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Real-Time Measurement of Electroosmotic Flow in Capillary Zone Electrophoresis

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A method is developed for measuring the electroosmotic (bulk) flow rate continuously in capillary zone electrophoretic separations, which is based on introducing a reference flow of solution containing a fluorescent marker into the running buffer downstream from the detection zone. This technique can determine changes as small as 1% in the flow rate during a separation. The response time of the measurement is ~1 s, and the dynamic range extends over more than an order of magnitude in flow rate. This method is able to correct for changes (run to run) in the migration times of analytes that are caused by alteration of the capillary surface during analysis of complex samples, such as human serum. In addition, this method makes more practical the use of adjusted migration indices for the general representation of migration data and enhances the accuracy and precision of analyses in capillary zone electrokinetic separations.

Separations of complex mixtures into their constituents by capillary zone electrophoresis (CZE) often depends on the presence of electroosmosis, a phenomenon that drives a plug-like bulk flow of electrolyte through the capillary column. This characteristic gives CZE the capability to resolve positive, negative, and neutral (with the addition of micelles, etc.) species with high efficiency in a single run. Because both the migration time and the resolution of compounds separated by CZE are dependent on the magnitude of electroosmotic flow,[1] its measurement and control are essential for characterizing the components of the analyzed mixture in a separation.

At present, an analyte is usually identified by a peak in an electropherogram according to its migration time (t_m). Because the migration time depends sensitively on the capillary wall ζ-potential (ζ_c), electric field (E), and temperature (T), all of which affect the electroosmotic velocity during separation, the use of t_m for the identification of peaks in CZE is not completely satisfactory. Although the relative migration,[2] the electrophoretic mobility (μ_{ep}),[2,3] the use of multiple internal standards,[4] and the adjusted migration index (AMI)[2] have recently been introduced to address this issue, it is not yet possible to compensate fully for changes that may occur in E, T, and ζ_c[5,6] during a single run. As a result, the quest for a solution to the problem of peak (analyte) identification continues.

One way of addressing the problem of variability in the electric field, temperature, and capillary surface conditions during a run is to measure the electroosmotic flow continuously and to subsequently compensate for the changes in the migration time. Previously, the t_m of a neutral marker[7] and the streaming potential[8] have been used to obtain the average electroosmotic flow rate during runs. Also, the changes in capillary current during separation have been monitored,[9] and variations in the flow rate have been recorded by carefully weighing the outlet buffer reservoir.[8,10] Unfortunately, none of these methods appear to be suitable for continuous measurement of the electroosmotic flow rate. Recently, a design based on a postcolumn mixing scheme with mechanical or electroosmotic pumping for measuring electroosmosis has been suggested, but the performance of the device has not been demonstrated.[11] Hence, alternate approaches are needed for monitoring electroosmotic flow to correct for variations in the migration time caused by changes in ζ_c, E, and T during a separation.

We describe here the design and performance of a method capable of measuring the electroosmotic flow rate in CZE in real time. The operating mechanism of the method is based on the dilution of a fluorescent dye solution that is mixed with the running buffer exuding from the outlet of the separation capillary. We show that this technique can detect as small as a 1% change in the electroosmotic flow rate during a run, has a fast response time (~1 s), and possesses a large linear dynamic range (over an order of magnitude). The utility of the method to correct for changes in the migration times of analytes is demonstrated through the analysis of human serum samples. In addition, we discuss the use of this technique in conjunction with AMI for the general representation of migration data in CZE.

EXPERIMENTAL SECTION

Capillary Zone Electrophoresis. The CZE system employed is similar to that described previously.[12] A 30-kV high-voltage power supply (Glassman High Voltage, Inc., Whitehorse Station, NJ) and a 360-μm o.d. × 75-μm i.d. fused-silica capillary tubing (81 cm total length; 63 cm effective length) (Polymicro Technologies, Inc., Phoenix, AZ) were used throughout. Injection was achieved hydrodynamically by

