Representing Primary Electrophoretic Data in the 1/Time Domain: Comparison to Representations in the Time Domain

Mathai Mammen, Ian J. Colton, Jeffrey D. Carbeck, Ryan Bradley, and George M. Whitesides

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

ANALYTICAL® CHEMISTRY

Reprinted from
Volume 69, Number 11, Pages 2165–2170

Anal. Chem. **1997**, *69*, 2165–2170

Representing Primary Electrophoretic Data in the 1/Time Domain: Comparison to Representations in the Time Domain

Mathai Mammen, Ian J. Colton, Jeffrey D. Carbeck, Ryan Bradley, and George M. Whitesides*

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

A plot of absorbance vs 1/time (the "1/time domain") is a more useful representation of the primary data in capillary electrophoresis than traditional plots of absorbance vs time (the "time domain") in a wide set of circumstances, especially when comparing electropherograms in which the rate of electroosmotic flow is not *precisely* the same. The quantity that is of fundamental interest in capillary electrophoresis (CE) is the electrophoretic mobility of an analyte. The electrophoretic mobility of a species is nonlinearly proportional to time and, therefore, not linearly represented in the time domain: that is, the distance between two peaks along the time axis is not linearly related to the difference in their electrophoretic mobilities. In contrast, the electrophoretic mobility is linearly proportional to 1/time, and the distance between two peaks along the 1/time axis is *linearly* related to the difference in electrophoretic mobilities. Plots in the 1/time domain are similar to the familiar plots in the time domain (each analyte is represented by a peak, and the order of peaks corresponds to the order in which these analytes reach the detector), but the spacing between the peaks corresponds linearly to differences in mobility. This article derives this useful, visually appealing, and broadly applicable plotting strategy and illustrates common situations in which these plots are more useful than plots in the time domain.

The current standard representation of primary data in capillary electrophoresis (CE) is a plot of absorbance as a function of time (the "time domain").[1] In such plots, the distance between two peaks along the x-axis is *nonlinearly* related to the difference in electrophoretic mobilities. In this paper, we discuss the advantages of an alternate format for representing primary data obtained using CE, in which absorbance is plotted against the negative reciprocal of time (-1/time, s^{-1}; the "1/time domain"). In plots in the 1/time domain, the distance between two peaks along the x-axis is *linearly* proportional to the difference in electrophoretic mobilities of these peaks; therefore, such plots are often more easily interpretable by *inspection* than plots in the time domain. None of the physical intuitiveness is lost in going from a time representation to a 1/time representation: each has peaks that correspond to compounds, and each has peaks in the same order that the compounds reach the detector. With minor changes in software, the primary output of an experiment (the raw, unprocessed data) can be in the 1/time domain.[2] We demonstrate that, in certain circumstances, plots in the time domain may actually be visually misleading and that plots depicting primary data in the 1/time domain are, in these cases, preferable. We illustrate the general utility of this plotting strategy using three examples: (i) portraying directly interpretable patterns in the mobilities of members of protein charge ladders, (ii) establishing trends in secondary markers in affinity capillary electrophoresis, and (iii) identifying peaks in complex mixtures when there are changes in electroosmotic flow (EOF).

1/TIME DOMAIN VS TIME DOMAIN

The electrophoretic mobility of an analyte, $\mu_{electro}$, is the fundamental quantity of interest in CE: $\mu_{electro}$ is the average velocity of an analyte per unit of electric field strength.[3-5] The characteristic value of $\mu_{electro}$ for an analyte is independent of the length of the capillary, the magnitude of the rate of EOF, and the magnitude of the electric field strength. The value of $\mu_{electro}$ is related theoretically to the charge, Z, on the analyte and is inversely proportional to its hydrodynamic drag (eq 1). The hydrodynamic drag is often given as a function of the mass of the molecule, M^{α} (where α varies between 0.5 and 1, depending on the class of molecule, and is typically 0.48 for proteins[6]); C_p is a proportionality constant (eq 1). Equation 2 gives an expression for $\mu_{electro}$ in terms of *experimentally* measurable quantities: L_{det} (m) is the length of the capillary from the inlet to the detector, L_{tot} (m) is the total length of the capillary, V (V) is the voltage applied across the capillary, and t_{eo} (s) and t_X (s) are the times taken for a neutral marker and a particular analyte (X) of interest to migrate from the inlet to the detector.

$$\mu_{electro} = C_p \frac{Z}{M^{\alpha}} \tag{1}$$

$$\mu_{electro} = \frac{L_{det}L_{tot}}{Vt_{eo}} - \frac{L_{det}L_{tot}}{V}\left(\frac{1}{t_X}\right) \tag{2}$$

$$\mu_{electro} = C_{exp} - D_{exp}\left(\frac{1}{t}\right) \tag{3}$$

The x-Axis in the 1/Time Domain Is Linearly Related to Electrophoretic Mobility. In cases where electropherograms are obtained under the same set of conditions (i.e., constant L_{det},

(1) St. Claire, R. L. *Anal. Chem.* **1996**, *68*, 569–586.

