Published in final edited form as:

J Sep Sci. 2007 December ; 30(18): 3255–3261. doi:10.1002/jssc.200700243.

Prediction of the location of stationary steady-state zone positions in counterflow isotachopheresis performed under constant voltage in a vortex-stabilized annular column

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Abstract

A theoretical model is presented and an analytical expression derived to predict the locations of stationary steady-state zone positions in ITP as a function of current for a straight channel under a constant applied voltage. Stationary zones may form in the presence of a countercurrent flow whose average velocity falls between that of a pure leader zone and of a pure trailer zone. A comparison of model predictions with experimental data from an anionic system shows that the model is able to predict the location of protein zones with reasonable accuracy once the ITP stack has formed. This result implies that an UP stack can be precisely directed by the operator to specific positions in a channel whence portions of the stack can be removed or redirected for further processing or analysis.

Keywords

Analytical; Constant voltage; Expression; Immobilization; Isotachopheresis

1 Introduction

ITP is a suitable fractionation and concentration technique for a variety of different sample types including food additives [1], drugs in biological samples [2], and recombinant proteins [3], and it is well suited for multiple scales ranging from microchip [4] to preparative applications [5]. Currently, there is a great deal of interest in using ITP in microchips due to their small sample size and rapid separation [4]. Electrophoretic separation techniques, including ITP, do not require high pressures, packings, or miniature pumping devices, which makes them ideal for use on microchips [6].

ITP is an electrophoretic separation method which utilizes a discontinuous buffer system [7,8] comprised of a leading electrolyte (LE) and trailing electrolyte (TE) having the highest and lowest mobilities, respectively, of all constituents in a target sample. A complex analyte sample is placed between the LE and TE, and some time after current is applied, those analytes with the same charge sign as the leading ion will stack themselves into a “train” of contiguous self-sharpening zones based on their electrophoretic mobilities. In the absence of a hydrodynamic counterflow, the ITP train will reach a constant, pseudo steady-state velocity that is governed by the mobility of the leading analyte and the conductivity of the LE.

At steady state, the concentration within each analyte zone increases until it is proportional to the concentration of the LE, which allows for simultaneous fractionation and concentration of dilute sample species. For this reason FTP is an attractive preconcentration step [9], which has been coupled online with CZE for many applications including monitoring protein formation [10], determining trace iodide in seawater [11], and determining drugs in serum [2]. The concentrating ability of ITP makes it a suitable candidate for use in processing low abundance species, which is one of the major problems facing proteomics research today [12].

There has been a significant amount of work done on computer modeling of ITP [13–16]. In 1983, Bier *et al.* [17] developed a general, nonlinear model for all electrophoretic transport modes including zone electrophoresis, IEF, and ITP. This model assumed a 1-D, isothermal system and was comprised of a set of nonlinear partial differential and algebraic equations, requiring numerical methods for quadrature. This model was modified in subsequent papers to account for various parameters including the effects of ionic strength on protein mobility [18,19], electroosmosis [20], and bulk flow [21].

The application of a bulk flow, countercurrent to isotachophoretic transport has been applied as early as 1966 by Preetz [22]. Counterflow applied at appropriate rates can immobilize the ITP train. Immobilization of the UP train has been used for recycling FTP of ovalbumin and lysozyme [23] and for CZE-ITP coupling [24].

In 1995, Thormann *et al.* expanded the model developed in 1983 by Bier *et al.* to investigate the impact of electroosmosis on ITP in capillaries [25]. EOF was assumed to be plug flow and was calculated based on the specific wall mobility, voltage gradient, pK of the wall, and pH of the solution. Their model provides concentration, pH , and conductivity profiles as a function of time. Computer simulation of an anionic ITP system showed that, under an applied current and with a counterflow of appropriate magnitude, the net velocity of the zones slowed asymptotically to zero. This demonstrated that a balance between electroosmotic counterflow and isotachophoretic migration leads to the evolution of a stationary steady-state zone in capillary ITP. Experiments performed in fused silica capillaries confirmed the formation of a stationary steady-state zone in ITP [25],

In the present work, it is shown that one can stop the ITP train at any chosen position within the channel. An algebraic model is derived which predicts the locations and breadth of these stationary steady-state zones in ITP. Unlike the models on counterflow ITP published to date, this model has an analytical solution which allows quick determination of zone position as a function of the current at a fixed voltage. To test the model predictions, ITP experiments were conducted in a preparative scale apparatus [26] from which data were obtained by measuring zone position and current for a series of counterflow velocities. As counterflow velocity is increased, the current increases proportionately, and the stationary position of zones moves closer to the cathode or anode for an anionic or cationic system, respectively. A comparison of theory with experiment shows that the model was consistently able to predict zone position within the 95% confidence interval of the data. The average difference between experimental and predicted zone positions was 18%.

