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Rapid simultaneous determination of glucagon and insulin by capillary electrophoresis immunoassays

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Abstract

A rapid capillary electrophoresis (CE) with laser-induced fluorescence (LIF) competitive immunoassay has been developed for the determination of glucagon in biological mixtures. In the assay, fluorescein-conjugated glucagon is mixed with the sample followed by addition of anti-glucagon. Free and antibody-bound, tagged glucagon could be separated in 3 s using CE to obtain quantitative determination of glucagon with a concentration detection limit of 760 p*M*. The assay was combined with a previously developed competitive immunoassay for insulin to produce a simultaneous immunoassay for both peptides. The method was used to determine glucagon content of islets of Langerhans.

Keywords: Glucagon; Insulin

1. Introduction

Antibody (Ab)–antigen (Ag) interactions have long been used for highly sensitive and selective immunoassays. However, individual photometric or radio immunoassays can take hours to complete and require lager sample volumes. While such assays can be performed in parallel formats for high throughput, the slow individual assays can limit utility in cases where rapid repetitive analyses are needed, e.g. chemical monitoring applications. Recently, significant progress has been made in development of rapid and sensitive immunoassays based on capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection [1–3]. Although CE is a serial technique, its ability to perform the analysis in <10 s and ease of automation has potential to improve speed and throughput for a variety of applications. In addition, high speed, separation power, and ease of automation of CE assays suggests that high throughput can be achieved by performing assays either simultaneously or in parallel. Throughput achieved using such configurations would be much higher than other approaches [4].

CE-LIF immunoassays can be performed in either a competitive or a non-competitive format. In a competitive assay, a fluorescently labeled antigen (Ag*) competes with Ag for binding to a limited amount of Ab. CE-LIF separation of the mixture produces two distinct fluorescent peaks corresponding to Ag* and Ag*–Ab complex, the intensities of which can be related to the original concentration of Ag. In a non-competitive assay, excess of fluorescently labeled antibody (Ab*) is added to

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the solution containing Ag. A CE separation of Ab* and Ag–Ab* allows for determination of concentration of Ag based on the complex peak.

Although in this work competitive assays are examined, it is recognized that both approaches have strengths and weaknesses and their use is dictated by the application. Compared to competitive assays, the non-competitive assays have larger linear dynamic range (LDR), are less dependent on binding properties of antibodies, and can detect cross-reactivity [5,6]. On the other hand, the competitive assay requires an easier separation since free Ag* must only be separated from Ag* bound to a large Ab. In addition, the difficult task of fluorescently labeling antibodies to produce a single homogeneous product is avoided in competitive immunoassays since only the antigen must be labeled. The advantages of competitive assays are especially apparent for the determination of small analytes such as peptides or small molecules.

An important advantage of CE-based immunoassays is that the separation step can be performed quickly, which not only allows for rapid analysis but also improves quantification. In immunoassays, non-covalent binding between Ab and Ag is governed by the equilibrium:

$$\mathrm{Ab} + \mathrm{Ag} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Ab{:}Ag}$$

where k_1 and k_{-1} are association and dissociation rate constants respectively; however, the equilibrium is disrupted as the constituents begin to separate. In a non-equilibrium environment, the half-life of the complex ($t_{1/2}$) is governed by:

$$t_{1/2} = \ln 2 / k_{-1}$$

This effect indicates that with increasing dissociation constant, the speed of separation becomes increasingly important for quantitative detection of the complex and successful application of CE immunoassays. The importance of speed of the separation has been implicit in several examples of using CE-LIF to obtain immunoassays with detection limits <1 n*M* [7–9]; however, it should be noted that many assays have been developed using slow separation conditions. These assays typically rely on either tighter binding or detection of just the free Ag* for quantitation.

In this work, we describe the development of a CE-LIF competitive immunoassay for the pancreatic peptide glucagon. Development of the assay was simplified by universality of the conditions developed previously including use of low conductivity high concentration buffers at slightly basic (7–8.5) pH, narrow-bore capillaries, application of high electric field, as well as use of previously developed methodology for labeling, purification, and detection of fluorescein-derivatized analytes [7]. Further optimization of separation buffer type and pH that allows application of high electric field and prevents adsorption of analyte to the inner capillary walls has been performed. In addition, flow-gated injections were used to improve reproducibility. Finally, we explored the potential of using CE for simultaneous immunoassays by combining a previously developed assay for insulin [7,10,11] with the new assay for glucagon.

