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# Examination of capillary zone electrophoresis, capillary isoelectric focusing and sodium dodecyl sulfate capillary electrophoresis for the analysis of recombinant tissue plasminogen activator

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**Abstract**

The microscale techniques of CZE, cIEF and SDS capillary electrophoresis have been evaluated for the analysis of a complex glycoprotein, recombinant tissue plasminogen activator (rtPA). A series of $\omega$-amino acid buffers (pH≈5) was found suitable for the CZE separation of rtPA on coated capillaries. rtPA could be resolved into a series of major and minor peaks in an $\epsilon$-aminocaproic acid buffer containing 0.01% (v/v) Tween 80. For cIEF, a two step method with pressure mobilization was utilized. Using a commercial instrument, either a polymer solution with a 50 $\mu$m I.D. capillary or narrow bore capillaries without a polymer solution (25 $\mu$m I.D.) were employed. rtPA was resolved into at least eight species within a p*I* range of 6.4–9.2 using Ampholine 3.5-10. Migration time precision for the major peaks ranged from 0.2% for CZE to ≤2–3% R.S.D. for cIEF. Total recovery of rtPA from the capillary was also demonstrated for both methods. Analysis of rtPA, rtPA Type I, rtPA Type II and the desialylated forms resulted in the expected elution profiles. Finally, the potential of SDS capillary electrophoresis using a coated capillary for an rtPA Type I/Type II purity assay was shown.

*Keywords:* Isoelectric focusing; Glycoproteins; Proteins; Plasminogen activator

## 1. Introduction

The large scale manufacturing of therapeutic proteins has necessitated the development of analytical methods for research and development, product characterization, consistency in manufacture and stability indicating assays. Glycosylation is one type of post-translational modification that requires monitoring because variation in the carbohydrate composition may significantly alter the properties of a protein e.g., biological activity, clearance, solubility and stability [1–3]. Capillary electrophoresis (CE) has the potential to become a significant method for the analysis of glycoproteins due to its high resolving power, quantitative capability, speed and ease of automation. This technique has been shown to be capable of analyzing the carbohydrate heterogeneity for several recombinant glycoproteins in their native form [4–7].

Currently, only separations of relatively simple, low-molecular-mass compounds have been successfully validated for CE [8]. In the case of small molecules, standard buffers can be used for a wide variety of samples. On the other hand, proteins exhibit large differences in p*I*, solubility, stability and overall charge complexity, each of which must be considered for method development.

rtPA, a therapeutic glycoprotein administered for

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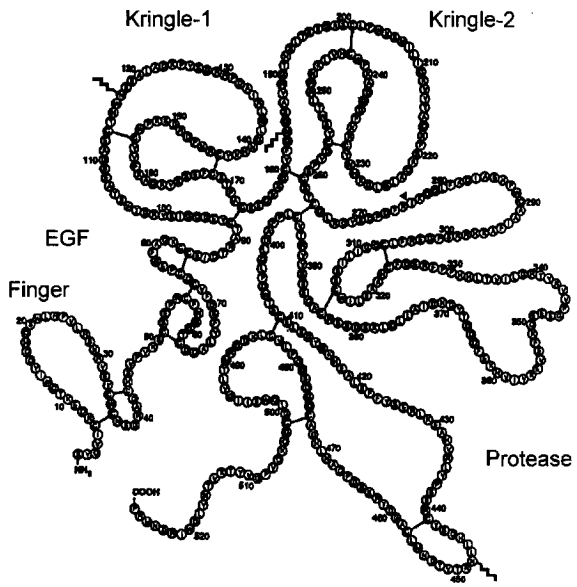


Fig. 1. Polypeptide structure of rtPA.

the treatment of myocardial infarction, is structurally detailed in Fig. 1 [9]. Of the two kringle domains, the kringle-2 domain contains a lysine binding site and is believed to control protein–protein interactions between rtPA and fibrin [10]. The presence of the binding site is supported by the fact that analogues of lysine, e.g. $\omega$-amino acids such as $\epsilon$-aminocaproic acid, are antifibrinolytic agents [11]. rtPA, with a molecular mass of $\sim 60 \times 10^3$, contains three N-linked glycosylation sites [12]. Two rtPA variants exist: Type I-glycosylated at Asn 117, 184 and 448 and Type II-glycosylated only at Asn 117 and 448. The carbohydrate structure at Asn 117 is of the high mannose type and the structures at Asn 184 and 448 are of the N-acetyllactosamine type. Differences in the number of sites glycosylated, the carbohydrate structure at each site, as well as variation in the sialic acid content of the N-acetyllactosamine carbohydrates at Asn 184 and 448 result in a variety of glycoforms. rtPA is present in either a one or two chain form, the two chain resulting from cleavage of the peptide bond between Arg 275 and Ile 276 by plasmin [13].

Analysis of rtPA glycoforms by capillary zone electrophoresis (CZE) has been hampered as the high salt solution required for its solubility is not compatible with CE methodology. The first reported CZE separation for rtPA did not resolve the glycoforms on a polyacrylamide coated capillary and a single peak was observed [14]. Subsequently, partial separation of the glycoforms was achieved [15,16]. Reproducibility and recovery data were not reported for any CZE separation of rtPA.

Capillary isoelectric focusing (cIEF) has the potential to be an effective tool for characterizing glycoforms based on differences in sialic acid content. This technique has been utilized for the analysis of hemoglobin, transferrin and monoclonal antibodies [17–20]. Poor migration time reproducibility was a major issue with rtPA analysis by cIEF using salt mobilization [15]. cIEF with mobilization by electro-osmotic flow (EOF) was also described. However, a constant trend toward faster migration times indicated deterioration of the capillary coating and a nonlinear pH vs. time plot was generated [21,22]. Recently, hydrodynamic mobilization has been successfully employed for cIEF of proteins to produce reproducible migration times and linear pH vs. time plots [18,23–25].

