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Separation of tetracycline and its degradation products by capillary zone electrophoresis

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ABSTRACT

The capillary zone electrophoresis of tetracycline and its degradation products is described. Various substances, including surfactants and metal ions, were employed as additives in the running electrolyte to improve the resolution. Using disodium ethylenediaminetetraacetate as an additive in phosphate buffer solution, tetracycline and its degradation products were completely separated from each other. The baseline separation obtained completely meets the needs for determining the contents of tetracycline, 4-epitetracycline, anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) in tetracycline samples. Further, two unknown substances co-existing with ATC and EATC were separated that had not been mentioned or separated before. Further investigations using other techniques are needed to determine the structures of these two unknown substances. The pH dependence of the migration behaviour of tetracycline and its degradation products was also explored. The migration behaviour of zwitterionic substances is so dependent on the pH of the buffer than the elution order may be changed with change in pH. The pH dependence of electroosmotic flow over the pH range 4–11 was also determined.

INTRODUCTION

Capillary zone electrophoresis (CZE) has been used in a variety of modes to separate diverse molecules as a complementary technique to HPLC. Small metal ions [1] and large nucleic acids [2], in addition to electrically neutral species such as chlorinated benzenes [3], have been successfully separated by CZE.

The migration of a solute in CZE is influenced by both the electroosmotic and electrophoretic mobilities. The electroosmotic flow allows solutes of opposite charges to migrate toward the detector end. The electrophoretic mobility may vary from one solute to another, which leads to different net velocities for the various analytes and brings about their separation. To induce different electrophoretic velocities for the solutes to be separated, reagents that may associate with the analytes either strongly or weakly are added to the buffer. These interactions between additives and solutes cause the solutes to migrate at different velocities owing to the difference in the magnitude of solute–additive association. The reagents that have been employed as buffer additives in CZE include surfactants [4–7], organic solvents [8–10], inorganic salts [11,12] and tetraalkylammonium salts [13]. In this work disodium ethylenediaminetetraacetate (EDTA) was used as the additive, by means of which tetracycline and its degradation products were successfully separated.

Tetracycline (TC) is one of the most important broad-spectrum antibiotics, and in acidic media (pH < 2) may be degraded to form anhydrotetracycline (ATC). In acidic solutions (pH 2–6), TC and ATC also epimerize to form 4-epitetracycline (ETC) and 4-epianhydrotetracycline (EATC), respectively [14]. The structures of these substances are shown in Table I.

Commercial tetracycline samples usually contain

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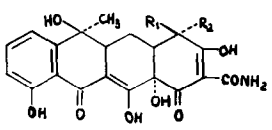
TABLE I

STRUCTURES OF THE FOUR TETRACYCLINES



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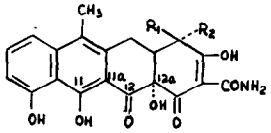

II

Compound	Abbreviation	No. on Figs.	Structure	R_1	R_2
Tetracycline	TC	1	I	$N(CH_3)_2$	H
4-Epitetracycline	ETC	2	I	H	$N(CH_3)_2$
Anhydrotetracycline	ATC	3	II	$N(CH_3)_2$	H
4-Epianhydrotetracycline	EATC	4	II	H	$N(CH_3)_2$

ETC, ATC and EATC, and differ widely in biological potency. Many papers concerning the determination and separation of tetracyclines by HPLC have been published, but the use of CZE has not previously been reported. This paper describes the separation of tetracycline and its degradation products, ETC, ATC and EATC, by CZE. The dependence of the migration behaviour of tetracyclines on the pH of the buffer medium was examined. The effect of disodium EDTA on the separation of tetracyclines was also studied. As will be shown, the selectivity of tetracyclines could be improved by the addition of EDTA. The mechanism by which EDTA improved the separation of tetracyclines is discussed.

EXPERIMENTAL

Apparatus

CZE was performed on an HPE 100 high-performance capillary electrophoresis system from Bio-Rad Labs. (Richmond, CA, USA), in the positive polarity mode, using an uncoated fused-silica capillary cartridge of 20 cm × 25 μm I.D. The applied voltage was 10 kV. Sample solutions were injected into the capillary by electromigration for 5 s at 1 kV. All analyses were carried out at ambient temperature (295–300 K). Detection was performed by monitoring the absorbance at 265 nm. The signal from the detector was processed with a C-R3A Chromatopac (Shimadzu, Kyoto, Japan).