2. Experimental

2.1. Chemicals

Unless stated otherwise, all chemicals used in our experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FITC-labeled insulin (Ins*) was purified by HPLC using Vydac protein C4 column (Chrom Tech, Apple Valley, MN, USA). Kreb's Ringer buffer (KRB) contained (in m*M*): 118 NaCl, 5.4 KCl, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 3 D-glucose, and 25 HEPES (*N*-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (pH 7.4). Monoclonal anti-insulin C-terminal peptide (E86211M, $K_d = 1$ n*M*) used for the assays was purchased from Biodesign International (Kennebunk, ME, USA). The antibody was diluted to 100 n*M* in 20 m*M* phosphate buffered saline with 0.5% sodium azide and stored at 4°C prior to use. Monoclonal anti-glucagon from mouse ascites fluid (clone K79bB10, $K_d = 1.6$ n*M*) was diluted to 500 n*M* in separation buffer and stored at 4°C prior to use. 5-(6)-carboxyfluorescein succinimidyl ester was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Preparation of fluorescein-labeled glucagon (Glu*)

The labeling reaction was initiated by addition of 0.1 ml of 10 mg ml^{-1} 5-(6)-carboxyfluorescein succinimidyl ester dissolved in anhydrous dimethylforamide (DMF) to 1 mg of glucagon dissolved in 0.9 ml of 100 m*M* carbonate buffer (pH 8.3). The mixture was incubated for 1 h at room temperature in the dark. Since glucagon has two possible labeling sites, three different products can be obtained from this reaction: two singly labeled and a doubly labeled glucagon. Singly-labeled glucagon was used in all experiments. Reaction products were purified by HPLC using Vydac protein C4 column (Chrom Tech, Apple Valley, MN, USA). The LC mobile phase composition was 69:31 mixture of 0.1% trifluoracetic acid and acetonitrile. Purified Glu* fractions were collected and their concentrations were estimated using visible absorption of fluorescein at 496 nm ($\varepsilon = 75\,000\ M^{-1}$ cm^{-1}).

2.3. Capillary electrophoresis

All fused-silica capillaries used in our experiments had 360 μm outer diameter (O.D.) with various lengths and inner diameters (I.D.'s) (Polymicro Technologies, Phoenix, AZ, USA). The CE apparatus used in this work was similar to that reported previously [6,7]. A low pressure bomb machined from clear Plexiglas was used to flow-inject the sample onto the flow-gated interface [12] (Fig. 1). The bomb was connected to the flow-gated interface using a 75 μm I.D. capillary. Analyte was pumped towards the flow-gated interface at a rate of 3 μl min^{-1} where it was washed with cross-flow of 0.1 ml min^{-1} supplied by the Dionex gradient pump (Smyrna, GA, USA). To make an injection, the cross-flow was stopped for 250 ms using an air actuated switching valve equipped with high speed switching accessory and digital interface (Valco Instruments, Houston, TX, USA). The separation capillaries used all were 12 μm I.D. Separation buffer for the majority of the experiments was 50 m*M* tricine (*N*-tris[hydroxymethyl] methylglycine) (pH 8.3); however, other buffers were also used as mentioned in the discussion section. High voltage

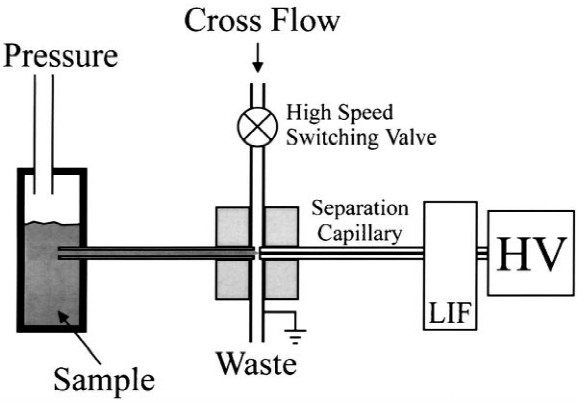

Fig. 1. Diagram of an instrumental setup used to perform CE-LIF with flow-gated injections. 10–15 p.s.i. of helium pressure was used to flow the sample towards a flow-gated interface. A cross-flow of 0.1 ml min^{-1} supplied by the Dionex gradient pump (Smyrna, GA, USA) washes the sample to waste at the interface. To make an injection, the cross-flow is stopped for 250 ms using high speed switching valve. During the time when the cross-flow is off, a sample can reach the inlet of the separation capillary where it is electrokinetically injected.

