a 10-ml volumetric flask. The determination was carried out by HPLC.

Thin-layer chromatography

A normal-phase HPTLC plate was predeveloped with saturated aqueous Na_2EDTA, then dried in air at room temperature for 1 h and activated at 130°C for 2 h before use. After the sample had been applied, the plate was developed with chloroform–methanol–5% aqueous Na_2EDTA (65:20:5) (lower phase). For reversed-phase TLC, the plate was developed with methanol–acetonitrile–0.5 *M* aqueous oxalic acid (1:1:4) (pH 3.0).

The developed normal-phase HPTLC plate was sprayed evenly with 0.2 *M* aqueous magnesium chloride and then air-dried at room temperature. The developed reversed-phase TLC plate was sprayed similarly and, after it had dried at room temperature, sprayed 10% TEA in methanol. The fluorescent spots of TCs produced with Mg^{2+} on both plates were observed under long-wavelength (360 nm) UV light.

High-performance liquid chromatography

A high-performance liquid chromatograph, equipped with a constant-flow pump (Shimadzu LC-5A, Kyoto, Japan), was used with a variable-wavelength UV detector (Shimadzu SPD-2AM), operated at 350 nm. The separation was performed on a Chemcosorb 3C8 column (3 μm, 75 mm × 4.6 mm, I.D.; Chemco, Osaka, Japan) with methanol–acetonitrile–0.01 *M* aqueous oxalic acid (2:3:16) (pH 3.0) as a mobile phase at a flow-rate of 1 ml/min at room temperature. For the determination of TCs, 100 μl of sample and standard solutions were injected.

RESULTS AND DISCUSSION

The previous tandem cartridge (Baker 10 C_{18} and COOH) system, followed by HPLC, is quite effective for the analysis of four TCs (OTC, TC, CTC and DC) in honey[5]. We therefore thought that a slight modification of this method would give satisfactory results for simultaneous analysis of seven residual TCs in honey, that could be carried out at most laboratories. Therefore, we planned to use TLC and HPLC as simple screening and precise determination methods, respectively.

The following conditions were optimized.

Elution of TCs from a COOH cartridge

In our previous study[5], four TCs could be eluted from a Baker-10 COOH cartridge with the mobile phase used for HPLC, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (2:3:6). The suitability of the mobile phase of the present HPLC method, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (2:3:16), pH 3.0, as an eluent for the Baker-10 COOH cartridge was investigated. After application of the TCs (25 μg of each) to the COOH cartridge in 1 ml of methanol, they were eluted with various volumes of the mobile phase and determined. The elution curves are shown in Fig. 1. All TCs were completely recovered from the COOH cartridge with 10 ml of the mobile phase. Therefore, we used 10 ml of this mobile phase as an eluent for the Baker-10 COOH cartridge in subsequent work.

Elution of TCs from a C_{18} cartridge

A Baker-10 C_{18} cartridge, containing 100 mg of packing material, was used for extraction of TCs from honey in the previous work[5]. However, a 500-mg Baker-10