(2) In this paper, we use the time data to obtain the 1/time data by simply mapping every absorbance point corresponding to a time point to a 1/time point. We carry out this transformation by taking a column of time points and applying a function $x \rightarrow -1/x$ using KaleidaGraph.

S0003-2700(96)01123-7 CCC: $14.00

L_{tot}, V), plots of absorbance vs 1/time are directly comparable to one another: that is, 1/time is proportional to mobility (eq 3). In eq 2, the values of t_X refer to the respective migration times of analytes; in eq 3, however, the values of t represent any given point in time during the electrophoresis run. C_{exp} and D_{exp} are constants that depend on the details of the experiment. Combining eqs 1 and 2 shows that the quantity $1/t$ is linearly proportional to the charge on the analyte, Z, and inversely proportional to its drag, M^{α} (eq 4).[7]

$$\frac{1}{t_X} = \frac{1}{t_{eo}} - \frac{VC_p}{L_{det}L_{tot}}\left(\frac{Z}{M^{\alpha}}\right) \quad (4)$$

The x-Axis in the Time Domain Is Not Linearly Related to Electrophoretic Mobility. Equation 3 shows that t is not linearly proportional to $\mu_{electro}$. Furthermore, combining eqs 1 and 2 to give eq 5 shows that t is ***nonlinearly*** related to both fundamental properties of that analyte, Z and M^{α}.

$$t_X = t_{eo}\Big/\left[1 - \frac{t_{eo}VC_p}{L_{det}L_{tot}}\left(\frac{Z}{M^{\alpha}}\right)\right] \quad (5)$$

Relative Values (but Not Absolute Values) on the x-Axis Can Be Compared in the 1/Time Domain. Equation 3 indicates that the absolute value of the position of a peak on the 1/time axis depends on the value of C_{exp}. Evaluating C_{exp}, however, requires analyzing the data and is, therefore, more cumbersome than using a simple 1/time plot, which requires no analysis. In a 1/time plot, the spacing between peaks is linearly proportional to differences in electrophoretic mobilities between analytes. Comparisons of different electropherograms in the 1/time domain can, therefore, be made visually by first manually aligning the different spectra as they are printed using an internal standard. Such manual corrections or comparisons are not possible in the time domain since the spacing between peaks is not directly proportional to mobility. We could take this logic one step further and determine the electrophoretic mobilities of each point in the time domain by determining the values of C_{exp} and D_{exp} in eq 3. We could then plot experimental data in a true "mobility domain" by plotting absorbance vs $\mu_{electro}$. The distance between peaks along the x-axis in such a plot would be precisely *equal* to the difference in electrophoretic mobilities (and not merely *proportional* to the difference, as in 1/time plots). In mobility plots, manual aligning of the spectra being compared would be unnecessary.

Electroosmotic flow (EOF) is the bulk flow of solution, in an electrical field, caused by a charge imbalance at the interface of the capillary containing the solution. The value of EOF is expressed as the time required for a neutral marker, t_{eo}, to migrate from the inlet of a capillary to the detector window, L_{det}. The value of t_{eo} may be affected by factors such as temperature, pH, ionic strength, and the nature of the capillary; the last is the most common. Plots in the 1/time domain confer two classes of advantages, which we will describe next.

