Determination of sulfadiazine and sulfamethoxazole by capillary electrophoresis with end-column electrochemical detection

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Capillary electrophoresis (CE) with end-column electrochemical detection (EC) of sulfadiazine (SDZ) and sulfamethoxazole (SMZ) is described. Under the optimum conditions, SDZ and SMZ were separated satisfactorily, and a highly sensitive and stable response was obtained at a potential of 1.1 V *versus* Ag/AgCl. Optimized end-column detection provides detection limits as low as 0.1 μM for both compounds, which corresponds to 0.024 and 0.021 fmol with peak efficiencies of 394 000 and 335 000 theoretical plates for SDZ and SMZ, respectively. The calibration graph was linear over three orders of magnitude. The relative standard deviations (n = 12) of peak currents and migration times were 2.3 and 2.7%, and 0.8 and 1.3%, respectively, for the two compounds. The proposed method was applied to the analysis of tablets and human urine samples with satisfactory results.

Introduction

Since its introduction over a decade ago by Virtanen,[1] Mikkers *et al.*[2] and Jorgenson and Lukacs,[3,4] capillary electrophoresis (CE) has typically been characterized by a minimal sample volume requirement, short analysis time and high separation efficiency.[5] It has become an attractive technique for the separation of charged molecules in the area of liquid-phase separations. It has been shown by Wallingford and Ewing[6] that electrochemical detection (EC) is advantageous for CE over methods such as UV detection, laser-based fluorescence detection and radioactivity detection. It has attractive features including high sensitivity, good selectivity and low cost. In addition, EC is very inexpensive to implement and maintain, and it can be applied to many biological compounds because they are electroactive.

Two modes of detection have been developed in CE-EC, *viz.*, 'off-column' and 'end-column' detection. For the off-column mode, to reduce the background, it is necessary to isolate the high voltage and current applied to the separation capillary from the small voltage and current measured in the electrochemical detection cell. The conductive junctions between the separation and detection capillary include porous glass joints,[7–10] Nafion joints,[11,12] cellulose acetate joints,[13] and palladium joints.[14,15] End-column detection is suitable for capillaries of 25 μm or less.[16] This design does not make use of a coupler to decouple the electrophoretic and detection currents. The electrode is placed at the outlet of the capillary. The primary advantage of this mode is its simple design and construction.

Sulfadiazine (SDZ) and sulfamethoxazole (SMZ) belong to the sulfonamide class of antibacterial compounds. They are used in the treatment of urinary-tract infections, pneumocystis pneumonia, chronic bronchitis, meningococcal meningitis, acute otes and toxoplasmosis.[17] Various methods have been reported for the detection of SDZ and SMZ including a photometric method,[18] the Bratton–Marshall method[19,20] a titrimetric assay method,[21] high-performance liquid chromatography, gas chromatography and gas chromatography-mass spectrometry.[22,23] However, these methods are tedious and time consuming. The use of micellar electrokinetic chromatography for the separation of sulfonamides and related impurities has been reported by Nickerson *et al.*[24] using UV detection. To the best of our knowledge, no reports of the electrochemical detection of SDZ and SMZ by CE have been published.

In this paper, the potential of end-column detection with a carbon fiber microdisc electrode was evaluated for SDZ and SMZ after their separation by CE. The determination of the analytes in tablets and human urine samples was also studied. The proposed method is simple, sensitive, rapid and convenient.

Experimental

Reagents

Sulfadiazine (SDZ) and sulfamethoxazole (SMZ) were obtained from Beijing Institute of Chemicals (Beijing, China). All chemicals were of analytical-reagent grade and used as received. The run buffer was made by mixing 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 to a certain pH value (pH > 4.5). When the pH was < 4.5, 10 mM H_3PO_4 was added to the buffer. Hence, when the pH changed, the ionic strength of the buffer at each pH value was unchanged. All solutions were freshly prepared with doubly distilled water and passed through a 0.45 μm membrane filter.

Apparatus

Electrophoresis in the capillary was driven by a high-voltage power supply (Spellman CZE 1000R, Plainview, NY, USA). A 40 cm length and 25 μm id, 320 μm od uncoated fused-silica capillary was used (Yongnian Optical Fiber Factory, Hebei, China). The capillary was flushed with 0.1 M sodium hydroxide solution overnight before use. Between each run, the capillary was rinsed with doubly distilled water, 0.1 M sodium hydroxide, doubly distilled water and run buffer for 2, 2, 2 and 5 min, respectively.

