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Separation and determination of the macrolide antibiotics (erythromycin, spiramycin and oleandomycin) by capillary electrophoresis coupled with fast reductive voltammetric detection

Separation and determination of erythromycin, spiramycin and oleandomycin by capillary zone electrophoresis coupled with fast reductive voltammetric detection using an Hg-film electrode was investigated in a simple aqueous phosphate buffer system. The influence of pH, concentration of phosphate, applied voltage, capillary length and dimension on the separation was examined and optimized. The entire separation of erythromycin, spiramycin, and oleandomycin was achieved in a 0.2 mol/L phosphate buffer system without organic modifiers. The electrochemical detection parameters, such as electrode material, applied waveform, scan rate, preconcentration potentials and preconcentration times, were investigated and discussed. This approach provides high separation efficiency and high sensitivity for all compounds, with detection limits ($3 \times$ peak-to-peak baseline noise) of 7.5×10^{-8} mol/L for spiramycin, and 3×10^{-7} mol/L for erythromycin and oleandomycin. The calibration plot of peak areas for each separated peak *vs.* concentration of analyte was found to be linear over three orders of magnitude.

Keywords: Erythromycin / Spiramycin / Oleandomycin / Capillary electrophoresis / Electrochemical detection / Fast cyclic voltammetry EL 3892

1 Introduction

Macrolides are important glycoconjugated antibiotics, and of these antibiotics erythromycin (ER), spiramycin (SP) and oleandomycin (OL) are widely used against many bacteria [1]. The structures of these compounds are similar (Fig. 1), and this is especially true for ER (pK_a 8.8 [11]) and OL (pK_a 8.7 [11]). Polarography/voltammetry has been used extensively as a sensitive analytical method, and has been reviewed by Smith and Vos [2]. Due to their similar redox properties, prior separation is required to differentiate among these compounds [3] when electrochemical detection is used. The separation of these antibiotics has been investigated widely by HPLC with UV detection [4–8] and with electrochemical detection based on their oxidation [9, 10].

Recently, there have been several reports on the separation of ER antibiotics by capillary electrophoresis (CE) with UV detection [11–14]. These CE separations with UV detection have been carried out in phosphate and borate buffer systems with an organic modifier, with resultant low sensitivity. An alternate approach that may offer improved detection was the use of fast cyclic voltammetric (CV) detection with an electrolyte system in which these compounds could be separated completely. We found that ER, SP, and OL could be separated completely in a simple phosphate solution by optimizing the CE separation parameters. A simple phosphate electrolyte was advantageous for electrochemical detection because the presence of organic modifiers influenced electrode behavior in applications of fast cyclic voltammetry, which is a detection method recently introduced for CE separation [15–17]. This CV detection approach can offer detection limits in the 10^{-8}–10^{-9} mol/L range for metal ions, and 10^{-6}–10^{-7} mol/L range for tetracyclines. We expect such sensitive CV detection approaches to improve the sensitivity for the macrolides, such as erythromycins. However, many factors have to be investigated before this approach can be recommended for analysis of erythromycins. The applied potential and the nature of the electrode material can have an important influence on detection. The sensitivity of CV detection can be enhanced by analyte accumulation, but it is uncertain whether these compounds will show an efficient preconcentration behavior.

While one would expect it to be possible to select suitable potential ranges to allow macrolides to be accumulated at an electrode surface, it is uncertain whether such compounds (*e.g.*, ER) would show as efficient a preconcentra-

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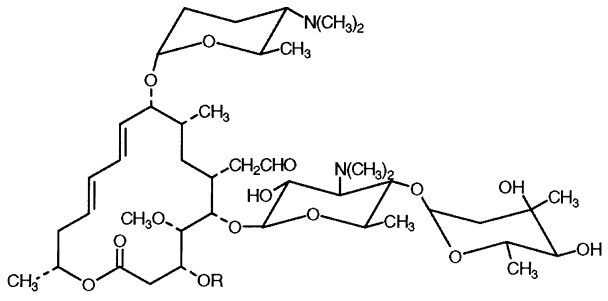
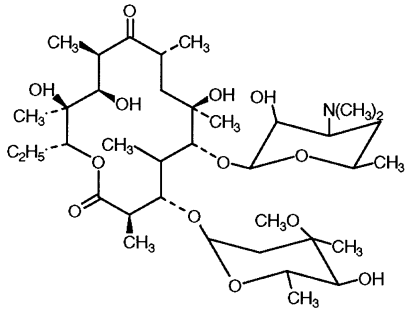
Abbreviations: **CV**, cyclic voltammetric; **ER**, erythromycin; **OL**, oleandomycin; **SCE**, saturated calomel electrode; **SP**, spiramycin

