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Optimization of capillary electrophoresis conditions for a glucagon competitive immunoassay using response surface methodology

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Abstract

The capillary electrophoresis (CE) conditions for a competitive immunoassay of glucagon were optimized for highest sensitivity of the immunoassay and resolution of the electrophoretic peaks using a Box-Behnken design. Injection time, voltage ramp time, and separation voltage were varied between three levels and two responses, bound-to-free (B/F) ratio of the immunoassay peaks and resolution between the peaks, were measured. Analysis of variance was applied to fit a predictive model, and a desirability function was used to simultaneously optimize both responses. A 10 sec injection, 1.6 min ramp time, and a 22 kV separation voltage were the conditions found when high B/F was given more emphasis than high resolution. To test the model, calibration curves of a glucagon immunoassay were measured at the optimum and least optimum CE conditions. Optimal conditions increased the sensitivity of the immunoassay by 388% compared to the least optimum conditions while maintaining adequate resolution.

Keywords

CE; Response surface methodology; Optimization; Glucagon; Competitive immunoassay

Introduction

Capillary electrophoresis (CE) has been used in affinity studies due to rapid analysis times, low reagent consumption, and multi-analyte capability [1]. In competitive immunoassays, an analyte of interest (Ag) competes with its fluorescently-labeled counterpart (Ag*) for binding to a limited amount of antibody (Ab). Electrophoretic separation of the immunoassay mixture allows detection of distinct peaks corresponding to bound (B) and free (F) Ag*. The ratio of B and F (B/F) can be used to quantify the amount of Ag [2].

Ideally, the magnitude of B/F is independent of the electrophoretic separation process. However, the detected CE peaks, and therefore the concentration of Ag determined, may be affected by factors that contribute to peak dispersion such as longitudinal diffusion, Joule heating, and wall adsorption [3]. By decreasing the time spent on the capillary, longitudinal diffusion is reduced. However, this reduction in capillary residence time is best achieved by decreasing the capillary length rather than increasing the electric field [4]. Higher separation voltages increase the electrophoretic velocity of the analytes, but also increase the current and Joule heating. This heating may affect the stability of the complex by disrupting the non-

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covalent interactions of antigen and antibody. Heating effects can be mitigated by lowering the current (for example, by decreasing the separation voltage or lowering the ionic strength of the buffer), or by enhancing heat dissipation (for example, by decreasing the surface area-to-volume ratio of the capillary, or a capillary cooling system); however, these options can be detrimental to separation efficiency [5]. A gradual increase rather than an abrupt application of the separation voltage (i.e., voltage ramp) has also been shown to improve separation efficiency by reducing thermal dispersion [6]. It has been reported that the limit of detection of a non-competitive immunoassay for insulin was significantly improved by increasing the voltage ramp time from 20 sec to 5 min [7]. This decrease in the limit of detection was due to the increased amount of bound Ab-insulin* detected with the long ramp times compared to short ramp times. However, increasing the ramp time also increases the separation time, which, as mentioned before, may be detrimental to the stability of Ab-Ag* complex.

We have developed a simultaneous capillary electrophoresis competitive immunoassay for insulin and glucagon using dual-color detection [8]. However, the immunoassay for glucagon exhibited a relatively low B/F in the absence of unlabeled glucagon, limiting the sensitivity and dynamic range of the assay. It was found that increasing the ramp time increased the B/F ratio for glucagon, although the cause of this finding was not fully investigated. This CE immunoassay could be improved by optimizing experimental conditions to achieve higher B/F, which should result in increased sensitivity, while also improving resolution of the Ab-glucagon* and glucagon* peaks. Exploring suitable combinations of electrophoretic variables through univariate screening would be expensive and time-consuming, as well as omitting interactions between variables that may exist. A multivariate approach that evaluates several factors at once is both more efficient and more accurate than a univariate screening process [9,10].

Response surface methodology is a multivariate optimization of one or more responses influenced by several variables. While several types of designs exist, the Box-Behnken design is more efficient compared to other response surface models such as central composite or full factorial designs, because it estimates the effects of individual variables, as well as two-factor and higher-order interactions, with a minimum number of analyses [11]. For example, only thirteen combinations of three factors at three levels are required to fit a model. The design space can be visualized as a cube, with one design point generated at each edge of the cube, and replicates incorporated at the center of the cube to estimate an error term independent of the fitted model. This design is an increasingly popular tool for optimization of analytical methods [12] and has been applied to several CE applications [13-23]. In addition, the Box-Behnken method has been applied to several affinity CE methods, for example, by predicting dissociation constants of carbonic anhydrase and its ligand as a function of injection time, capillary length, and applied voltage [24]. Also, buffer pH, concentration, and applied voltage were varied to optimize peak efficiencies, migration time, and resolution of leucine enkephalin and its immuno complex [25].