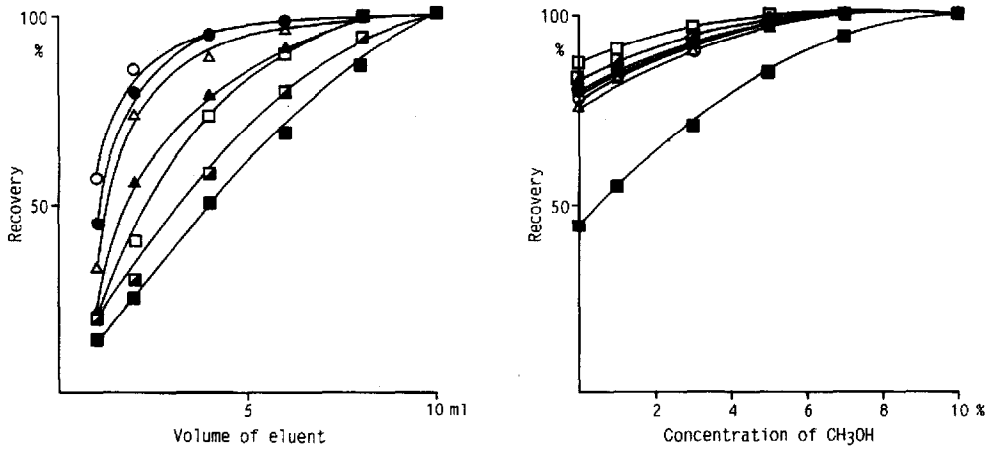


Fig. 1. Elution curves for seven tetracyclines from a Baker-10 COOH cartridge. After application of TCs (25 μg of each) in 1 ml methanol, they were eluted with various volumes of the mobile phase, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (2:6:16), pH 3.0, and then the recoveries were calculated. TCs; ○, MINO; ●, OTC; △, TC; ▲, DMCTC; □, CTC; ■, MTC; □, DC.

Fig. 2. Effect of methanol concentration on the recovery of seven tetracyclines from a Baker 10 C_{18} cartridge. After application of TCs (25 μg of each), they were eluted with 50 ml of ethyl acetate containing various amounts of methanol, and then 5 ml of the eluate were applied to a Baker-10 COOH cartridge. The TCs were eluted with the mobile phase used for HPLC, and then determined. For symbols, see Fig. 1.

C_{18} cartridge was used in the present work, because the capacity of the previous cartridge was not sufficient for extraction of TCs from 25 g of honey. Although ethyl acetate was the most effective eluent of TCs for 100-mg Baker-10 C_{18} cartridges, in the case of the 500-mg Baker-10 C_{18} cartridges the TCs were not eluted completely. When we investigated the analysis of four TCs in animal liver by TLC[6], we found that a mixture of methanol and ethyl acetate was effective for eluting TCs from a 500-mg Baker-10 C_{18} cartridge. Because we believed that this mixture would also be

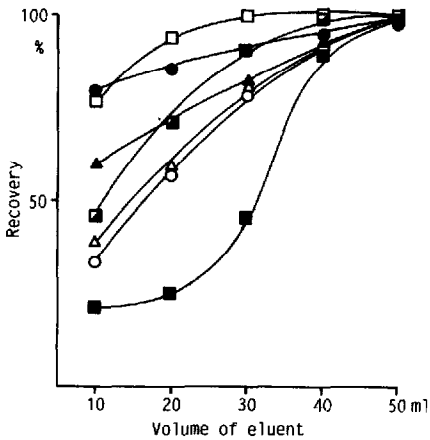


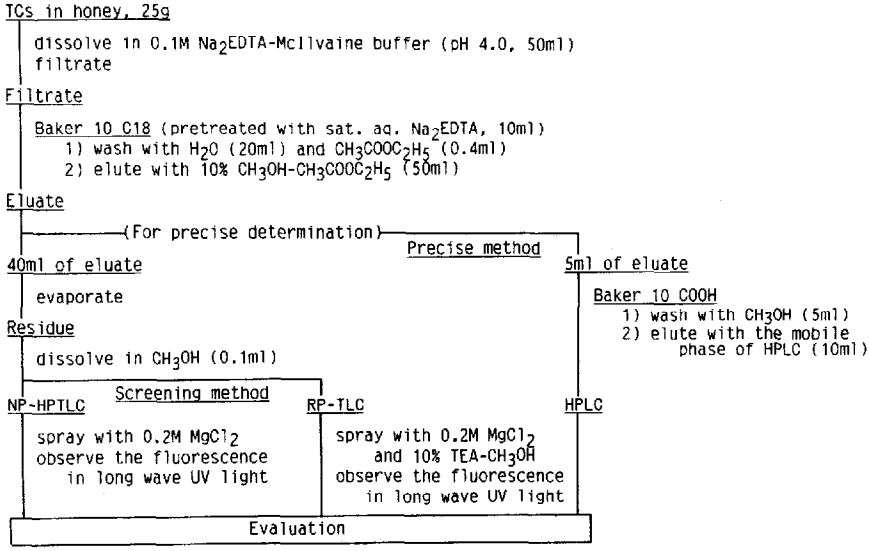
Fig. 3. Effect of the volume of 10% methanol in ethyl acetate solution on the recovery of seven tetracyclines from a Baker-10 C_{18} cartridge. Procedure as in Fig. 2. For symbols, see Fig. 1.

