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# Rapid one-step capillary isoelectric focusing method to monitor charged glycoforms of recombinant human tissue-type plasminogen activator

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**Abstract**

A rapid (< 10 min) one-step capillary isoelectric focusing (cIEF) method was developed to monitor charged glycoforms of recombinant human tissue-type plasminogen activator (rt-PA). Focusing takes place between the detector and the anode and the electro-osmotic flow (EOF) sweeps the separated glycoforms past the detector, towards the cathode. The separation uses a neutral coated capillary and hydroxypropylmethylcellulose (HPMC) to reduce the EOF to a constant and reproducible value. The method uses an ampholyte mix with a 50:50 ratio of pH 5–8 and pH 3–10 ampholytes in 4 *M* urea and 0.1% HPMC to produce maximal resolution whilst maintaining protein solubility during focusing. The electropherograms were compared to isoelectric focusing (IEF) slab gels of samples of intact rt-PA. In both cases approximately ten charged species could be detected. Data analysis indicated that the intra-assay precision was < 5% for peak migration times and < 10% for normalized peak areas. The number of charged species detected by each of the two methods was consistent for samples of intact rt-PA, rt-PA types I and II and for neuraminidase-digested rt-PA. Overall the data indicate that the automated cIEF method can be an adjunct to slab-gel IEF in the characterization and routine analysis of recombinant glycoproteins.

## 1. Introduction

A major challenge in the quality control of recombinant glycoproteins is the analysis and monitoring of the heterogeneity of the constituent oligosaccharides. To date only hydrolytic assays for total neutral sugars and sialic acid content are routinely used, methods which are too simplistic to ensure the level of consistency required for a pharmaceutical product. The need for more specific methods has grown as the importance of the carbohydrate moiety of glycoproteins has become known. This is of particular importance with glycoproteins used as therapeutics with respect to their half-life and potency in vivo [1].

Recombinant human tissue-type plasminogen activator (rt-PA) is a fibrin-specific plasminogen activator which has been approved for the treatment of myocardial infarction. rt-PA is a glycoprotein consisting of 527 amino acids, with a polypeptide molecular weight of 59 000. The carbohydrate structures of the Chinese hamster ovary (CHO)-derived rt-PA have been elucidated [2]. The molecule has three N-glycosylation sites, positions 117, 184 and 448 and exists as two main variants, designated type I rt-PA and type II rt-PA. Type I contains N-linked oligosaccharides at all three sites while type II is

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only glycosylated at positions 117 and 448. Position 117 contains high mannose oligosaccharides exclusively, while positions 184 and 448 principally contain N-acetyllactosamine-type oligosaccharides. The N-acetyllactosamine-type oligosaccharides contain different amounts of sialic acid attached to the terminal galactose residues. Oligosaccharides at position 184 contain significant amounts of mono-, di-, and tri-sialyl residues while position 448 contains disialyl oligosaccharide as the predominant structure. The different numbers of sialic acid residues result in a number of different glycoforms of the parent molecule, or microheterogeneity, with corresponding variability in the isoelectric point (p*I*).

The traditional method for the analysis of charge heterogeneity of recombinant proteins has been by the techniques of ion-exchange chromatography and isoelectric focusing (IEF). In slab-gel IEF, glycoforms of the intact protein containing increasing amounts of sialic acid can be separated and visualized as discrete bands with increasingly acidic isoelectric points. Although this method can be used to characterize the heterogeneity and monitor the production consistency of recombinant proteins, it has the disadvantages of long and labor-intensive analysis times producing at best semi-quantitative data. A cIEF method for the fractionation of rt-PA glycoforms has previously been published using a two-step method (focusing followed by salt mobilization) [3]. However, this cIEF method gave poor peak shape, especially for the more basic glycoforms and was not compared to slab-gel isoelectric focusing patterns.

In this paper we based the successful development of a rapid capillary isoelectric focusing (cIEF) method on an existing slab-gel IEF method for rt-PA and on recently documented so-called one-step cIEF methodology [4,5]. In this one-step cIEF method focusing takes place between the detector and the anode, and the electro-osmotic flow (EOF) sweeps the separated glycoforms past the detector, towards the cathode. The two techniques were compared in order to demonstrate the applicability of the routine use of this cIEF method in addition to, or in place of, the traditional slab-gel IEF method.