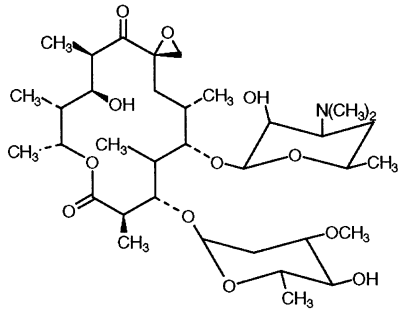
 0173-0835/00/0707-1349 $17.50+.50/0



	R
Spiramycin I	H
Spiramycin II	$COCH_3$
Spiramycin III	COC_2H_5



Erythromycin



Oleandomycin

Figure 1. Structures of SP, ER and OL

tion behavior. In addition, the nature of the electrode material, the applied potential, and the scan rate can have an important influence on redox behavior, and these aspects must also be investigated. In this paper we report on an investigation on the feasibility of the CE separation in a simple phosphate buffer system coupled with fast CV detection. The influence of pH, phosphate concentration, applied voltage, capillary length, and dimension on the separation was investigated. The electrochemical detection parameters, such as electrode material, applied waveform, scan rate, preconcentration potentials, and preconcentration times, were investigated.

2 Materials and methods

2.1 Chemicals

ER, SP, and OL were obtained from Sigma (Oakville, ON, Canada). All reagents were of analytical grade and were used without further purification, and deionized-distilled water was used throughout. Prior to the separation and electrochemical measurements all buffer solutions (about 200 mL) were purged with pure nitrogen for 15 min, and then transferred to the small buffer reservoirs 1 h later. The NaH_2PO4 solution was adjusted to the desired pH values with 5 mol/L NaOH. 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 ER, SP, and OL were prepared in methanol, 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 (Model RHR30PN30; Spellman High Voltage Electronics, 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 and 50 μm ID (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 introduced into the capillary by elevating the capillary to a 10 cm height for 15 s and CE polarity was positive (detection cell was grounded). 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 (SCE, miniature model; Fisher Scientific, Ottawa, Canada) was used as reference electrode. The working electrode consisted of an Hg-film (6 μm thick)/Au 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 HCl_4 solution [18]. The Hg-film electrode was washed with water and operating buffer solution, and then aligned to the capillary outlet at a distance of 30 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 waveform used for application of the potential to the electrodes consisted of an initial constant-potential period (400 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 an initial potential of –1.0 V to a vertex potential of –1.8 V and then reversed to –1.0 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 & 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; the reduction current was defined as negative one), 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 from the background electrolyte. The time required for the integration depended on the scan rate and the selected potential range. More detailed descriptions of data acquisition and processing are given elsewhere [15, 16]. The CV charge response gave better S/N than the CV current, and was thus used as response for most of the discussed data.

3 Results and discussion

3.1 CE separation

The influence of pH values in a phosphate buffer on the migration times is shown in Fig. 2. In the pH range studied (6.5–8.2), SP, ER, and OL should exist as positively charged species; their electrophoretic mobilities were in the same direction as the electroosmotic flow (EOF), and thus they migrated faster than the EOF. The migration order was SP, ER, and OL. In the pH range lower than 7.3, with increasing pH the migration times became longer values, and the separation or ER and OL was poor while the resolution of SP and ER was very good. Between pH 7.3 and 7.8, the migration times decreased slightly with increasing pH values, and the migration time differences between SP, ER, and OL were the largest, resulting in complete separation of the three compounds. At the range of pH larger than 7.8, migration times increased, and migration time differences became smaller, peaks overlapped gradually, and resolution became worse. Thus, pH 7.5 was chosen as working pH for further studies.

The influence of phosphate concentration on the separation was also investigated; this parameter can also influence electroosmotic flow and the current produced in the capillary. The highest buffer concentration was prepared by adjusting 0.2 mol/L phosphate with 5 mol/L NaOH to pH 7.5, then diluting to the desired concentration with water; the pH values of these buffer solutions were all close to pH 7.5. As shown in Fig. 3, the resolution was strongly influenced by the buffer concentration. At low buffer concentrations the peaks of SP, ER and OL were entirely overlapped, and only one peak for these three compounds could be observed. With increasing buffer concentrations the resolution was markedly improved, and complete resolution of three compounds was achieved at 0.2 mol/L phosphate. While there is a linear relationship between $1/(C)^{1/2}$ (C, concentration of buffers) and the migration times of the three compounds and the EOF, the slightly different relationship for these three compounds resulted in good resolution. These results show that a moderate increase in phosphate concentration results in good resolution of these compounds, which