In order to handle the high salt concentration present in rtPA samples, a micellar method was developed for purity assessment [26]. SDS capillary electrophoresis for molecular mass determination was also demonstrated and found useful for monitoring the plasmin digestion of rtPA [26].

In this paper, we show that CZE and cIEF methods can be developed for analysis of a complex glycoprotein such as rtPA to the level of being validatable. Protein adsorption, protein recovery and method reproducibility are essential parameters that must be evaluated for validation and are addressed with respect to rtPA. SDS capillary electrophoresis of rtPA is also briefly explored.

## 2. Experimental

### 2.1. Instrumentation and materials

Beckman P/ACE systems 2100 or 5500 (Fullerton, CA, USA) with System Gold version 8.10 were used for all electrophoretic experiments. Analytical grade buffers [$\beta$-alanine (BALA), $\gamma$-aminobutyric acid (GABA), $\epsilon$-amino-*n*-caproic acid (EACA), $\epsilon$-amino-*n*-valeric acid, and $\epsilon$-amino-*n*-caprylic acid] and

Tween 80 were obtained from Fluka (Ronkonkoma, NY, USA). Urea (ultra pure grade) was from ICN Biomedicals (Costa Mesa, CA, USA). Ampholine 3.5-10 and Pharmalyte 3-10 as well as peptide standards ($Gln^{11}$-amyloid $\beta$-protein fragment 1-28 and human $\beta$-endorphin) were purchased from Sigma (St. Louis, MO, USA). Servalyte 3-10 was obtained from Crescent Chemical (Hauppauge, NY, USA). Fused-silica capillaries for in-house modification were purchased from Polymicro Technologies (Phoenix, AZ, USA). Mill-Q water (Millipore, Bedford, MA, USA) was used to prepare all buffer and sample solutions. Recombinant tissue plasminogen activator (rtPA) was manufactured at Genentech. Separation of the rtPA glycoforms into the Type I and Type II variants and treatment with plasminogen (+P) or plasminogen and neuraminidase (+P/N) were conducted as described previously [21].

### 2.2. Capillary zone electrophoresis

Fused-silica as well as polyacrylamide (PAA) and polyvinyl alcohol (PVA) coated capillaries were utilized [27,28]. The specific capillary tube diameters and lengths are included in the figure captions. Sample injection was accomplished by the P/ACE at low pressure (3.5 kPa). The applied voltage was in the normal polarity mode, and protein absorbance was monitored at 214 nm. The separation buffers were prepared by maintaining the *n*-aminocarboxylic acid concentration at 200 m*M* and buffering with 50 m*M* acetic acid.

### 2.3. Capillary isoelectric focusing

The eCAP cIEF 3-10 Kit (Beckman Instruments, Fullerton, CA, USA) was adapted for rtPA analysis by adding urea to the manufacturer's polymer solution to the desired mass/volume percentage [36% (w/v) urea/ polymer solution]. Ampholyte (4 $\mu$l) and sample (20 $\mu$l) were added to this denaturing polymer solution (100 $\mu$l), and thoroughly mixed. The mixture was centrifuged for 10 min at 4 000 *g*. eCAP Neutral Capillaries, 50 $\mu$m I.D., with a total length of 27 cm and an effective length of 20 cm were used. The anolyte and catholyte were 91 m*M* phosphoric acid in polymer solution and 20 m*M* sodium hydroxide solution, respectively. cIEF experiments were performed by filling the capillary with the sample solution, focusing at 13.5 kV for 2 min, and mobilizing with low pressure (from anolyte to catholyte) while the voltage remained applied. The detection wavelength was set at 280 nm. High pressure rinses (138 kPa) of 10 m*M* phosphoric acid (1 min) and water (2 min) were performed between each analysis.

For cIEF experiments involving narrow bore capillaries, 25 $\mu$m I.D. capillaries were coated with PVA [28]. All samples and solutions were prepared as previously stated except water replaced the polymer solution. Focusing was performed at 25 kV for 5 min, followed by mobilization with low pressure. The voltage remained applied during mobilization. Slab gel IEF was performed on rtPA samples as described previously [21].

### 2.4. SDS capillary electrophoresis

The samples were prepared and analyzed using the SDS 14-200 kit from Beckman Instruments. In general, 100 $\mu$l of the sample buffer (0.12 *M* Tris–HCl 1% SDS, pH 6.6) was combined with 85 $\mu$l of the sample, 5 $\mu$l of $\beta$-mercaptoethanol and 10 $\mu$l of the internal standard, orange G. After mixing, the samples were boiled for 10 min and cooled on ice for 3 min. All samples were analyzed on an eCAP SDS coated capillary, 100 $\mu$m I.D, 20 cm effective length and 27 cm total length. Samples were introduced into the capillary by applying low pressure for 30 s. The separation was performed in the polymer buffer solution supplied in the kit (SDS 14-200 gel buffer) with an applied voltage of 8.1 kV (reverse polarity), and the protein zones were detected at 214 nm.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Nunc microtiter plates were coated overnight (>12 h) at 2–8°C with affinity purified goat anti-rtPA diluted in 0.05 *M* sodium carbonate buffer pH 9.6. Plates were then washed with phosphate-buffered saline, pH 7.4 containing 0.05% Tween 20 (PBS–Tween) and blocked for 1 h with 200 $\mu$l PBS–Tween containing 0.5% bovine serum albumin (PBS–BSA). Following another wash with PBS–Tween, 100 $\mu$l of standards, samples and controls