useful as an eluent in the present study, the required concentration of methanol and the volume of the mixture was examined. After application of the TCs (25 μg of each) to the C_{18} cartridge, they were eluted with 50 ml of ethyl acetate containing various amounts of methanol, and then 5 ml of the eluate (total volume 50 ml) were applied to a Baker-10 COOH cartridge. The TCs were eluted with the mobile phase used for HPLC and then determined. All TCs were completely recovered from the C_{18} cartridge by 10% methanol in ethyl acetate. Typical elution curves are shown in Fig. 2. When various volumes of this mobile phase were used as eluents in the same manner, good recoveries of all TCs from the C_{18} cartridge were obtained with $\geqslant$ 50 ml (Fig. 3). Thus in subsequent experiments, 50 ml of 10% methanol in ethyl acetate employed as the eluent for the 500-mg Baker-10 C_{18} cartridge.

Quantitative analysis of TCs by HPLC

On the basis of the preliminary studies described above, a precise analytical method for seven TCs in honey was proposed (Scheme 1). For its elaboration, TCs (25 μg of each) were added at each step and then the recoveries were calculated (Table I). The recoveries of additions 1, 2 and 3 represent those of overall, at the C_{18} and the COOH cartridges steps and at the COOH cartridge step, respectively. Comparison of the recoveries shows that those of TCs after the filtration, from the C_{18} and COOH cartridges were almost 100, 95–97 and 90–95%, respectively. Slight reductions in the recoveries of MTC and MINO at the COOH step were indicated. However, when comparing the chromatograms of honey extracts before and after the COOH cartridge clean-up step, the peaks of interfering substances were found to be eliminated at this step (Fig. 4). This step is essential for the quantitation of the seven residual TCs in honey.

As the results of six replicates, the overall recoveries of seven TCs from honey spiked at a level of 1.0 ppm, their coefficients of variation and detection limits are shown in Table II. Typical chromatograms of extracts from honey are shown in Fig.



Scheme 1. Systematic analytical method for seven residual tetracyclines.

TABLE I

RECOVERY OF TETRACYCLINES FROM HONEY AT EACH STEP

TCs (25 μg) were added at each step. Results of three replicates.

	Recovery (%)		
	Addition 1	*Addition 2*	*Addition 3*
MINO	83– 89	84– 88	88– 90
OTC	92– 94	92– 94	95– 97
TC	88– 94	88– 94	98–100
DMCTC	85– 92	84– 92	98–100
CTC	94–100	95–100	99–100
MTC	79– 87	80– 88	87– 90
DC	93–100	95–100	95–100

5. These results indicate that the present HPLC method in combination with Baker-10 C_{18} and COOH cartridge clean up is applicable to the practical analysis of seven TCs in honey with good recovery, higher accuracy and better detection limit in comparison with the previously reported methods[3,4]. Therefore, we recommend the present method for the precise quantitative analysis of residual TCs in honey.