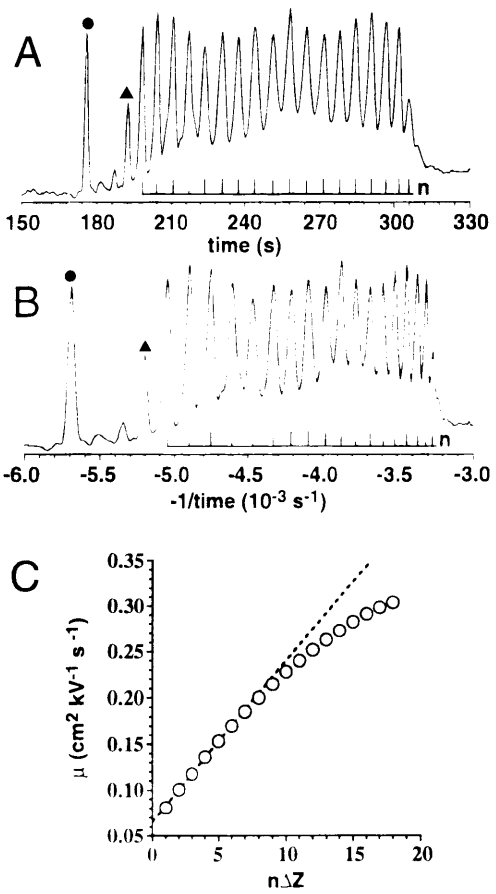


Figure 1. Electropherogram of a charge ladder of carbonic anhydrase II in (A) the time domain and (B) the 1/time domain. The members of the charge ladder appear to be separated by an equal spacing when the data are plotted in the time domain. When the data are represented in the 1/time domain, the spacing between members decreases as a function of extent of conversion. The electropherogram plotted in the 1/time domain reveals the same information as a plot of μ vs $n\Delta Z$ (C) but does not require any data analysis. ●, The peak due to p-methoxybenzyl alcohol, used as a neutral marker in these experiments. ▲, The peak due to native carbonic anhydrase II. This experiment was run at 25 °C, 15 kV, using 25 mM Tris−192 mM Gly buffer, pH 8.3, and detection wavelength λ = 214 nm. The capillary tubing was 47 cm long, with a distance of 40 cm from inlet to detector and an inner diameter of 50 μm.

Apparent Spacing of Peaks in the 1/Time Domain Is Proportional to Differences in Electrophoretic Mobility. In Figure 1, we show plots of a charge ladder of carbonic anhydrase II in both the time domain (A) and the 1/time domain (B).[8,9] This ladder was made by random partial acetylation of lysine ϵ-amino groups on the surface of the protein. At neutral pH, there is a unit charge difference between each successive member of a charge ladder but only a relatively small change in mass. Because eq 5 is a nonlinear equation in Z, it is difficult to predict rapidly the pattern of peaks for this mixture of proteins. Experimentally, the absorbance vs time plot gives an approximately equal spacing between members of the charge ladder. Equation 4 is a linear equation relating the migration time of an analyte peak to its

(3) Mammen, M.; Gomez, F.; Whitesides, G. M. *Anal. Chem.* **1995**, *67*, 3526–3535.
(4) Kuhr, W. G.; Monnig, C. A. *Anal. Chem.* **1992**, *64*, 389–407.
(5) Chu, Y.-H.; Avila, L. Z.; Gao, J.; Whitesides, G. M. *Acc. Chem. Res.* **1995**, *28*, 461–468.
(6) Gao, J.; Whitesides, G. M. *Anal. Chem.*, in press.
(7) CE data expressed in the 1/time domain have a bit-to-bit correspondence with the same data expressed in the time domain. As an example from a related field, IR spectra may be expressed either in wavelength (micrometers) or in frequency terms (cm^{-1}) without any loss of information.
(8) Gao, J.; Mrksich, M.; Gomez, F. A.; Whitesides, G. M. *Anal. Chem.* **1995**, *67*, 3093–3100.
(9) Gao, J.; Mammen, M.; Whitesides, G. M. *Science* **1996**, *272*, 535–537.