2 Materials and methods

2.1 Chemicals

BSA, bovine hemoglobin (Hb), and epsilon-amino-*n*-caproic-acid (EACA) were obtained from Sigma (St. Louis, MO). Trizma base was purchased from Invitrogen (Carlsbad, CA), hydrochloric acid (HCl) from Fisher Scientific (Hampton, NH), and bromophenol blue from

BioRad (Hercules, CA). Nanopure water came from a Barnstead Thermolyne (Dubuque, IA) Nanopure® Infinity UV/UF system.

2.2 Electrolyte and sample preparation

The LE was 0.01 M HCl adjusted to pH 9.5, and the TE was 0.06 M EACA adjusted to pH 10. The pH of the LE and TE were adjusted using Trizma base, which also served as the counterion. So that the BSA band was visible, bromophenol blue, which binds to BSA, was added to the LE in a 1:1 molar ratio to make a 10 mg/mL “bluealbumin” solution. Bovine hemoglobin was dissolved in LE to make a 10 mg/mL Hb solution.

2.3 Instrument

ITP experiments were performed in a vortex-stabilized electrophoresis apparatus designed by Ivory and Gobie [27] which has recently been described in detail [26]. Briefly, the apparatus has a complementary grooved boron nitride rotor inside a Plexiglas® stator, which together forms an annulus in which the separation takes place. Spinning the rotor at 50 RPM creates stable vortices in the annulus that are similar in structure to, but distinctly different from, Taylor vortices. The vortices prevent axial mixing of the fluid due to natural convection and provide improved heat and mass transfer *via* radial circulation of the vortices.

Cooling was provided to the apparatus by circulating a Syltherm XLT coolant oil (Dow, Midland, MI) at 10°C through the inside of the hollow rotor using an MGW Lauda RC3 circulator (Brinkman, Westbury, NY). A Spellman SL300 power supply (Hauppauge, NY) provided the power to the electrodes and counterflow was applied using a Buchler Instruments multistatic® pump (Lenexa, KS).

2.4 ITP procedure

In each experiment, the anode was located at the bottom of the column and the cathode at the top with LE and TE used as analyte and catholyte, respectively. Figure 1 shows a picture of the apparatus with relevant parts marked. Due to the high pH of the system, all of the ions being separated were anions; therefore, the electrophoretic movement of the sample ions was toward the anode located at the bottom of the column.

Before each run, the bottom half of the column was filled with LE using a syringe mounted on a port at the anodic end of the column. The top half of the annulus was filled with TE using a syringe mounted at the center of the column. Then, a protein sample containing 30 mg BSA and 30 mg Hb at a concentration of 10 mg/mL in LE was injected between the two electrolytes. After the sample was injected, a constant electric field was applied across the column and a counterflow was applied to the annulus by pumping LE into the bottom of the column.

2.5 Theory

Our model assumes that the system is at steady state, which means that the TCP train is fully formed and that it has reached its final, stationary position. It includes only the sample species being separated, the leading and trailing ions, and a single counterion. It is assumed that each zone is pure, meaning that a zone contains only one coion and one counterion. Overlap between bands due to dispersion, as well as salt effects and double layer effects are neglected.

A unique characteristic of ITP is that all species move at the same speed once steady state has been reached. The velocity of each species moving in an electric field is calculated by multiplying its electrophoretic mobility by the electric field strength; therefore in ITP,

$$\mu_{LE} E_{LE} = \mu_j E_j = \mu_{TE} E_{TE} \quad (1)$$

where μ_j is the electrophoretic mobility of the j^{th} component and E_j is the electric field strength in the j^{th} zone. The subscripts LE, TE, and j represent the LE, TE, and each of the sample species in their respective zones. The electrophoretic mobility of a species is dependent on many factors including temperature, ionic strength, and pH but, in this derivation, they are assumed to be constant within each zone.