The construction of the CE-EC system was carried out in-house.[25] End-column detection was employed by using a wall-jet configuration. Detection was effected in the amperometric mode using a three-electrode system with a carbon fiber microdisc (33 μm diameter) electrode, a Ag/AgCl (saturated KCl) reference electrode and a platinum auxiliary electrode. Potential control and current output were provided by a PAR Model 400 amperometric detector (EG&G, Princeton Applied Research, Princeton, NJ, USA). The data collection of the electropherograms was provided by a Philip's computer configured as a Gilson 715 HPLC system controller software.

Cyclic voltammetry was carried out with a Model 832 computerized voltammetric analyzer (CH Instruments, Cordova, TN, USA) using the same three-electrode system as described above.

Sample introduction was accomplished by an electromigration system and the volume injected was calculated in the continuously filling mode by recording the time required for the sample to reach the detector.

Electrode preparation

The carbon fiber microdisc electrodes were constructed with 33 μm diameter carbon fiber. A 3 cm length of carbon fiber was carefully inserted into a fused-silica capillary (75 μm id, 360 μm od, 15 mm length) until it was just protruding from the other end of the capillary. A drop of transparent epoxy glue was applied to both ends of the capillary to secure the carbon fiber. The end of the capillary with the longer length of carbon fiber exposed was introduced into a glass capillary (0.5 mm id, 2 mm od) such that the other end was exposed to a length of 7 mm, and the capillary was sealed with carbon fiber powder which provides electrical connection. The open end of the glass capillary was enclosed with epoxy glue. With one end of the carbon fiber fixed with epoxy glue, the other end of the electrode was polished on abrasive papers, then with 1.0, 0.3, 0.1 and 0.05 μm α-alumina on a polishing cloth, and finally ultrasonicated in doubly distilled water for 5 min.

Sample preparation

The synergistic bisulfanilamide tablets were crushed to a fine powder and dissolved in 0.1 M HCl. The solution was filtered through a membrane filter (0.45 μm). This stock sample was diluted further with the electrophoretic medium of 10 mM phosphate buffer (pH 5.0) before injection.

Human urine samples were diluted immediately with 10 mM buffer (urine : buffer = 1 : 3) and filtered through a 2 μm pore size filter. The resulting solution was injected directly on to the capillary column.

Results and discussion

Cyclic voltammetry

Fig. 1 shows the cyclic voltammogram of SDZ (b) and SMZ (c) in 10 mM phosphate buffer (pH 5.0) obtained at the carbon fiber microdisc electrode. It can be seen that both SDZ and SMZ yield irreversible oxidation waves at *ca.* 1.1 V (*versus* Ag/AgCl).

CE-EC of SDZ and SMZ

Hydrodynamic voltammograms (HDVs). Fig. 2 illustrates the hydrodynamic voltammograms (HDVs) for SDZ and SMZ obtained by CE-EC with the carbon fiber microdisc electrode. When the applied detection potential varies from 0.7 to 0.9 V, very low current responses of SDZ and SMZ are obtained. However, the current responses increase rapidly when the applied detection potential is higher than 0.9 V and reach a plateau at 1.1 V. Because high potentials give rise to a higher background current, 1.1 V was selected for the simultaneous determination of SDZ and SMZ. Under the optimized conditions, a highly sensitive and stable response was obtained.

Effect of separation voltage (E_s). The separation voltage (E_s) exerts an influence on the number of theoretical plates (N) and the peak current of the analytes (i_p). The effect of E_s on N and i_p was investigated. As shown in Fig. 3, i_{pSDZ} and i_{pSMZ} increase as E_s increases from 10 to 20 kV, reach a maximum at 20 kV and then decrease at E_s values higher than 20 kV. N_{SDZ} and N_{SMZ} show a similar behavior. To obtain large i_p and high N, a separation voltage of 20 kV was selected.