## 2. Experimental

### 2.1. Materials

All chemicals were of analytical reagent grade. Hydroxypropylmethylcellulose (HPMC) and neuraminidase (E.C. 3.2.1.18, from *Clostridium perfringens*) were obtained from Sigma (St. Louis, MO, USA). Ampholytes (pharmalytes) ranges pH 3–10 and pH 5–8 and p*I* standards were obtained from Pharmacia LKB (Piscataway, NJ, USA). N,N,N′,N′-Tetramethylethylenediamine (TEMED), urea (electrophoresis grade) and 30% acrylamide/bis solution 19:1 (5% C) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Urea and acrylamide were deionized using a mixed-bed resin prior to use. rt-PA was manufactured in-house, types I and II rt-PA were separated by affinity chromatography using a lysine resin [6].

### 2.2. Capillaries

Unless otherwise stated the capillary routinely used was an eCAP™ neutral capillary from Beckman (Fullerton, CA, USA). μSIL® DB™-1, DB™-17 and DB™-WAX capillaries were from J&W Scientific (Folsom, CA, USA). CElect-H150 and CElect-P150 capillaries were from Supelco (Bellefonte, PA, USA). The BioCap capillary was from BioRad (Hercules, CA, USA).

### 2.3. Isoelectric focusing

Isoelectric focusing was performed using 0.4 mm thickness, 4% acrylamide gels containing 8 *M* urea and 2.4% (w/v) pH 3–10 and 5–8 ampholytes in the ratio of 65:35. A 20-μg amount of protein or 10 μl of standards (prepared according to the manufacturer's recommendation) were applied to paper applicators

placed 1–2 cm from the anode. The gel was focused for 2 h at 4°C, first at a constant power of 10 W until the voltage reached 1600 V, then at constant voltage. The anolyte was 1 *M* phosphoric acid and the catholyte 1 *M* NaOH. After focusing the gel was fixed with 5% sulfosalicylic acid–10% trichloroacetic acid (w/v) and then stained with Coomassie Brilliant Blue.

### 2.4. Capillary isoelectric focusing

cIEF was performed using a Beckman P/ACE 5510 (Fullerton, CA, USA). The internal diameter of the capillary was 50 $\mu$m, the total length was 27 cm; 20 cm from the inlet to the detector and 7 cm from the detector to the outlet. During analysis the temperature of the capillary was maintained at 20°C, the reagents and samples were at ambient temperature. The anolyte was 10 m*M* phosphoric acid and the catholyte 20 m*M* NaOH. The capillary was rinsed for 1 min with water then filled for 2 min with sample. The separation was achieved using reversed polarity, i.e. the anolyte at the outlet, at a constant voltage of 500 V/cm. The absorbance was measured at 280 nm with detection complete in less than 10 min. After each analysis the capillary was rinsed for 1 min with 0.1 *M* HCl, 1 min with 10 m*M* phosphoric acid and 1 min with water. For later experiments the post-separation HCl wash was excluded and the phosphoric acid wash extended to 2 min. In these cases a single 1-min HCl wash was performed at the beginning of each day. rt-PA samples were routinely diluted to give final concentrations of 125–250 $\mu$g protein/ml, 4 *M* urea, 0.1% (w/v) HPMC, 7.5% (v/v) TEMED and 3% (w/v) pH 3–10 and pH 5–8 ampholytes in the ratio 50:50. The final salt concentration of samples was 50 m*M* or less.

### 2.5. Removal of N-acetylneuraminic acid

Sialic acid residues were removed from rt-PA by treatment with neuraminidase. rt-PA was digested for 4 h with neuraminidase at 37°C in 0.2 *M* arginine-phosphate, 0.1% Tween 80, pH 7.2, with 0–0.003 units enzyme/ml rt-PA. After incubation samples were stored on ice before analysis.