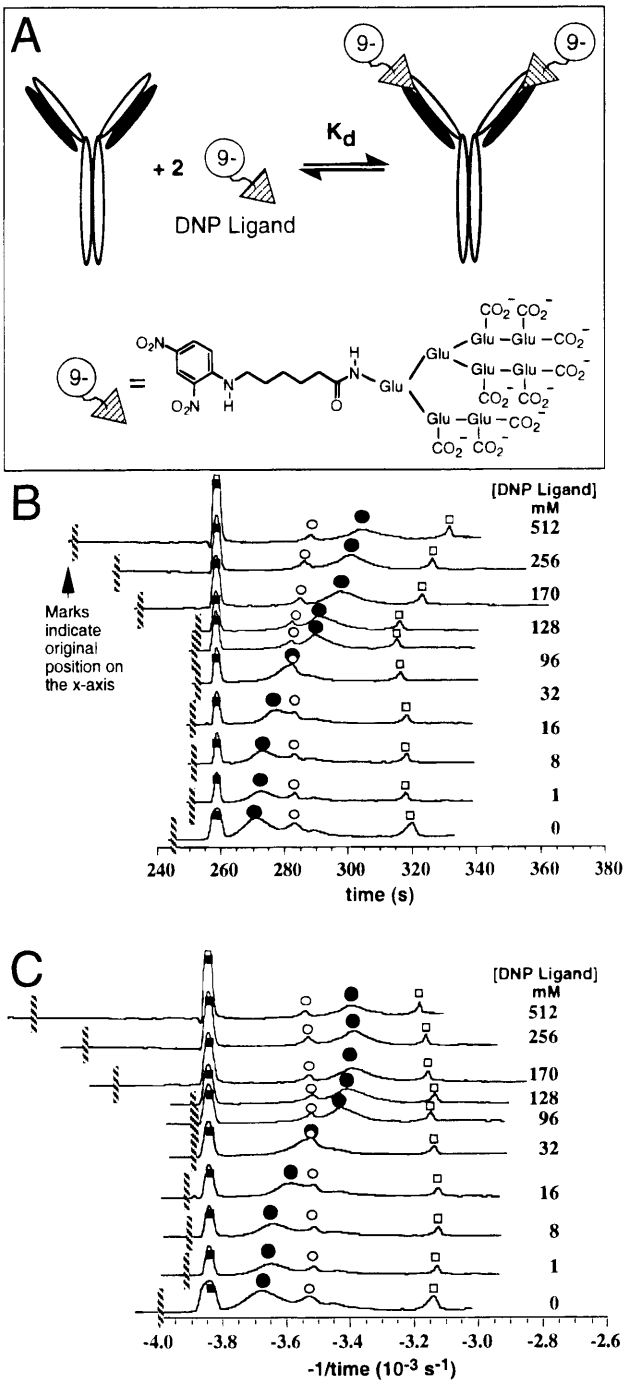


Figure 2. (A) Affinity capillary electrophoresis (ACE) experiment involving an anti-DNP IgG antibody and a (9−)-DNP ligand. Electropherograms from this ACE experiment in (B) the time domain and (C) the 1/time domain. The vertical hatch marks indicate the relative position of the electropherogram at 245 s in the time domain and -4.1×10^{-3} s^{-1} in the 1/time domain. The electropherograms plotted in the 1/time domain reveal that the mobilities of the secondary markers do not change appreciably as a function of antigen concentration. The same information can be obtained by plotting the calculated electrophoretic mobilities of the markers as a function of [(9−)-DNP]. Using mesityl oxide (MO, 20 μM) (■) as an indicator of rate of EO flow and carbonic anhydrase B (CAB, 1 mg/mL) (○) and α-lactalbumin (LA, 1 mg/mL) (□) as internal references, electropherograms of IgG_{2b} (0.6 mg/mL) (●) are obtained as a function of increasing concentration of a negatively (9−) charged ligand containing a dinitrophenyl (DNP) group. The capillary tubing was 57 cm long, with a distance of 47 cm from inlet to detector and an inner diameter of 50 μm. The conditions of the experiment were as follows: $V = 30$ kV; current, 20 μA; buffer, 25 mM Tris, 192 mM glycine (pH 8.3), with 0.5 M quinuclidine propanesulfonate and 10 mM K_2SO_4 added to reduce adsorption of protein to the capillary wall; detection at $\lambda = 200$ nm; $T = 25 \pm 2$ °C.

charge and mass. This equation predicts that the spacing between peaks in an electropherogram plotted in the 1/time domain should be directly proportional to the charge and inversely porportional to the mass of successive members of the charge ladder. When the data are plotted in the 1/time domain, the spacing between members of the charge ladder clearly *decreases* with extent of derivatization. This decrease suggests either that the charge increment is decreasing or that the hydrodynamic drag (proportional to M^{α}) is increasing with extent of acetylation. The reasons in either case are probably complicated and include the possibility of differential shielding of differently charged surfaces, changes in hydrodynamic drag on acetylation, incomplete ionization of a highly negative surface, and slight expansion of the body of the protein due to electrostatic repulsion between like charges. We are actively exploring these possibilities.

The important information, provided by the curvature in Figure 1C,[8,9] is lost when the data are plotted in the time domain, which may lead the experimenter to draw incorrect conclusions. The conventional plot in the time domain, in this instance, is *visually misleading*.

