The Electrophoretic Mobility of Tripeptides as a Function of pH and Ionic Strength: Comparison with Iontophoretic Flux Data

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Capillary electrophoresis (CE) is an extremely efficient separations tool which can also be used to determine fundamental molecular parameters, e.g., the electrophoretic mobility of a molecule. We have studied the changes in the CE estimated electrophoretic mobility of thyrotropin releasing hormone (TRH) as a function of pH and ionic strength. Further, we have used CE to estimate the mobilities of two synthetic analogs of TRH to examine the behavior of positive (basic) and negative (acidic) peptides under the conditions of this work. These data were then compared with literature values of iontophoretic flux of these molecules under similar formulation conditions. Our results suggest that CE could potentially assist formulation optimization for the iontophoretic delivery of peptides.

KEY WORDS: iontophoresis; capillary electrophoresis; TRH; formulation; electroosmosis.

INTRODUCTION

Iontophoresis is the electrically assisted motion of charged molecules across the skin. While a few clinical applications of this non-invasive drug delivery scheme have continued over the years, notably the application of pilocarpine for diagnostic purposes (1-3) and the application of lidocaine and/or dexamethasone for analgesic purposes (3-6), this technique has not replaced more traditional drug delivery routines. Recently, there has been resurgent interest in iontophoresis due in large part to the growth in biotechnology and the utility of peptides and proteins as therapeutic agents. These drugs are generally not administerable via the oral route and could profit from the many potential benefits of iontophoretic delivery as pointed out by Chein, et al. (3). Interest in iontophoresis of these types of relatively large molecules has moved research in this area away from the transport of small, charged species to that of molecules which are generally not responsible for significant current carrying capabilities.

Capillary electrophoresis (CE) is an analytical separations technique which depends upon small differences in molecular electrophoretic mobilities (charge/mass ratios) for the separation of different species (7-9). Further, a net solvent flow, electroosmosis, which results from the electrical double layer established at the capillary solution interface, generally carries all molecules (i.e., cations, anions, and neutrals) through the capillary for detection at the far end of the separation. This technique has been used for a wide variety of separations including peptides (10), proteins (11,12), amino acids (13), polystyrene nanoparticles (14), oligonucleotides (15,16), and conventional drugs (17).

As originally pointed out by Abramson and Gorin (18) and later confirmed both theoretically and experimentally by Pikal (19) and Pikal and Shah (20,21) and recently reviewed by Pikal (22), a theoretical relationship exists between CE and iontophoresis based upon fundamental physical processes which result in unidirectional solvent flow (electroosmosis), and molecular migration (electrophoresis). Our hypothesis for this work was that CE estimated mobilities might be useful in formulation optimization (i.e., pH, ionic strength) of the iontophoresis of peptides.

This hypothesis has been tested via CE determination of the mobility of thyrotropin releasing hormone (TRH) over a range of pH and ionic strength as well as the mobilities of two synthetic peptide analogs of TRH under more specific conditions. *In vitro* iontophoretic penetration studies of these substrates across human cadaver skin have been carried out using similar formulations and reported elsewhere (23-25). A comparison of the results from those studies with the CE derived mobilities determined in this work supports the notion that mobilities estimated by CE could be useful in formulation optimization for iontophoresis of peptides.

MATERIALS AND METHODS

All CE data were gathered using a self built instrument and capillaries obtained from PolyMicro Technologies, Phoenix, AZ. The capillaries were 50 um i.d. and 350 um o.d. and varied from 75-110 cm long with detection occurring 40-60 cm from the high voltage end. The peptides under investigation and riboflavin, the neutral marker used to evaluate the coefficient of electroosmotic flow (μ_{eo}), were detected by UV absorbance at 225 nm using the CV[4] Capillary Electrophoresis Detector, manufactured by Isco, Lincoln, NE. The CE apparatus contains a relay box which allows for timed injections, a plastic box, purchased from IsoLabs, Inc., Akron, OH, and was modified in-house to meet safety specifications (i.e., safety interlocked for operator protection from accidental electric shock). High voltage was supplied from a Bertan Associates model 210-50R high voltage power supply (Bertan Associates, Hicksville, NY). All sample injections were carried out electroosmotically as detailed by Ewing, et al. (7), using a 1 or 2 sec voltage pulse at the running voltage; usually either 25 or 30 kV. Electropherograms were recorded using a Kipp and Zonen BD-41, 2-pen strip chart recorder.

