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Capillary electrophoresis of some tetracycline antibiotics coupled with reductive fast cyclic voltammetric detection

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Abstract

The separation and quantitative performance parameters for tetracycline, chlortetracycline and oxytetracycline antibiotics were investigated by capillary zone electrophoresis coupled with fast cyclic voltammetric detection. Optimization of pH and complexation with a boric acid–sodium tetraborate buffer provided good resolution of all compounds. Detection by electrochemical reduction using fast on-line cyclic voltammetric detection with a Hg-film μm-electrode gave detection limits (2×peak-to-peak baseline noise) of $7 \cdot 10^{-7}$ mol/l for tetracycline and chlortetracycline, and $1.5 \cdot 10^{-6}$ mol/l for oxytetracycline. The influence of electrode material, potential range and scan rate was examined and discussed. Optimal electrochemical detection was obtained at a Hg-film electrode with a waveform that consisted of an initial constant potential of −0.6 V for 200 ms followed by a cyclic voltammetry (CV) scan at 300 V/s from −0.6 V to a vertex potential of 1.7 V. The analytical signal was obtained by plotting the integrated values of the CV current from each applied waveform as a function of time. The calibration plot (peak areas) for each separated peak was found to be linear over three-orders of magnitude.

Keywords: Cyclic voltammetry; Electrochemical detection; Detection, electrophoresis; Pharmaceutical analysis; Antibiotics; Tetracyclines

1. Introduction

Tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC) are important antibiotics widely used to control bacterial infections in both humans and animals, and are extensively utilized as animal feed additives [1,2]. Their structures and pK_a values in Table 1 show that these compounds are very similar. Polarography/voltammetry has been used extensively as a sensitive analytical method, and has been reviewed by Siegerman [3] and Smith and Vos [4]. Due to their similar redox properties, prior separation is required to differentiate among these compounds [5] when electrochemical detection is used. The separation of tetracycline antibiotics has been investigated extensively by high-performance liquid chromatography (HPLC) with UV detection [6–10], and recently with electrochemical detection (amperometric detection by oxidation) [11,12]. The peaks of these compounds tend to tail and exhibit low efficiency due to interaction with the residual silanol groups on silica-based packing materials. Thus, polymer-based columns have been used for separation of these compounds, but long separation times are required [9,10].

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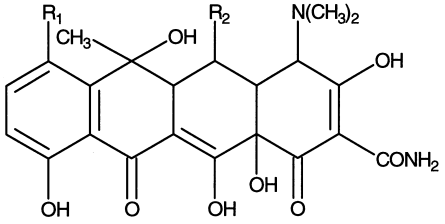
Recently there have been some reports on the separation of tetracycline antibiotics by capillary electrophoresis (CE) with UV detection [13–23]. Most of these aqueous CE separations have been carried out in carbonate and phosphate buffer systems, but there have been at least two studies reported for micellar electrokinetic capillary chromatography (MECC) [17,18]. Tjørnelund and Hansen [23] have stated that it is difficult to separate mixtures of TC, CTC and OTC completely in such aqueous buffer systems even when using MECC. All of these reported studies have used UV detection. An alternate approach that may offer improved detection and separation is the use of fast cyclic voltammetric detection with borate electrolyte systems.

Boric acid–sodium tetraborate electrolytes are of interest because they might complex with the analytes, which have structures that are similar to carbohydrates, and such complexation could possibly improve selectivity and resolution. A borate–phosphate electrolyte has been used in a micellar electrokinetic separation [18], but only one borate concentration was used and there appeared to be no recognition that it might be possible to use borate complexation to adjust resolution. A simple borate electrolyte would also be advantageous for electrochemical detection as the presence of surfactants would influence electrode behavior in applications of fast cyclic voltammetry (CV), which is a detection method recently introduced for CE separation [24,25] of metal ions. This CV detection approach can offer detection limits in the 10^{-8} to 10^{-9} mol/l range for metal ions, but there are a number of factors that must be investigated before this approach could be recommended for organic analytes such as tetracyclines. The previous studies of CV detection [24,25] were for metal ions that could be preconcentrated by deposition at the electrode surface. While one would expect that it may be possible to select suitable potential ranges to allow certain organic compounds to accumulate at an electrode surface, it is uncertain whether water-soluble compounds, such as tetracyclines, would show as efficient a preconcentration behavior. In addition the applied potential and the nature of the electrode material can have a important influence on redox behavior, and these aspects must also be investigated.