## 3. Results and discussion

### 3.1. Development of cIEF

Development of the cIEF method for rt-PA consisted of an adaptation of the cIEF methods by Mazzeo et al. [4] and Pritchett [5] with consideration of known rt-PA slab-gel IEF requirements. rt-PA is a very hydrophobic protein and the loss of salt during either dilution or IEF causes extensive precipitation. For this reason IEF in both slab gels and capillaries must contain urea. For slab gels a final concentration of 8 *M* urea was used whereas for cIEF only 4 *M* was required. The urea concentration was kept to a minimum to avoid urea crystallization in the sample, or capillary during analysis. Original separations also included reduced Triton X-100 to aid solubilization, but this was found to be unnecessary. Later separations included HPMC to suppress any changes in EOF and aid the reproducibility of multiple injections using a single capillary. The choice of ampholytes (Pharmacia pharmalytes) was made to compare directly with the slab-gel method as a means of conserving the slab-gel IEF profile.

The effect of different ratios of ampholytes is shown in Fig. 1. The ratio giving optimal resolution and separation was determined to be a 50:50 mix of pH 5–8 and pH 3–10 ampholytes. This is close to the 65:35 ratio for these ampholytes used in the slab-gel IEF and is consistent with the known p*I* range of rt-PA measured in urea containing IEF gels as pH 6.2–7.9. A typical electropherogram is shown in Fig. 2.

Other factors investigated included the concentration of TEMED and the concentration of protein. TEMED acts as a basic blocker and upon focusing occupies the end of the capillary between the detector and the inlet. We found that altering the TEMED concentration in the range 3.75–7.5% (v/v) showed very little effect on either resolution or overall elution time. Use

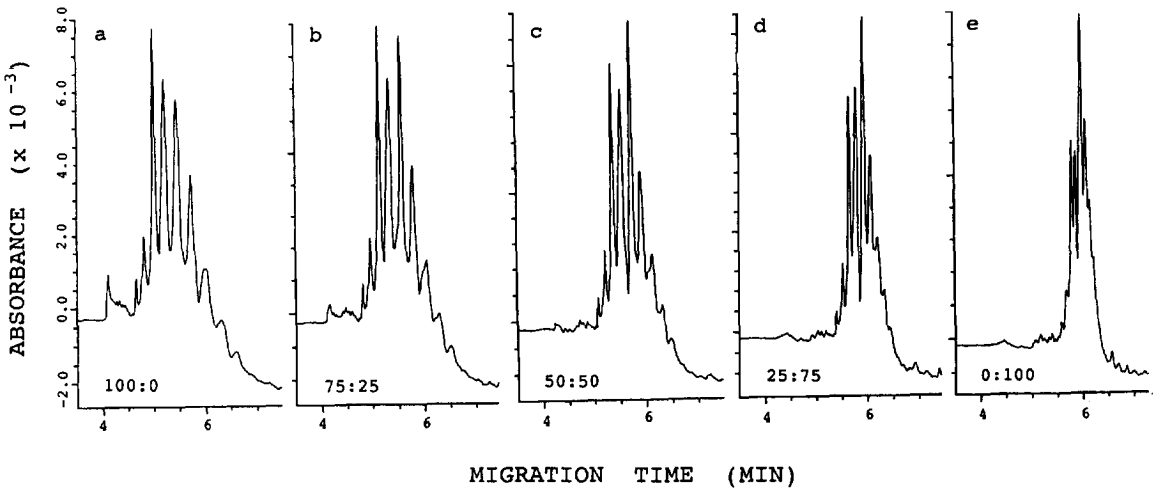


Fig. 1. Effect of changing the ratio of the ampholytes used from 100% pH 5–8 to 100% pH 3–10. The use of increasing amounts of pH 5–8 ampholytes, up to 50%, resulted in increasing resolution and separation of peaks. Values above 50% resulted in a loss of peak shape on the acidic side of the electropherogram (a–c). The use of increasing amounts of pH 3–10 ampholytes (c–e) resulted in decreasing resolution of the rt-PA peaks as the bands became compressed.

of concentrations in excess of 25% (v/v) lead to an overall loss of resolution and distortion of the pH gradient as evidenced by extremes in baseline response. Protein concentrations between 5 and 500 $\mu$g/ml were evaluated. Higher protein concentrations resulted in higher signals but also in loss of resolution. Lower concentrations gave the best resolution but suffered