It is further assumed that a single counterion, represented by subscript m , is present in varying concentrations in each zone in order to ensure electroneutrality within that zone. Equations (2a–c) represent electroneutrality for an LE zone, a single sample zone, and a TE zone, respectively

$$z_{LE} c_{LE} + z_m c_{mLE} = 0 \quad (2a)$$

$$z_j c_j + z_m c_{mj} = 0 \quad (2b)$$

$$z_{TE} c_{TE} + z_m c_{mTE} = 0 \quad (2c)$$

where z represents charge valence and c represents concentration. Since the concentration of the counterion varies within each zone, a second subscript is used to specify the zone that is being considered, for example c_{mLE} is the concentration of the counterion, m , in the leading zone.

The current in the j^{th} zone can be described by the equation

$$i_j = \sigma_j E_j A \quad (3)$$

where i_j is the current, σ_j is conductivity, E_j is electric field strength, and A is the cross-sectional area of the separation chamber, which is assumed constant along the channel. By expanding the conductivity in terms of the ionic concentrations, we can write for the current in each zone.

$$i_{LE} = F(z_{LE} \mu_{LE} c_{LE} + z_m \mu_m c_{mLE}) E_{LE} A \quad (4a)$$

$$i_j = F(z_j \mu_j c_j + z_m \mu_m c_{mj}) E_j A \quad (4b)$$

$$i_{TE} = F(z_{TE} \mu_{TE} c_{TE} + z_m \mu_m c_{mTE}) E_{TE} A \quad (4c)$$

where F is Faraday's constant. The current in each discrete zone is the same, thus

$$i_{LE} = i_j = i_{TE} \quad (5)$$

The following equations for the total voltage drop across the column and total length of the column, respectively, for a system of n components in our sample are

$$V_T = E_{LE} L_{LE} + \sum_{j=0}^n E_j L_j + E_{TE} L_{TE} \quad (6)$$

$$L_T = L_{LE} + \sum_{j=0}^n L_j + L_{TE} \quad (7)$$

where L is the length of a zone and the subscript, T, represents total.

The concentration of each component in its respective zone is

$$c_j = \frac{M_j}{L_j A} \quad (8)$$

where M_j is the mass of species j in that zone.

In fully developed ITP, the plateau concentration in each zone is dependent on the concentration of the LE. By solving Eqs. (1)–(5), one obtains the following relationships for the plateau concentration of the TE and sample species [28]

$$\frac{c_{TE}}{c_{LE}} = \frac{z_{LE}(\mu_{LE} - \mu_m)\mu_{TE}}{z_{TE}(\mu_{TE} - \mu_m)\mu_{LE}} \quad (9a)$$

$$\frac{c_j}{c_{LE}} = \frac{z_{LE}(\mu_{LE} - \mu_m)\mu_j}{z_j(\mu_j - \mu_m)\mu_{LE}} \quad (9b)$$

From this solution it is seen that the plateau concentration of each zone is dependent only on the properties of the LE, the particular zone species, and the counterion, and is independent of the other species present in the system. This shows that the plateau concentration of a sample zone does not depend on the amount of sample present or on the electric field. From the relationship given in Eq. (8) one can see that the length of the sample zone increases proportionally with increasing mass. Therefore, once a species achieves its plateau concentration, zone length can be used as a direct measure of its total mass.

By solving Eqs. (1), (2), and (4)–(8) simultaneously, algebraic expressions are obtained for the length of each zone, which allows prediction of the position of each band interface in the

sample train. Here, the position of the train is defined as the interface between the LE and the first sample zone relative to the front edge of the separation chamber. To predict this position, Eqs. (1)–(8) were solved to obtain the length of the leading zone as a function of the current

$$L_{LE} = - \frac{i_{LE} \mu_{LE}^2 \left[\sum_{j=0}^n \frac{\alpha_j}{\gamma} + L_T \left(\frac{\mu_m}{\mu_{LE}} - 1 \right) \right] + F V_T \mu_{TE} \gamma (\mu_{LE} - \mu_m)^2}{i_{LE} (\mu_{LE} - \mu_m) (\mu_{LE} - \mu_{TE})} \quad (10a)$$

where

$$\alpha_j = M_j z_j \left(1 - \frac{\mu_{TE}}{\mu_j} \right) \left(1 - \frac{\mu_m}{\mu_j} \right) \quad (10b)$$

and

$$\gamma = z_{LE} c_{LE} A \quad (10c)$$

The length of the sample zones can be calculated from the following:

$$L_j = \frac{M_j z_j \mu_{LE} (\mu_j - \mu_m)}{\gamma \mu_j (\mu_{LE} - \mu_m)} \quad (11)$$