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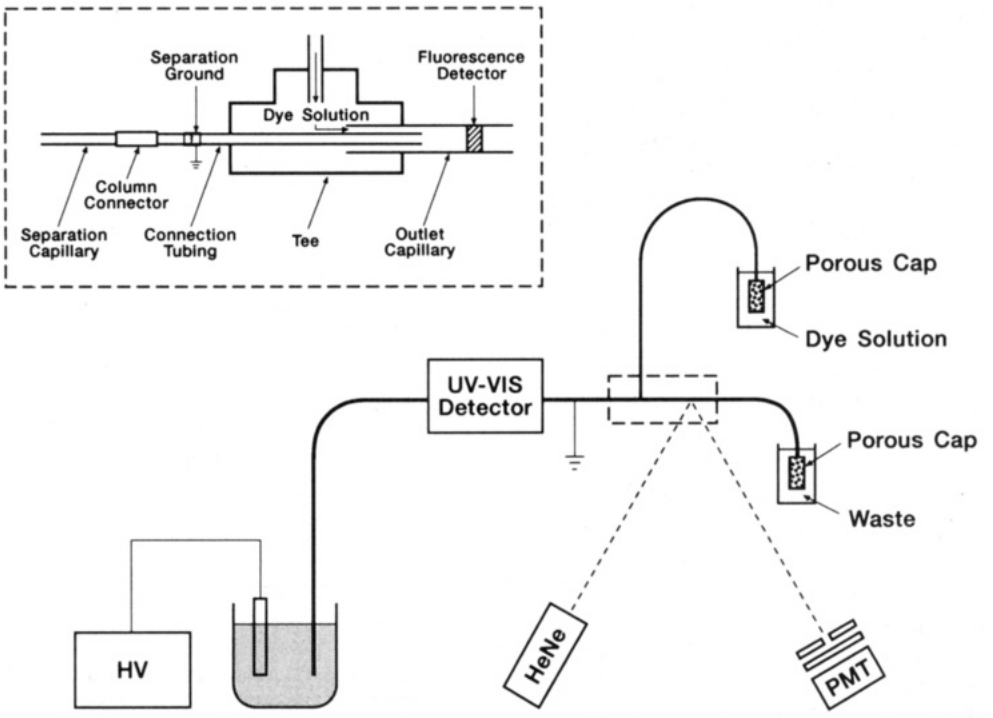

Figure 1. Schematic of apparatus used to measure electroosmotic flow.

raising the injection end of the capillary to a height of 5 cm relative to the detection end for 30 s. The running buffer utilized was 10 mM sodium borate solution at pH 9.1, and the capillary was pretreated with a 0.1 M sodium hydroxide solution prior to each day of analysis. Detection of analytes was accomplished with a UV–vis absorbance detector (Model CV⁴, Isco, Inc., Lincoln, NE) operating at 254 or 330 nm. Data were acquired through LabCalc (Galactic Industries Corp., Salem, NM) and stored on a PC/AT clone.

Measurement of Electroosmosis. The schematic of the system used to measure the electroosmotic flow is depicted in Figure 1. The detection end of the 75-μm-i.d. separation capillary was connected to a 1.5 cm long, 75-μm-i.d. grounding capillary via a "zero dead volume" capillary column connector (Polymicro Technologies Inc., Phoenix, AZ). To obtain a leakproof connection of the capillaries in the union, they were covered with a small piece of Teflon tape. This covering rendered permanent sealing unnecessary and resulted in a detachable system so that the reloading of separation capillaries could be readily performed. The other end of the grounding capillary was directed into a buffer reservoir where the electrical circuit was completed by on-column grounding. This grounding was achieved by creating a crack in the capillary to permit the migration of ions between the externally grounded buffer and the interior of the capillary.[13] The cracked part of the capillary was protected by a 365-μm-i.d. Nafion tubing (Perma Pure Products, Tom's River, NJ) to prevent obstruction of the channel resulting from misalignment of the capillary tubing on either side of the crack. The whole assembly was glued with waterproof epoxy onto a standard glass microscope slide, which was immersed in the ground buffer reservoir made from a 1-mL polyethylene vial. The last 3 mm of the 1.5-cm section of the capillary subsequent to the crack and emerging from the ground buffer reservoir was etched to an o.d. of approximately 80 μm with hydrofluoric acid and then inserted into a 150-μm i.d. × 360-μm o.d. fused-silica capillary. The junction was enclosed in a tee-shaped container with a third fused-silica tubing (365-μm i.d. × 435-μm o.d.) already attached. The assembly housed in the tee-shaped container resembled the design of a postcolumn derivatization apparatus for fluorescence detection.[14]