Plotting in the 1/Time Domain Allows Visual Indentification of Peaks in a Complex Mixture. Using the standard plot, changes in EOF prevent one from directly comparing the electropherograms from different runs. As an example, in Figure 2B, we show a series of stacked electropherograms from an affinity capillary electrophoresis experiment involving a monoclonal IgG antibody and its ligand.[3] When we manually overlay the spectra using the neutral marker as a reference, we cannot simultaneously overlap the peaks corresponding to the neutral marker and the secondary markers, α-lactalbumin and carbonic anhydrase, due to changes in EOF. A plot of absorbance as a function of 1/time (Figure 2C) permits a more reasonable alignment of both the neutral *and* the secondary marker peaks. When the peaks corresponding to the antibody and carbonic anhydrase are close together, it becomes difficult to identify each peak. Plots in the 1/time domain simplify this identification greatly. Another advantage of the plot in the 1/time domain is that it clearly indicates the concentration at which a protein is completely saturated with ligand; by following the IgG peak in the stacked electroperograms, a binding isotherm is traced out. Due to changes in EOF, the extent of complexation is not clear with the plot in the time domain. Such immediate visual information is useful prior to performing Scatchard analysis to determine binding constants of proteins for ligands.

Figure 3 depicts three electropherograms obtained using laser-induced fluorescence (LIF) detection ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm).[10] The first electropherogram, in the time domain (Figure 3B), represents a sample containing acylated derivatives of insulin formed by allowing insulin to react with 5-carboxyfluorescein succinimidyl ester (F-NHS) in solution. The three amines on insulin react randomly with F-NHS; the positive charge on each acylated amine group is neutralized, while negative charge is introduced from the carboxylate and phenolate groups of the acylating agent. The second electropherogram, in the time domain (Figure 3C), shows the products formed when insulin was first adsorbed to a self-assembled monolayer (SAM) of hexadecanethiolate and allowed to react with F-NHS. The third electropherogram, in the time domain (Figure 3D), is a coinjection of

(10) Colton, I. J.; Gao, J.; Brittain, S.; Mrksich, M.; Whitesides, G. M. Manuscript in preparation.

