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Reversed-phase ion-pair chromatographic analysis of tetracycline antibiotics
Application to discolored teeth

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Abstract

A high-performance liquid chromatographic method with diode array detection was developed to simultaneously separate tetracycline antibiotics and applied to the analysis of discolored teeth. By a reversed-phase ion-pair chromatographic system using pentanesulfonate as a counter ion, minocycline, oxytetracycline, tetracycline and demeclocycline were eluted in this order, and they showed base-line separation within 9 min. When using oxytetracycline as an internal standard, the quantitative ranges were between 2.5 ng/ml and 7.5 μg/ml. Powdered dentine (10 mg) and enamel (40 mg) prepared from discolored primary teeth were sonicated in 0.25 ml of 10 m*M* HCl containing oxytetracycline (0.75 μg/ml) and 50 m*M* EDTA-2Na, thereafter the supernatants were chromatographed. Eluates from both discolored tooth samples were identified as minocycline based on diode array spectra of their peaks, while minocycline was not detected in any samples from nondiscolored normal teeth, indicating that discoloration of the tested teeth was due to minocycline incorporated into dentine and enamel. Replicate quantitative analyses of the identical tooth substances showed that intra- and inter-assay C.V.s were 2.63 and 4.95% for dentine, and 5.42 and 10.88% for enamel. Application of the developed method to nine discolored teeth revealed that the incorporated minocycline ranged from 20.13 to 84.62 ng/mg of dentine and 0.89 to 7.87 ng/mg of enamel.

Keywords: Tetracyclines

1. Introduction

As undesirable side-effects, tetracycline antibiotics not only discolor the primary and permanent teeth but also cause hypoplasia in developing teeth when they are administered to infants, mothers during pregnancy and children under 12 years [1–3]. It has also been suggested that discoloration by tetracyclines occurs in adult dentition [4,5]. We recently encountered child patients with the primary and permanent teeth discolored dark grey–brown. Such discoloration was suspected to result from the chemotherapy with a certain tetracycline antibiotic because any other causatives were negative. An

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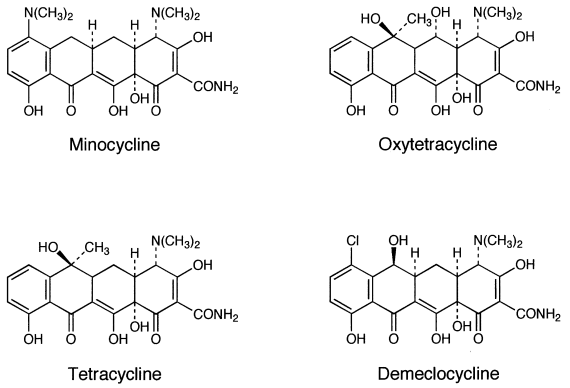

Fig. 1. Structures of tetracycline antibiotics.

analytical method for tetracyclines in the teeth was required to assess if tetracycline antibiotics were actually present in the tooth matrix and if so, which and how much tetracycline was incorporated into dentine and enamel.

Different methods by high-performance liquid chromatography (HPLC) have been previously reported to analyze tetracycline antibiotics [6–11]. However, their applications are limited to tissues [6,8–11] and body fluids [7]. In addition, previous HPLC separation showed tailing of the tetracycline peaks on chromatograms [7,8,11].

In the present study, an HPLC method was developed to simultaneously separate four representative tetracycline antibiotics (Fig. 1). Since the tested tetracyclines commonly possess an amino group to form an ion-pair with an anionic counter ion, they were chromatographed by a reversed-phase ion-pair system using alkyl sulfonate. After optimization of the HPLC conditions, the proposed method was applied to the identification and quantitative analysis of tetracycline antibiotics incorporated into dentine and enamel of the discolored teeth obtained from patients.

2. Experimental

2.1. Reagents and chemicals

Minocycline hydrochloride and demeclocycline hydrochloride, and tetracycline hydrochloride and oxytetracycline hydrochloride were purchased from Sigma (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively. Stock solutions (1.0 mg/ml of each) were prepared every month by dissolving the standards in 10 m*M* HCl. They were then stored at 4°C. They were diluted as required with 10 m*M* HCl before use. Sodium alkyl sulfonates with different chain-lengths (carbon number: 2 to 8) were of ion-pair chromatographic grade (Tokyo Kasei, Tokyo, Japan). Acetonitrile of HPLC grade was obtained from Kishida (Osaka, Japan). All other reagents were of the highest analytical grade available. Water was redistilled by an all-glass apparatus after purification by a Milli-RO water purification system (Nihon Millipore, Tokyo, Japan).