The polyimide coating on the section of the 150-μm i.d. capillary, which overlapped with the outlet of the 75-μm i.d. capillary, was removed with warm concentrated sulfuric acid to expose the bare fused silica. After mounting the whole assembly (tee junction) onto a piece of Teflon for ease of handling, the section containing the 150-μm-i.d. capillary was tilted at approximately Brewster's angle with respect to the beam (543.5 nm) from a 1-mW HeNe laser (PMS Electro-Optics, Boulder, CO). A 1-cm focal length lens (Melles Griot, Irvine, CA) was used to focus the beam into the interior of the 150-μm i.d. capillary, and two OG-570 long-pass filters (Melles Griot, Irvine, CA) were employed for the rejection of scattered light. Other details of the laser-induced fluorescence (LIF) detection scheme are given elsewhere.[15]

A 0.1 mM solution of 2′,7′-dichlorofluorescein (dye) made up in the running buffer was forced by gravity into the tee-shaped container through the 360-μm-i.d. capillary by raising the dye reservoir 5 cm above the container. The 150-μm i.d. capillary extended for a length of 3 cm from the junction with its end terminated in a 0.5-in.-diameter porous phase separator (cap) (Altair Gases of the West, San Jose, CA) which is totally immersed in a waste buffer vial. The use of the porous cap was found necessary to minimize noise from turbulence caused by the movement of liquid in the capillary.

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Reagents. Reagent grade sodium hydroxide and sodium tetraborate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). 2′,7′-Dichlorofluorescein was acquired from Eastman Kodak Co. (Rochester, NY), and human serum powder, dansyltyrosine, dansylisoleucine, and mesityl oxide were obtained from Sigma Chemical Co. (St. Louis, MO). All solutions were prepared in water purified with an Ultra-Pure water system (Millipore, Bedford, MA) and were filtered with 0.22-μm cutoff cellulose acetate filters (Alltech Associates, Deerfield, IL) before use.

RESULTS AND DISCUSSION

Because it is important that the modifications and the attachments to the separation capillary do not have any detrimental effects, we decided to compare the performance of an unmodified capillary with that of one that had been modified. In our apparatus (as is typical), electroosmotic flow is directed toward the cathodic (detection) end. Hence, subsequent to the termination of the electric field near the end of the grounding capillary, electroosmotic pressure forces the otherwise static fluid in the remainder of the grounding capillary into the 150-μm-i.d. outlet capillary where it mixes with the dye solution. 2′,7′-Dichlorofluorescein was chosen for our purposes because a homologous compound, fluorescein, is known not to adhere to the wall of the capillary.[16] Moreover, the 2′,7′-dichloro derivative possesses a higher overall quantum efficiency for fluorescence than fluorescein does when excited at 543.5 nm. The termination of the electric field prior to the outlet capillary is necessary to allow the use of a relatively large inner diameter. This procedure is also found to minimize nonlinearity in the fluorescence response. As previously noted for electrochemical detection in CZE, the presence of 1–3 cm of static buffer zone subsequent to the on-column grounding does not give rise to a noticeable loss of efficiency.[13] The employment of the concentric design has an added advantage. Only a low pressure is required to maintain adequate flow of the dye solution into the mixing zone. Hence, little back-pressure is generated in the separation capillary, as has been demonstrated by the high separation efficiency achievable in CZE with a sheath-flow cuvette-based LIF detector.[17] As shown in Figure 2, the efficiency attained with our design is comparable to that of an unmodified capillary. Figure 2 also shows that the on-column grounding and postcolumn mixing schemes employed here do not give rise to significantly adverse effects on separation efficiency.

The hydrodynamics of the sheath flow are well-characterized.[18] Assuming that the flow rate of the dye solution is small compared to that of electroosmosis and that photobleaching is not serious (since the laser power used is low), the intensity (I_f) of the collected fluorescence emitted by the dye in the mixing zone upon excitation can be expressed as

$$I_f = (KQ_d/v_{eo})C_o \qquad (1)$$

where Q_d, v_{eo}, C_o, and K represent the volumetric flow rate of the dye solution, the electroosmotic velocity, the concentration of the dye solution, and an instrumental constant determined by the excitation intensity, collection efficiency, fluorescence quantum yield of the dye in the mixing zone, and geometrical factors, respectively. Because v_{eo} should be linearly proportional to the electrophoretic current (J),[7,19–21] we verified the validity of eq 1 by plotting the inverse of the fluorescence intensity ($1/I_f$) versus the capillary current (shown in Figure 3). A linear relationship was observed (R = 0.999) between current values corresponding to an electroosmotic velocity of ~0–18 cm/min (for the 75-μm i.d. separation capillary used in the experiment). This range of flow rate is sufficient for most situations typical in practical analyses.