Injection system. Injection was effected by electromigration. The electrokinetic injection volume, V_i, is given by $V_i = E_i \times t_i \times V_{capillary}/E_s \times t_m$,[26,27] where E_i is the injection voltage, E_s the separation voltage, t_i the time of injection, t_m the migration time of the analyte, and the volume of the capillary, $V_{capillary}$, is given by the volume of a cylinder of length L and inner diameter d: $V_{capillary} = \pi d^2 L/4$. The number of theoretical plates, N, is calculated according to the following equation: $N = 5.54 \times (t_m/W^{1/2})^2$,[26,27] where $W^{1/2}$ is the width at half-peak

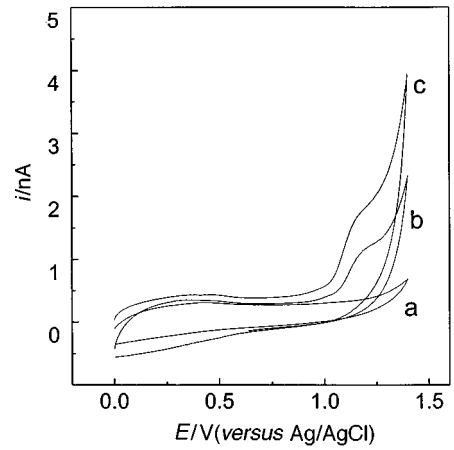

Fig. 1 Cyclic voltammograms of 10 mM phosphate buffer of pH 5.0 (a), 1 mM SMZ (b) and SDZ (c) at the carbon fiber microdisc electrode. Scan rate: 50 mV s^{-1}.

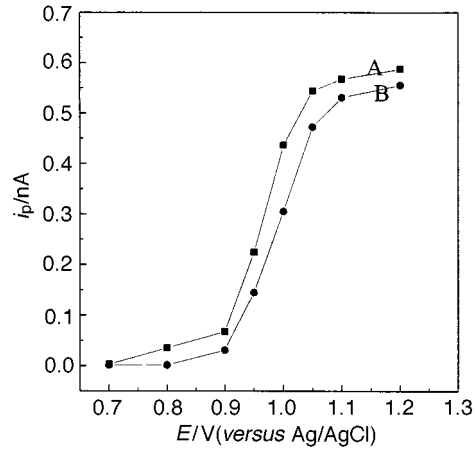

Fig. 2 Hydrodynamic voltammograms of 50 μM SDZ (A) and SMZ (B) at the carbon fiber microdisc electrode. Conditions: 10 mM sodium phosphate buffer of pH 5.0; separation capillary, 25 μm id, length, 40 cm; separation voltage, 20 kV; sample injection, 10 s at 5 kV.

height on the electropherogram. The influence of injection time, t_i, and injection voltage (E_i) was also studied. Fig. 4(a) shows the dependence of i_p and N on E_i. It can be seen that i_{pSDZ} and N_{SMZ} are almost proportional to E_i from 2 to 5 kV. However, i_{pSMZ} and N_{SDZ} are proportional to E_i in the range 2–4 kV; i_{pSMZ} then reaches a plateau and N_{SDZ} decreases at an E_i of 5 kV. In order to obtain a higher i_p for SDZ and SMZ, and also to obtain a considerably larger N for these compounds, E_i was fixed at 5 kV in the CE–EC experiments. Fig. 4(b) shows the effect of injection time (t_i) on i_p and N. N_{SDZ} and N_{SMZ} decrease slowly in the range 5–25 s. The i_p of SDZ and SMZ increases proportionally to t_i between 5 and 15 s. When t_i is longer than 15 s , i_{pSMZ} does not change significantly; i_{pSDZ} increases slightly and then decreases. This is because an excessively long t_i results in an excessively large injection volume and leads to band broadening. Hence, a value of 10 s for t_i was chosen.

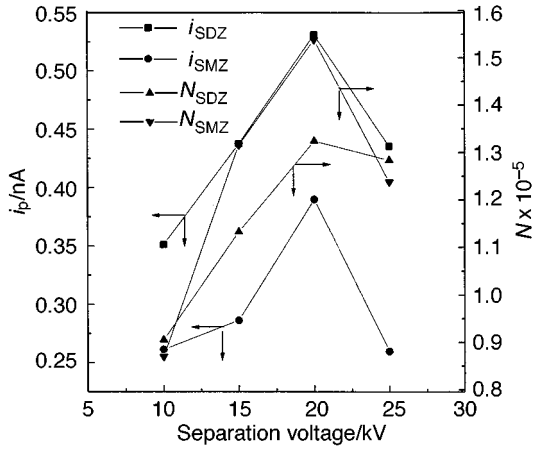

Fig. 3 Effect of separation voltage on i_p and N of 50 μM SDZ and 25 μM SMZ. Conditions: 10 mM sodium phosphate buffer of pH 5.0; separation capillary, 25 μm id, length, 40 cm; sample injection, 10 s at 5 kV; detection potential, +1.1 V *versus* Ag/AgCl.