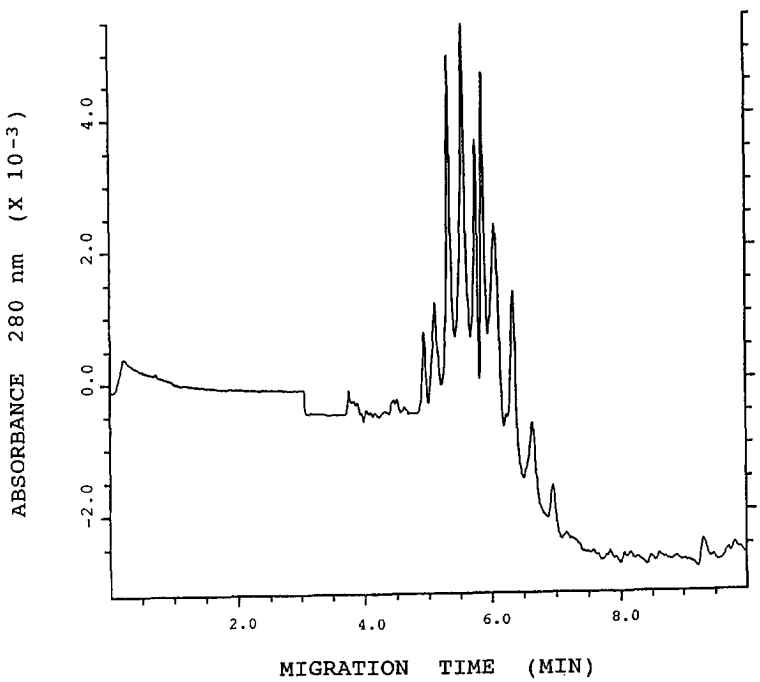


Fig. 2. A typical cIEF electropherogram of rt-PA showing the resolution and separation of ten peaks of increasing acidity. Analysis conditions were as described in Experimental.

from baseline noise, probably arising from residual absorbance by some of the ampholytes at 280 nm. Considering resolution and signal-to-noise ratio, the optimal protein concentration was determined to be in the range 125–250 μg/ml..

Using the optimal conditions intra-assay precision was calculated for six sequential injections of a single sample of rt-PA. The relative standard deviations (R.S.D.s) for the peak migration times and normalized peak areas of each of the ten peaks resolved are reported in Table 1. For migration time the R.S.D.s ranged from 2.3 to 3.1%, for area the R.S.D.s ranged from 0.6 to 10.4%, values within acceptable limits for a quality control assay.

A number of other coated capillaries were investigated for their resolving capabilities using this method, the results are shown in Fig 3. All of the capillaries, with the exception of the μSIL DB-WAX, gave equivalent or better resolution compared to the neutral coated capillary routinely used, indicating that the separation is very rugged. The success with so many alternate coatings could be credited to the presence of urea and HPMC in the separation buffer. The presence of urea seems to eliminate any solubility problems the protein may have, in particular at its isoelectric point that would lead to loss of resolution. The poorer resolution seen with the μSIL DB-WAX could be attributed to the coating which, even in the presence of 4 *M* urea, is able to exert some effect on the rt-PA molecule. The presence of HPMC depresses the inherent EOF which would otherwise cause loss of resolution, allowing the use of coatings which have greater EOF values than the neutral capillary.

Table 1
Intra-assay precision for six replicate injections of rt-PA using CE-IEF

| Peak | R.S.D. | |
|---|---|---|
| | Migration time (min) | Normalized area[a] |
| 1 | 2.3 | 10.4 |
| 2 | 2.4 | 8.3 |
| 3 | 2.5 | 2.3 |
| 4 | 2.6 | 1.3 |
| 5 | 2.7 | 1.6 |
| 6 | 2.9 | 1.6 |
| 7 | 2.8 | 0.6 |
| 8 | 2.9 | 2.6 |
| 9 | 3.1 | 3.3 |
| 10 | 3.1 | 2.5 |

[a] Normalized peak areas were calculated as: (individual peak area/total peak area) · 100%.