Although eq 1 reveals that the sensitivity of the device is improved by having a large Q_d, the comparable inside diameters

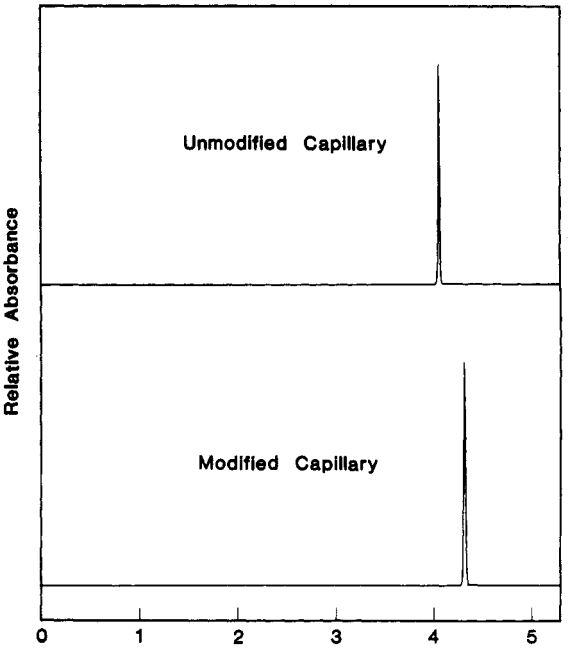

Figure 2. Comparison of separation efficiencies obtained with modified and unmodified capillaries. Mesityl oxide is used as the analyte. The separation voltage is 25 kV. Other details are given in the text.

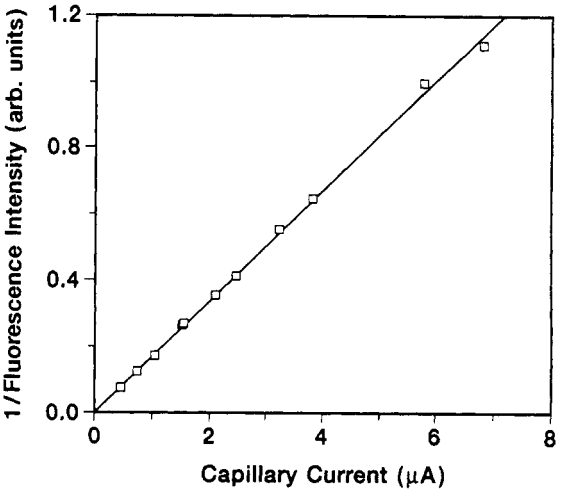

Figure 3. Plot of the inverse of the measured fluorescence intensity versus the capillary current.

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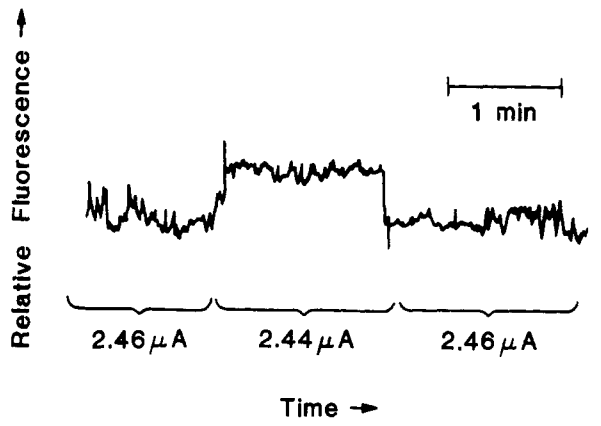

Figure 4. Plot of fluorescence intensity versus time for different capillary currents.

of the separation (75 μm) and receiving (150 μm) capillaries used here result in a large K and, hence, render the use of large Q_d's unnecessary. This conclusion is substantiated by the fact that the method can detect as small as a 1% change in v_{eo}, as revealed by observing the change in fluorescence intensity by changing the applied voltage (and, hence, the current) (Figure 4). Since the current of our naturally air-cooled CZE system is observed to fluctuate by roughly 1% every few seconds, the inherent fluctuations in J and, hence, in v_{eo} prevent us from identifying the smallest change in electroosmotic flow that can be detected with our design. Nonetheless, the minimal level of change in v_{eo} that can be detected with our device would ultimately be limited by flicker-noise on the excitation light, as has been the case for indirect fluorometric detection in CZE.[16] When higher levels of performance are required, the employment of semiconductor lasers as excitation sources with suitable dyes may prove to be advantageous. Although we have utilized LIF in this work, it should be noted that other detection schemes (e.g., absorbance) may also be applicable for the measurement of electroosmotic flow, especially in cases where analytes interfere with the fluorescence response.