power supply was CZE 1000R from Spellman High Voltage Electronics (Plainview, NY, USA). Detection was accomplished using a LIF detector described elsewhere [6,7].

2.4. Data acquisition and analysis

Data acquisition and analysis was accomplished by software written in-house using National Instruments Lab Windows (Austin, TX, USA). Data was acquired at 400 Hz and filtered with a 10 ms low pass filter. Peak width, migration time, theoretical plates, and skew were calculated using statistical moments [13]. For the calibration curves, it was assumed that quantum yield of the complex is the same as that of the free Glu*.

2.5. Flow-injection immunoassay for glucagon

The glucagon immunoassay was performed using a 7 cm long capillary. (4.3 cm injection to detection). Samples contained sample glucagon, 3 μl of 187 n*M* solution of Glu* and 5 μl of 500 n*M* solution of anti-glucagon. Additionally, 10 μl of 50 n*M* solution of fluorescein was added to the mixture as internal standard. Separation buffer was used to adjust final

sample volume to 200 μl. Samples were incubated for 3 min before analysis and injected electrokinetically for 3 s at 1 kV. Ultrafast immunoassay was performed using a 5 cm long capillary (effective length 1.8 cm) with applied electric field of 2400 $V\,cm^{-1}$. In this case, 20 μl of 700 n*M* solution of Glu* was mixed with sample glucagon and 20 μl of 500 n*M* solution of anti-glucagon in glass microvial. Injections were made by application of flow-gating; the flow was stopped for 250 ms. Final concentrations of analytes are given in the Figs. 3 and 4.

2.6. Simultaneous immunoassay of insulin and glucagon

Simultaneous assays were performed using a capillary with 4.3 cm effective length (total length was 7 cm) and flow-gated injections. Samples contained 10.1 n*M* Ins*, 2.8 n*M* Glu*, 12.5 n*M* anti-glucagon, and 0.5 n*M* anti-insulin (final volume of 200 μl). In both cases, 10 μl of 50 n*M* solution of fluorescein was added to the sample as an internal standard. Standard concentrations were 0, 5, 10, 30, and 50 n*M* of each species.

2.7. Islet content analysis

Islets of Langerhans were isolated from CD-1 mice following ductal injection with collagenase XI and placed in RPMI culture medium (GIBCO, Gaithersburg, MD, USA). In less than one hour after isolation, islets were washed twice using each KRB [14] and separation buffer. Islet contents were extracted using previously described procedure [15]. Briefly, islets were picked out of wash solution, placed into conical glass micro-vials containing 15 μl of CE separation buffer, and sonicated for 30 min. After sonication, 25 μl of 0.18 *M* solution of HCl in ethanol was added to each vial. Solutions were thoroughly mixed and incubated for 18 h. Vials containing disrupted islets were dried using gentle stream of helium to evaporate ethanol and HCl. Remaining residue was reconstituted using separation buffer. Necessary amounts of Ins* and Glu* were added to the solution followed by addition of antibodies to produce final concentration as developed for the simultaneous assay described in Section 2.6. The final volume was brought to 200 μl using separation buffer. The solution was incubated for 3 min at room temperature and analyzed.