were added to the wells of the microtiter plate and incubation continued for 2 h at ambient temperature with agitation. Plates were washed to remove unbound material and 100 $\mu$l of horseradish peroxidase labelled rabbit anti-rtPA were added to each well of the microtiter plate. Following a second incubation for 1 h at ambient temperature, the plates were washed with PBS–Tween and 100 $\mu$l of enzyme substrate added (one 5 mg tablet of orthophenylene diamine in 12.5 ml PBS containing 4 m*M* hydrogen peroxide), and incubation was continued for 20–25 min. The reaction was stopped by the addition of 100 $\mu$l 2.25 *M* sulfuric acid to each well. Absorbance was measured at 492 nm on an SLT 340 EAR AT plate reader (SLT Labinstruments, Research Triangle Park, NC, USA). Standard curves were fitted using a four parameter fitting program written at Genentech based on the nonlinear least squares algorithm of Marquardt [29]. rtPA concentrations for samples were obtained by interpolation on the standard curve. Controls were monitored for consistency of assay performance.

## 3. Results and discussion

### 3.1. Capillary zone electrophoresis

A significant issue in protein analysis by CZE is adsorption onto the surface of the capillary wall [30]. The most effective way to suppress protein adsorption has been to modify the capillary surface with a hydrophilic coating. Bare fused-silica capillaries and capillaries covalently modified with linear PAA and PVA were evaluated for the CZE separation of rtPA, Fig. 2. An EACA buffer (pH=5.1) was used as it was previously reported that addition of this zwitterion to the run buffer improved the separation of rtPA glycoforms [15]. With uncoated capillaries, elution of the glycoforms was not initially observed, indicating that rtPA was strongly adsorbed to the fused-silica surface at this pH. Only after five injections did several very broad peaks begin to appear (data not shown). The PAA and PVA coatings helped to minimize adsorption of rtPA to the capillary surface, as indicated by the elution of the glycoforms with the first injection. Sharper peaks and a somewhat better separation was found with the

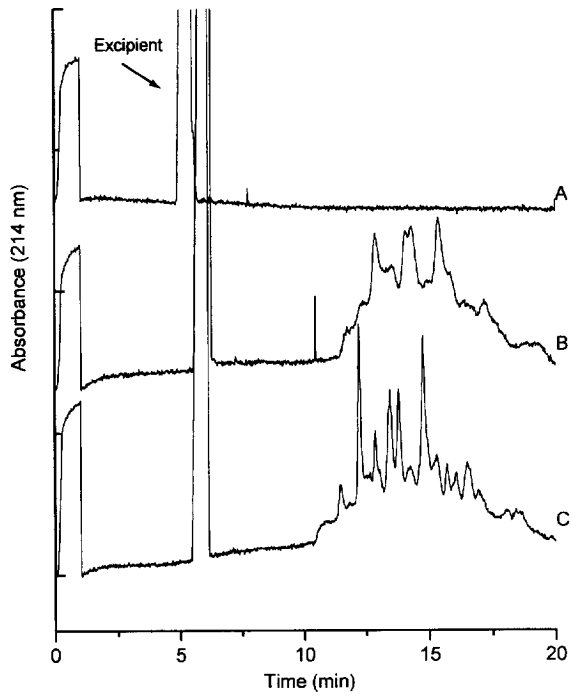


Fig. 2. Comparison of uncoated and coated capillaries for the CZE separation of rtPA. (A) fused-silica, (B) polyacrylamide coated and (C) polyvinyl alcohol coated. Capillary: 75 $\mu$m I.D.; $L$=37 cm; $l$=30 cm. Separation conditions: 0.2 *M* EACA/ 50 m*M* acetic acid (pH 5.1); $E$=405 V/cm; $T$=25°C, normal polarity. Injection: 6 s by pressure (3.5 kPa). Sample: 1 mg/ml rtPA in formulation buffer. Detection: 214 nm.

PVA coated capillary (Fig. 2C), suggesting that the proteins adsorb less to the PVA surface than to the PAA coating (Fig. 2B). Based on these results, all CZE separations of rtPA glycoforms were performed on PVA coated capillaries.

In an attempt to optimize the separation further, $\omega$-amino acid buffers similar to EACA were tested for the CZE separation of rtPA, Fig. 3. The $\omega$-amino acid buffers differed only in the carbon chain length by single methylene groups. BALA had the lowest pH (pH 4.6) of the series, due to the proximity of the charged groups and, as a result, this buffer system gave the fastest separation. No peaks could be observed with GABA (pH 4.9), even though the carbon chain length and pH of the buffer lie between the BALA and EACA systems. Aminocarboxylic acid buffers with longer carbon chains, e.g. $\epsilon$-amino-*n*-valeric acid (pH 4.75) and $\epsilon$-amino-*n*-caprylic acid

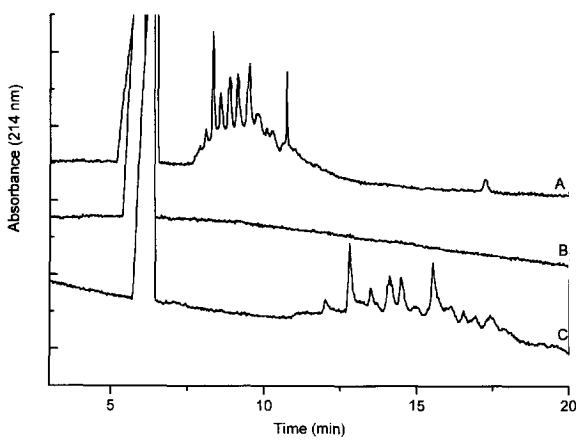


Fig. 3. CZE separation of rtPA with $\omega$-amino acid separation buffers. (A) 0.2 *M* BALA/ 50 m*M* acetic acid (pH=4.6), (B) 0.2 *M* GABA/ 50 m*M* acetic acid (pH=4.9) and (C) 0.2 *M* EACA/ 50 m*M* acetic acid (pH=5.1). Capillary: PVA coated, 50 $\mu$m I.D. Separation conditions: $E$=540 V/cm. Injection: 12 s by pressure. All other conditions are the same as Fig. 2.