2.2. Tooth samples

Teeth were obtained from child siblings seeking dental treatment for discoloration of both primary and permanent teeth at the Hospital of Asahi University School of Dentistry (Hozumi, Japan). Nine shed primary teeth were selected randomly. Dentine and enamel were powdered using an agate mortar and pestle (100–200 mesh).

2.3. Sample preparation

Elution procedure was performed using a glass tube with a cap (50×5 mm I.D.), which was silanized to avoid the adsoption of analytes onto the glass surface [12]. Powdered dentine (10 mg) or enamel (40 mg) was immersed in 0.25 ml of 10 m*M* HCl containing oxytetracycline (0.75 μg/ml) and 50 m*M* EDTA-2Na which were added to the glass tube together with EDTA-2Na (10 mg). After vortex-mixing for 3 s, the immersion solution was sonicated for 5 min in a USC-150 sonicater (Kimura, Osaka, Japan). After centrifugation (1200 *g*, 1 min), the supernatant was filtered through a pore-size of 0.22 μm. An aliquot (40 or 100 μl) of the filtrate was subjected to HPLC. This procedure was repeated at least five times in quantitative analyses.

2.4. HPLC analysis

The HPLC system consisted of an LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan), a KMT-30A autosampler (Kyowa Seimitsu, Tokyo, Japan), a

Shim-pack CLC-C_8 (M) column (250×4.6 mm I.D., particle size 5 μm, Shimadzu) placed in a thermo-controller (Kyowa Seimitsu) and an SPD-M10AVP diode array detector (Shimadzu) controlled by an FMV-5133D5 personal computer (Fujitsu, Tokyo, Japan). The mobile phase consisted of acetonitrile–50 m*M* sodium phosphate buffer, pH 1.75 (26:74, v/v), to which 10 m*M* sodium pentanesulfonate was added. The mobile phase was delivered at a flow-rate of 1.0 ml/min and at a column temperature of 50°C. In routine analyses of the teeth, eluates from the column were detected at 354 nm. The tetracycline concentrations were determined based on the peak area ratios to oxytetracycline used as an internal standard by reference to the calibration graphs prepared as described in Section 2.6.

2.5. Optimization of HPLC conditions

Standard tetracycline antibiotics (5.0 μg/ml of each) were chromatographed as described in Section 2.4 by varying the type (carbon number: 2 to 8) and concentration (0 to 20 m*M*) of sodium alkyl sulfonates, the phosphate buffer pH (1.5 to 2.5) and the acetonitrile concentration (22 to 38%, v/v). The chromatographic conditions were optimized by calculating capacity and resolution factors. The results are expressed by the means of different determinations (n=2–3).

2.6. Analytical evaluation

To evaluate the quantitative range, calibration graphs were prepared by plotting the mean peak area ratios (n=2–4) of each antibiotic to oxytetracycline against the known concentrations. Standard minocycline, tetracycline and demeclocycline solutions (2.5 ng/ml to 10.0 μg/ml of each) were chromatographed as described in Section 2.4.

The intra- (n=6) and inter-assay C.V.s (n=4) were determined by quantitatively analyzing the identical dentine and enamel samples as described in Sections 2.3 and 2.4.

3. Results and discussion

Discoloration of the tested teeth was suspected to be due to the treatment with a certain kind of tetracycline antibiotic during the formative period of the teeth. Therefore, reversed-phase ion-pair chromatography was first studied to separatively analyze the representative tetracyclines (Fig. 1) and identify an antibiotic incorporated into the teeth as a discoloring agent.