In this paper we report on a study of the feasibility of the CE separation of TC, CTC and OTC in a boric acid–sodium tetraborate buffer system coupled with fast CV detection. The detection parameters investigated included applied waveform, scan rate, preconcentration potentials, and electrode material. In addition, peak resolution was examined as a function of the concentration of borate.

Table 1
Structures and pK_a values for of TC, CTC and OTC



Compound	R_1	R_2	pK_a [16]
Tetracycline	H	H	3.46, 7.39, 9.59, 12.10
Chlortetracycline	Cl	H	3.60, 7.52, 9.88, 10.40
Oxytetracycline	H	OH	3.57, 7.49, 9.44, 10.5

2. Experimental

2.1. Chemicals

TC, CTC and OTC (all in hydrochloride form) were obtained from Sigma (Oakville, Canada). Sodium tetraborate and boric acid were obtained from BDH (Toronto, Canada). All reagents used were of analytical grade and were used without further purification; distilled–deionized water was used throughout. Prior to the separation and electrochemical measurements all buffer solutions were purged with pure nitrogen for 15 min, and then transferred to the small buffer reservoirs. The buffer solution consisted of 0.1 mol/l borate containing 1 mmol/l Na_2EDTA (added to prevent the complexation of metal ions with TC, CTC and OTC), and was adjusted to the desired pH values with 0.5 mol/l boric acid. Benzoquinone was used as a neutral marker for the measurement of electroosmotic flow (EOF) by its reduction peak at −1.4 V. Stock solutions of 1 mg/ml TC, CTC and OTC were prepared in buffer solution (pH=8.7) and diluted with the operating buffer solutions as necessary.

When stored at 4°C, these stock solutions could be used over a period of three days.

2.2. Apparatus and procedures

The output of a 30 kV power supply (Spellman, High-Voltage Electronics, Model RHR30PN30, Plainview, NY, USA) was placed in a Plexiglas box equipped with a safety switch on the access door. The separation was performed in a fused-silica capillary, 80 cm×50 μm I.D (Polymicro Technology, Phoenix, AZ, USA). Prior to use the capillaries were rinsed with 1 mol/l HCl (this HCl wash is only necessary for new capillaries) for 10 min, 0.1 mol/l NaOH for 15 min, water for 5 min, and finally with operating buffer for 10 min. The samples were hydrodynamically introduced into the capillary by elevating the capillary to a 10-cm height for 15 s.

Electrochemical detection was carried out with a three electrode system in a Faraday cage. The counter electrode was a Pt wire (surface area about 0.5 mm^2) and a saturated KCl calomel electrode (Miniature model, Fisher Scientific, Ottawa, Canada) was used as a reference electrode. The working electrode consisted of a Hg-film (~6 μm thick) μm-electrode, which was prepared by computer-controlled electrodeposition of a layer of Hg onto a 25 μm Au disk electrode under constant-current conditions in a 0.1 mol/l $Hg_2(ClO_4)_2$ and 1 mol/l $HClO_4$ solution [26]. The Hg-film electrode was washed with water and operating buffer solution, and then aligned to the capillary outlet at a distance of 50 μm with a micropositioner. If the electrode was kept in the operating buffer solution, it was stable for two days. During the course of a day the operating buffer solution does not need to be changed, but fresh buffer should be used at the start of each day. The periodic wave form used for application of the potential to the electrodes consisted of an initial constant-potential period (200 ms) followed by a triangular CV portion. The initial constant-potential region was used for analyte preconcentration. In the CV portion, the potential was scanned from a initial potential of −0.6 V to a vertex potential of −1.7 V and then reversed to −0.6 V. Detection was controlled with a Pentium/16.0 MB RAM IBM personal computer equipped with a PCL-818 high-performance data acquisition card (B and C Microsystems, Sunnyvale, CA, USA) to collect data at specific time periods and to display the data. Two types of signal were used for analytical response; one was the CV current response (the maximum current in each voltammogram), and the other one was expressed in terms of CV charge response. CV charge was obtained by integrating the current in the electrode response over a certain potential range after subtraction of signal obtained for the background electrolyte. The time required for the integration depended on the scan rate and the selected potential range; the reduction current was defined as negative current. More detailed descriptions of data acquisition and processing has been given elsewhere [24,25]. CV charge response gave better S/N than CV current, and thus it was used as response for most of the discussed data.