### 3.2. Comparison of cIEF and slab-gel IEF methods

To validate the replacement of slab-gel IEF by cIEF a comparison of the data from the two methods was made using three sets of samples: intact rt-PA representing the most heterogeneous sample, purified type I and type II rt-PA representing the two major variants of rt-PA, and neuraminidase-digested rt-PA representing samples with a range of carbohydrate concentrations.

A typical cIEF electropherogram of intact rt-PA is shown in Fig. 2, while Fig. 4 shows the same sample analyzed by slab-gel IEF. Inspection of the gel shows that approximately ten major bands can be detected while the same number of peaks are resolved by cIEF. This data indicates that comparable resolution and separation was achieved by the two methods.

cIEF electropherograms of purified rt-PA types I and II are shown in Fig. 5, while Fig. 6 shows the same samples analyzed by slab-gel IEF. Glycosylation at the additional N-linked glycosylation site which occurs in type I rt-PA but not in type II rt-PA results in higher concentration of sialic acid for this variant. This difference can be seen by cIEF as a greater number of peaks of increasing acidity when compared to the number and acidity of peaks for type II rt-PA. Overall the number and relative p*I* of peaks detected by cIEF for the two samples was comparable with the number and relative p*I* of bands detected by slab-gel IEF.

When considering appropriate methodologies for the quality control analysis of charged glycoproteins one of the most important properties of

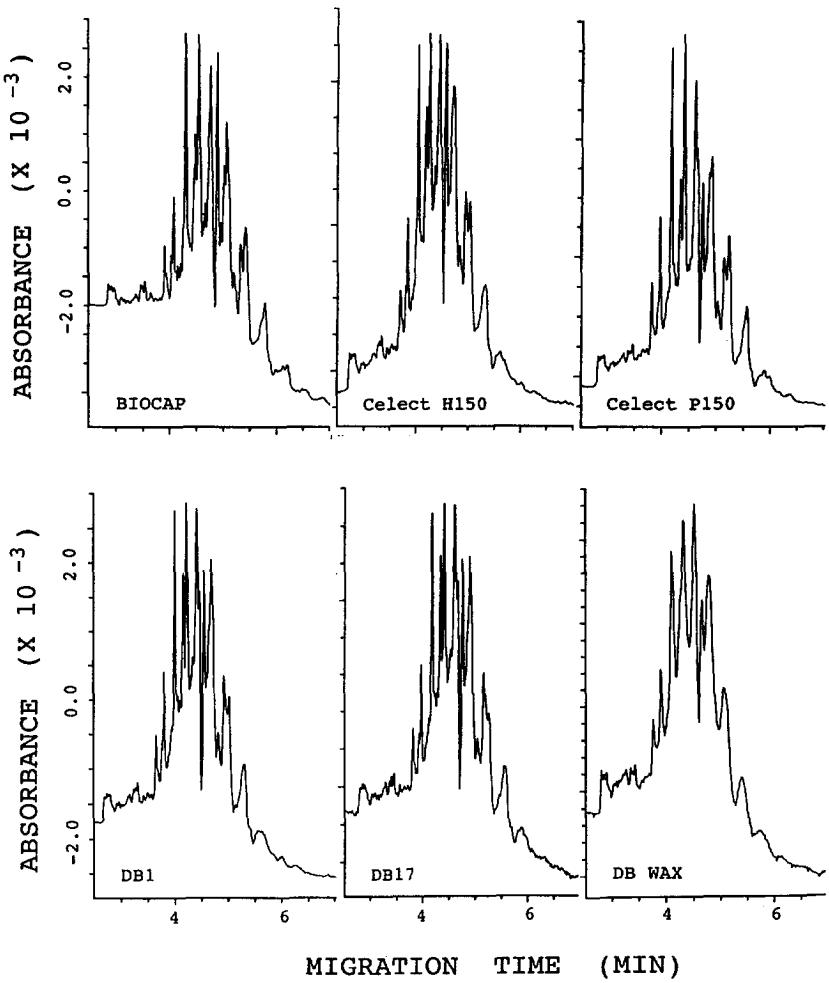


Fig. 3. The separation of rt-PA charged glycoforms was routinely performed using a neutral capillary (eCAP™). This figure shows that a number of other capillaries could also be used including: two neutral capillaries, the CElect-P150 with a hydrophillic phase and the BioCap, capillaries with a moderately hydrophobic phase, the CElect-H150 and the μSIL® DB™-17, and a capillary with a very hydrophobic phase the μSIL® DB™-1. Only a capillary with a high polarity, the μSIL® DB™-WAX gave an inadequate separation which may have resulted from the interaction of rt-PA with the polyethylene glycol phase used in this column.