3. Results and discussion

3.1. CE-LIF based immunoassay for glucagon

Previous work on an immunoassay for insulin has demonstrated that rapid separation is important for successful separation of antibody-bound and free antigen. Similar experimental conditions were used for development of the glucagon immunoassay. An important step in any CE-based technique is selection of a separation buffer that allows application of high electric field and at the same time prevents analytes from interacting with the inner wall of the capillary. Performance of the separation buffer is even more significant for rapid immunoassay since factors that affect dissociation rate of the complex, such as column temperature and interactions with the walls or buffer itself, can directly affect detection limit of the assay. Several different buffers were examined as summarized in Table 1. As shown in the table, only buffers with pH above 7.4 allowed reasonable efficiencies for the Glu* peak to be obtained, presumably due to prevention of adsorption of Glu* and the associated tailing of the Glu* zone (note the difference in skew as a function of pH). Increasing the pH above 8.3 tended to decrease resolution of bound and free Glu* zones. This effect was due to both poorer efficiency for Glu* and to increasing the rate of dissociation as evidenced by bridging between the bound and free Glu* zones. These results illustrate that increasing the pH is not necessarily advantageous for immunoassays, as it usually is with protein separations, since the change in pH can affect the binding reaction. The tricine buffer was clearly the best buffer of all those tested. Fig. 2 depicts a representative separation obtained using tricine. The effect of tricine was not limited to just pH as it performed considerably better than TAPS-AMPD (TAPS-(*N*-tris[Hydroxymethyl]methyl-3-aminopropanesulfonec acid), AMPD-2-amino-2-methyl-1,3-propanediol) at the same pH (see Table 1). The tricine appeared to be especially good at preventing adsorption of both Glu* and the complex peak. With TAPS-AMPD, the baseline continued to

Table 1
Summary of electrophoretic data for Glu* and Glu*–Ab complex with different electrophoresis buffers[a]

pH	Buffer	Glu* Efficiency (plates m^{-1})	Glu* skew	Resolution of Glu*/Ab–Glu*
7.4	10 m*M* Phosphate	6000	0.23	N.D.[b]
7.4	100 m*M* HEPES	16 000	0.42	N.D.[b]
8.3	150 m*M* TAPS-AMPD	44 000	0.16	1.8
8.3	50 m*M* tricine	495 000	0.01	2.2
9.0	50 m*M* CHES[c]	165 000	0.02	1.1
10.2	50 m*M* Borate	353 000	0.01	0.9

[a] All separations for buffer optimization utilized effective separation length of 3 to 4 cm and $E = 2400$ V cm^{-1}. Samples were injected electrokinetically at 1 kV for 3 s.
[b] Not Determined because of poor peak shape for Glu*.
[c] CHES-(2-[*N*-Cyclohexylamino]-ethanesulfonec acid).

rise and ghost peaks were observed when the complex was repeatedly injected on to the column. Since this effect was not observed with just Glu*, it was concluded that the antibody was adsorbing in this buffer. These results highlight the important issues in selecting both pH and buffer type in optimizing an immunoassay.

While bound and free Glu* could be separated using tricine buffer with high efficiency and reproducible migration times (<0.5% RSD), we found that peak areas were highly irreproducible with RSD's of 15–20% during repeated electrokinetic or hydrodynamic injections performed manually. This irreproducibility appeared to be primarily due to the difficulty of controlling injections on short columns for fast separations.

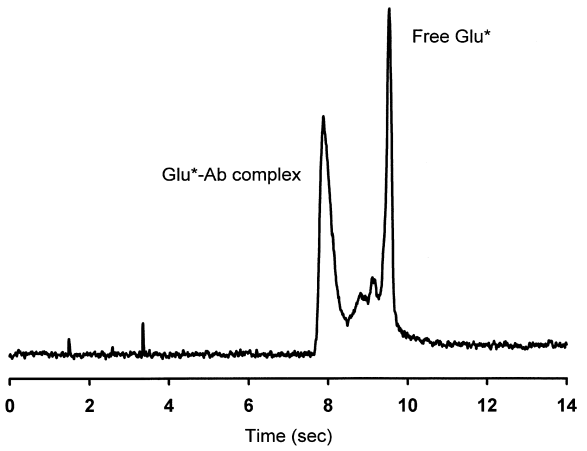

Fig. 2. Separation of the free Glu* from Ab-Glu* complex. In this experiment, 7.5 n*M* Glu* and 22 n*M* antibody were used. A 6 cm long (effective, 9 cm total) capillary was used in this case with $E = 2000$ V cm^{-1}, electrokinetic injections at 1 kV for 3 s.