3. Results and discussion

3.1. CE separation

Previous reported CE separations of TC, CTC and OTC did not produce well resolved peaks in aqueous buffers [13–23]. Since these compounds have several OH-groups, borate buffers were investigated. Borates have been used intensively for carbohydrate separations, and the complexation between borate and polyhydroxy compounds has been reported and utilized to improve the resolution of such compounds [27,28]. It was expected that even if the interaction between borate and tetracyclines were weak, it would provide improved separation. A borate–phosphate electrolyte has been used in a micellar electrokinetic separation [18], but only one borate concentration was used and there appeared to be no recognition that it might be possible to use borate complexation to adjust resolution. The influence of pH values in a borate buffer on the migration times is shown in Fig. 1. At lower pH values tetracyclines likely exist as zwitterions, and above ≈pH 5.5 keto-enol ionization should lead to a net negative charge. Thus at the starting pH in this study, the species should be negatively charged, and in the pH range of 8.5 to 9.5 a further increase in overall negative charge occurs (pK_a values given in Table 1) most likely due to deprotonation of the dimethylamino

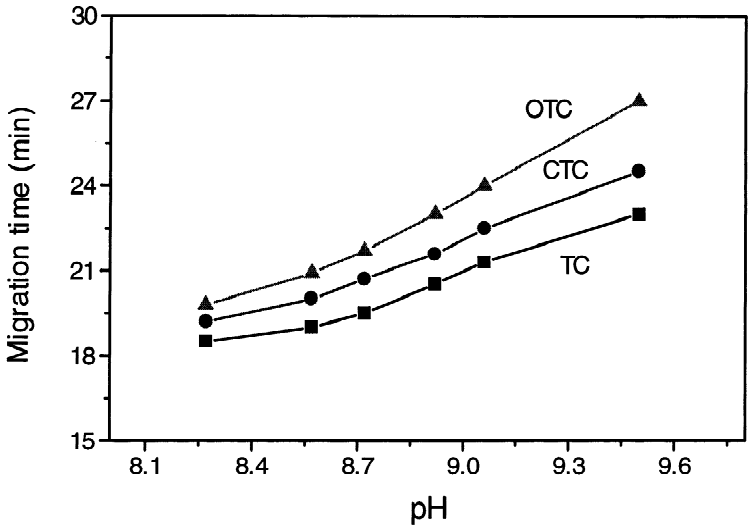


Fig. 1. Influence of pH value on migration time. Separation voltage: 20 kV over 80 cm×50 μm capillary; background electrolyte, 0.01 mol/l boric acid–borate including 1 mmol/l EDTA; CV potential, −0.6 to −1.7 V; scan rate, 300 V/s; TC, CTC: $1 \cdot 10^{-4}$ mol/l, OTC, $2 \cdot 10^{-4}$ mol/l; hydrodynamic injection for 15 s.

group. Thus the electrophoretic mobilities of the tetracyclines were in a direction opposite to the EOF, and as the results in Fig. 1 show, migration times became longer with increasing pH values in the range studied; complexation with borate could also contribute to the overall negative charge on the analytes, and this aspect is discussed below. Changes in EOF were not a significant factor in these changes in migration times since EOF (measured by the reduction peak of benzoquinone) increased only slightly (a few seconds). Fig. 1 also shows that the pattern of the influence of pH on the electrophoretic mobility of each compound was similar, but none the less offered an improved separation for all components with increasing pH. Separation was complete at pH≥8.7, and thus pH 8.7 was chosen for further studies.