the method is its demonstrated ability to differentiate samples which are known to be heterogeneous with respect to their charged carbohydrate content. To further demonstrate this for cIEF, samples greatly differing in their charged carbohydrate content were generated by the increasing removal of sialic acid residues by increasing concentrations of neuraminidase. A series of electropherograms of rt-PA digested with increasing concentrations of the enzyme is shown in Fig. 7, while Fig. 8 shows the same samples analyzed by slab-gel IEF. As sialic acid residues are increasingly removed from the rt-PA molecule there is a concomitant decrease in the number and acidity of the peaks detected by cIEF and the number and acidity of bands detected by slab-gel IEF. The two major bands remaining after incubation with the highest concentration of neuraminidase did not appear to be related to sialic acid as incubation for extended

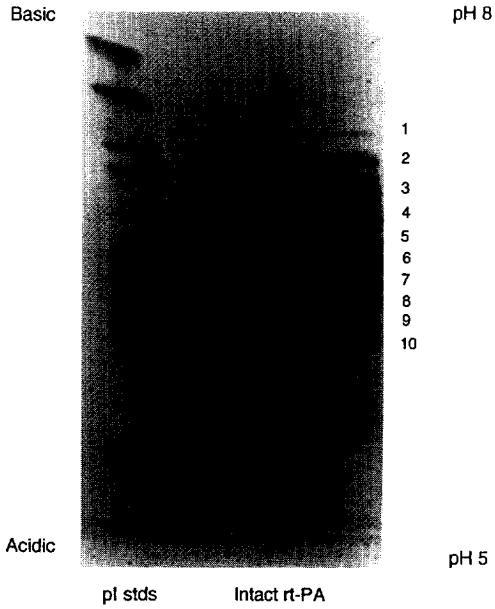


Fig. 4. An IEF slab gel of the same sample of rt-PA as analyzed in Fig. 2 showing the separation and resolution of ten major bands of increasing acidity. Analysis conditions were as described in Experimental.

periods of time at the highest concentration of enzyme used did not decrease their number (data not shown). Inspection of the data for the two methods indicated an excellent correlation with the same number of peaks or bands being detected by each method at every concentration of enzyme used.

## 4. Conclusion

Recent advances in CE methodology have allowed us to develop a fast and rugged cIEF method for the analysis of the charged carbohydrate heterogeneity exhibited by a recombinant glycoprotein, rt-PA. The ease of use, automation and short analysis time of this cIEF method offers great advantages over the current slab-gel IEF methodology. In addition to these advantages cIEF also offers a great reduction in the volume of reagents used, in particular the elimination of the use of the neurotoxin acrylamide