In this report, we build on our previous work of a CE immunoassay for glucagon by attempting to increase the sensitivity of the assay and increase the resolution of Ab-glucagon* and glucagon* peaks. We hypothesized that the heat induced by the voltage ramp to the separation voltage was degrading the Ab-glucagon* peak, thus the larger B/F observed at the increased ramp times. A Box-Behnken design was used to explore different injection times, ramp times, and separation voltages for the optimal conditions of B/F and resolution.

Materials and methods

Chemicals and reagents

Sodium tetraborate and sodium phosphate monobasic were from Thermo Fisher Scientific (Waltham, MA). Ethylenediaminetetraacetic acid (EDTA) and sodium hydroxide were from EMD Chemicals (San Diego, CA). Bovine serum albumin (BSA) was from MP Biomedicals (Solon, OH). Hydrochloric acid was from BDH (Deer Park, NY). Fluorescein, Tween-20, glucagon, and monoclonal antibody to glucagon from mouse ascites fluid (clone K79bB10, $K_d = 1.6$ nM) were from Sigma-Aldrich (St. Louis, MO). FITC-glucagon was purchased from CHI Scientific (Maynard, MA). All solutions were prepared using ultrapure deionized water (Barnstead, Dubuque, IA).

Capillary electrophoresis

All experiments were conducted using a P/ACE MDQ CE instrument with an LIF detector, using a 3 mW argon-ion laser as the excitation source (Beckman, Fullerton, CA). Data were collected at an acquisition rate of 4 Hz and analyzed using Beckman System Gold 32Karat™ software. A 50 μ m inner diameter capillary (Polymicro Technologies, Phoenix, AZ) was used with a 10 cm effective length (30 cm total length). Before the optimization experiments, the capillary was rinsed with 0.1 M HCl, 0.1 M NaOH, and deionized water for 5 min at 40 psi, and then conditioned with the separation buffer for 10 min at 40 psi. Between each run, the capillary was rinsed with 0.1 M NaOH and separation buffer for 0.5 min at 40 psi. The sample storage compartment and the capillary cartridge were held at a constant temperature of 20°C for all separations.

Sample preparation

The separation buffer was 20 mM borate, pH 9.3. Samples were prepared in 20 mM NaH_2PO_4 , 1mM EDTA, pH 7.4 supplemented with 10 μ M BSA, and 0.1% w/v Tween-20. In instrument repeatability studies, the sample was 50 nM fluorescein. In optimization experiments, final concentrations of 40 nM FITC-labeled glucagon (Glu*), 5 nM unlabeled glucagon (Glu), and 20 nM anti-glucagon antibody (Ab) were prepared in a total sample volume of 40 μ L. Calibration curves were obtained using 40 nM Glu* and 20 nM Ab, with unlabeled Glu varied from 5 to 300 nM. Samples were injected by applying 10 kV, with injection time, ramp time, and separation voltage settings varied as described in text.

Experimental designs

Design Expert® 7 (DE7) software (StatEase, Minneapolis, MN) was used to produce experimental designs and perform all statistical analysis. The performance of the CE instrument was investigated using a full factorial design where the reproducibility of peak characteristics over a range of instrument settings was evaluated. Three factors were varied over two levels, giving a 2^3 design with 8 combinations of settings. Three replicates were performed to estimate the variability of the instrument. The factors investigated were injection time, separation voltage, and voltage ramp time. Levels examined were either low or high: 4 and 10 sec for injection, 0.8 and 1.6 min for voltage ramp time, and 16 and 22 kV for voltage. Data from the full factorial design were integrated to determine peak area and migration time of fluorescein at various experimental conditions.