Chemicals and reagents

Phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate and disodium EDTA were of analytical-reagent grade from Beijing Chemical Reagent Co. (Beijing, China). TC was a National Reference Standard (China) for potency assay and ETC, ATC and EATC were reference substances for purity testing by TLC inspection according to the Chinese Pharmacopoeia.

Procedures

Phosphate buffers in the pH range 2–9 were prepared by mixing solutions of phosphoric acid, sodium dihydrogenphosphate and disodium hydrogenphosphate, all of concentration 0.02 *M*, in appropriate proportions. TC, ETC, ATC and EATC were freshly dissolved in 0.02 *M* phosphate buffer (pH 2.2) to give a series of test solutions of concentration 0.05–0.2 mg/ml. Solutions of neutral solutes (20 mg/ml methanol, 0.001 mg/ml phenol and 0.01 mg/ml benzene) for monitoring electroosmotic flow were prepared in the same way as for tetracyclines. Each value of the migration time in the figures was the average of three repeated runs, the R.S.D. being from 0.3% to 3%.

RESULTS AND DISCUSSION

pH dependence of the separation of tetracyclines

Electroosmotic flow in fused-silica capillaries is influenced by the pH of the medium because the

dissociation of the silanol groups on the fused-silica capillary surface is pH dependent. The electrophoretic flow of a solute is related to its charge, size, etc. Tetracyclines are amphoteric in aqueous solution, the charge and sign being dependent on the pH of the solution. Therefore, the pH of the running electrolyte is important for the separation of tetracyclines. Electroosmotic flow transports all the solutes at the velocity of the medium solution, which shortens the migration time. To determine the electroosmotic velocity, a neutral substance such as methanol [15] or phenol [16] was added to the sample solutions. In our experiments a mixed sample solution of methanol, phenol and benzene was injected into the capillary and detection was performed at 190 nm. These three different components wih different structures and hydrophilic characters always coeluted in the pH range 2–9, which shows that neither of the neutral components undergoes interaction with the capillary wall and their migrations were cuased solely by the electroosmosis, thus providing a means for the determination of the electroosmotic flow.

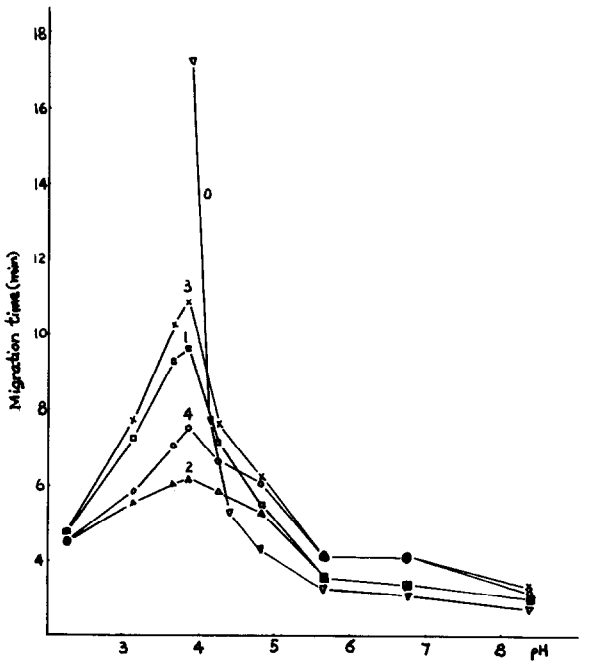

Fig. 1. Dependence of migration time of tetracyclines and neutral solutes on the pH of the buffer in CZE. The numbers on the plots corespond to the solutes in Table I; in addition, 0 is used to designate the mixture of neutral solutes. Conditions: capillary, 20 cm × 50 μm I.D., uncoated; buffers, 0.02 *M* phosphate solutions of various pH; applied voltage, 10 kV; current, 17–20 μA; electroinjection, 1 kV for 5 s; temperature, ambient (295–300 K); detection wavelength, 265 nm for tetracyclines and 190 nm for the neutral solutes.