(pH 4.95), were also investigated. The separations obtained with these buffers were comparable to the EACA separation (data not shown).

An expanded view of the major peaks from the rtPA separations with EACA and BALA showed that almost every peak was non-gaussian. For a specific glycoform with a given number of sialic acid moieties, heterogeneity from the distribution of neutral carbohydrates as well as from the position of the sialic acids can exist and may be the cause of the split and shouldered peaks that are observed. Another possible cause for the partial resolution of the variants was deamidation (see Section 3.2).

One factor, other than pH, that may have influenced the rtPA separation shown in Fig. 3 is the interaction between the $\omega$-amino acid analogues and the lysine binding site of rtPA. While the amino group of the analogue interacts with the Asp residues at the center of the hydrophobic kringle-2 binding domain, the carboxylic acid group remains exposed [10]. These zwitterion buffers may assist in separation by preventing individual rtPA molecules from interacting with lysine residues on other molecules and thus precipitating, or by reducing interactions with the capillary surface. It is possible that GABA did not interact with the lysine binding site as strongly as the other analogues since it was the only $\omega$-amino acid buffer investigated that did not elute the glycoforms.

To ensure that proteins are completely eluted, protein recovery from the CE system was determined. Recovery is an important issue for an analytical method used in the characterization of recombinant proteins as complete recovery is essential to the accuracy of the analysis and reproducibility of the method. In these experiments, the eluted amount of rtPA was determined by ELISA after electrophoretic collection, Table 1. The recoveries were between 50 and 80%, depending on sample and buffer system. While the BALA buffer gave a higher value for the Type I variant, EACA yielded a higher value for the Type II variant. In all cases, however, recovery was not optimal as a measurable loss of protein occurred during the electrophoretic separation. In an attempt to increase recovery, 0.01% (v/v) Tween 80, a non-ionic detergent was added to the separation buffer. As shown in Table 1, addition of this detergent essentially achieved complete recovery

Table 1
Recovery of rtPA after CZE separation determined by ELISA

| Buffer | Recovery (%) | | |
|---|---|---|---|
| | rtPA | rtPA Type I | rtPA type II |
| 200 m*M* BALA/ 50 m*M* HOAC | 65 | 79.9 | 70 |
| 200 m*M* EACA/ 50 m*M* HOAC | 52.4 | 51.8 | 70.7 |
| 200 m*M* BALA/ 50 m*M* HOAC and 0.01% (v/v) Tween 80 | 100 | 99 | 108 |
| 200 m*M* EACA/ 50 m*M* HOAC and 0.01% (v/v) Tween 80 | 107 | 107 | 125 |

Separation: PVA coated, 50 $\mu$m I.D., $l$=20, $L$=27 cm, $V$=15 kV, $T$=25°C, normal polarity. Detection: 214 nm. The rtPA glycoforms from five runs were collected electrophoretically in a single vial containing 0.4 ml run buffer. Collected fractions were diluted with 0.4 ml ELISA buffer and analyzed accordingly.

of the glycoprotein. Therefore, in the following CZE separations of rtPA, Tween 80 was added to the buffer to provide an optimized separation medium. Run to run migration time reproducibility for rtPA injected from formulation buffer was determined to be better than 0.2% R.S.D. ($n$=27) with an EACA buffer system containing 0.01% (v/v) Tween 80. This reproducibility allows for routine, direct analysis of samples in the formulation buffer.

Using the optimized buffer system and PVA coated capillaries, plasminogen treated rtPA, Type I and Type II variants as well as plasminogen/ neuraminidase treated rtPA were analyzed by CZE, Fig. 4. Plasminogen treatment of rtPA rendered the sample homogeneous by ensuring that all of the rtPA was in the two chain form. A complex separation pattern was observed for rtPA as it was composed of both variants. Analysis of Type I showed a broad distribution of numerous unresolved peaks while Type II was mainly composed of a smaller number of sharper peaks which had faster migration times than the Type I glycoforms. The longer migration time for the Type I forms was likely a result of the higher number of sialic acid residues of this variant. Although the Type I and Type II variants had the same concentration, Type I exhibited lower peak intensities possibly due to the increased heterogeneity of the variant or residual interaction with the capillary wall. Removal of sialic acid heterogeneity by neuraminidase treatment resulted in a collapse of the pattern into a nonuniform broad peak with increased electrophoretic mobility. The significant amount of heterogeneity which remained after neuraminidase treatment may result from differences in the neutral carbohydrate content and deamidation (see Section 3.2).