Optimization of the immunoassay for highest bound-to-free peak area ratio and resolution was performed using the Box-Behnken optimization design. Again, injection time, ramp time, and separation voltage were varied over three levels, presented in Table 1. Five replicates of the centerpoint were included in the design. Data from the 3-factor, 3-level Box-Behnken design were integrated to determine peak area (A), peak width (W), and migration time (MT) for the

bound Glu*-Ab complex and free Glu* peaks. The responses were calculated as described in Eq. (1) and (2).

$$B/F = \frac{A_{bound}}{A_{free}} \quad (1)$$

$$Resolution = \frac{2(MT_{free} - MT_{bound})}{(W_{free} + W_{bound})} \quad (2)$$

Results and discussion

In our previous work on a CE immunoassay for glucagon, it was found that an increased ramp time produced a larger B/F than a short ramp time. We hypothesized that the heat produced during the ramp to the separation voltage would decrease the Ab-Glu* peak. This decrease could be countered by injecting more of the analyte, although a loss in resolution of the B and F would result as well. To fully optimize the immunoassay conditions, a multivariate approach was taken that would produce the highest B/F at a low concentration of unlabeled glucagon (and therefore a high sensitivity of the immunoassay) while maintaining a high resolution of Ab-Glu* and Glu*. The factors and levels were selected based on electrophoretic intuition as well as from the previous work as most likely to influence responses of interest, namely the peak area ratio of antigen-antibody complex (bound) to free antigen, and resolution between bound and free peaks.

Repeatability of the CE instrument

The repeatability of the instrumental parameters was important to determine, as it had to be shown that the instrument itself would not generate significant experimental changes in the B/F. The repeatability of the CE instrument was tested using a full factorial design by varying the instrumental parameters used in further optimization experiments. The relative standard deviations (RSD) for the 3 replicates of each run type are shown in Figure 1. The RSD were found to range from 2.0 to 5.8 % for peak area and 0.12 to 0.66 % for migration time across the experimental conditions investigated. The variation in instrumental performance was considered to be within the range of error typically observed in CE data [5]. It was therefore assumed that variation observed in the B/F and resolution in later optimization experiments could be attributed to changes in experimental conditions and not to a lack of repeatability of the instrument.

Analysis of model design

The responses optimized were the B/F and resolution of the Ab-Glu* and Glu* peaks. The model used in this study to fit the data produced from the Box-Behnken design was the quadratic model. The generalized form of this model is shown in Eq. (3).

$$Y = \beta + A + B + C + (AB) + (AC) + (BC) + A^2 + B^2 + C^2 + E \quad (3)$$

Where Y was the response (B/F or resolution), β the model coefficient, A the injection time, B the ramp time, C the separation voltage, and E the residual error. Terms in parentheses were interactions between factors, and squared terms were the quadratic effects of the factors. Analysis of variance (ANOVA) partitioned the total variation in the data into the variation due to the factors and to random error. These components of variation were then used to calculate

an F-value, a test statistic for the null hypothesis (no effect due to that factor). The calculated F-value was compared to a tabulated F-distribution to generate a result called “Prob>F” (p). If p was less than 0.05, then the effect of that factor was significant. Model reduction, which pooled non-significant terms ($p > 0.10$) with the residual error by backward elimination regression, was applied to find the best fit for each response. Terms of borderline significance ($0.05 < p < 0.10$) and terms required to maintain hierarchy were retained in the model. The final reduced quadratic model for each response was described by the equations shown in (4) and (5).

$$B/F = A + B + C + (AC) + A^2 + E \quad (4)$$

$$Resolution = A + B + C + A^2 + B^2 + E \quad (5)$$

The ANOVA table for the reduced quadratic models for each response (Table 2) showed that the injection time (factor A), the interaction of the injection time with separation voltage (factor AC), and the quadratic effect of injection time (factor A^2) were significant terms in the B/F model, while the ramp time (factor B) was borderline significant. Separation voltage (factor C) was not significant, but was retained in the model to allow estimation of the interaction term between injection time and separation voltage. In the resolution model, all three main effects (factors A, B and C) as well as the quadratic term for injection time (factor A^2) were significant, while the quadratic term for ramp time (factor B^2) was borderline significant.