In order to improve injection reproducibility and investigate the potential for automation, we used flow-gated injections that allow sample to be electrokinetically injected onto the CE column from a continuous flowing stream [12]. Fig. 3 shows representative electropherograms obtained using a 4.3 cm long separation capillary with flow-gated injections with different concentrations of unlabeled glucagon. The flow-gated interface dramatically improved reproducibility with the free Glu* peak having an RSD of 3.8% for all concentrations of glucagon tested (at least 20 electropherograms for each test); however, variability of the complex peak was still high at 10% RSD. Under these conditions, quantification of glucagon was best determined from the area of the free Glu* peak. The limit of detection (LOD) for the assay was estimated to be 4 n*M* by multiplying the standard deviation of the blank peak area for Glu* (no glucagon added) by 3 and dividing by the slope of the calibration curve. (Calibration curve constructed from standards of 5, 10, 50, 150 n*M* glucagon all analyzed five times). This concentration detection limit corresponds to a mass detection limit of 640 zmol given the 0.16 nl injection volume.

Several explanations could be proposed for continuing variability in the area of the complex peak. First, inspection of the electropherograms in Figs. 2 and 3 reveals that two peaks migrate before the free Glu* peak. These peaks appear only with addition of antibody to Glu* solutions and decrease with addition of unlabeled glucagon which indicates that both peaks are complexes of antibody and Glu*. Since a

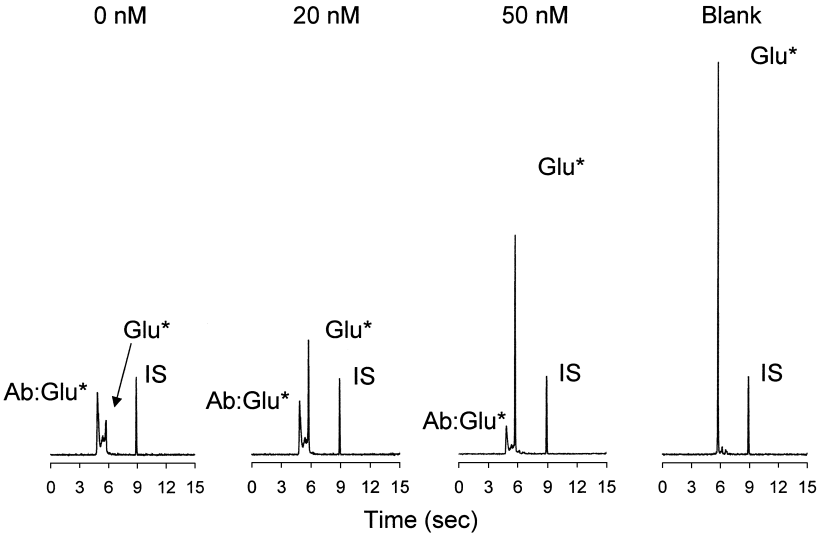

Fig. 3. Rapid CE-LIF based competitive immunoassay for glucagon using 4.3 cm long capillary. Solutions contained 2.8 n*M* Glu*, 12.5 n*M* anti-glucagon (Ab), 2.5 n*M* fluorescein used as internal standard (IS), and 50, 20, or 0 n*M* glucagon as indicated in the figure. A blank electropherogram contained only Glu* and I.S. is shown for comparison. ($E = 2400$ V cm^{-1}, injections were 250 ms long).

whole antibody was used in this experiment, these peaks may represent antibody with one or two Glu* bound. (Note that in calculation of resolution indicated in Table 1, only the first peak was used). It is suspected that some of the variability in the complex peak area is due to dynamic equilibrium between antibodies bound to one or two Glu* molecules. Broadening of the complex peak and formation of multiple complexes has been reported previously when whole antibodies were used in CE-immunoassays [9]. Second, commercial preparations of glucagon have been reported to contain multiple derivatives of glucagon mono- and di-desamidoglucagon and *N*-terminal degraded glucagon [16]. Previously reported MALDI analysis indicated presence of methionine sulfoxide and doubly oxidized tryptophan side-chain [17]. Some of these derivatives were reported to be less effective in immunoassays [18,19], which could create various dynamic equilibria leading to irreproducibility of the complex peak. Finally, despite our efforts in buffer selection, adsorption of the complex to the inner capillary walls could contribute to irreproducibility of the complex peak area.