The temporal response of the device is illustrated in Figure 5 where the behavior of the fluorescence signal as a function of abrupt changes in the electrophoretic current (by changing the applied voltage) is monitored. It is seen that the fluorescence signal responds faithfully to changes in flow rate (as revealed through the current) within 1–3 s. Because typical migration times in CZE range from 10 to 30 min, the error caused by the temporal response of our device is less than 1%.

To test the utility of our device for correcting variations in the migration times of analytes resulting from a change in the electric field, we chose the separation of two dansylated amino acids as model analytes for study. In Figure 6, the fluorescence signal from our device (dashed line) is superimposed upon the absorbance signal of the analytes from the detector (solid line). As shown in Figure 6a, the fluorescence signal remains constant for the duration of the first run. (The initial drop in fluorescence response is caused by the applied voltage being changed from 0 to the separation voltage of the run.) However, the same does not apply to the case depicted in Figure 6b where the electric field is decreased from 246 to 136 V/cm at 6.8 min into the run (right after detection of the

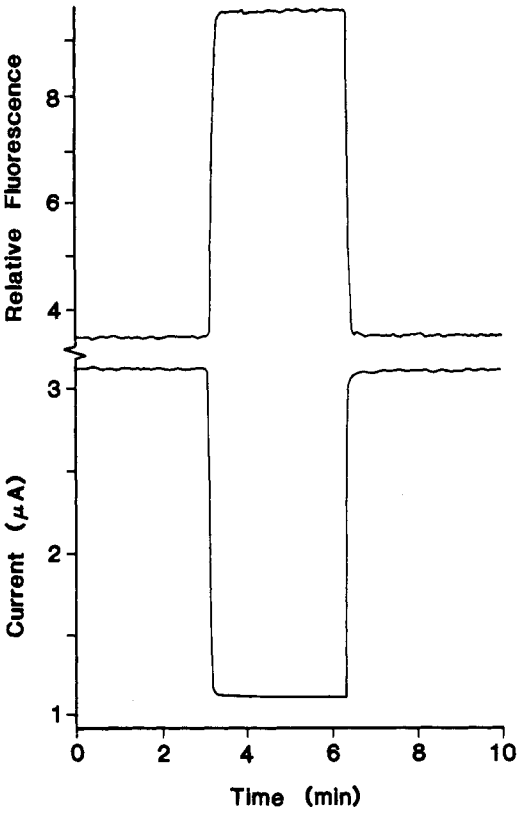

Figure 5. Plot depicting the temporal response of the measurement technique by comparison of the fluorescence intensity and the capillary current as a function of time.

first peak). As expected, the change in the electroosmotic flow rate from the decrease in the electric field is recorded in the fluorescence signal and leads to a 10% increase in the migration time of dansylisoleucine (peak 2). Using the fluorescence signal measured continuously during both runs, we can easily compensate for the changes in the migration times of the analytes resulting from the change in the electric field. The results are summarized in Table 1. It is seen that the migration times for the pair of analytes from both runs agree to within 2% after normalization with the fluorescence signal. This agreement indicates that our method can be used to compensate for changes in the migration times of analytes resulting from variations in the electroosmotic flow rate induced by changes in the electric field during separation. Because the actual electroosmotic flow rate is measured, our method should also correct for variations in the flow rate even in the presence of variations in temperature. Moreover, no internal standardization is necessary.