Since the results from the pH study did not clearly differentiate between resolution enhancement from differences in pK_a values and borate complexation, the influence of borate concentration on the separation was also investigated; this parameter can also influence EOF and the current produced in the capillary. The highest buffer concentration was prepared by adjustment of 0.15 mol/l borate with 0.75 mol/l boric acid to pH 8.7, then diluted to the desired concentration with water; the pH values of these buffer solutions were all very close to pH 8.7. As shown in Fig. 2, the resolution was strongly influenced by the buffer concentration. At low buffer concentrations the peaks of CTC and OTC were entirely overlapped, and only one peak for CTC and OTC could be observed. With increasing buffer concentrations migration times increased >three-fold relative to EOF, the resolution was markedly improved, and complete resolution of three compounds was achieved at 0.1 mol/l borate. The improvements observed in resolution, the differential changes in resolution, and changes in migration times relative to EOF all suggest that complexation with borate was occurring. These results show that the use of borate electrolytes results in good resolution of these compounds, which is difficult to do in other electrolytes studied previously [13–19]. Thus 0.1 mol/l borate was chosen as the working buffer concentration for further study. Although this is a rather large ionic strength for CE separations, the currents obtained (~50 μA) did not cause unusual band broadening effects or cause bubble formation.

3.2. Electrochemical detection

Initial cyclic voltammetric investigations were done in stationary borate solutions in the pH range of

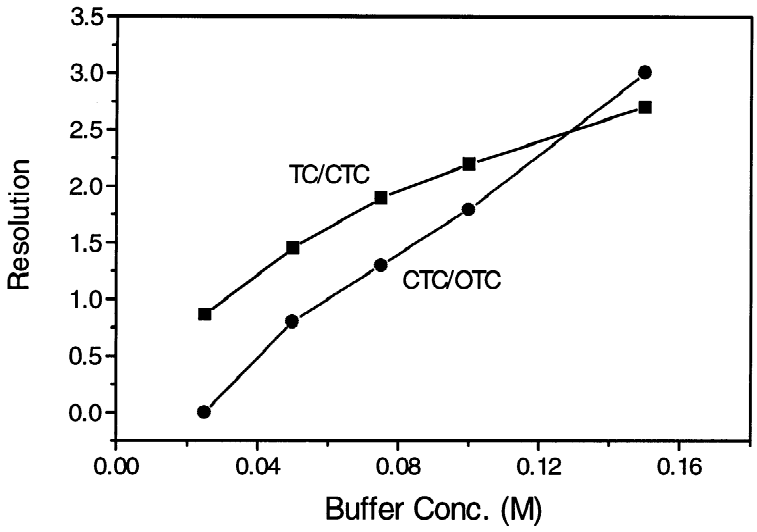


Fig. 2. Influence of buffer concentration on resolution. pH=8.7; other conditions as in Fig. 1.

8.0 to 9.6. Initial studies with Pt, Ag, Cu and Au electrodes did not provide useful results for analytical applications, and in particular were unsuitable because the cathodic potential range available at these metal μm-electrodes did not permit the reduction of the tetracycline compounds. However, studies with a Hg-film μm-electrode, which had a potential window down to −2.0 V, showed that all three compounds exhibited a reduction peak between −1.4 to −1.5 V (vs. SCE). Thus a vertex potential of −1.7 V was used. No corresponding oxidation peaks were observed due to the irreversibility of the reductions [5]. Since the difference in reduction potentials for these three compounds is very small (<50 mV), it is not possible to clearly differentiate between these three compounds by voltammetric methods alone.