The actual and predicted responses of the model are shown in Figure 2. As seen, the actual responses correlated well to the reduced quadratic model used to predict the responses. The quality of fit (R^2) of the curves was 0.956 and 0.877 for B/F and resolution, respectively. Although the central point in the Box-Behnken design had 5 replicates, a single replicate was performed for each other point in the design. Three separate sets of experiments from the same design were performed and produced results with the same trend as the presented results, although with different B/F values (data not shown). Over the time course of the experiment, the degradation of the protein samples was found to introduce more errors unrelated to the factors under investigation when attempts at more replicates were performed. With the low RSD of the centerpoints (6% and 5% for B/F and resolution, respectively) and the good fit of the responses to the model, we felt confident that the presented results of a single block were reflective of the variations due to the factors tested in the design.

Response surface mapping

The ability to examine the entire predictive model in a three-dimensional plot was a significant benefit of the response surface methodology as the run conditions that provided optimal and non-optimal responses were easily distinguished. B/F was the largest with long injection time, long ramp time, and high separation voltage although, as mentioned before, only the injection time (factor A), the interaction of the injection time with separation voltage (factor AC), and the quadratic effect of injection time (factor A^2) were significant. This large dependence on the injection time was likely due to the effect longitudinal diffusion had on the concentration of the reagents in the sample plug. For example, when the injection time was small, a small sample plug was introduced into the capillary, which could broaden and reduce the concentration of the reagents with a concomitant decrease in the B/F. If a large sample plug was injected, longitudinal diffusion would have less of an effect on the concentration of the reagents in the sample plug thereby having less of an effect on the B/F. While statistically insignificant, the trend in the results indicated that a high ramp time and separation voltage (E-field) were beneficial to a high B/F, possibly due to less heating and dilution of the samples.

under these conditions compared to short ramp times and low separation voltages, respectively. The response surface showing optimum B/F at a separation voltage of 22 kV is displayed in Figure 3A.

Resolution was also optimized as a lack of this response would hinder analysis. Resolution was greatest with short injection time, long ramp time, and lowest separation voltage. The response surface for optimum resolution obtained at a constant voltage of 16 kV is shown in Figure 3B. It should be noted that the trends of the response curves for both B/F and resolution shown in Figures 3A and 3B were the same at all voltages investigated.

Desirability function

Due to the opposing influence of injection time on B/F and resolution, a balance between these two responses is necessary depending on the relative importance of each response in the immunoassay being performed. For example, if the separation of Ab-Ag* and Ag* is facile, a long injection time should be made to ensure that the largest B/F is obtained. On the other hand, if the Ab-Ag pair has a small K_d (resulting in tight binding of the Ab-Ag pair and a subsequent high B/F), and separation of the two peaks is difficult, a short injection time should be performed to obtain the highest resolution.

To quantify this opposing effect of injection time on B/F and resolution, the data were further analyzed using a simple interface for numerically optimizing multiple responses. A desirability function, D , transformed predicted values from individual response surfaces into a dimensionless scale, d_i , ranging from $d = 0$ (unacceptable) to $d = 1$ (most desirable). D was the geometric mean of the scaled response surface values as described in Eq. (6).

$$D=(d_1 \times d_2 \times \dots d_m)^{1/m} \quad (6)$$

A three-dimensional plot of D vs the model terms represented the conditions that optimized all responses. Each response can be weighted by relative importance in the desirability function on a scale of 1 to 5, with 1 as least important and 5 as most important. As previously mentioned, we assumed the sensitivity of the competitive immunoassay would be increased if the initial B/F point on the calibration curve was large. Therefore, B/F was weighted more heavily than resolution in the desirability function. Figure 4 shows the response surface for D in which B/F was given a relative importance of 5 and resolution given a relative importance of 3. The map indicated that the optimum combination of B/F and resolution was obtained with 10 sec injection, 1.6 min voltage ramp time, and 22 kV separation voltage. Conversely, the least optimal combination was found with 4 sec injection, 0.8 min ramp time, and 22 kV separation voltage. As these conditions resulted from a predicted model, it was important to test the validity of these optimum and least optimum experimental parameters on the performance of the glucagon immunoassay.