In the application of CZE to the practical analysis of samples, a problem of great concern is the change in the analyte migration time caused by the adsorption of concomitants from the sample matrix to the capillary wall, thereby giving rise to an altered ζ_c. To complicate matters further, no assurance can be given that the overall ζ_c remains constant throughout a run. Therefore, methods relying on internal standardization cannot be used to address the problem of changing ζ_c during a separation. Because the electrophoretic current is dominated by contributions from the movement of ions far away from the inner wall of the capillary, changes in the electroosmotic current are not easily detectable. Hence, measuring the

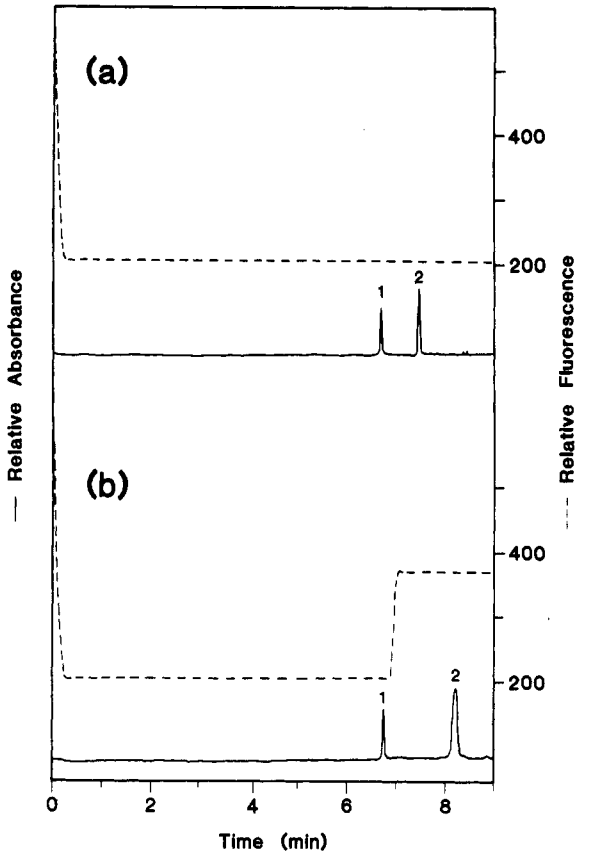

Figure 6. Electropherograms showing separation of dansyltyrosine (peak 1) and dansylisoleucine (peak 2) superimposed with the fluorescence signal. Part a shows the constant voltage run (20 kV) while part b shows the run in which the voltage is decreased from 20 to 11 kV at 6.8 min into the run. Absorbance detection is performed at 330 nm. Other details are given in the text.

Table 1. Comparisons of Corrected and Uncorrected Values for μ_{ep} from Separation of Dansylated Tyrosine and Isoleucine

	μ_{ep} (10^4 cm^2 V^{-1} s^{-1})			
	constant potential		step potential	
	uncorrected[a]	corrected[b]	uncorrected[a]	corrected[b]
dansyltyrosine	−1.00	−1.01	−1.02	−1.02
dansylisoleucine	−1.66	−1.67	−2.19	−1.68

[a] Calculated from $(L'/E)(1/t_m - 1/t_{eo})$. [b] Calculated from $(L'/E_o t_{eo})[(t_{eo}/I_{eo})(\int_0^{t_m} dt/I_f)^{-1} - 1]$; see Appendix A for derivation.

electrophoretic current during separation provides little useful information on ζ_c.

The ability to monitor electroosmotic flow accurately and continuously opens up however the possibility of accounting for variations in ζ_c resulting from changes during a separation. In the absence of variations in temperature and electric field strength, it can be shown that the electrophoretic mobility (μ_{ep}) is given by (see Appendix A)

$$\mu_{ep} = \frac{L'}{E t_m}\left(1 - \frac{I_{eo}}{t_{eo}}\int_0^{t_m} \frac{dt}{I_f}\right) \qquad (2)$$

where L', E, t_m, and I_f denote the distance between the injection end and detection region of the separation capillary, electric field, analyte migration time, and fluorescence signal from the device. In eq 2, t_{eo} and I_{eo} serve as external calibration factors that may be obtained before running the unknown