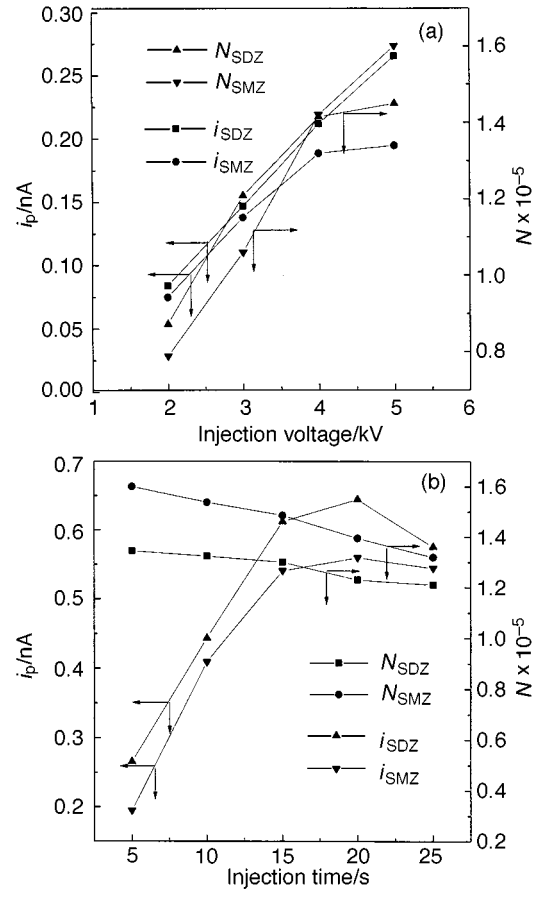

Fig. 4 Effect of injection voltage (a) and injection time (b) on i_p and N of 25 μM SDZ and SMZ. Conditions: 10 mM sodium phosphate buffer of pH 5.0; separation capillary, 25 μm id, length, 40 cm; separation voltage, 20 kV; detection potential, +1.1 V *versus* Ag/AgCl.

Effect of pH. The effect of pH on separation and detection was also investigated from pH 3.0 to 9.0 at intervals of about 0.5 pH unit. With the optimum values of detection potential (+1.1 V), E_s (20 kV), t_i (10 s) and E_i (5 kV), the current responses of SDZ and SMZ were almost constant. Their migration times became shorter with increasing pH, while Δt_m ($\Delta t_m = t_{mSMZ} - t_{mSDZ}$) became shorter also. When the pH was higher than 6.0, SDZ and SMZ could not be separated satisfactorily. Thus, pH 5.0 was selected for high separation efficiency.

Detection limit and reproducibility. Under the optimum conditions, the detection limit and detection reproducibility were also studied for SDZ and SMZ in CE-EC. Electrokinetic injection for 10 s at 5 kV was used. A linear working range was observed from 0.2 to 100 μM and from 0.13 to 100 μM for SDZ and SMZ, respectively. The detection limit (S/N = 3) for both SDZ and SMZ was 0.1 μM (0.024 and 0.021 fmol, respectively) with peak efficiencies of 394 000 and 335 000 theoretical plates for SDZ and SMZ, respectively.