The pH dependence of the migration time of tetracyclines and the neutral solutes is shown in Fig. 1. The electroosmotic flow velocity indicated by the neutral solutes increases with increase of pH, which can be ascribed to the increasing dissociation of silanol groups on the capillary wall. The migration times of the neutral solutes changed sharply near pH 4.

Tetracyclines have three functional groups relevant to acid–base equilibrium in aqueous solution, namely a dimethylamino group (D, pK_a = 9.5), a phenolic diketone group (P, pK_a = 7.7) and a tricarbonylmethane group (T, pK_a = 3.3) [17]. When the pH of the buffer was below the isoelectric point, tetracyclines were positively charged and migrated towards the negative electrode in the same direction with electroosmotic flow due to the protonated D group, and consequently eluted before the neutral solutes, as shown in Fig. 1. In contrast, at pH above the isoelectric point, owing to the dissociation of the T and P groups, the negatively charged tetracyclines eluted after the neutral solutes. According to our experimental CZE results, the isoelectric points of tetracyclines are in range pH 4.0–4.5, which are below the calculated value of pH 5.5.

The dependence of the migration time of a solute is the sum of the dependences of its electrophoretic movement and electroosmotic movement. At pH < 4, the migration time of tetracyclines increased with increase in pH, which shows that a decrease in the electrophoretic movement of tetracyclines had occurred as positive ions had overcome the increase in electroosmotic flow. However, at pH > 4.5, the increase in the electroosmotic flow became dominant and consequently the migration times decreased with increase in pH.

It can be seen from Fig. 1 that the pH of the running buffer could dramatically affect the separation of tetracyclines and the optimum pH value was in range 3.5–4.5, near their isoelectric points. A typical electropherogram of tetracyclines at pH 3.9 is shown in Fig. 2.

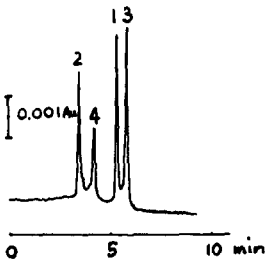

Fig. 2. Electropherogram of tetracycline and its degradation products at the best pH value 3.9. Other conditions as in Fig. 1 and solute numbers as in Table I.

Effect of EDTA on the separation of tetracyclines

To improve the resolution of tetracyclines, metal ion additives that may chelate with them were tested. When $CuSO_4$, $ZnSO_4$ and $MgSO_4$ were added to the buffer, no improvement in resolution was achieved. When EDTA was added to the phosphate buffer, a dramatic improvement in the resolution was observed. Complete separation of TC, ETC, ATC and EATC was achieved with relatively sharp peaks by addition of 0.005 *M* EDTA to 0.02 *M* phosphate buffer (pH 3.9), as shown in Fig. 3. Their resolution is sufficient to be able to determine unequivocally the contents of TC, ETC, ATC and EATC in samples. In comparison with the HPLC trace obtained by US Pharmacopeia method [18], the results shown in Fig. 3 have the advantages of better resolution and shorter analysis time, in addition to the simplicity of CZE.

EDTA is a well known complexing reagent,

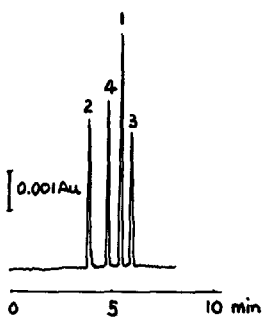

Fig. 3. Separation of tetracycline and its degradation products by CZE with 0.005 *M* EDTA as additive in 0.02 *M* phosphate buffer (pH 3.9). Other conditions as in Fig. 1 and solute numbers as in Table I.