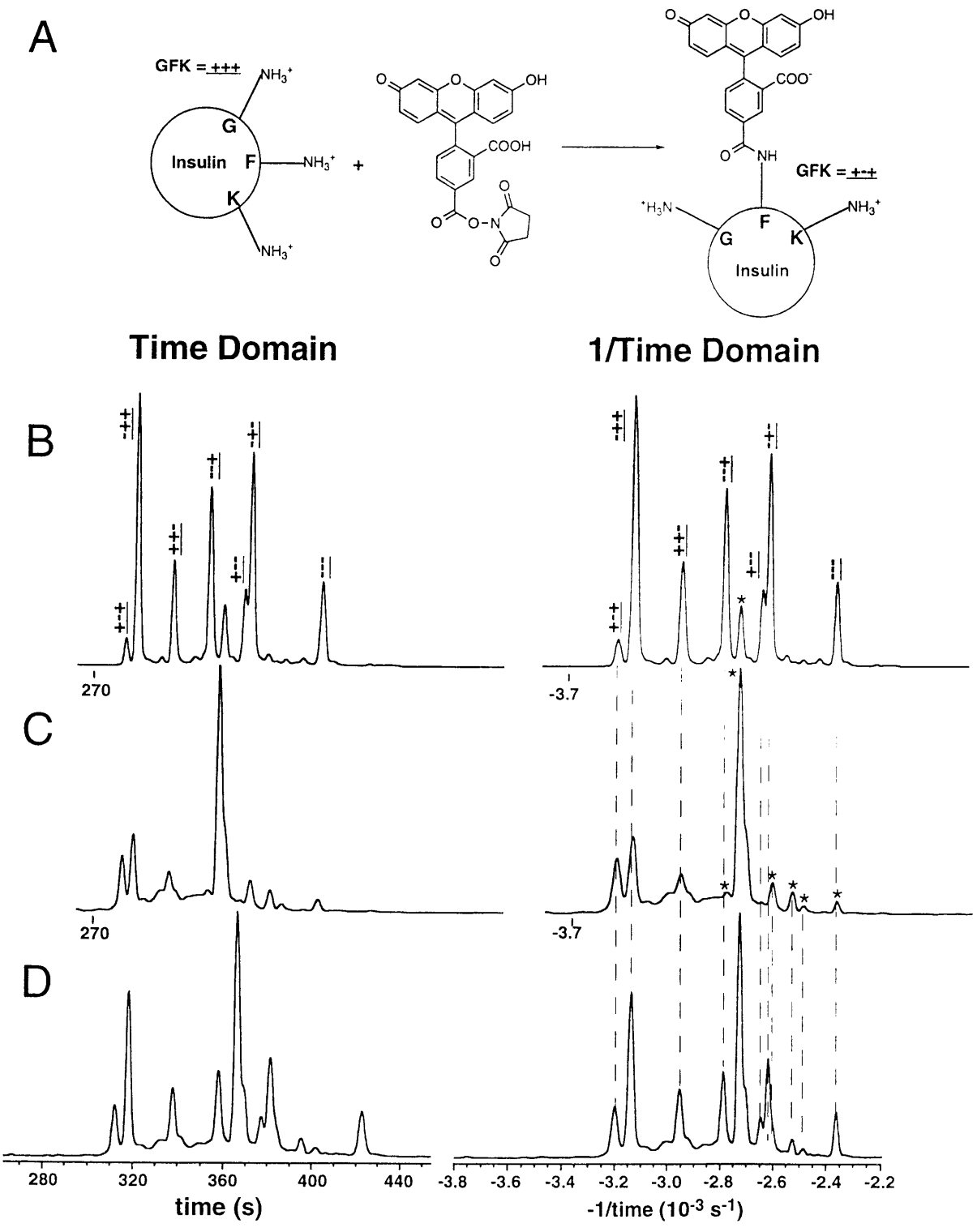


Figure 3. (A) Insulin was adsorbed to a monolayer of hexadecanethiolate on gold and allowed to react with 5-carboxyfluorescein succinimidyl ester. The letters G, F, and K are abbreviations for the amino acid residues of glycine, phenylalanine, and lysine, respectively. In the example shown, native insulin is symbolized by +++, while the monoacylated phenylalanine derivative is symbolized by +−+; the "+" signifies an unmodified amino residue, while the "−" signifies an amino group modified with negatively charged fluorescein. (B) Electropherograms of a standard set of acylated derivatives of insulin formed by allowing native insulin to react with 5-carboxyfluorescein succinimidyl ester in 100 mM phosphate buffer, pH 6.8, both in the time and the 1/time domains. (C) Electropherograms of products obtained upon acylation of insulin adsorbed to a self-assembled monolayer of hexadecanethiolate using 5-carboxyfluorescein succinimidyl ester, in both the time and the 1/time domains. (D) Electropherograms of a coinjection of (A) and (B) in both the time and the 1/time domains. Electropherograms in (B), (C), and (D) in the time domain could not be aligned properly due to changes in the nature of the capillary that resulted in changes in EOF. Plotting the data in the 1/time domain permits us to align the electropherograms and facilitates peak identification. The asterisks in the electropherograms denote peaks that either did not overlap with any of the peaks from the standard set of acylated derivatives of insulin or had coincident mobilities with some of the standard peaks. These peaks were discounted as fluorescent impurities on the basis of an experiment involving an anti-insulin antibody that complexes specifically with insulin and its acylated derivatives. Experiments were run at 25 °C, 15 kV, using 25 mM tris, 192 mM glycine (pH 9.4) running buffer; LIF detection, λ_{ex} = 488 nm, λ_{em} = 520 nm. The capillary tubing was 47 cm long, with a distance of 40 cm from inlet to detector and an inner diameter of 50 μm.