Calibration curves for glucagon immunoassay

To establish the sensitivity of the immunoassay, B/F Glu* was measured with increasing amount of unlabeled Glu. Calibration curves were produced using the optimal and least optimal conditions predicted from the optimization design (Figure 5A). Representative electropherograms produced with 40 nM Glu*, 5 nM Glu and 20 nM Ab are shown in Figure 5B. Standard deviation ($n = 4$) were larger using the optimal set of experimental conditions because resolution between bound and free peaks was slightly worse, which led to a greater source of variance in peak integration. Nevertheless, the B/F ratio at optimum conditions was consistently higher, providing improved sensitivity and a larger dynamic range for the assay compared to sub-optimum conditions. By fitting a linear curve to the first three points of each

calibration curve, the sensitivity of each immunoassay could be found from the slope of the fitted line. The curve from the optimized conditions had a slope of -0.0128 nM^{-1} , while the curve from the least optimum conditions had a slope of -0.0033 nM^{-1} , yielding a 388% increase in the sensitivity of the immunoassay under optimized conditions compared to non-optimized conditions. The calibration curves therefore confirmed the prediction and validity of the optimization model.

Conclusions

These experiments were undertaken to optimize the CE conditions for a competitive immunoassay of glucagon as previous experiments had shown that the sensitivity of the method was limited. We hypothesized that Joule heating produced during the ramp to the separation voltage was reducing B/F and a longer ramp time would allow for efficient heat dissipation and a higher B/F. After analysis of the experiments, although B/F increased with increasing ramp time, the effect was not significant, thus our hypothesis was not confirmed. Injection time was found to significantly affect B/F ratio with the highest injection times resulting in the highest B/F. Optimum resolution was obtained with the smallest injection time and a desirability curve was used to optimize the two responses.

The potential of the approach described here enables efficient optimization of the glucagon immunoassay, maximizing the amount of information obtained while minimizing consumption of expensive immunoreagents. The Box-Behnken design provides information about the relative significance of main effects, as well as information about interaction effects that cannot be predicted by univariate techniques. The model results are easily interpreted and visualized in response surface plots. Moreover, the statistical methods are readily transferable to any analytical technique, thus streamlining sequential investigations and simplifying the development of multi-analyte applications.

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Fig. 1. Variation in fluorescein peak characteristics for different instrument settings, given as injection time (sec), ramp time (min), and separation voltage (kV), respectively.

b)**Actual Resolution**

Fig. 2. Scatter plots representing model fit for a) B/F, and b) resolution. Distance from any point to diagonal line of fit is equal to the residual error for that point.

Fig. 3. Response surface plots for a) B/F and b) resolution as a function of injection time (A) and ramp time (B). The response surface for B/F is given for a 22 kV separation voltage and the response surface for resolution is given for a 16 kV separation voltage.

Fig. 4. Response surface plot showing desirability, D , as a function of injection time and ramp time for a constant 22 kV separation voltage, indicating optimal conditions at high injection times and high ramp times.

Fig. 5.

a) Calibration curves obtained at optimal (grey: 10 sec injection time, 1.6 min ramp time, 22 kV separation voltage) and least optimal (black: 4 sec injection time, 0.8 min ramp time, 22 kV separation voltage) conditions using Glu* and Ab at concentrations of 40 nM and 20 nM respectively, with unlabeled Glu varied from 5 to 300 nM, showing improved sensitivity at optimum conditions; b) Representative electropherograms from optimal and least optimal conditions for 40 nM Glu* with 20 nM Ab and 5 nM Glu, showing improved B/F but poor resolution for the optimum conditions versus low B/F and better resolution for the non-optimal conditions.

Table 1

Experimental factors and levels used in the Box-Behnken design

Factors	Level (-)	Level (0)	Level (+)
Injection time (A) (sec)	4	7	10
Ramp time (B) (min)	0.8	1.2	1.6
Separation voltage (C) (kV)	16	19	22

Table 2

ANOVA table for the reduced quadratic model

Source	Df ^(a)	Response: B/F ratio			Response: Resolution		
		Sum of squares	F-value	Prob>F	Sum of squares	F-value	Prob>F
A	1	0.9640	323	< 0.0001	0.2890	68.9	< 0.0001
B	1	0.0132	4.43	0.0591 ^(c)	0.0260	6.20	0.0301
C	1	0.0003	0.11	0.7510 ^(b)	0.1390	33.2	0.0001
AC	1	0.0458	15.3	0.0024			
A ²	1	0.0293	9.80	0.0096	0.0278	6.61	0.0260
B ²	1				0.0215	5.13	0.0447 ^(c)
Model	5	1.0500	70.5	< 0.0001	0.5010	23.9	< 0.0001
Error	7	0.0267	2.46	0.2011 ^(b)	0.0353	1.85	0.2890 ^(b)

^(a) Degrees of freedom for each source of variation

^(b) Not significant

^(c) Borderline significant