Electrophoretic mobilities (μ_{ep}) and coefficients of electroosmotic flow (μ_{eo}) were calculated in the normal manner (8) from the measured migration times of the neutral marker, riboflavin, and that of the peptide substrates of interest using the following equations:

$$\mu_{eo} = [(L_1)(L_2)]/[(V)(t_r)] \quad (1)$$

$$\mu_{Total} = [(L_1)(L_2)]/[(V)(t_p)] \quad (2)$$

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$$\mu_{ep} = \mu_{Total} - \mu_{eo} \quad (3)$$

where L_1 = length of capillary (cm), L_2 = length to the detector (cm), V = voltage (Volts), t_r = migration time of the neutral (sec), and t_p = migration time of the peptide (sec). These coefficients have units of $cm^2/(V*sec)$ and are independent of experimental parameters, i.e., capillary dimensions (L_1, L_2, diameter) and the voltage gradient. This allows for direct comparison of these coefficients in different buffer systems of varying ionic strengths and pH's and between capillaries. It is useful to point out that reproducibility of electroosmotic flow is normally within 1% coefficient of variation (26).

Riboflavin was obtained at 98% purity from Aldrich Chemical Co. All buffer species and TRH were purchased from Sigma Chemical. Milli-Q water (18 Mohm*cm) was used throughout all CE experimentation. The synthetic tripeptides were a gift of Mr. Mark Bobko, Sterling Winthrop, Inc., consisting of desamino-tyrosinyl-lysinyl-prolinamide (dTLP) and desamino-tyrosinyl-glutamyl-prolinamide (dTGP).

RESULTS AND DISCUSSION

CE is carried out in very narrow diameter capillaries (2 μm < i.d. < 100 μm i.d.) made of fused silica at voltage gradients of up to 400 V/cm (7-10). In the normal, anodal configuration, a large positive voltage is imposed across the capillary from the starting end to the detector end of the capillary which is held at electrical ground. Molecules move through the capillary under the influence of this large potential gradient by the sum of two forces, electrophoretic mobility and electroosmotic flow. The anodal configuration results in solvent flow from the positive end of the capillary to the ground end as shown in figure 1a. The magnitude and direction of this flow are a function of the nature of the capillary material (27). Inasmuch as silica/water interfaces are negative over readily accessible pH ranges, the positive counterions within the double layer cause the solution to move away from the positive electrode (i.e., the anode). The solution pH influences this flow by altering the magnitude of the negative charge on the capillary wall. Ionic strength effects the induced solvent flow by masking the charge of the double layer and thereby diminishing the driving force for bulk solvent flow (18).

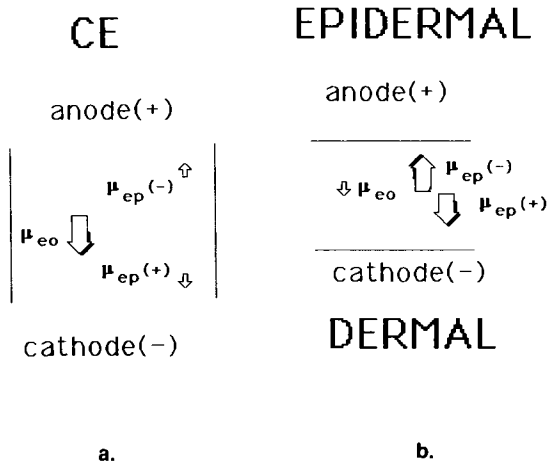

Fig. 1. A vectoral illustration of the direction and relative magnitudes of electroosmotic flow and electrophoretic mobility in a) capillary electrophoresis and b) iontophoretic delivery.

Charged molecules can electrophoretically move in concert with this flow (i.e., cations) or against this flow (i.e., anions); yet, in either case these ions are generally transported to the ground end of the capillary by the overwhelming magnitude of the induced solvent flow. This is represented mathematically by equation 4 (8)

$$U = \mu_{Total}(E) = (\mu_{ep} + \mu_{eo})(E) \quad (4)$$

where U is the molecular velocity in cm/sec, E is the voltage gradient (Volts/cm), and μ_{Total} is the total mobility ($cm^2/(V*sec)$). It is clear from equation 4 that as long as the absolute value of the negative electrophoretic mobility of an anion is less than the magnitude of μ_{eo}, the anion will be transported through the capillary under these normal (anodal) conditions, albeit at longer times than either cations or neutral molecules. This is generally the case for CE where univalent ions possess μ_{ep} values of 1 to 2 (μm*cm)/(V*s) and μ_{eo} values can range from 4 to 9 (μm*cm)/(V*s) (table I, figure 1a). If the absolute value of μ_{ep} of the anion does exceed the value of μ_{eo}, then the net motion of the anion will be towards the starting end of the capillary (the anode) and it will not traverse the capillary under these conditions. This corresponds to the normal mode of iontophoresis where values of μ_{eo} are estimated to be less than 1 (μm*cm)/(V*s) (figure 1b) and thus the absolute value of μ_{ep}(anion) is greater than the value of μ_{eo} in the skin.