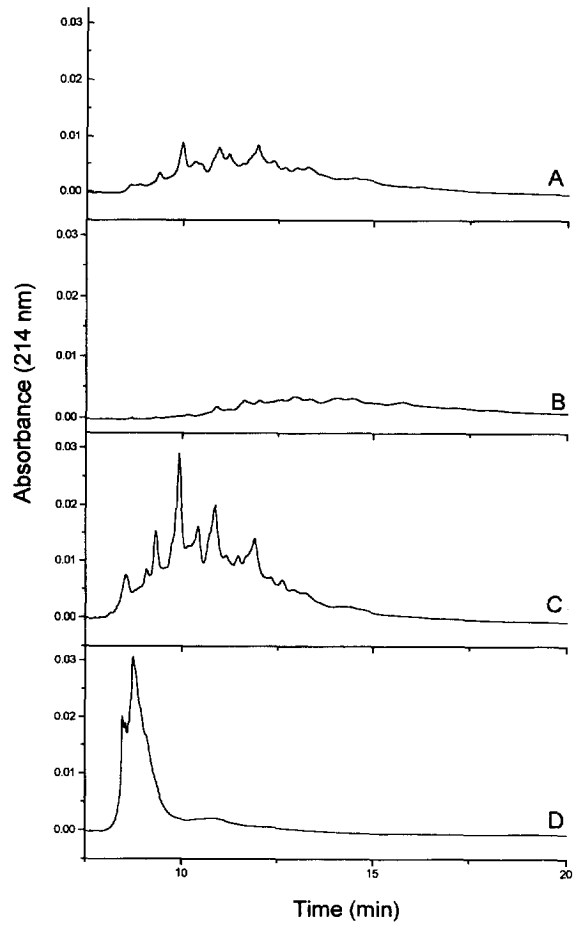


Fig. 4. Separation of plasminogen treated rtPA variants and plasminogen/neuraminidase treated rtPA by CZE. (A) rtPA (+P), (B) rtPA Type I (+P), (C) rtPA Type II (+P) and (D) rtPA (+P/N). Capillary: $L$=47; $l$=40 cm. Separation conditions: 0.2 $M$ EACA/ 50 m$M$ acetic acid (pH=5.1) with 0.01% (v/v) Tween 80, $E$=532 V/cm. Injection: 15 s by pressure. All other conditions are the same as Fig. 3.

### *3.2. Capillary isoelectric focusing*

Recombinant glycoproteins are often evaluated by isoelectric focusing because variants differing by a single charge (sialic acids or charged amino acids) can be easily resolved. In addition, the concentration effect which is inherent from the focusing in the technique enables minor charged variants to be detected. Thus, cIEF has the potential to be an important tool for protein characterization. The cIEF method presented here utilized coated capillaries that minimize electro-osmotic flow and required two steps, (1) focusing of the sample–ampholyte mixture into narrow zones and (2) pressure mobilization of the zones with an applied field to allow for detection. This mobilization approach offers the advantages of a linear pH vs. time calibration and good migration time reproducibility. Two related approaches are demonstrated for the control of the linear flow velocity on a commercial instrument which has a fixed mobilization pressure (3.5 kPa). The first utilizes a commercially available cIEF kit with 50

$\mu$m I.D. eCAP coated capillaries and a polymer solution to increase the solution viscosity. An alternative method, which employs 25 $\mu$m I.D. PVA coated capillaries to increase column resistance and thus eliminate the polymer additive, was also briefly studied.

To prevent rtPA from precipitating during cIEF analysis a strong solubilizing agent, urea, was required [15]. Although the mixing of high concentrations of urea with polymer solution in the commercial kit reduced the original polymer concentration, the viscosity of the resulting urea/polymer solution was similar to the original polymer solution (data not shown).

Three commercially available ampholytes often used for isoelectric focusing are Ampholine, Pharmalyte and Servalyte. The distribution of the low-molecular-mass amphoteric substances in the pH gradient and their composition may vary [31]. The effect of the these ampholytes on the cIEF separation of rtPA dissolved in formulation buffer was investigated, Fig. 5. Ampholine 3.5-10 gave the sharpest peaks and separated rtPA into several major and minor peaks. The glycoforms were not sufficiently resolved with Pharmalyte 3-10. Although approximately 8 peaks were obtained with Servalyte 3-10, the peaks were broader than those with Ampholine 3.5-10, and the signal-to-noise ratio was lower. It was concluded that Ampholine 3.5-10 gave the best separation of the glycoforms under the given separation conditions.

As in the case of CZE, we evaluated the protein recovery for the cIEF separation. rtPA was collected from a cIEF separation using Ampholine 3.5-10 and analyzed by ELISA. The average percent recovery was 93% ($n=3$), indicating that, within the limits of experimental error, full protein recovery was attained.

Run to run absolute migration time reproducibility for the four major rtPA glycoforms was ~2–3% R.S.D. ($n=17$). It should be noted that the system may be sensitive to temperature variation as the polymer solution viscosity, which determines the migration times, is strongly affected by temperature (~2%/°C). Correction for run to run migration time differences was achieved by incorporating internal standards into the rtPA sample/ampholyte mixture. Recently, peptides were described as standards for