3.2. Ultrafast determination of glucagon

Utilization of the flow-gated interface allowed us to use capillaries as short as 1.8 cm and separations performed in 3.5 s as shown in Fig. 4. Compared to the separations on the longer column, faster separations showed a larger second complex peak. We suspect that this complex peak is an Ab bound with two Glu* molecules. Possible reasons for the increase in size of this peak compared to longer runs are (1) faster separation results in less dissociated complex and (2) change in concentrations of Ab* and Glu* resulted in more of the complex being formed. The larger 2nd complex peak overlapped more with the free Glu* peak. Despite that, reproducibility of the overall area of both complex peaks during rapid separations was improved 5-fold to 2% RSD. This improvement allowed us to prepare a calibration curve based on bound to free ratio for the assay performed using 1.8 cm capillary (Fig. 5). A detection limit for glucagon of 760 p*M* was calculated from this calibration. This detection limit is slightly higher than that reported for detection of insulin [20] which could be explained by weaker

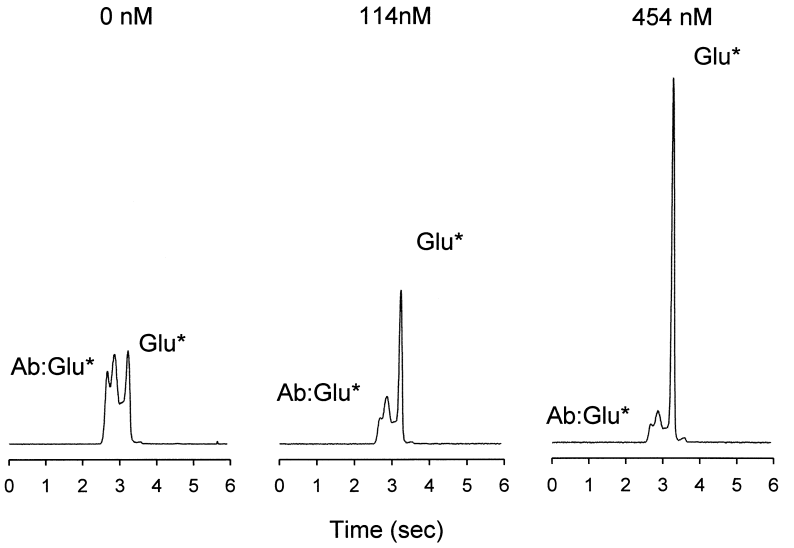

Fig. 4. Rapid CE-LIF based competitive immunoassay for glucagon using 1.8 cm long capillary. Solutions contained 70 n*M* Glu*, 50 n*M* anti-glucagon, and 454, 114, or 0 n*M* glucagon. Internal standard is not needed since the ratio of the bound to free is used for quantitation. Presence of multiple complex peaks is probably due to formation of singly and doubly bound complexes with whole antibodies. ($E = 3400$ V cm^{-1}, injection time was 250 ms).

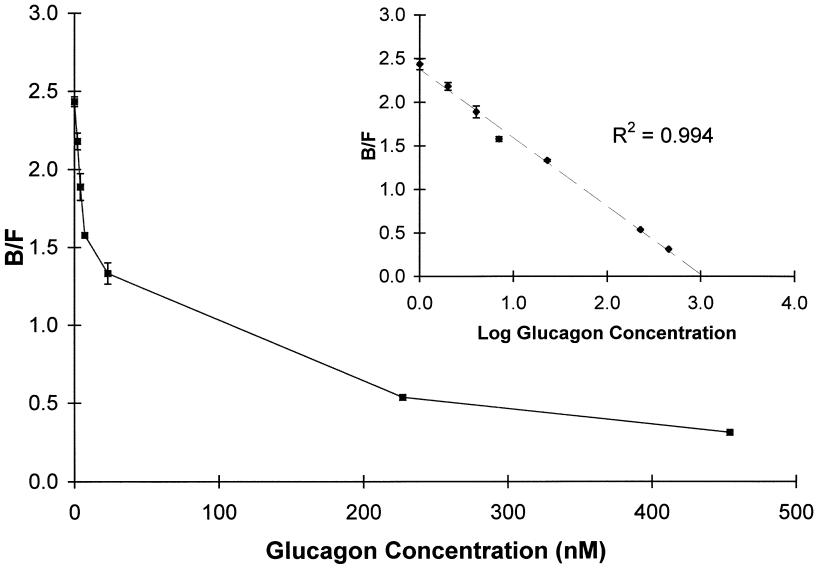

Fig. 5. A calibration curve for the detection of glucagon obtained using conditions described in caption for Fig. 2. Insert shows the sample calibration performed on a semi-log scale in the linear region of the calibration. A detection limit of 760 p*M* was determined for this calibration.