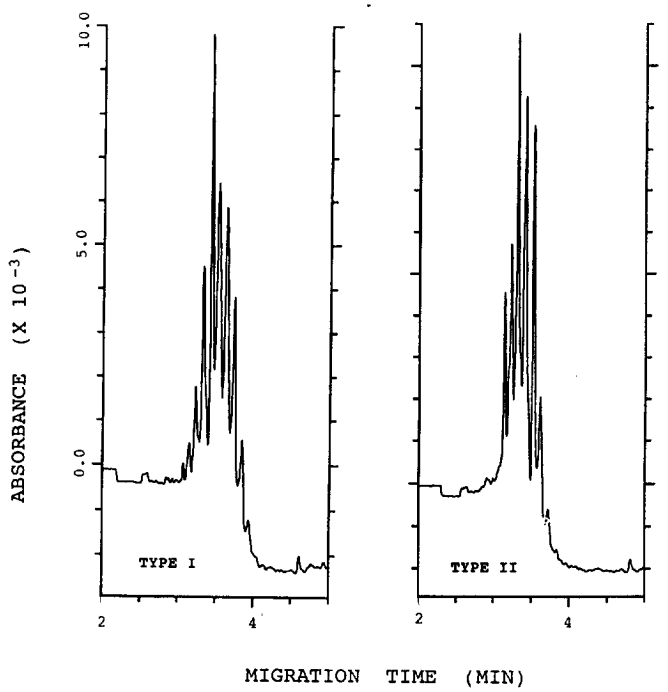


Fig. 5. cIEF electropherograms of the two major glycosylation variants of rt-PA, type I and type II. Type I rt-PA has sialic acid containing carbohydrate at two positions while type II has only one. The presence of additional sialic acid residues in rt-PA type I can be seen by cIEF as a greater number of peaks with increasing acidity compared to the number and acidity of the peaks for the rt-PA type II sample. Analysis conditions were as described in Experimental.

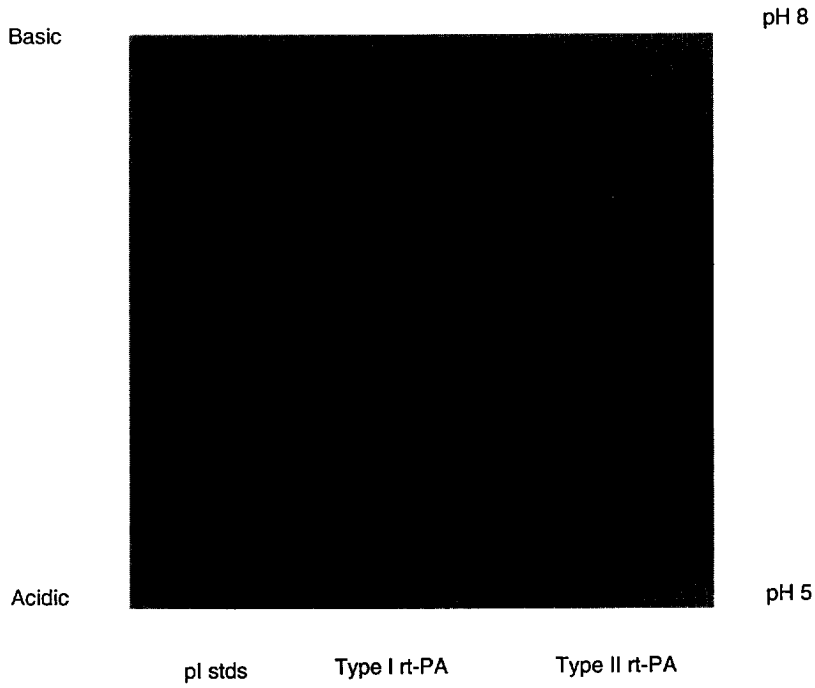


Fig. 6. An IEF slab gel of the same samples of rt-PA (in duplicate) as analyzed in Fig. 5. Type I rt-PA has sialic acid containing carbohydrate at two positions while type II only has it at one. The presence of additional sialic acid residues in rt-PA type I can be seen as a greater number of bands with increasing acidity compared to the number and acidity of bands for the rt-PA type II sample. Analysis conditions were as described in Experimental.