Previous studies have shown that the adsorption of tetracyclines on hanging-mercury drop electrodes resulted in enhanced signals by eight- to ten-fold for preconcentration times of 90 s at −0.6 V, compared to the response without preconcentration [5], and thus the effect of preconcentration time on the CV charge was examined. For CE the preconcentration time is limited by the narrow peak size, as increases in the time spent for preconcentration would eventually reduce the number of data points to less than 10/peak, which would affect resolution and reproducibility. The effect of potential on preconcentration was also studied over the range of −0.2 to −1.0 V. The optimum results, obtained with a preconcentration time of 0.2 s at a potential of −0.6 V, showed that the signal could be increased by 30–50%. Thus 0.2 s and −0.6 V were chosen as the preconcentration conditions for other measurements. With a preconcentration time of 0.2 s application of this technique would be limited to peaks that are ≥2 s in width to ensure proper peak definition and to minimize instrumental peak broadening.

In CE separations coupled with fast cyclic voltammetric detection high scan rates are used. Therefore it is important to examine the dependence of the detector response on the scan rate since this influences both analyte signal and background noise. The dependence of CV current and CV charge on the scan rate was examined over the range of 25 to 1000 V/s. The CV current of the three compounds depended linearly on the scan rate in the range of 25 to 500 V/s. In the higher scan range the CV current increased only slightly. The scan rate also influenced the CV peak potentials. With a increase of scan rate from 25 to 500 V/s, the reduction peak potential of these compounds was shifted towards a more negative potential by 40 to 60 mV, due to slow charge transfer kinetics and the ohmic drop. This influence of scan rate on the CV current was similar to that reported earlier [25,29]. As shown in Fig. 3, CV

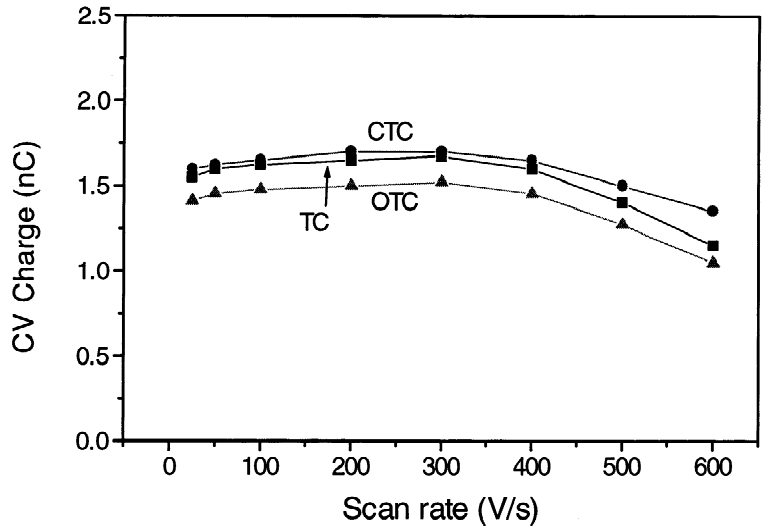


Fig. 3. Influence of scan rate on CV charge at CE peak maximum. pH = 8.7; other conditions as in Fig. 1.

charge changed slightly with scan rate over the range of 25 to 400 V/s, and then decreased at higher scan rates because these compounds were not completely reduced at high scan rates, a result most likely from their slow charge-transfer kinetics. The highest ratio of S/N was obtained at scan rate between 200 and 400 V/s for these compounds, and was two- to three-times higher than those obtained at lower scan rates. It should be noted that currents measured in our experiments were proportional to the scan rate in this range, and higher currents were less influenced by the separation current in capillary electrophoresis and other environmental noise. Thus 300 V/s was chosen as working scan rate. CV charge response gave better S/N than CV current, thus it was used as response for most of the discussed data, and electropherograms were obtained by plotting CV charge vs. migration time. When CV current was used for analytical response, the sensitivities were five- to eight-fold lower than those for CV Charge.