Detection of TCs by TLC with spray reagents

In previous work[7], we reported that good separations among MINO, CTC, DC, MTC and OTC and among MINO, OTC, TC, DMCTC and CTC were obtained in normal-phase HPTLC and reversed-phase TLC, respectively. The combination of these techniques enabled the identification of seven TCs. Therefore, both TLC sys-

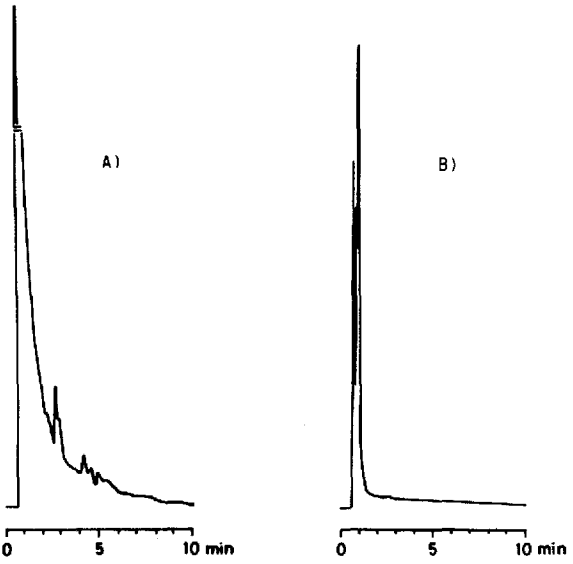


Fig. 4. Comparison of chromatograms before (A) and after (B) clean up on a Baker-10 COOH cartridge. For chromatographic conditions, see Experimental.

TABLE II

RECOVERY OF SEVEN TETRACYCLINES FROM FORTIFIED HONEY

Recovery of TCs from 25 g of honey fortified at a level of 1.0 ppm. Results of six replicates.

	MINO	*OTC*	*TC*	*DMCTC*	*CTC*	*MTC*	*DC*
Recovery (%)	85.7	92.8	91.7	87.8	99.6	83.7	97.0
C.V. (%)	2.1	0.9	2.5	2.9	2.7	4.3	2.7
Detection limit ppm	0.02	0.02	0.02	0.02	0.05	0.05	0.05

tems were used for the detection of TCs with spray reagents in the present work. Our previously established Fast Violet B salt–pyridine method[8] and the magnesium chloride–TEA method reported by Szabó *et al.*[9] were mainly examined. At first, the detection limits of TCs on both plates were investigated with the spray reagents. As shown in Table III, the formation of fluorescent spots under UV light (360 nm) after spraying the normal-phase HPTLC plate with 0.2 *M* magnesium chloride was the most sensitive (MINO, 0.03 μg; others, 0.01 μg). Although the use of this spray reagent followed by 10% TEA in methanol could not detect MINO on both plates, this method gives enough sensitivity for other TCs (0.01 μg). Other combinations of TLC and spray reagents did not give satisfactory results. Therefore, we chose the combinations of 0.2 *M* aqueous magnesium chloride and normal-phase HPTLC, and 0.2 *M* aqueous magnesium chloride followed by 10% TEA in methanol and reversed-phase TLC for detection of MINO, CTC, DC, MTC and OTC and for the detection of OTC, TC, DMCTC and CTC in honey, respectively.

When such TLC methods are applied to the analysis of TCs in honey, the final sample solution should be concentrated because of the low sensitivity. However, the final sample solution in the above double cartridge system (Baker 10 C_{18}/COOH) for