The current can be adjusted by varying the counterflow rate applied to the system. At steady state the counterflow velocity will be equal to and opposite to the electrophoretic velocity of the zones. Substitution of the counterflow velocity, v_{CF} , into Eq. (4a) and simplification using the electroneutrality condition (Eq. 2a), yields a direct relationship between current and counterflow velocity:

$$i_{LE} = v_{CF} F \gamma \left(\frac{\mu_m}{\mu_{LE}} - 1 \right) \quad (12)$$

Upper and lower limits of the counterflow rate appropriate for stopping the zones within a separation chamber can be estimated by substituting Eq. (12) for the current in Eq. (10a) for a system with no sample species, and solving for the counterflow velocity:

$$v_{CF} = \frac{V_T \mu_{TE} \mu_{LE}}{L_{LE} (\mu_{LE} - \mu_{TE}) - L_T \mu_{LE}} \quad (13)$$

The length of the LE zone in Eq. (13) can then be set to zero and to the total length of the separation chamber to approximate a suitable range of counterflow velocities.

In order to make quantitative predictions using this model, the electrophoretic mobilities and charges of all species, the masses of all sample species, and the concentration of the LE

must be specified. The cross-sectional area and length of the separation chamber and the voltage across the separation chamber are also required.

3 Results and discussion

ITP was performed in a vortex-stabilized electrophoresis apparatus as described above. For each experiment, the voltage was set at a constant value of 2.50 kV and the current was allowed to adjust to its steady-state value depending on the conditions set. A set of counterflow rates ranging from about 0.002 to 0.03 cm/s were used to control the current. As the counterflow velocity is increased, the steady-state position of the sample train is shifted towards the cathode, and the LE fills more of the annulus. Since the LE has a higher conductivity than the other species in the column, the current will increase as the counterflow velocity is increased, according to Eq. (12). The minimum and maximum counterflow rates were chosen such that no part of the sample train would migrate beyond the anode or cathode.

The system was allowed up to 12 h to reach steady state at each counterflow rate. When the sample was first introduced, and the voltage applied, distinct protein bands with sharp front and rear boundaries formed within 30 min. The sample train then began moving toward its steady-state position and arrived there approximately 5 h after the start of the experiment. Considering the fact that separation times decrease as applied voltage is increased, this length of time is comparable to preparative IEF experiments performed in the same apparatus, where hemoglobin was focused over a 90 min period at 10 kV [29].

Figure 2 shows a photograph of the contiguous protein zones at two steady-state positions. At an experimental pH of 9.5, blue albumin has a greater electrophoretic mobility than hemoglobin and therefore forms the front protein zone. The figure of the sample train at 9.6 mA shows a clear band between the blue albumin and hemoglobin bands which is most likely BSA which does not have bromophenol blue bound to it. As the current was increased, the edges of both sample zones became sharper, which is seen in Fig. 2. However, as the sample train neared the cathode, there was some dispersion of the Hb zone. This dispersion is likely due to the fact that the fluid flow near the cathode is disturbed by the presence of an opening for the electrode housing. No data were collected above a current of 24.1 mA because the Hb zone began exiting the system at this point.

Modeling was performed by solving the set of nonlinear algebraic equations (Eqs. 1–8), which describe steady-state ITP. An analytical solution was obtained for the length of the LE zone as a function of the current. By choosing to model the length of the LE zone, the position of the beginning of the sample train is obtained. If desired, one could also solve for the length of each individual zone (Eq. 11) to predict the position of each zone interface.

A system consisting of only an LE and a TE was initially modeled, using Eq. (10), to illustrate the response of zone position to current. From this solution it is shown that, as the current is increased *via* increasing the counterflow velocity, the fraction of the annulus that is filled with LE increases asymptotically. With the addition of 30 mg of two sample proteins, the resulting curve has the same shape as that of a system with no sample, but is shifted down.

Figure 3 shows the length of the leading zone for both a system with only a leading and trailing zone and for the same system with two sample species added. Figure 3 is based on a system with an annulus length of 29 cm, a cross-sectional area of 0.685 cm², and a constant voltage of 2.50 kV. The relevant properties of all species in the system are shown in Table 1.