Table I. Flux and Mobility Values of TRH at pH = 4 and 8[a]

Ionic strength (mM)	μ_{Total}[b]	μ_{eo}[b] (μm*cm/(V*sec))	μ_{ep}[b]	Flux[c] (μg/hr/cm²)
pH = 4				
1.2	6.00	4.63	1.37	
6.0	4.56	3.19	1.37	
21.0	3.43	2.06	1.37	
60.0	3.04	1.67	1.37	99.5
200.0	(1.90)[e]			38.8
600.0	(1.37)[e]			14.9
pH = 8	(μ_{eo})[d]			
1.2	9.00			
6.0	8.10			
10.0	7.01			
21.0	6.14			
60.0	4.94			57.2
100.0	5.04			
120.0	4.25			
200.0	(3.95)[e]			7.3
600.0	(2.75)[e]			3.7

[a] Flux measurements carried out with pH = 7.4 HEPES buffered saline in the receptor solution, 8 hour measurements (24).
[b] μ_{eo} estimated from the migration time of neutral riboflavin.
[c] Across human cadaver skin from reference 24.
[d] At pH = 8, TRH is neutral and thus $\mu_{eo} = \mu_{Total}$.
[e] Data points estimated from the linear relationship between μ_{Total} and Ln(ionic strength). $\mu_{Total} = -0.78[\text{Ln(ionic strength)}] + 6.04$, $r^2 = .993$, pH = 4. $\mu_{Total} = -1.04[\text{Ln(ionic strength)}] + 9.43$, $r^2 = .967$, pH = 8.

There is general agreement that iontophoretic delivery occurs through shunt pathways in the skin (3,18,23,24). That is, rather than transit the highly resistive stratum corneum, currents and thus ions and polar neutral molecules pass through the pores of the skin including hair follicles, sweat ducts, and in some cases intercellular spaces. These delivery channels are not composed of fused silica like the capillaries used in CE and can be as small as a few nanometers in diameter. They are, however, similar to CE capillaries in that they are net negatively charged under normal pH conditions. Inasmuch as many drugs are cations at physiological pH values, the anodal configuration is the "normal" mode of iontophoresis much as the anodal configuration is the normal mode of CE (figure 1 a,b).

The thrust of this report is that CE derived mobilities of tripeptides can be used to guide formulation optimization of the iontophoretic delivery of those peptides.

The Effects of pH and Ionic Strength on Flux and Mobility

TRH has a pK_a of 6.2 such that at lower pH values it is protonated and positively charged and at higher pH values it is deprotonated and neutral. Table I shows the electrophoretic mobilities of TRH and the corresponding coefficients of electroosmotic flow evaluated by CE at several ionic strengths for both pH = 4 and 8 using the same buffer species in the donor solution as reported by Burnette and Marrero (23). Table I also lists published *in vitro* iontophoretic flux values of TRH observed at several ionic strengths using these same buffer species in the donor solutions at pH = 4 and 8 (23,24).

There are several important trends illustrated by the data in table I. First, both the values of the coefficient of electroosmotic flow (CE data) and the flux values for TRH are inversely related to the ionic strength. On the other hand, the electrophoretic mobility of TRH (at pH = 4) is not altered over the range of ionic strength examined in this work. Secondly, the value of μ_{eo} increases from pH = 4 to pH = 8 consistent with earlier CE reports (7,8,11). However, the flux values at equal ionic strengths decrease from pH = 4 to pH = 8. While these data may appear to be inconsistent, they are reflective of small μ_{eo} values in the skin. CE mobilities are dominated by electroosmotic flow (figure 1a). In the skin, the electrophoretic mobility of the ion is the major contributor to the observed flux (figure 1b). Hence, while the rate of electroosmotic flow increases in the skin from pH = 4 to pH = 8, that increase is overwhelmed by the loss of the electrophoretic mobility of the TRH molecule as it becomes neutral over the same pH range. While the Stokes-Einstein relationship indicates an inverse impact of molecular size on the diffusion coefficient and hence on passive transport, the diffusion of a molecule becomes insignificant in the presence of electroosmotic flow. This is just the reason that CE demonstrates such extraordinarily selective separations. Thus, within limits, if a molecule has a charge equal to zero, its mobility will not generally show a dependence on size or shape and will effectively be equal to zero also.