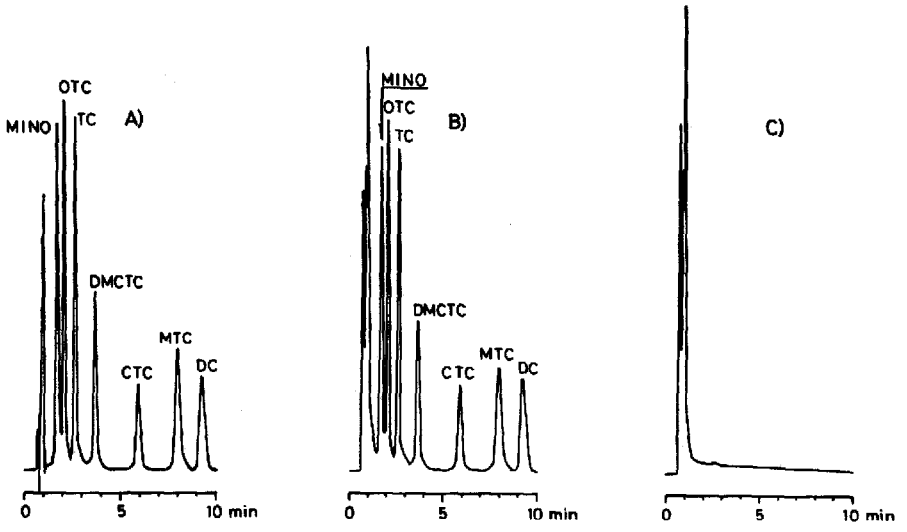


Fig. 5. Typical chromatograms of honey extracts. (A) standard of TCs (10 ng); (B) fortified honey (1.0 ppm); (C) blank of honey. For chromatographic conditions, see Experimental.

TABLE III

DETECTION LIMITS (μg) OF TETRACYCLINES BY TLC WITH DIFFERENT SPRAY REAGENTS

nd = Not detected.

Detection reagent	*Normal-phase HPTLC*		*Reversed-phase TLC*	
	MINO	*Others**	*MINO*	*Others**
1% Fast Violet B and pyridine	0.1	0.03	0.1	0.03
0.2 *M* Magnesium chloride	0.03	0.01	nd	nd
0.2 *M* Magnesium chloride and 10% TEA in methanol	nd	0.01	nd	0.01
0.2 *M* Magnesium chloride and pyridine	0.1	0.05	0.1	0.05

* OTC, TC, CTC, DC, DMCTC and MTC.

precise quantitative analysis contained oxalic acid, so it could not be concentrated. Further, direct elution from the C_{18} cartridge with 10% methanol in ethyl acetate without any washing of the cartridge gave many interfering spots from honey on the TLC plates. Therefore, washing of the C_{18} cartridge with 0.4 ml of ethyl acetate before elution of TCs was tried. As a result, TCs at a level of 0.1 ppm in honey could be detected on the normal-phase HPTLC plate with 0.2 *M* aqueous magnesium chloride as the spray reagent; further, except for MINO, they could be detected at a level of 0.05 ppm on the reversed-phase TLC plate with 0.2 *M* aqueous magnesium chloride followed by 10% TEA in methanol. The washing of the C_{18} cartridge with 0.4 ml of ethyl acetate is essential to detect residual TCs in honey with good sensitivity. In comparison with previously reported methods[3,4], a simpler, more rapid, more reliable and more sensitive screening method for residual TCs in honey could be established (screening method in Scheme 1).

Systematic simultaneous analysis of residual tetracyclines in honey

As shown in Scheme 1, we were able to establish a simultaneous analytical system for seven residual TCs in honey by a combination of the TLC methods and the HPLC method described above. The normal-phase HPTLC and reversed-phase TLC are used as initial screening methods for MINO, CTC, DC, MTC and for OTC, TC, DMCTC and CTC, respectively, after extraction of TCs from honey by the Baker-10 C_{18} cartridge. When spots of TCs were identified by these methods, their precise quantitation could be achieved using HPLC after Baker-10 COOH clean up. Because this system is simple, rapid and reliable, it can readily be employed in most laboratories.

CONCLUSIONS

In order to establish a system for the simultaneous analysis of seven residual TCs in honey which can readily be applied in every laboratory, simple TLC methods and a precise HPLC method were investigated as screening and determination assays, respectively. By a combination of these methods, the simultaneous analytical system shown in Scheme 1 has been established. The TLC screening method can detect TCs

at a level of 0.1 ppm in honey without the need for special equipment, and the precise quantitation by HPLC can determine TCs with good recovery (83.7–99.6%) and coefficient variation (0.9–4.3%). Therefore, we recommended this analytical system for the analysis of seven residual TCs in honey.

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