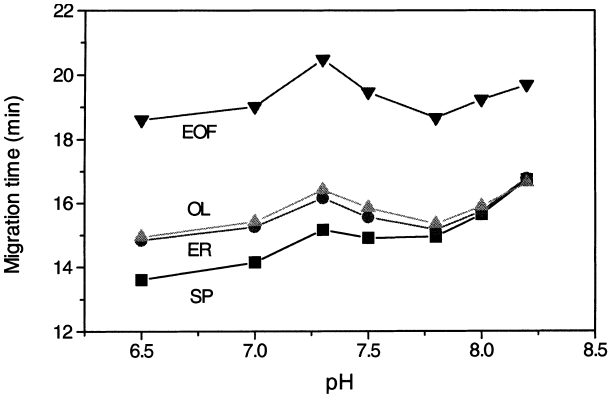


Figure 2. Influence of pH value on migration time. Separation voltage, 12.5 kV over 80 cm × 50 μm capillary; background electrolyte, 0.2 mol/L; CV potential, –1.0 to –1.8 V; scan rate, 50 V/s; SP, 1×10^{-5} mol/L, ER and OL, 4×10^{-5} mol/L; hydrodynamic injection for 15 s.

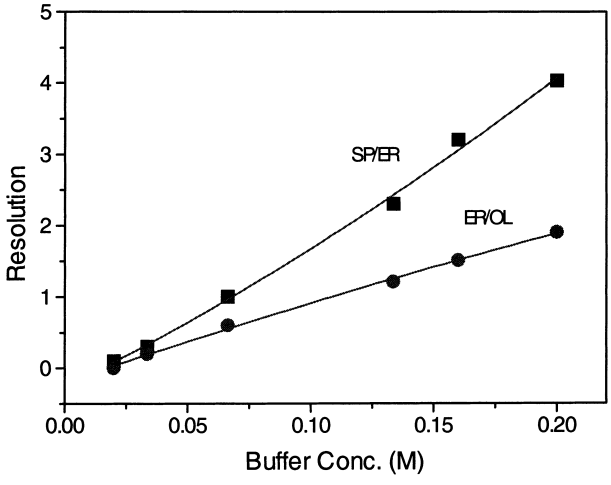


Figure 3. Influence of buffer concentration on resolution. pH 7.5; other conditions as in Fig. 1.

was not achieved in other studies [11–14]. Thus 0.2 mol/L phosphate was chosen as the working buffer for further study. Although this is a rather large ionic strength for CE separation, the currents obtained (~ 80A) did not cause unusual band broadening effects or cause bubble formation. The influence of other important parameters, such as applied voltage and capillary length and dimensions, was also investigated and optimized. For an 80 cm capillary with 50 μm ID, the optimal applied voltage was found to be 12.5 kV.

3.2 Electrochemical detection

Initially, CV was investigated in stationary phosphate solutions in the pH range of 6–9 using different electrodes. Pt, Ag, Cu and Au electrodes did not provide useful results for analytical applications; in particular, they were unsuitable because the cathodic potential range available at these metal m-electrodes did not permit the reduction of these compounds. However, studies with an Hg-film 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 –1.8 V was used. No corresponding oxidation peaks were observed due to the irreversibility of the reductions [3]. Since reduction potentials for these three compounds are very close (reduction potential < 50 mV), it is impossible to clearly differentiate between these three compounds by voltammetric methods alone.

It was expected that these three compounds could be absorbed on an Hg-film μm-electrode, and therefore 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 0.4 s at a potential of –0.4 V, showed that the signal could be increased by 50–70%. Thus 0.4 s and –0.4 V were chosen as the preconcentration conditions for other measurements.

In CE separations coupled with fast CV 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 10–400 V/s. The CV current of the three compounds depended linearly on the scan rate in the range of 10–100 V/s. In the higher scan range the CV current increased only slightly. The scan rate also influenced the CV peak potentials. With an increase of scan rate from 10 to 400 V/s, the reduction peak potential of these compounds was shifted towards a more negative potential by 40–60 mV due to slow charge transfer kinetics and the ohmic drop. This influence of scan rate on the CV current was similar to those reported earlier [16, 17]. The CV charge changed slightly with scan rates over the range of 25–100 V/s, and then decreased at higher scan rates because these compounds were not completely reduced at high scan rates, probably as a result of their slow charge transfer kinetics. The highest S/N was obtained at a scan rate of 50 V/s for these compounds, and was 2–3 times higher than those obtained at the other scan rates. Thus, 50 V/s was chosen as a working scan rate. CV charge response gave a better S/N than the 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 10-fold poorer than those for CV charge.