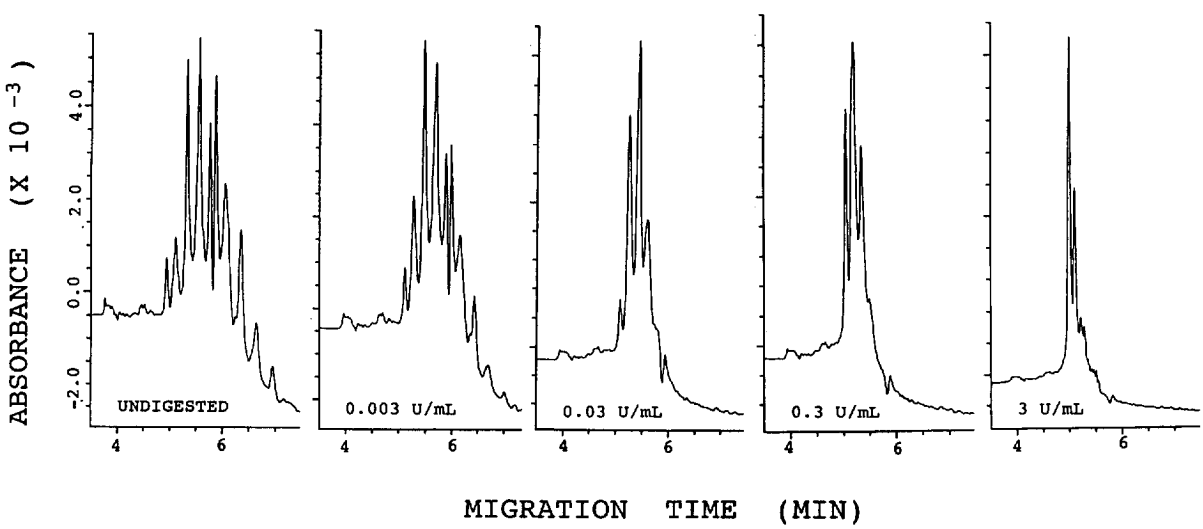


Fig. 7. cIEF electropherograms of rt-PA digested with increasing concentrations of neuraminidase. As sialic acid residues are sequentially removed there is a concomitant decrease in the number and acidity of peaks detected by cIEF. Enzyme incubation conditions and cIEF analysis conditions were as described in Experimental.

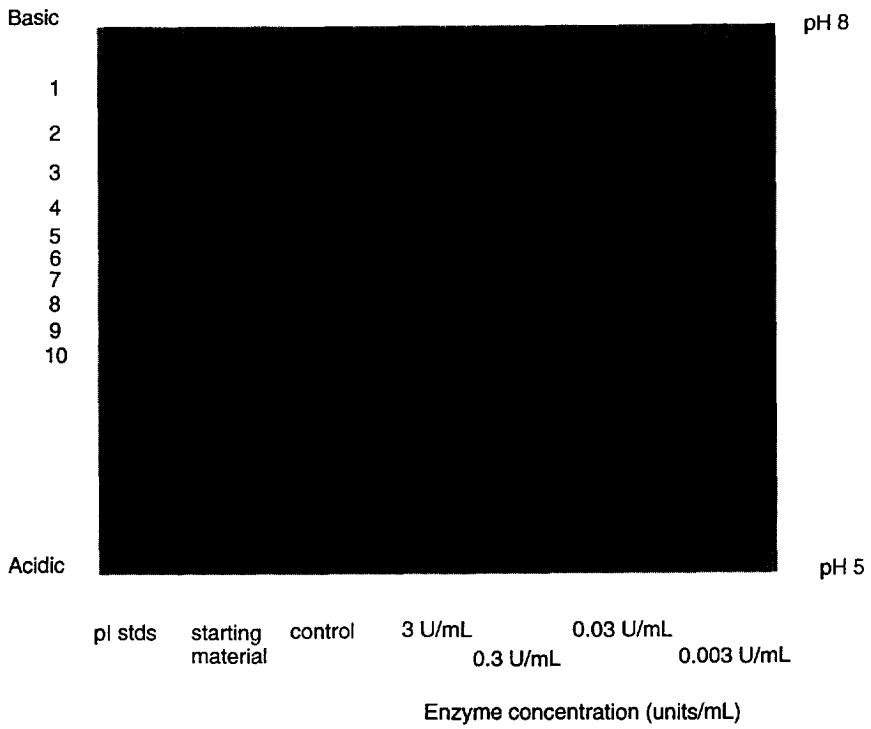


Fig. 8. IEF slab gel of the same samples of rt-PA as analyzed in Fig. 7. As sialic acid residues are sequentially removed there is a concomitant decrease in the number and acidity of bands detected by IEF. Enzyme incubation conditions and IEF analysis conditions were as described in Experimental.

and the large volumes of noxious stain/destain solutions required for slab-gel IEF methods. Most importantly the excellent correlation of cIEF to the slab-gel IEF, as demonstrated in this paper, provides a strong argument for its adoption for the routine quality control analysis of the consistency of recombinant glycoproteins.

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