dissociation constant of the antibody used ($K_{d\ Glucagon} = 1.6$ n*M*, $K_{d\ Insulin} = 1.0$ n*M*).

3.3. Simultaneous CE-LIF Immunoassay for Glucagon and Insulin

As mentioned earlier, a rapid competitive CE-immunoassay for insulin has been developed in our laboratory [7]. This assay was combined with the assay for glucagon for simultaneous determinations. Initial attempt to resolve two complex peaks using 4.3 cm long (effective) capillary was not successful. In attempt to further improve separation, separation column length was increased to 7.3 cm while keeping the electric field the same, as illustrated on Fig. 6. Electropherogram A shows a separation of Glu* and Ins*. Several small peaks around Ins* are impurities from the Ins* solution. In electropherogram B, antibodies against insulin and glucagon were added giving rise to complex peaks. Finally, electropherograms C and D demonstrate an effect of different concentrations of glucagon and insulin on the sizes of free and bound peaks of Glu* and Ins*. Several factors could contribute to poor separation between complexes. First, both antibodies used in the assay are IgGs and have similar electrophoretic properties that would make it difficult to separate them using short capillaries. Second, formation of multiple complexes between antibody and antigens, as mentioned earlier, may lead to broader peaks preventing complete separation. Third, bridging resulting from dissociating complex creates tailing in both complexes, which further complicates separation. Since the increase in the length of the capillary did not produce adequate separation of the complexes, the capillary length was cut back to 4.3 cm. The simultaneous calibration curves based on area measurements of free Glu* and Ins* peaks are shown on Fig. 7. Detection limits of 4.3 n*M* for

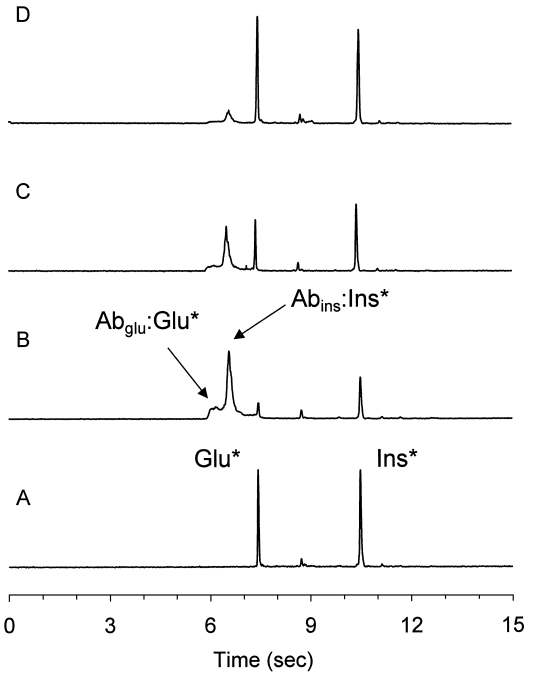

Fig. 6. Simultaneous CE-LIF immunoassay for insulin and glucagon. Separation is performed in 7.3 cm long capillary, $E = 2200$ V cm^{-1}, injections were 250 ms. (A) Separation of 2.8 n*M* Glu* and 10.1 n*M* Ins*. (B) Separation of a mixture of same concentrations of Ins* and Glu* with 12.5 n*M* anti-glucagon and 0.5 n*M* anti-insulin. Identification of the complex peaks was performed in the separate experiment (not shown). (C) 25 n*M* glucagon and insulin added to the mixture described in B. (D) 100 n*M* glucagon and insulin added to the mixture described in B.