When using a C_8 column and different alkyl sulfonates, the separation of four tetracycline antibiotics was influenced by the type of counter ions as shown in Fig. 2. The capacity factors of all tetracyclines increased with lengthening alkyl chains

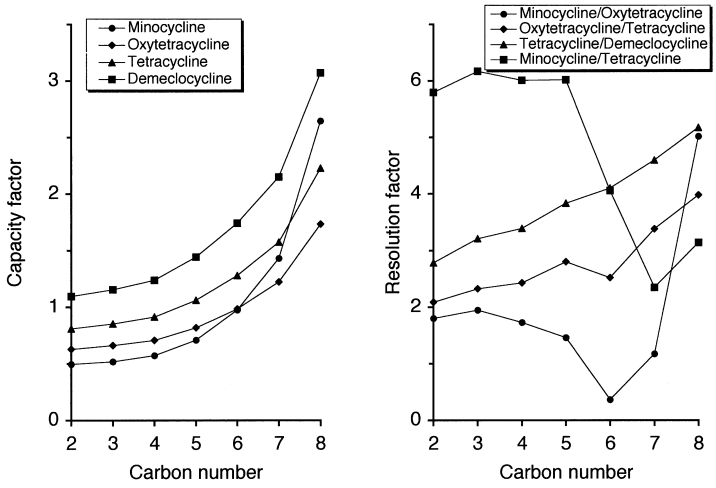

Fig. 2. Effect of the carbon number of alkyl sulfonates used as a counter ion on the capacity and resolution factors.

(carbon number varying from 2 to 8), leading to enhancement of the retention. Minocycline especially showed a characteristic increase in capacity factors. Although minocycline was eluted first using shorter alkyl sulfonates (carbon number: 2 to 5), its elution order was delayed with increasing length of chain. Such changes may be due to a dimethylamino group at the 7-position which is present only in minocycline, but not in the other tetracyclines. That substituent possibly ionizes in the acidic mobile phase and additionally contributes to the ion-pair formation, resulting in greater retention on the solid-phase. The separation among tetracyclines was improved with an increase of the carbon number. However, the resolution factors between minocycline and oxytetracycline or tetracycline decreased using counter ions of carbon number 6–8 because of the significantly enhanced retention of minocycline. As a compromise between rapid separation and high resolution, pentanesulfonate was chosen as counter ion.

The retention and separation of tetracycline antibiotics changed by varying concentrations of sodium pentanesulfonate as shown in Fig. 3. The capacity factors of all tetracyclines increased with an increase of concentration, reaching a plateau over 15 m*M*. The separation of minocycline and oxytetracycline became poor with increasing concentrations because of the greater effect of pentanesulfonate concentrations on minocycline. Therefore, the concentration of 10 m*M* was chosen.

The other chromatographic conditions such as acetonitrile concentration and buffer pH of the mobile phase were similarly optimized as described in Section 2.5. Using optimal conditions, the elution order was minocycline, oxytetracycline, tetracycline and demeclocycline (Fig. 4). Their base-line separation was completed within 9 min. Although several reversed-phase HPLC methods were previously reported, their chromatograms showed tailing of the tetracycline peaks [7,8,11]. In the present HPLC separation, however, the peak tailing was suppressed by employing the ion-pair chromatographic system.

When using oxytetracycline as an internal standard, the calibration graphs showed a good linearity in concentrations ranging from 2.5 ng/ml to 7.5 μg/ml for minocycline, 10.0 ng/ml to 7.5 μg/ml for tetracycline and 25.0 ng/ml to 7.5 μg/ml for demeclocycline. The regression equations were found to be $y=1.2383x+0.0929$ ($r^2=0.998$) for minocycline, $y=1.3037x+0.1109$ ($r^2=0.9976$) for tetracycline and $y=0.9886x+0.0426$ ($r^2=0.9979$) for demeclocycline.

Solubilization of tooth substances is essential for the quantitative analysis of the drugs incorporated in dentine and enamel. Although a demineralization method has been proposed for tooth and bone matrix [13], its procedural conditions, such as heating and