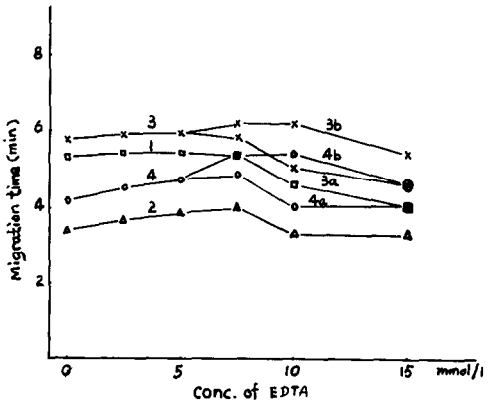

Fig. 4. Effect of EDTA concentration on the separation of tetracycline and its degradation products by CZE. Other conditions as in Fig. 3. Solute numbers as in Table I; in addition, 3a, 3b, 4a and 4b correspond to ATC-a, ATC-b, EATC-a and EATC-b, respectively.

which acquires a negative charge in aqueous solution near pH 4 and may interact with the positively charged tetracyclines to form ion pairs. These interactions lead to a decrease in the electrophoretic mobility of tetracyclines as positive ions, and as a result the migration times gradually increase with increasing EDTA concentration below 0.0075 *M*, as shown in Fig. 4. However, at EDTA concentrations > 0.0075 *M*, the variation of the migration time became equivocal owing to the splitting of the ATC and EATC peaks.

TC, ETC, ATC and EATC could be completely separated using EDTA concentrations from 0.002 to 0.005 *M*. However, at higher EDTA concentrations each peak corresponding to ATC and EATC split into two with nearly the same peak areas as shown in Figs. 4 and 5. The ATC samples employed here contained a small amount of its opposite epimer EATC, and the EATC samples contained some ATC. As Fig. 5A shows for ATC samples, the second peak relating to ATC, is larger and the smaller peak (the first peak) relates to EATC, whereas for EATC samples as shown in Fig. 5B, the first peak, relating to EATC, is larger and the smaller peak (the second peak) relates to ATC. However, at 0.0075 *M* EDTA, each of the ATC and EATC peaks divided into two, as shown in Fig. 5C and D. These phenomena cannot yet be interpreted, but they may tentatively explained as follows.