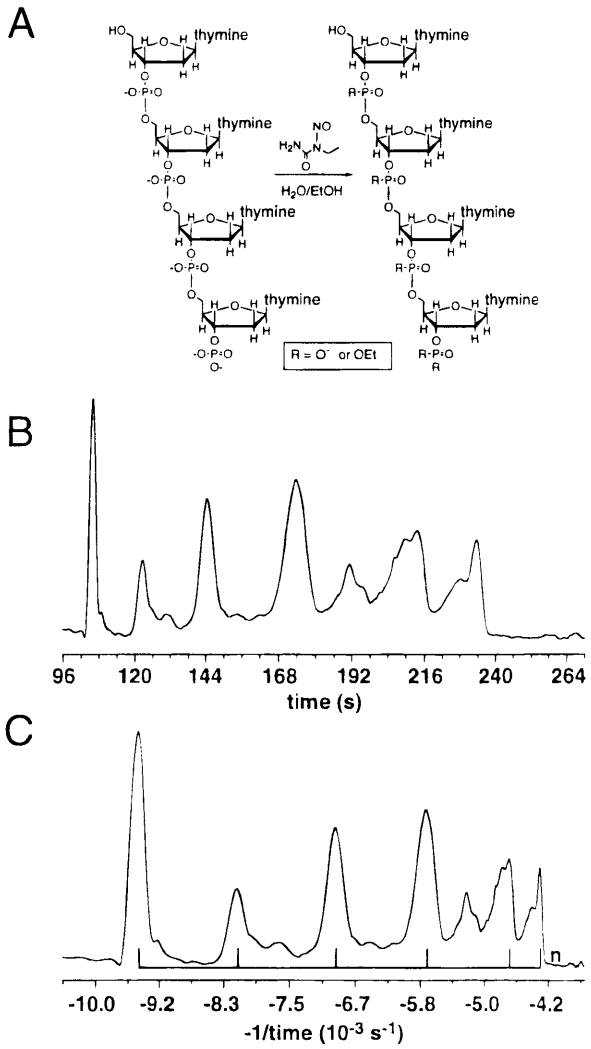
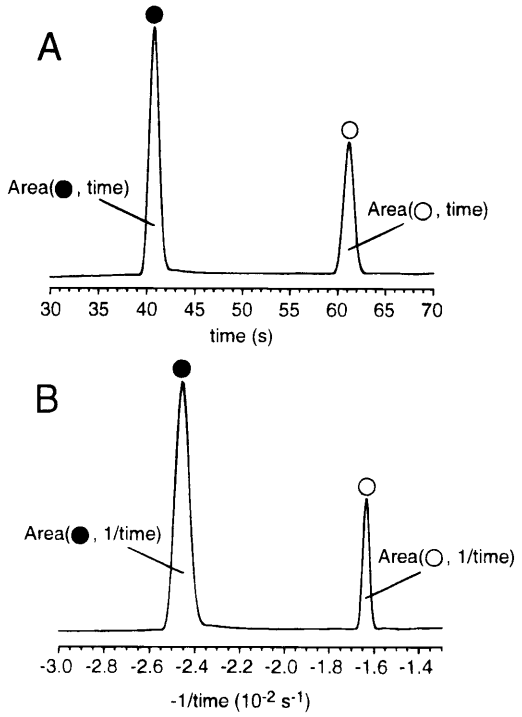


Figure 4. (A) Charge ladder of oligothymidylic acid, $(p(dT)_4)$, generated by ethylating the oxygens of phosphate on $p(dT)_4$, thereby neutralizing a variable number of negative charges. Electropherograms of a crude reaction mixture of a charge ladder of $p(dT)_4$ in both (B) the time domain and (C) the 1/time domain. Plotting the data in the 1/time domain facilitates assignment of the members of the charge ladder. The CE experiments were run at 25 °C using 50 mM sodium cacodylate buffer, pH 8, $\lambda = 254$ nm. The capillary tubing was 27 cm long, with a distance of 20 cm from inlet to detector and an inner diameter of 50 μm.



Peak	Corrected Area of Peak: time domain	Corrected Area of Peak: 1/time domain
●	$\frac{\text{Area}(\bullet, \text{time})}{t_{migration}(\bullet)}$	$\text{Area}(\bullet, 1/\text{time}) \times t_{migration}(\bullet)$
○	$\frac{\text{Area}(\circ, \text{time})}{t_{migration}(\circ)}$	$\text{Area}(\circ, 1/\text{time}) \times t_{migration}(\circ)$

Figure 5. Electropherogram of *p*-methoxybenzyl alcohol (●, neutral marker) and *p*-methoxybenzoic acid (○, one negative charge) in both (A) the time domain and (B) the 1/time domain. The total area of a peak in *neither* representation directly indicates the quantity of material on the capillary. The observed peak area of the analyte in the 1/time domain is given by Area (○, 1/time), and that in the time domain is given by Area (○, time). In the 1/time domain, the amount of material is proportional to Area (○, 1/time) *multiplied* by the time of migration. In the time domain, the amount of material is proportional to Area (○, time) *divided* by the time of migration.