Sarpotdar (25) has reported the effects of pH on iontophoretic flux using a basic tripeptide analog of TRH. The compound, desamino-tyrosinyl-lysinyl-prolinamide (dTLP), exhibits a single ionization process at pH = 10.5 for the free lysine side chain. Thus, at pH < 9, this molecule is positively charged. In the absence of electroosmotic flow, the flux of this molecule would be predicted to remain unchanged from pH = 4 to 8; however, as pointed out by Sarpotdar, a dramatic increase in flux is observed over this very pH range (25). CE estimation of the electrophoretic mobility of dTLP as well as the magnitude of the coefficient of electroosmotic flow over this pH range is shown in figure 2. These CE data support the hypothesis that the increase in flux results from the increase in electroosmotic flow over this pH range (25).

The work of VanOrman, et al. (26), demonstrated that μ_{eo} is linearly dependent upon the ln(ionic strength). Values of mobility at ionic strengths of 200 and 600 mM can be estimated from this relationship using the existing data from 1.2 to 60 mM (pH = 4) and from 1.2 to 120 mM (pH = 8) (table I). These data and the estimated mobilities are supportive of a relationship between iontophoretic flux and total mobility at least as far as trends with varying ionic strength. In further support of the hypothesis, we have attempted to illustrate the potential of CE as a predictive tool with respect

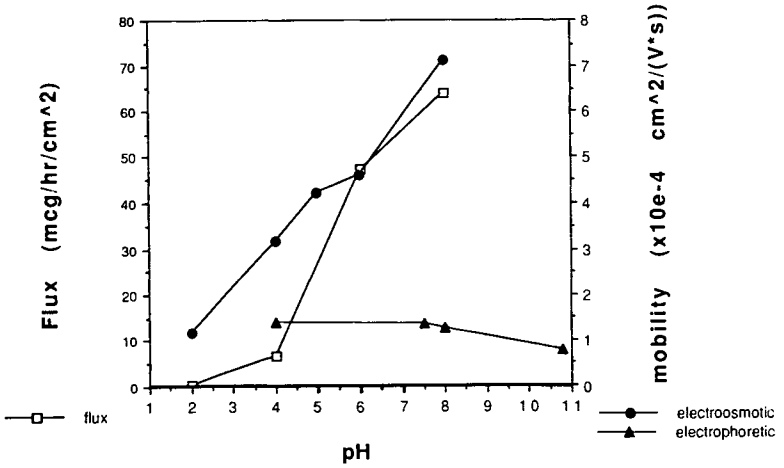

Fig. 2. Variation in the flux of and the electrophoretic mobility of dTLP with pH and the corresponding coefficients of electroosmotic flow. Flux data from reference 25.

to iontophoresis by relating the flux measurements for three tripeptides at pH = 8; TRH, dTLP, and the corresponding acid analog, desamino-tyrosinyl-glutamyl-prolinamide (dTGP) as reported by Sarpotdar, et al. (24,25,28) with their CE estimated electrophoretic mobilities (figure 3). The acid analog (dTGP) exhibits a negative charge at pH > 5. Thus, under anodal conditions, dTGP is not transported across the skin. We have used the flux determined under cathodal conditions as "negative" anodal flux for the purpose of this figure (figure 3). The straight line through the data in figure 3 is simply to guide the readers eye. There is no apriori reason to expect this to be a linear relationship and the paucity of data do not afford a determination of a definitive mathematical relationship.

Certainly, these limited data make any quantitative conclusions regarding the relationship between CE determined mobilities and iontophoretic flux values untenable. Nevertheless, use of CE as a tool for the optimization of iontophoresis is beginning to make its way into the literature. For example, Heit, et al., used CE to study the ability of luteinizing hormone releasing hormone to be iontophoresed (29). Work described herein would also suggest that a relationship exists between CE determined mobilities and flux values.