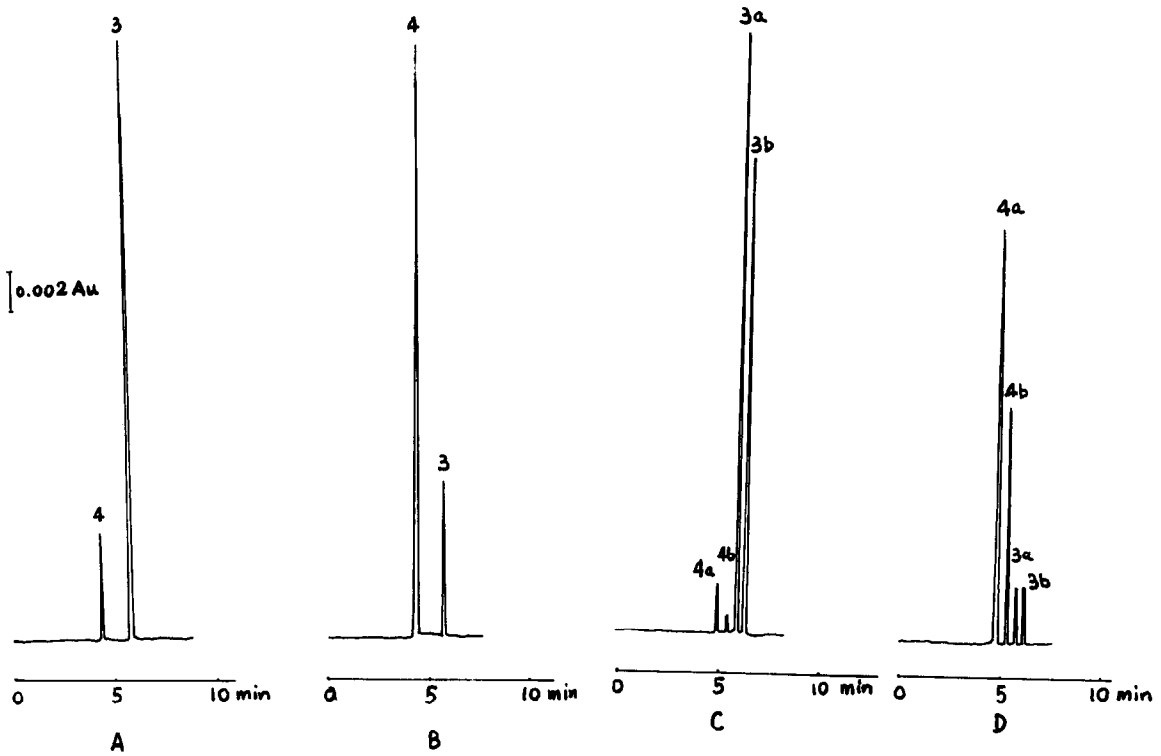

Fig. 5. CZE of ATC and EATC samples with EDTA as additive in 0.02 *M* phosphate solution (pH 3.9). (A) ATC samples, EDTA concentration 0.0025 *M*; (B) EATC samples, EDTA concentration 0.0025 *M*; (C) ATC samples, EDTA concentration 0.0075 *M*; (D) EATC samples, EDTA concentration 0.0075 *M*. Other conditions and solute number are as in Fig. 4.

The splitting of ATC and EATC peaks cannot be generated either with the injection technique or by a mismatch in the pH of the sample and running buffer, because the same phenomenon did not occur for the TC and ETC peaks simultaneously. The experimental results therefore suggest that there are two major components with about the same concentration in each of the ATC or EATC samples. For ATC samples (Fig. 5C), the responses of the last two peaks are greater than those of the first two, whereas for EATC samples (Fig. 5D), the result is exactly the reverse. Hence the last two peaks correspond to the two components relating to ATC samples, which we name ATC-a and ATC-b. The first two peaks correspond to the two components relating to EATC samples, named EATC-a and EATC-b. These four unknown degradation products of TC have not been mentioned or separated by any technique previously. It can be seen from Fig. 4 that ATC-a co-eluted with ATC-b and EATC-a co-eluted with EATC-b when the EDTA concentrations were below 0.005 *M*. At 0.015 *M* EDTA, EATC-a co-eluted with TC and EATC-b

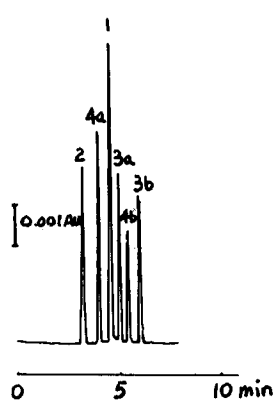

Fig. 6. Separation of tetracycline and its five degradation products by CZE with 0.01 *M* EDTA as additive in 0.02 *M* phosphate buffer (pH 3.9). Other conditions and solute numbers as in Fig. 4.

co-eluted with ATC-a. TC and its five degradation products, ETC, ATC-a, ATC-b, EATC-a and EATC-b, could be successfully separated from one another using 0.01 *M* EDTA, as shown in Fig. 6.

The determination of the structures of the four unknown substances requires further study by other techniques. We consider that ATC-a and ATC-b or EATC-a and EATC-b may be pairs of isomers. The difference in structure between such pairs of isomers perhaps lies in the exchange of positions of hydroxy and carbonyl groups at C-11 and C-12, or the stereo direction of the hydroxy group at C-12a.

CONCLUSIONS

The pH of the buffer is a critical factor for the separation of zwitterionic substances, the charge and sign of which are considerably affected by the pH. The migration behaviour and selectivity of these substances are so dependent on pH that the elution order may be changed. The optimum pH for the separation of TC and its degradation products was in range 3.5–4.5, slightly below the isoelectric point of TC of pH 5.5. However, even at the optimum pH complete separation was not achieved in phosphate buffer without any additive. The addition of EDTA to the buffer yielded a baseline separation of TC and its degradation products. The EDTA buffer system is suitable for the determination of the contents of TC, ETC, ATC and EATC in one analysis. Further, each of the ATC and EATC samples was separated into two large peaks and two small peaks at higher EDTA concentration, which had not been reported previously. The structures of the compounds represented by these four peaks need further study by other techniques.

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