Figure 4 shows cyclic voltammograms of ER 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 16 ms to complete, but since there was a 400 ms preconcentration period in each waveform, each of the CVs recorded represent 432 ms intervals across the ER peak. The voltammetric behavior of these compounds under CE conditions

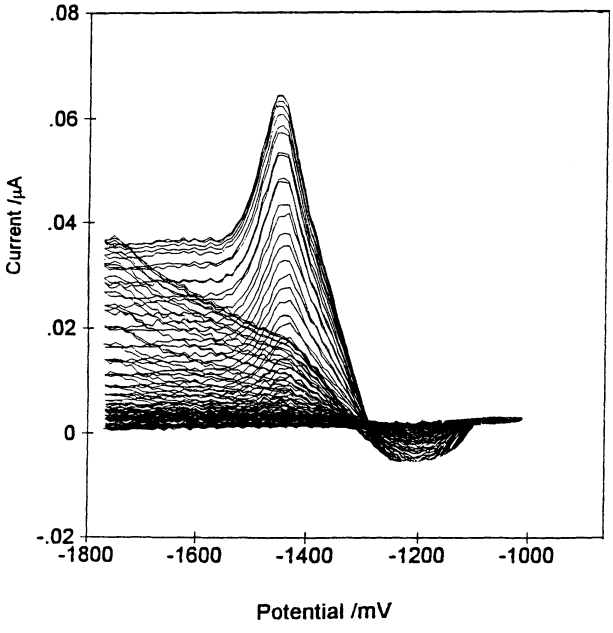


Figure 4. Electropherovoltammogram of ER recorded across CE peak; pH 7.5; ER, 4×10^{-5} mol/L; each scan corresponds to 16 ms; other conditions as for Fig. 1.

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, this 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. The reduction peak current of CV for the first scan is the largest, and the current decreased gradually at further scans. A representative electropherogram for the separation of the compounds using fast scan voltammetric detection is shown in Fig. 5. All compounds were well resolved. The efficiency was high for CE separation, and the theoretical plates were in the range of $2.0–3.0 \times 10^5$.

3.3 Analytical performance

The following optimum conditions were chosen for evaluation of quantitative performance: separation voltage, 12.5 kV over 80 cm 50 m capillary; background electrolyte, 0.2 mol/L phosphate; pH 7.5; CV potential, –1.0 to –1.8 V; scan rate, 50 V/s; preconcentration time and potential, 0.4 s and –0.4 V. 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 1×10^{-7} to 1×10^{-5} mol/L for SP and 4×10^{-7} to 5×10^{-5} mol/L for ER/OL, and gave good correlation coefficients (0.993–0.999). Since correlation coefficients can be misleading, linearity was also evaluated by plotting the response factor (response/concentration) *versus* concentration [19]. The maximal deviation in this plot was ± 6%. The detection limits, based on 3 peak-to peak noise (10 σ), were found to be 7.5×10^{-8} mol/L for SP, 3×10^{-7} mol/L for ER and OL. 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 5×10^{-6} mol/L. The RSD

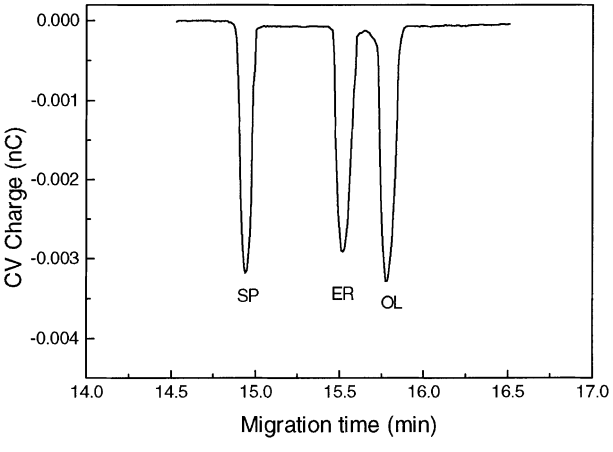


Figure 5. Electropherogram of SP, ER, and OL; pH 7.5; other conditions as in Fig. 1.

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} mol/L) [11–14].

4 Concluding remarks

In conclusion, a selective separation and sensitive detection of SP, ER, and OL can be achieved by CE coupled with reductive fast cyclic voltammetry in a simple phosphate buffer solution. Relative to other reported procedures for these compounds, this approach offers greatly improved detection limits and improved resolution without organic modifiers. 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.

We thank the Natural Science and Engineering Research Concil of Canada for financial support of this work.

Received August 16, 1999; in revised form January 26, 2000

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