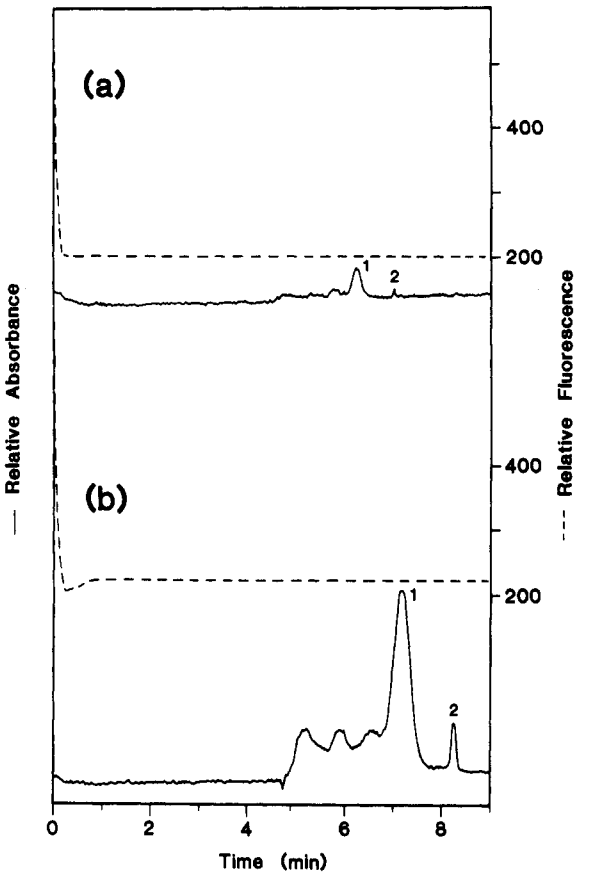

Figure 7. Electropherograms showing analyses of human serum at two different concentrations, superimposed with the fluorescence signal. Part a: 100-fold dilution; part b: 10-fold dilution. Detection is conducted at 254 nm. The separation voltage is 25 kV.

samples. As a consequence, no internal standardization is necessary.

Figure 7a,b depicts electropherograms and fluorescence signals from the injection of human serum at 100- and 10-fold dilutions, respectively. It can be seen that the fluorescence signal from the injection of the 10-fold diluted sample is larger than that from the less concentrated sample, indicating that the electroosmotic flow rate has been reduced in this case caused, possibly, by the adsorption of matrix concomitants to the capillary wall. This behavior is consistent with both the migration times observed here and the conclusions of an earlier study on the effects of proteins on electroosmotic flow in CZE.[5] Interestingly, the fluorescence signal in the case involving the more concentrated sample changes continuously in the first 35 s of the run subsequent to the decay of the fluorescence signal caused by the rise of the electric field. We have not observed similar behavior when low concentration samples are used (Figures 6 and 7). These figures indicate that a significant time delay exists before a steady overall ζ_c is reached when certain concentrated samples are injected. An understanding of this phenomenon may eventually lead to improved methods for minimizing analyte–wall interactions and increasing reproducibility in CZE.

The ability of our device and eq 2 to extract useful and accurate information from runs involving a continuously changing ζ_c is illustrated in Table 2. It is seen that even with the relatively large difference between the migration times of each analyte in the electropherograms, nearly identical corrected μ_{ep}'s (~2% difference) are obtained with our method.

Table 2. Comparisons of Corrected and Uncorrected Values for μ_{ep} from Analysis of Human Serum at 10- and 100-fold Dilutions

	μ_{ep} (10^4 cm^2 V^{-1} s^{-1})			
	100× dilution		10× dilution	
	uncorrected[a]	corrected[b]	uncorrected[a]	corrected[b]
component 1	−2.33	−2.37	−3.08	−2.42
component 2	−2.96	−3.00	−3.70	−3.04

[a] Calculated from $(L'/E)(1/t_m - 1/t_{eo})$. [b] Calculated from eq 2.

The utility of our device is not limited to cases involving either a change (within or between runs) in ζ_c or E. It may also be used to relate results from separations performed in the presence of simultaneously varying ζ_c, E, and/or T. To realize this advantage, the effects of changes in ζ_c, E, and T on the electroosmotic flow rate must be decoupled. This decoupling necessitates the measurement of both the electrophoretic current and the electroosmotic flow rate simultaneously. It has been established that AMI is independent of changes in ζ_c, E, and/or T between runs, but only E and/or T during runs.[2] With the ability to measure accurately the electroosmotic flow rate in real time, the use of AMI may now be extended to the case involving a changing ζ_c during separations. For this purpose, we can express AMI as (see Appendix B)

$$\mathrm{AMI} = \left(\int_0^{t_m} \frac{i}{L}\,dt\right) \Big/ \left(1 - \frac{I_{eo}}{t_{eo}} \int_0^{t_m} \frac{dt}{I_f}\right) \tag{3}$$

where i and L denote the electrophoretic current density and distance between the injection end of the separation capillary and the location of the on-column ground on the grounding capillary. The use of our device and eq 3 should render AMI independent of variations in E, T, and ζ_c during and/or between runs.