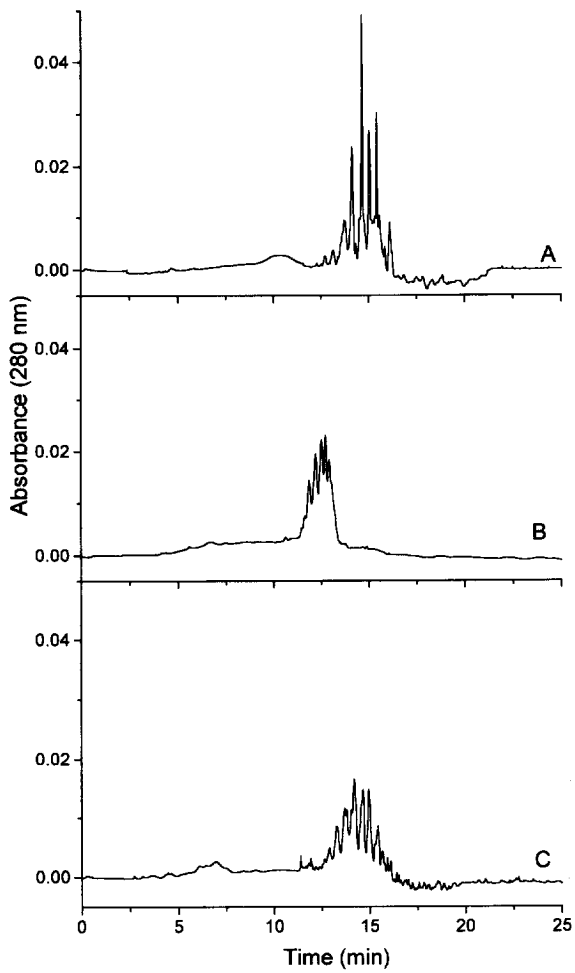


Fig. 5. Comparison of ampholytes for the cIEF separation of rtPA. (A) Ampholine 3.5-10, (B) Pharmalyte 3-10 and (C) Servalyte 3-10. Capillary: eCAP neutral coated 50 $\mu$m I.D., $L=27$ cm; $l=20$ cm. Separation conditions: anolyte, 91 m$M$ phosphoric acid in gel buffer; catholyte, 10 m$M$ sodium hydroxide; focusing, 500 V/cm for 5 min; mobilization, 500 V/cm with low pressure. Sample: 0.32 mg/ml rtPA, 4.8 $M$ urea and 1.3% ampholyte. Detection: 280 nm.

isoelectric focusing under denaturing conditions and their p$I$ values were determined in the presence and absence of urea [32]. Two of these peptides, Gln″ amyloid B-protein fragment (p$I$ 6.4 in 8 $M$ urea) and human $\beta$-endorphin (p$I$ 9.2 in 8 $M$ urea), were chosen based on rtPA slab gel results and used in the present work to bracket the rtPA glycoforms separated by cIEF, Fig. 6. Using these peptides, the following equation was used to calculate the relative

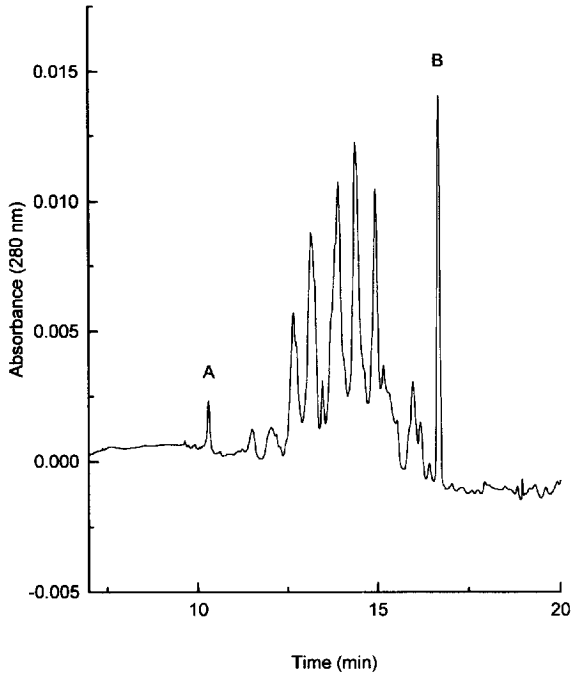


Fig. 6. cIEF separation of rtPA with (A) human β-endorphin (p*I* 9.2) and (B) $Gln^{1}$ amyloid B-protein fragment 1-28 (p*I* 6.4) as p*I* markers. Sample: 0.16 mg/ml rtPA, 0.04 mg/ml human β-endorphin and 0.04 mg/ml $Gln^{1}$ amyloid B-protein fragment 1-28, 4.8 *M* urea and 1.3% Ampholine 3.5-10. All other conditions are the same as Fig. 5.

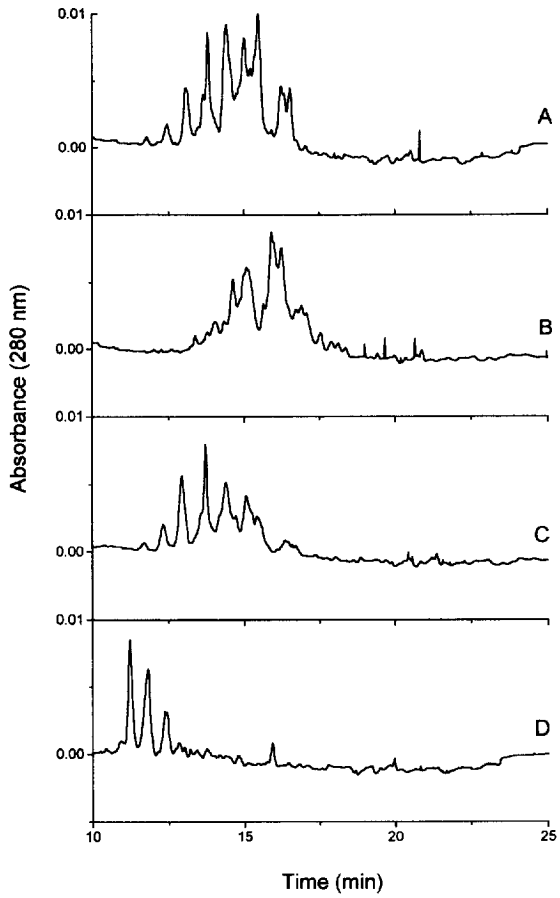


Fig. 7. cIEF of plasminogen treated rtPA variants and plasminogen/ neuraminidase treated rtPA. (A) rtPA (+P), (B) rtPA Type I (+P), (C) rtPA Type II (+P) and (D) rtPA (+P/N). Samples: 0.16 mg/ml rtPA, 4.8 *M* urea and 1.3% Ampholine 3.5-10. All other conditions are the same as Fig. 5.

migration of the glycoforms: $(t_{\text{glycoform}} - t_{\beta\text{-endorphin}})/(t_{\text{amyloid B-protein}} - t_{\beta\text{-endorphin}})$, where $t$ is the elution time. The correction resulted in a relative run to run migration time reproducibility of ~0.25–1% R.S.D. ($n=17$).