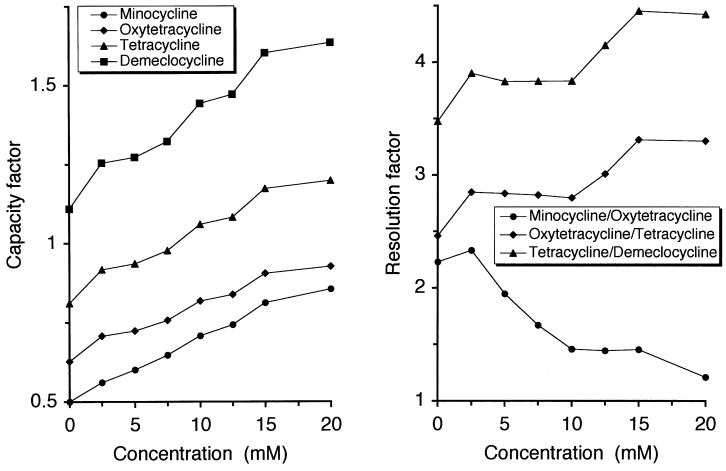

Fig. 3. Effect of the concentration of sodium pentanesulfonate on the capacity and resolution factors.

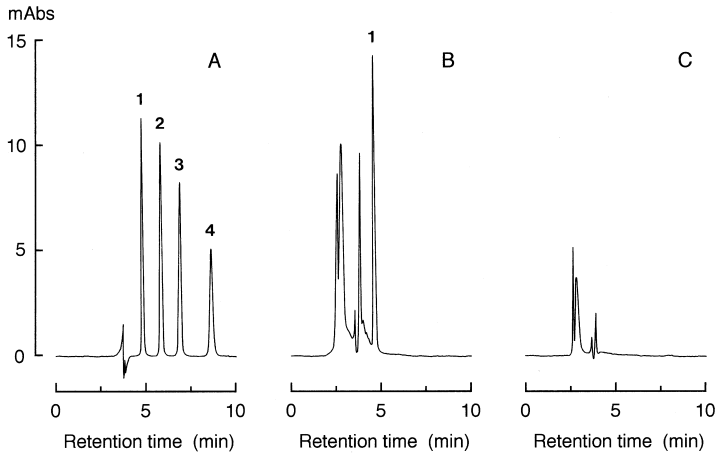

Fig. 4. Chromatograms obtained from standard and dentine samples. (A) Standard tetracyclines (0.5 μg/ml of each), (B) dentine (10 mg) from discolored tooth and (C) dentine (10 mg) from nondiscolored normal tooth. Detection at 354 nm. Peaks: 1=minocycline, 2=oxytetracycline, 3=tetracycline and 4=demeclocycline.

long immersion time, affect the stability of tetracycline antibiotics, and it does not achieve full solubilization of tooth substances. Tetracyclines chelate calcium ions to form the insoluble complexes and remain in calcifying tissues, producing a grey–brown discoloration of teeth [5,14,15]. Since this complex formation occurs under alkaline conditions (pH range from 8 to 10) but not under acidic conditions [16], dissociation of the calcium-complex to solubilize analytes in the discolored teeth was performed by sonicating powdered dentine and enamel in 10 m*M* HCl in the presence of EDTA. Chromatograms obtained after sonication of dentine samples for 5 min are shown in Fig. 4. One major peak was eluted from all the discolored teeth, but not from nondiscolored normal teeth. The same elution characteristics were also found in the analyses of enamel samples. The eluate from both dentine and enamel was identified as minocycline based on the diode array spectra and capacity factor of the peak. These results indicate that discoloration was caused by minocycline incorporated into the tooth matrix.

Minocycline eluted from the discolored teeth was analyzed by repeating the sonication procedure. The elution profiles of four different teeth are shown in Fig. 5, which were obtained by plotting the peak area ratios of the eluted minocycline to oxytetracycline for every 5 min sonication. In both dentine and enamel samples, the peak area ratios drastically decreased depending on the number of sonications. At least five consecutive sonications lead to almost completed solubilization sufficient to quantitatively analyze minocycline in both tooth matrices.

Dentine and enamel were sonicated in the presence of oxytetracycline as an internal standard,

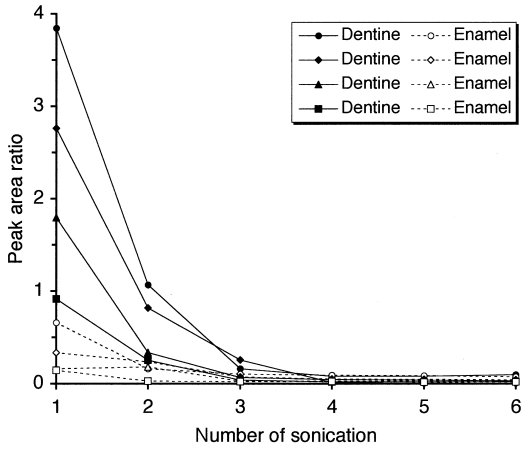

Fig. 5. Elution profiles of minocycline from dentine and enamel. Four different dentine (10 mg) and enamel (40 mg) samples were sonicated and for every 5 min sonication, the peak area ratios of the eluted minocycline to oxytetracycline were plotted against the number of sonications.