This paper has emphasized the use of the addition of fluorescent molecules into the flow as a means of determining continuously the electroosmotic flow rate. This information can be used readily for the identification of peaks in CZE. The utility of the method is not restricted to this use. It is conceivable that the technique may be suitable for functioning as an electroosmotic flow sensor used in a feedback control system (e.g., by using pressure or external electric fields[22,23]) so that resolution and analysis time can easily be optimized.

ACKNOWLEDGMENT

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APPENDIX A: DERIVATION OF EQUATION RELATING ELECTROPHORETIC MOBILITY AND FLUORESCENCE INTENSITY

External Calibration of Device. Equation 1 may be expressed as

$$(dx/dt) = (KQ_dC_o)/I_f \tag{4}$$

where x is the distance that a neutral species has traveled at time t. By measuring I_f during an external calibration run when only a neutral marker is injected, integration of eq 4 from the start of the run until the appearance of the peak corresponding to the neutral marker at t_{eo} gives

$$KQ_dC_o = (L'I_{eo})/t_{eo} \tag{5}$$

where I_{eo} is the time-weighted average of I_f before the appearance of the peak from the neutral marker.

Equation Relating μ_{ep} to I_f for Runs with Varying E. In general, the migration velocity of an analyte in CZE is given by

$$\frac{dy}{dt} = \frac{dx}{dt} + \mu_{ep}E \tag{6}$$

where y and E represent the distance that the analyte has traveled and the electric field strength used in the run, respectively. If we assume that μ_{eo} does not change with time during the run (ζ_c remains constant) and substitute eq 5 into eq 4, we get

$$dx/dt = L'(I_{eo}/t_{eo})(1/I_f) \tag{7}$$

Let E_o be the electric field used during the calibration run. By noting that $\mu_{eo} = (L'/t_{eo}E_o)$ and $dx/dt = \mu_{eo}E$, we obtain (from eq 7)

$$E = L'(I_{eo}/t_{eo})(1/I_f)(1/\mu_{eo}) \tag{8}$$

and

$$E = E_o(I_{eo}/I_f) \tag{9}$$

By substituting eqs 7 and 9 into eq 6, we can get

$$dy/dt = [L'(I_{eo}/t_{eo})(1/I_f)] + [\mu_{ep}E_o(I_{eo}/I_f)] \tag{10}$$

Integrating eq 10 between $t = 0$ and t_m and $y = 0$ and L', we obtain the equation in footnote b of Table 1.

Equation Relating μ_{ep} to I_f for Runs with Varying ζ_c. After substituting eq 5 into eq 4 and integrating the resulting equation from the start of the run to the appearance of the analyte peak at t_m, eq 2 is obtained. Note: Inspection of eq 2 shows that variations in dx/dt during separation are recorded in the time integral of $1/I_f$.

APPENDIX B: DERIVATION OF EQUATION FOR AMI

As shown by Lee and Yeung[2]

$$\frac{dy}{dt} = \frac{\epsilon i}{k\eta}\left[\zeta_c + \frac{2\zeta_a}{3} f(\kappa a)\right] \tag{11}$$

where ϵ, k, η, ζ_a, and $f(\kappa a)$ denote the dielectric constant of the medium, buffer conductivity, buffer viscosity, ζ-potential of the analyte, and a factor dependent upon the shape and size of the analyte molecule, respectively. ζ_c is related to the magnitude of the electroosmotic flow whereas $\zeta_a f(\kappa a)$ is related to that of the electrophoretic component. As the proportionality factor between dy/dt and i is relatively insensitive to temperature,[2,19-21] the use of the proportionality factor accounts for the generality of AMI for specifying an analyte in CZE. Once again, by integrating eq 11 from the start of a run to the appearance of the analyte peak at t_m, the following

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equation ensues:

$$L' = \frac{\epsilon}{k\eta}\int_0^{t_m} i\zeta_c \, dt + \frac{2\epsilon\zeta_a f(\kappa a)}{3k\eta}\int_0^{t_m} i \, dt \qquad (12)$$

where the first part on the right-hand side represents the distance traveled by an imaginary neutral marker and contains time-dependent contributions from both i and ζ_c. The second part on the right-hand side of the same equation gives the distance that the analyte would have migrated in the absence of electroosmotic flow. Hence, by measuring i and v_{eo} simultaneously, it is possible to decouple the contributions from E and ζ_c to t_m during a separation. Finally, by combining eqs 4, 5, and 12 and noting the definition of AMI,[2] we obtain eq 3.

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