Fig. 4 shows cyclic voltammograms of CTC recorded across its CE peak. Each of the curves in Fig. 4 is a complete CV taken at different time intervals as the peak migrated past the electrode. Each CV took about 2 ms to complete, but since there was a 200 ms preconcentration period in each waveform, each of the CVs recorded represent 202 ms intervals across the CTC peak. The voltammetric behavior of these compounds under CE conditions was similar to that in stationary solution; only reduction peaks without corresponding oxidation peaks were observed. Since the different curves in Fig. 4 represent a wide range of analyte concentrations, these result also shows that this CV approach can provide important information about the electrochemical behavior of the analyte even at the low concentrations used for analytical applications. A representative electropherogram for the separation of the compounds using fast scan voltammetric detection is shown in Fig. 5. The results in Fig. 5 show that all compounds were well resolved into symmetrical peaks. While the efficiency is rather low for a CE separation (12 000 to 50 000 theoretical plates), this is likely associated with the rate of the borate complexation equilibria; efficiencies for application of similar detection procedures to metal ions gave much higher efficiencies obtained in our laboratories. If required, further improvements in resolution are possible by a change in borate concentration (see Fig. 2), or by a simultaneous optimization of both pH and borate concentration.

3.3. Analytical performance

The following optimum conditions were chosen for evaluation of quantitative performance: sepa-

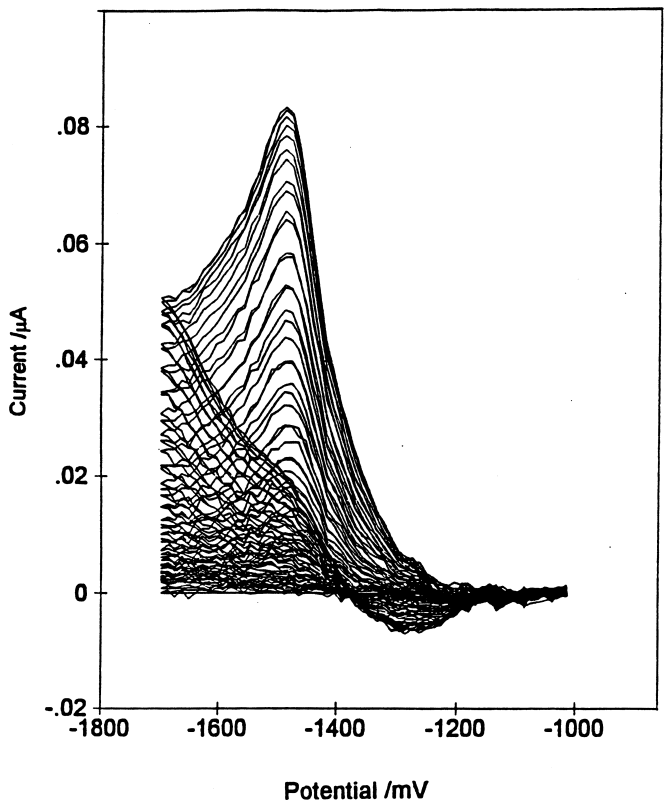


Fig. 4. Voltammograms of CTC recorded across CE peak. pH=8.7; CTC: $1\cdot10^{-4}$ mol/l; each scan corresponds about 2 ms; other conditions as in Fig. 1.