cIEF analysis with the 50 μm capillaries and the polymer additive was applied for the analysis of plasminogen treated rtPA, rtPA Type I and rtPA Type II, as well as plasminogen/neuraminidase treated rtPA, Fig. 7. Plasminogen treated rtPA was separated into approximately 8 peaks, several of which were not well resolved. Analysis of the two variants showed that each type gave a different separation pattern. Type I was separated into four main peaks composed of numerous unresolved species while Type II revealed approximately 7 slightly heterogeneous peaks. The glycoforms for the Type I variant eluted later than those of Type II indicating that Type I was more acidic. As expected, treatment of rtPA with neuraminidase simplified the cIEF pattern by removing the charge heterogeneity on the glycoprotein caused by the sialic acids. The same cIEF pattern for all neuraminidase/plasminogen treated rtPA samples (data not shown) indicated the elimination of all sialic acid microheterogeneity and revealed the presence of additional charge heterogeneity that may possibly originate from deamidation of asparagine residues.

In order to assess the results obtained with the cIEF method, the same samples were analyzed by slab gel IEF, Fig. 8. Although different ampholytes were used in the slab gel experiment, there was general agreement between the two methods in that the patterns were in the same p*I* region. The slab gel resolved the plasminogen treated rtPA into approxi-

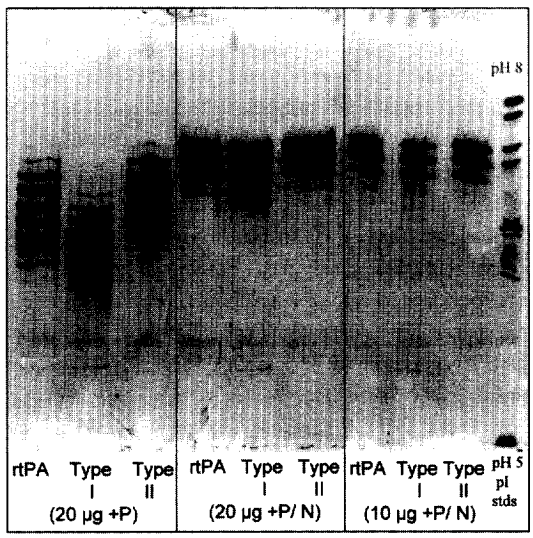


Fig. 8. Slab gel IEF of rtPA variants and neuraminidase treated rtPA. Gel: 0.4 mm thickness, 4% acrylamide with 8 *M* urea and 2.4% (w/v) Pharmalyte 5-8. Focus: 2 h at 4°C. Initially, constant power at 10 W until 1600 V. At 1600 V the separation was switched to constant voltage mode. Anolyte: 1 *M* phosphoric acid. Catholyte: 1 *M* sodium hydroxide. Fix: 10% sulfosalicylic acid/ 5% trichloroacetic acid (w/v). Stain: Coomassie brilliant blue.

mately 10 bands and each variant into approximately 8 bands with the Type I variant having more acidic p*I* values than Type II. The cIEF separation of the glycoforms produced the same trend, e.g. the more acidic variant being Type I. Correlating the number of bands obtained with the slab gel to those in the capillary format was not possible and may be attributed to the amount of protein used for analysis. The total amount of protein loaded onto the capillary for cIEF, 85 ng, was more than 200 times less than that used for slab gel IEF. The two techniques produced identical results for the neuraminidase/ plasminogen treated samples, giving three major bands.

Comparison of the cIEF and CZE results showed the difference in the separation mechanism between the two methods. While CZE separated according to mass and charge under native conditions, cIEF separated only according to charge under denaturing conditions. One of the most striking examples was for the Type I variant. CZE analysis showed that this variant consisted of a large number of peaks in low abundance, as indicated by the low absorbance for each peak at 214 nm. For cIEF, a few heterogeneous peaks, with an absorbance equivalent to the other cIEF separations of rtPA were obtained.

Using a 25 $\mu$m I.D. polyvinyl alcohol coated capillary, cIEF separations of rtPA without polymers were briefly explored. For a capillary with a fixed length and for a given mobilization pressure, a reduction in the internal diameter by a factor of two results in a four-fold increase in the column resistance [33]. Although the linear flow velocity for the 25 $\mu$m I.D. capillary was higher than that obtained for the 50 $\mu$m I.D. capillaries with a polymer solution, separation of glycoforms was possible. Long term reproducibility for four of the major peaks was >2% R.S.D. over 90 runs, and it can be expected that even higher reproducibility could be achieved by incorporating an internal standard. However, the loss in signal due to the decreased path length in the narrower capillary may be problematic when detecting low protein concentrations or proteins that contain few aromatic amino acids.

In summary, both cIEF and CZE primarily separate the glycoforms according to sialic acid content. One factor which differentiates the two techniques, other than the separation mechanism, is the use of native conditions for CZE and denaturing conditions for cIEF. As compared to slab gel IEF, cIEF provides a comparable separation of the variants and offers the advantage of quantitation, automation and an increase in the speed of analysis. Although multiple forms of rtPA were successfully separated by CZE and cIEF, the identity of the component(s) that comprise each peak remains unknown. Further work is necessary to elucidate their structure.

### *3.3. SDS capillary electrophoresis*

Denaturing SDS capillary electrophoresis with polymer additives for sieving has been used to estimate the molecular weight of proteins and can provide data similar to SDS polyacrylamide gel electrophoresis (PAGE). In the production and control of therapeutic proteins, SDS-PAGE is used to evaluate purity as well as to determine the presence of non-dissociable aggregates of the product. CE methods would have the same advantages described above for cIEF over the slab gel methods currently used. Initially, attempts were made to perform SDS

capillary electrophoresis of rtPA with bare fused-silica capillaries. The rtPA peak was broad and migrated much later than would be expected for its molecular weight, suggesting that even in the presence of SDS, this glycosylated protein adhered to the capillary surface.