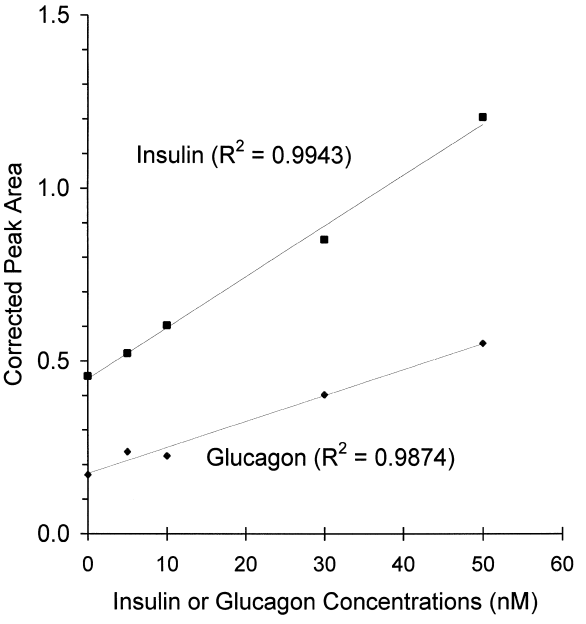

Fig. 7. Simultaneous calibration curves for detection of insulin and glucagon prepared in the linear region of the calibration. Assay conditions are described in the legend of Fig. 6.

glucagon and 5.5 n*M* for insulin were obtained. Lower detection limits could most likely be obtained using a 1.8 cm long capillary; however, difficulties with resolution precluded us from using this condition. Error bars of 1 standard deviation ($n=10$) were placed on each of the points (Fig. 7); however, they are too small to be observed. Given the high reproducibility of an assay, the deviation from the line for some standards most likely resulted from difficulties with accurate pipetting of derivatized Ins* and Glu*. Stock solutions of some constituents of an assay contained 30% acetonitrile which affected pipette accuracy.

3.4. Measurement of glucagon content of islets

Measurement of glucagon content was performed using conditions developed for the simultaneous assay. Groups of five islets were prepared as described in the Materials and Methods sections and analyzed for glucagon content. Measured concentrations were in the range between 8 and 18 n*M* glucagon, which is well within linear region of the calibration curve. We have determined that each islet contained 1.9±0.6 ng glucagon (errors are given as standard error of the mean). This value is in good agreement with published value of 1.5 ±0.5 ng [20]. Additionally, all prepared samples, including calibration standards, were measured in less than 30 min which demonstrates significant improvement in analysis time compared to RIA. Simultaneous measurement of glucagon and insulin content of the islets was complicated by the fact that the islets contain much more insulin than glucagon. The published value for insulin content of the mouse islets is ~48 ng [14]. Using experimental conditions described above, these values would require measurements of 1–3 n*M* glucagon and 40–60 n*M* insulin in the same assay. Although it is possible to adjust concentrations of Ins* and antibody to make the measurement possible, overlap of a large Ab–Ins* complex peak with free Glu* peak made it impossible to simultaneously measure insulin and glucagon content of the islet in a single separation. Insulin content of the islets was measured by itself in the separate experiment and was determined to be 58±13 ng which compares well with the published value.

4. Conclusion

CE-LIF based immunoassay described in this work demonstrates that methodology, which includes application of the short separation distances, high electric fields, and low-conductivity buffers, for a CE-immunoassay could, with minor modifications, be transferred from one analyte to the other. Some of the important factors in development of rapid CE-immunoassay are proper choice of separation buffer and pH and reproducible injection techniques. As we have found, adsorption to the inner capillary walls of the capillary columns may be detrimental to the assay; thus, optimization of separation buffer to prevent this effect is required. Additionally, adsorption of reagents to pipette tips and to inner walls of sample vials during sample preparation significantly affects reproducibility and precision of the assay. Therefore, careful choice of materials and treatment for tips and vials may further increase reproducibility. Finally, in order to obtain best detection limits, it is important to choose an antibody with smallest dissociation constant possible.

Application of flow injection to rapid CE-immunoassays provides a simple means for future automation. Combination of fast separations demonstrated with ease of conjugation to a commercial autosampler can significantly increase throughput of analysis of biological samples. Using the described assay, we determined glucagon with results comparable to those obtained by RIA but in a fraction of the time required for RIA. Multi-analyte analysis in a single electropherogram was also demonstrated by CE immunoassay.

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