Limitations of CE with Respect to Iontophoresis

Iontophoresis is a complex process. One which cannot easily be rationalized via simple physical parameters. CE does have a relationship to the physical aspects of iontophoresis as demonstrated above. However, CE cannot address any of the physiological or toxicological aspects of this mode of drug delivery. For example, results from these laboratories (figure 4) and others (30) suggest that the addition of ethanol to the buffer in CE results in a rapid decrease in the rate of electroosmotic flow with increased ethanol concentration. Yet, the addition of ethanol to iontophoretic formulations often enhances transdermal transport (3,31). This inconsistency suggests a physiological basis for the effect of ethanol which overpowers the loss of electroosmotic flow.

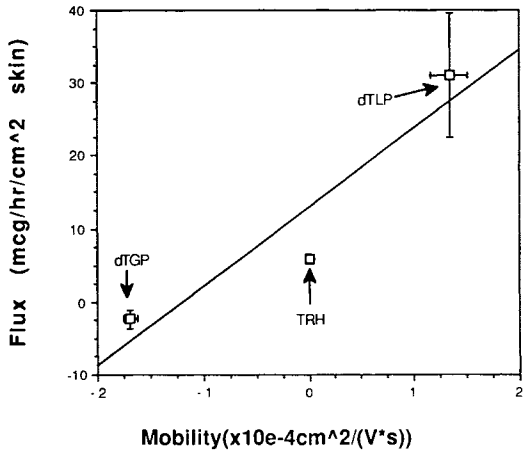

Fig. 3. Flux of three tripeptides at pH = 8 versus corresponding electrophoretic mobilities of those tripeptides. Flux data from references 24 and 25.

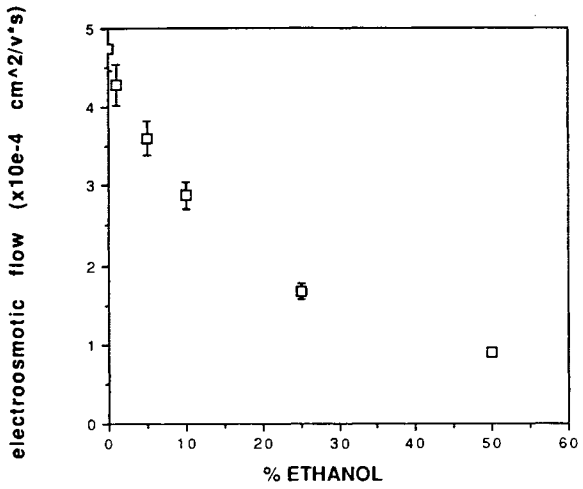

Fig. 4. Variation of the coefficient of electroosmotic flow with increasing ethanol content in the running buffer. The solution was buffered at pH = 8 with a phosphate, citrate, NaCl solution at a constant ionic strength of 60 mM. Electroosmotic flow was estimated from the migration time of neutral riboflavin.

A further limitation with respect to the use of CE for optimization of these processes is that the ionic strengths often used in iontophoretic studies to maintain pH during the course of the administration are too high for the instrumental technique of CE. These excessive buffer concentrations result in current levels in the capillary which generate enough heat to boil the solvent and thus disrupt the experiment. As the push for lower ionic strength buffers and polymeric buffers grows, albeit prompted by different mechanistic reasons, this is anticipated to become less of a problem (28).

Lastly, the magnitude of electroosmotic flow in a capillary is dependent upon the composition of the capillary. This is a direct result of the dependence of the charge on the walls of the capillary (i.e., pore) upon composition and the corresponding pH profile (32). Obviously, the composition of the skin bears little resemblance to fused silica other than the fortuitous similarities in zeta potential and isoelectric points. It is just these two factors which provide the physical similarities observed between these techniques. Of course, any solution component which modifies the capillary (pore) walls will effect the magnitude of electroosmosis. In fact, adsorption of a cationic surfactant onto the capillary walls reverses the direction of electroosmotic flow (17). These kinds of chemical controls may prove to be useful in future iontophoresis formulation studies. It is clear, however, that CE is not able to act as a "screen" for potential candidates for iontophoresis due to the complex biological aspects of this delivery technology.

CONCLUSIONS

Capillary electrophoresis can provide estimates of both the electrophoretic mobility of molecules of interest and the induced solvent flow, electroosmosis, under various formulation conditions. The electrophoretic mobilities of TRH determined by CE demonstrate trends with pH and ionic strength that compare well with those of published values of TRH iontophoretic flux under similar formulation conditions. While CE lacks any indication of physiological or tox-

icological effects, there may be a semiquantitative relationship between *in vitro* flux measurements and CE mobility measurements with TRH.

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