the first and second samples. Due to changes in EOF between the second and third runs, we were unable to compare directly the corresponding electropherograms plotted in the time domain. Corrections for changes in EOF may be made by calculating and comparing the electrophoretic mobilities for each emergent peak from the two runs. Since, however, we did not have a water-soluble, fluorescent neutral marker with appropriate excitation and emission wavelengths, these calculations could not be made conveniently. The proportionality between 1/time and mobility allowed us to compensate for EOF changes; plotting the data in the 1/time domain permitted us to overlay all three electropherograms. Comparison of the electropherograms in the 1/time domain revealed the formation of three monoacylated derivatives of insulin, $\underline{+-+}$, $\underline{-++}$, and $\underline{++-}$ (see Figure 3 caption for peak labeling) on the surface of the SAM. Some fluorescent impurity peaks labeled with an asterisk were coincident with certain of the acylated derivatives of insulin.[11]

Figure 4 shows electropherograms of a charge ladder of oligothymidylic acid $(d(pT)_4)$ in the time and 1/time domains, respectively.[12] These ladders were made by ethylating the oligonucleotide on the phosphate oxygens using *N*-nitroso-*N*-ethylurea;[13,14] this alkylation neutralizes a negative charge. Since there were many peaks in the crude reaction mixture, it was difficult to identify the peaks that corresponded to the actual members of the charge ladder and to distinguish these peaks from unidentified impurities. Plotting absorbance as a function of $-1/$time allowed the members of the charge ladder to be assigned visually. Plotting the data in the 1/time domain shows the appropriate separation of a charge ladder comprised of four members differing from each other by an approximately integral

(11) An experiment involving the specific reaction of acylated members of insulin with anti-insulin antibody (Sigma I-2018) showed that only the monoacylated derivatives of insulin were formed on the SAM.
(12) Carbeck, J.; Bradley, R. J.; Whitesides, G. M. Manuscript in preparation.
(13) Rhaese, H.-J.; Freese, E. *Biochim. Biophys. Acta* **1969**, *190*, 418–433.
(14) Sun, L.; Singer, B. *Biochemistry* **1975**, *14*, 1795–1802.

unit of charge but only minimally in hydrodynamic drag.[15] When the data are represented in the time domain, peak assignment is not possible.

PEAK SHAPE IN THE TIME AND 1/TIME DOMAIN

In CE, the analyte moves past the detector at different velocities. The greater the velocity, the narrower the appearance of the peak in the traditional time domain (Figure 5). The peak areas for different peaks are, therefore, not directly comparable. To compare the areas of peaks, the observed areas are first multiplied by the velocity of the moving peak (or, equivalently, divided by its time of migration). The corrected peak areas are then meaningful and can be compared to one another. This effect is entirely independent of any mechanism leading to broadening of the band on the capillary. In the 1/time domain, this trend is reversed: the apparent peak areas must be *divided* by the apparent velocity (or, equivalently, multiplied by the time of migration) to obtain the true peak area. Again, the corrected peak areas are then directly comparable.

CONCLUSIONS

Plotting absorbance as a function of 1/time improves the interpretability of data from CE. Plots in the 1/time domain allow direct comparison of raw spectra by overlaying of a marker peak (for example, a neutral marker) and comparison of relative values of 1/time (that is, relative differences in mobility) of the remaining peaks. Such a plotting strategy is qualitatively different than previously suggested methods.[16] A 1/time plot can be the primary output from an experiment. Here we have shown two sorts of advantages of plots in the 1/time domain over those in the time domain: the plots in the 1/time domain present information that would have otherwise been overlooked in a time domain plot (e.g., spacing between members of a charge ladder as a function of n), and the plots in the 1/time domain aid in rapid visual peak identification when a complex mixture of analytes is being analyzed. We suggest that plots in the 1/time domain have more interpretable information than those in the time domain and are, therefore, generally preferred.

Currently, raw data from electrophoretic runs must be imported to a plotting application in order to display the data in the 1/time domain. Therefore, the only current disadvantage of plotting primary data in the 1/time domain is that it may not be convenient. CE software must be modified to accommodate this alternate plotting format.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health, Grant GM51559.

Received for review November 5, 1996. Accepted March 3, 1997.[⊗]

AC961123Y

(15) The last peak to migrate represents the modification of the terminal phosphate residue that has a pK_a of ~7; neutralization of this residue, therefore, results in only a partial charge separation from its adjacent peak, using a running buffer of pH 8.

(16) Rathore, A. S.; Horvath, C. *J. Chromatogr. A* **1996**, *743*, 231–264.

[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1997.