ration voltage, 20 kV over 80 cm×50 μm capillary; background electrolyte, 0.1 mol/l borate including 1 mmol/l EDTA; pH=8.7; CV potential, −0.6 to −1.7 V; scan rate, 300 V/s; preconcentration time and potential, 0.2 s and −0.6 V. As shown in Table 2, under these conditions calibration plots for these compounds with CV charge as the analytical signal were found to be linear over the concentration range from 10^{-6} to 10^{-4} mol/l, and gave good correlation coefficients (0.994–0.999). Since correlation coefficients can be misleading, linearity was also evaluated by plotting the response factor (response/concentration) versus concentration [30]. The maximal deviation in this plot was ±6%. The detection limits, based on 2×peak-to-peak noise (~ 10σ), were found to be $7\cdot10^{-7}$ mol/l for TC and CTC, and $1.5\cdot10^{-6}$ mol/l for OTC. The reproducibility was evaluated as the relative standard deviation (RSD) of the migration time and CV charge for five consecutive injections at a concentration level of $5\cdot10^{-5}$ mol/l.

Table 2
Quantitative data

Compound	Slope	Intercept	Detection limit (mol/l)	Linear range (mol/l)
TC	1.69 nC/10^{-4} mol/l	0.0029	$7\cdot10^{-7}$	$1\cdot10^{-6}$–$5\cdot10^{-4}$
CTC	1.71 nC/10^{-4} mol/l	0.0031	$7\cdot10^{-7}$	$1\cdot10^{-6}$–$5\cdot10^{-4}$
OTC	0.75 nC/10^{-4} mol/l	0.0017	$1.5\cdot10^{-6}$	$2.5\cdot10^{-6}$–$8\cdot10^{-4}$

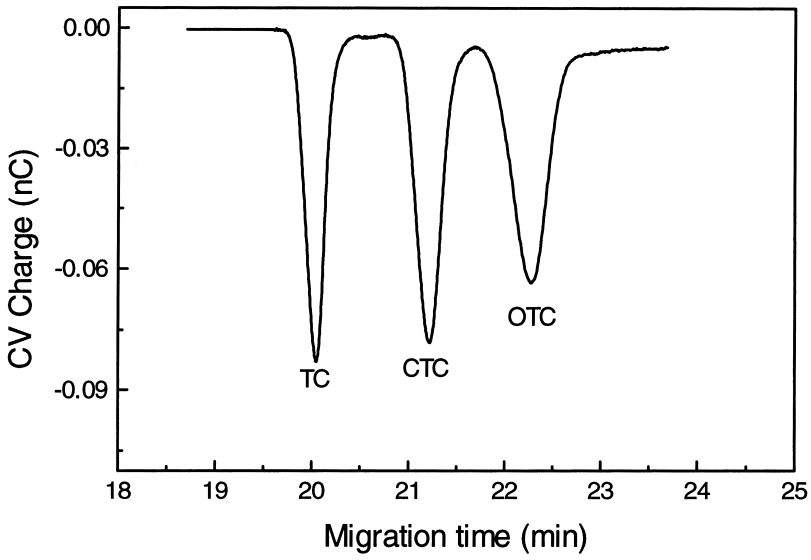


Fig. 5. Electropherogram of TC, CTC and OTC. pH = 8.7; other conditions as in Fig. 1.

The RSD values for the migration times for these compounds and for the CV charge were found to be $<1\%$ and $<5\%$, respectively. These results indicate that separation and detection with CE coupled with fast cyclic voltammetry using a high scan rate are reproducible for the measurement of these compounds, and that this method is much more sensitive than results reported for UV detection (detection limits 10^{-4} to 10^{-5} mol/l) [13–23].

In conclusion, a selective separation and sensitive detection of TC, CTC and OTC can be achieved by CE coupled with reductive fast cyclic voltammetry in a borate buffer solution. Relative to other reported procedures for these compounds, this approach offers greatly improved detection limits and improved resolution. Previous studies of fast CV detection were based on the use of analytes that could be effectively preconcentrated via deposition at the electrode. These studies show that such approaches are also viable for tetracyclines, and it is possible these approaches may also apply to a wider range of water-soluble organic analytes. While the analytical performance factors suggest that such an approach could offer an attractive and selective analysis technique for such compounds, further studies with real samples are obviously needed.

Acknowledgements

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