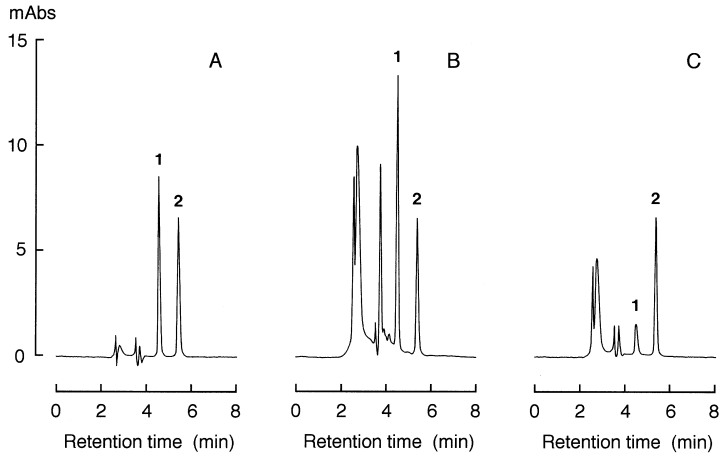

Fig. 6. Chromatograms obtained from standard and tooth samples. (A) standard minocycline (0.75 μg/ml), (B) dentine (10 mg) from discolored tooth and (C) enamel (40 mg) from discolored tooth. Detection at 354 nm. Peaks: 1=minocycline and 2=oxytetracycline.

thereafter minocycline was quantitatively analyzed. Chromatographic results obtained from the first sonication for 5 min are shown in Fig. 6. While unknown front peaks appeared, minocycline and oxytetracycline were analyzed without significantly interfering peaks.

Analytical reproducibility was evaluated by analyzing replicate dentine and enamel samples prepared from the same discolored tooth either on the same day (intra-assay, $n=6$) or on different days (inter-assay, $n=4$). The intra- and inter-assay C.V. values were 2.63 and 4.95% for dentine and 5.42 and 10.88% for enamel, respectively.

Minocycline was determined by totalizing the amount of minocycline eluted after five consecutive sonications. The quantitative results of dentine and enamel from nine discolored teeth are shown in Table 1. The incorporated minocycline ranged from 20.13 to 84.62 ng/mg of dentine and 0.89 to 7.87 ng/mg of enamel. The quantitative values were found to increase with increasing discoloration of the teeth. The administered tetracycline antibiotics first diffuse into the mineralization site of dentine, where tetracyclines are subjected to the complexing reaction with calcium ions, and are thereafter retained firmly [5,14,15]. Therefore, minocycline content is greater in dentine than in enamel.

Chemotherapy with tetracycline antibiotics during the formative period of the teeth is known to discolor the primary and permanent teeth as an adverse side-effect, resulting in an esthetic problem [1–5]. A fluorescent microscopic method is generally applied to trace tetracyclines in dental hard tissues [17–21]. However, it has neither quantitatively dealt with the amounts of tetracyclines incorporated into the teeth nor independently analyzed each individual tetracycline. The proposed method will be a useful tool for the determination of tetracycline antibiotics retained in teeth and the employed ion-pair separation would be applicable to the separative analysis of different tetracyclines in other biological samples.

Table 1
Minocycline in dentine and enamel of the discolored teeth

Discolored teeth	Minocycline content (ng/mg)	
	Dentine	Enamel
Lower molar	84.62	4.46
Lower canine	65.05	5.93
Upper molar	66.09	3.06
Lower molar	61.92	1.70
Lower canine	54.19	7.87
Upper incisor	38.56	1.82
Upper molar	28.66	1.43
Upper incisor	25.32	0.89
Upper incisor	20.13	0.94

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