Detection reproducibility was determined from 12 consecutive injections of a standard containing 10 μM SDZ and SMZ. If no pre-treatment was carried out on the electrode and the separation capillary before each run, the reproducibility was very poor. The current response decreased rapidly, the migration time became longer and Δt became even shorter. After five injections, the decrease in the current response became slower and i_p reached a fairly constant value. Carbon fiber microelectrodes have been widely used in CE-EC, but the electrode surface is easily fouled by the solution. Fouling of the electrode often results in variations in sensitivity or reproducibility and sometimes blocks the charge transfer process completely. In order to obtain reproducible and well defined electrochemical behavior, activation of the carbon fiber microelectrode is required. Electrochemical treatment has been used successfully for the activation of carbon fiber microelectrodes.[28–30] A reactivation process was performed by means of cyclic potential scanning for 2 min between −0.5 and 1.5 V (*versus* Ag/AgCl) at a scan rate of 100 mV s^{-1} and then maintaining the detection potential for 3 min. The reproducibility was greatly improved through the reactivation process. As a major factor resulting in a change in the sensitivity and migration time is the adsorption of analytes on the walls of the separation capillary, which also changes the electro-osmotic flow, the separation capillary was rinsed successively with doubly distilled water, 0.1 M sodium hydroxide solution and doubly distilled water for 2, 2 and 2 min, respectively. It was then equilibrated with the run buffer for at least 5 min before each run. Thus, the variations in peak height and migration time were 2.3 and 2.7%, and 0.8 and 1.3%, for SDZ and SMZ, respectively.

Application. Fig. 5A shows a typical electropherogram obtained for SDZ and SMZ under the optimum conditions. The two compounds were well separated and sharp peaks were obtained with high efficiency. To ascertain the potential of the method, the determination of SDZ and SMZ in tablets and in urine samples from healthy volunteers was also performed. The electropherogram of the tablets is shown in Fig. 5B. Fig. 5C is the electropherogram of a blank urine sample and Fig. 5D is the electropherogram of urine spiked with a standard solution of SDZ and SMZ. After the volunteers had taken the tablets for 10 h, urine samples were collected and analysed; see Fig. 5E. From Fig. 5C and 5E, it can be seen that after being metabolized in the body, some of the SDZ and SMZ was excreted unchanged. Both

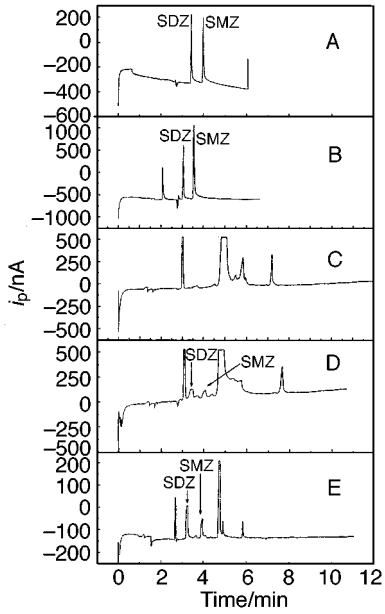

Fig. 5 Typical electropherogram of 50 μM SDZ and SMZ standard solution (A); synergisic bisulfanilamide tablets (B); pure urine sample (C); SDZ and SMZ were added to C (D) and urine sample after taking the tablets (E). Conditions: all urine samples were diluted with run buffer (urine : buffer = 1 : 3); run buffer, 10 mM phosphate buffer of pH 5.0; separation capillary, 25 μm id, length, 40 cm; separation voltage, 20 kV; sample injection, 10 s at 5 kV; detection potential, +1.1 V *versus* Ag/AgCl.

compounds were separated from other compounds in urine. Hence, the proposed method is very useful for detecting SDZ and SMZ in urine samples and has clinical relevance. In addition, the method is simple, rapid, sensitive and convenient. Interestingly, it was found that the peaks of SDZ and SMZ obtained from standard solutions (Fig. 5A) were very sharp, whereas band broadening was observed in real samples (Fig. 5D and 5E). The experiments were repeated and the same results were obtained. The reason for the band broadening is the high salt (ionic strength) content of the sample matrix *versus* that of the run buffer, which affects the peak shape of SDZ and SMZ.

Conclusion

This study has demonstrated the end-column electrochemical detection of SDZ and SMZ by capillary electrophoresis with a carbon fiber microdisc electrode. Under the optimized conditions, SDZ and SMZ were separated within 5 min with separation efficiencies of up to 390 000 and 335 000 theoretical plates, respectively. The detection limit was 0.1 μM for both compounds, which corresponds to 0.024 and 0.021 fmol for SDZ and SMZ, respectively. The method also exhibited high reproducibility of peak currents and migration times and can be used to detect SDZ and SMZ in real samples.

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