Datasets were collected for three experiments (E1–E3), with a BSA/Hb sample as described above. Model predictions are compared to the experimental data in Fig. 4 which shows that the model accurately predicts that, as the current increases due to an increase in counterflow velocity, the length of the leading zone increases asymptotically. A statistical analysis of the data was conducted using the Matlab 7.0 curve-fitting tool to calculate a 95% prediction interval (PI) [30] for the future response of experimental data. This interval includes the error in estimating the mean and the variation in the data to predict that 95% of future data collected for zone position as a function of current will fall between the upper and lower limits. Although the predicted LE zone length comes near the upper limit of the 95% PI for currents between 5 and 7 mA, all points in the model are within the 95% PI, which shows that it is able to predict the position of the steady-state stationary zone to within experimental variation.

4 Concluding remarks

The model presented in this paper produces an analytical solution which can be used to accurately predict the positions of the stationary steady-state zones formed during ITP. By varying the countercurrent velocity, within the calculated bounds, the ITP train can be immobilized at any position within the channel. We have shown that these positions can be predicted as a function of the current, given a constant voltage, which gives us the ability to stop the train at any point in a channel.

From these results, it appears that the assumptions made in the development of our model are valid for this apparatus under our experimental conditions. One assumption is that dispersion is negligible. In free-flow electrophoresis, conductivity gradient instability has been reported, which causes significant dispersion [31]. The agreement between the model and our experimental results in the present work show that the vortex stabilized electrophoresis apparatus stabilized the fluid and decreased dispersion. Experimental results showed that dispersion was more pronounced at low currents but, even in this region, the model is able to predict the behavior of the stationary steady-state zone with reasonable accuracy.

Temperature effects were not considered in this model. Since Joule heating is the product of the electric field strength with the current and since we are working with a constant current, then the temperature of zones will increase as electrophoretic mobility decreases [28]. Therefore, there will be temperature variations during separation. Species parameters, such as pK and electrophoretic mobility are dependent on temperature as well as other variables including ionic strength. Neither temperature nor ionic strength were measured directly or predicted for this experiment. Values used as input parameters were those found in literature for a temperature of either 20 or 25°C and an ionic strength ranging from 0 to 0.01 M. Our results show that our model was able to predict stationary steady-state zone positions in agreement with experimental data, despite neglecting temperature variation.

Acknowledgments

This material is based upon work supported in part by the National Institute of General Medical Sciences/National Institutes of Health (grant no. GM008336) and the Chemical Engineering Department at Washington State University.

Abbreviations

EACA	epsilon-amino- <i>n</i> -caproic-acid
LE	leading electrolyte

TE trailing electrolyte

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Figure 1.

Photograph of the vortex-stabilized electrophoresis apparatus used for ITP. At the beginning of each ITP experiment the annulus was filled with TE (1) and LE (3). Sample was then injected between the electrolytes (2). Counterflow (4) was applied at the anodic end of the apparatus, countercurrent to isotachopheretic migration.

Figure 2.
Photograph showing the isotachopheretic separation of 30 mg BSA and 30 mg Hb at a voltage of 2.50 kV and a current of (A) 4.3 and (B) 9.6 mA.

Figure 3.

Model predictions of the length of the LE zone as a function of current comparing a system containing only an LE and TE to a system with 30 mg BSA and 30 mg Hb added.

Predictions are made for a system with a constant applied voltage of 2.50 kV. The LE is 0.01 M HCl, pH 9.5, and the TE is 0.06 M EACA, pH 10.0.

Figure 4.

Comparison of model predictions to experimental data for the length of the LE zone. The experiment was conducted three times in order to obtain datasets, represented by E1 through E3. The dashed line shows the 95% prediction interval (PI) for the experimental results, based on data analysis. Model input parameters were identical to the experimental conditions when possible. Literature values of properties were used for parameters not directly measured during the experiment, shown in Table 1.

Table 1

Parameters used for model calculations

Zone	Species	Charge	Mobility ^{a)}
Leading	Cl ⁻	-1	-7.91 [7]
Trailing	EACA	-0.14 ^{b)} [32]	-0.40 ^{c)} [33]
Sample 1	BSA	-25 [34]	-3.25 [35]
Sample 2	Hb	-12 [36]	-1.20 ^{c)} [37]
Counterion	Tris	0.96 ^{b)} [32]	2.84 ^{c)} [33]

^{a)} Net electrophoretic mobility in units of $\text{m}^2\text{V}^{-1}\text{s}^{-1} \times 10^8$.

^{b)} Calculated based on pK values from cited reference.

^{c)} Calculated by multiplying the absolute mobility from the cited reference by the charge.