The feasibility of a CE method for quantitating Type I and Type II in rtPA was demonstrated with a commercial kit that used a polymer for sieving and a coated capillary, Fig. 9. Exposure of plasminogen treated rtPA samples to $\beta$-mercaptoethanol resulted in cleavage of the disulfide bond that holds the A chain (Gly 1–Arg 275) and the B chain (Ile 276–Pro 527) together. Plasminogen treated rtPA was separated into three polypeptide chains, a B chain and two A chains (with and without N-acetyllactosamine carbohydrate at Asn 184). The B chain which consists of the protease portion of rtPA, migrated first and was easily identified as it was present in all of the samples analyzed. Peaks 2 and 3, which corresponded to the two A chains, were identified by comparing the separation of rtPA and its variants. Only two peaks were observed for the Type I variant, with the later eluting peak being the A chain-Type I polypeptide. Analysis of plasminogen treated rtPA Type II showed that the sample contained both A chain polypeptides, with the Type II polypeptide, peak 2, being the major A chain constituent. Since glycosylation is known to affect the activity of rtPA (the Type II variant has a 30–50% higher activity than Type I [34]), a method to determine the variant composition of rtPA will be valuable.

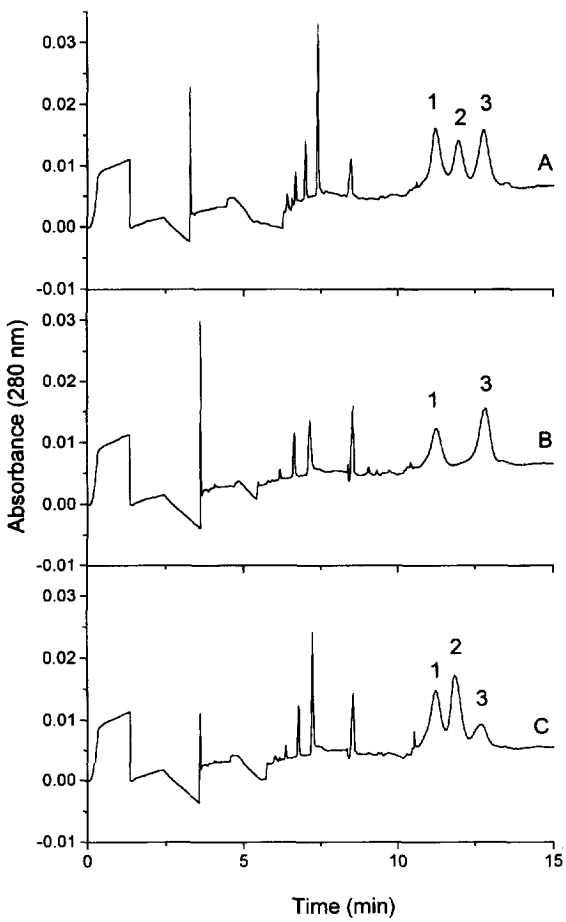


Fig. 9. SDS capillary electrophoresis of plasminogen treated rtPA variants. (A) rtPA, (B) rtPA Type I and (C) rtPA Type II. (1) B-chain-protease, (2) A-chain-Type II polypeptide and (3) A-chain-Type I polypeptide. Capillary: eCAP neutral coated 100 $\mu$m I.D.; $L$=27 cm; $l$=20 cm. Separation Conditions: SDS 14-200 gel buffer; $E$=300 V/cm. Injection: 30 s by pressure. Sample: 0.425 mg/ml rtPA in SDS sample buffer with $\beta$-mercaptoethanol and orange G*. Detection wavelength: 214 nm.

## 4. Conclusion

This paper addressed several issues that are important for the validation of CE methods used in the characterization of recombinant proteins. These issues include protein recovery, which is essential to ensure accurate and reproducible analyses, and migration time reproducibility, which greatly facilitates validation and implementation of the methods.

An important aspect of the CZE and cIEF methods was that rtPA was directly analyzed from its formulation buffer, thus eliminating the need for sample clean-up. CZE with $\omega$-amino acid buffers and polyvinyl alcohol coated capillaries provided a fast procedure for the separation of rtPA variants. Addition of 0.01% (v/v) Tween 80 to the separation buffer provided full protein recovery from the capillary and excellent migration time reproducibility ($\leq$0.2% R.S.D.). cIEF, using a polymer solution and 50 $\mu$m I.D. capillaries, or narrow bore capillaries (25 $\mu$m I.D.) without polymer, resulted in high elution time reproducibility for the glycoforms. Addition of urea to the cIEF sample solution was necessary for protein solubilization and for essentially full protein

recovery from the capillary. The modification of the commercial kit with urea may be used as a general protocol for samples that are difficult to solubilize.

The high migration time reproducibilities and protein recovery demonstrate conclusively that the CZE and cIEF methods can be used for the routine analysis of glycoform heterogeneity. The feasibility of using SDS capillary electrophoresis with a coated capillary for the quantitation of rtPA variants was also illustrated and puts forth the possibility of replacing the time consuming PAGE method with CE. Taken together, this report has demonstrated the potential of CE for the routine analytical characterization of a complex protein such as rtPA. This conclusion can be important since analytical methods such as reversed-phase HPLC and PAGE employ large volumes of toxic reagents which require careful storage and disposal. Future work will focus on the identification of the separated components by either direct coupling with mass spectrometry or through collection followed by enzymatic digestion and analysis with HPLC and/or mass spectrometric analysis.

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