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Fractionation of the human recombinant tissue plasminogen activator (rtPA) glycoforms by high-performance capillary zone electrophoresis and capillary isoelectric focusing

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ABSTRACT

This paper reports the fractionation of recombinant human tissue plasminogen activator (rtPA) glycoforms, a complex mixture to demonstrate the high resolving power of capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF). rtPA is a glycoprotein with a complex carbohydrate structure. The electropherograms and IEF patterns have been discussed in light of the known carbohydrate structures of rtPA. rtPA was treated with neuraminidase which removes the sialic acids from the carbohydrate chains. The desialylated rtPA was analyzed by both CZE and IEF and the results were compared to those of untreated rtPA. The usefulness of CZE and cIEF in the characterization of glycoproteins proteins is also discussed.

INTRODUCTION

Zone electrophoresis and isoelectric focusing in the slab-gel format are well established high resolution separation techniques for proteins. Capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) are their counterparts in the capillary format. Giddings [1] has shown that the upper limit of N (number of theoretical plates) for electrophoretic separations is proportional to the applied voltage. So in theory, higher applied voltages result in narrower peaks. The highly efficient heat transfer from small diameter capillaries allows the application of high voltages and results in high resolution and short analysis times [2]. The purpose of this paper is to evaluate the resolving power of cIEF and CZE, using the glycoforms of rtPA as a test mixture.

Glycoforms are glycoproteins sharing an identical polypeptide but differing with respect to the structure, location and incidence of individual oligosacccharides. It is becoming increasingly obvious that protein glycosylation patterns may be important – if not essential – to their function or therapeutic efficacy. The oligosaccharides can influence a protein's clearance rate, its specific ativity and its immunogenicity [3].

Recombinant human tissue plasminogen activator (rtPA) is a fibrin-specific plasminogen activator which has been approved for the treatment of myocardial

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infarction. It has a polypeptide molecular mass of about 60 000 and consists of 527 amino acids. The carbohydrate structures of Chinese hamster ovary (CHO)-derived rtPA have been elucidated [4]. The amino acid sequence of rtPA has four potential N-glycosylation sites, as predicted by the consensus sequence Asn–X–Ser/Thr [5]; these residues are: 117, 184, 218 and 448. rtPA is not glycosylated at Asn-218 (X is a proline residue). rtPA exists as two glycosylation variants designated Type I and II. Type I rtPA is glycosylated at asparagine residues 117, 184 and 448 whereas Type II rtPA is glycosylated only at asparagine resides 117 and 448. The nature of the carbohydrate as Asn-117 was found to be high mannose oligosaccharide. At ASN-448 the carbohydrates comprise biantennary, 2,4-branched triantennary, 2,6-branched triatennary and tetraantennary N-acetyllactosamine oligosaccharides. In Type I rtPA, the carbohydrates as Asn-184 are of the same complex types of oligosaccharide as at Asn-448. All of the hybrid and N-acetyllactososamine-type oligosaccharides can contain sialic acid attached to galactose. This paper reports the results of the application of capillary electrophoretic methods to the resolution of a complex mixture of similar forms of a protein. Both free zone and isoelectric focusing methods have been succesfully applied using columns whose inner surface have been covalently coated with polymer.

EXPERIMENTAL

All capillary electrophoresis was performed using the HPE 100 high-performance capillary electrophoresis system from Bio-Rad Labs. (Hercules, CA, USA). All the capillaries were purchased from Bio-Rad Labs. The capillaries were enclosed in microsampler cartridges. The capillaries used in the CZE and IEF experiments were 20 cm and 14 cm long respectively. The internal surfaces of the capillaries were coated with a covalently bonded linear polymer and the internal diameters of the capillaries were 25 μm. Detection was performed by UV at 200 nm in the CZE mode and at 280 nm in the IEF mode.

Sample preparation

rtPA (approximately 5 mg/ml) was obtained by adding a suitable amount of water for injection (WFI) into a vail of lyophilized Activase rtPA. Type I and Type II rt-PA were obtained by partially separating rtPA into two fractions on a lysine-Sepharose column using an arginine gradient [6]. Type I was collected from the leading edge of the first peak and Type II was collected from the trailing of the second peak. They were concentrated by Centriprep and then dialysed into 10 m*M* sodium phosphate buffer, pH 2.5. The final sample concentrations were 2.85 mg/ml and 3.05 mg/ml, for Type I and Type II rtPA respectively. The Type I/Type II mixture sample was prepared by mixing equal volumes of the above samples. The dialysed samples were then diluted to obtain an approximate concentration of 0.5 mg/ml rtPA in 2% ampholyte (pH 6–8) containing 2% 3-[(3-cholamidopropyl)dimethylammonio] 1-propanesulfonate (CHAPS) and 0.36 mg/ml of urea. The IEF calibration mixture was obtained from Pharmacia LKB (Piscataway, NJ, USA) and reconstituted with an ampholyte solution (pH 3–10) containing N,N,N′,N′-tetramethylethylenediamine (TEMED) as a spacer. The final concentrations of the ampholyte and TEMED were 1 and 0.4%, respectively.

IEF conditions

The capillary was first washed with 100 μl of 10 m*M* phosphoric acid and then rinsed with 100 μl of deionized water. The sample (10–20 μl) was introduced into the capillary. The purge block was first washed with 1 ml of deionized water and then with 1 ml of catholyte (20 m*M* sodium hydroxide). A microcentrifuge tube (the anode reservoir) was filled with the anolyte (10 m*M* phosphoric acid) and inserted into the microsampler housing. The focusing voltage and time were 12 kV and 2 min. At the end of the focusing step, the purge block was purged with 1 ml of the mobilizer (10 m*M* sodium hydroxide and 80 m*M* sodium chloride). The mobilizing voltage was set at 8 kV.

CZE conditions

The capillary was washed with 100 μl of 10 m*M* phosphoric acid and then rinsed with 100 μl of the running buffer. The purge block reservoir was then flushed with 1 ml of buffer. Volumes of 10–50 μl of sample was added to a microcentrifuge tube and placed into the microsampler housing and the loading cycle started. The loading voltage was 8 kV and loading time was 8 s. The sample was then replaced by buffer and the run started. The run voltage was set at 6 kV. The rtPA sample was the same as described above in the IEF section. The running buffer consisted of 0.1 *M* ammonium phosphate pH 4.6 with 0.01% Triton X-100 (chemically reduced to minimize UV absorbance) and 0.2 *M* ε-aminocaproic acid (EACA).

RESULTS AND DISCUSSION

Since the total volume of a 14 cm × 25 μm capillary is only about 60 nl, the difficulties associated with collection and characterization of proteins separated by capillary electrophoretic methods lead to the need for calibration of the system by markers of known properties. Fig. 1 is the cIEF of a mixture of some p*I* markers to illustrate the features of a typical IEF pattern. cIEF is a three-step process. In the first

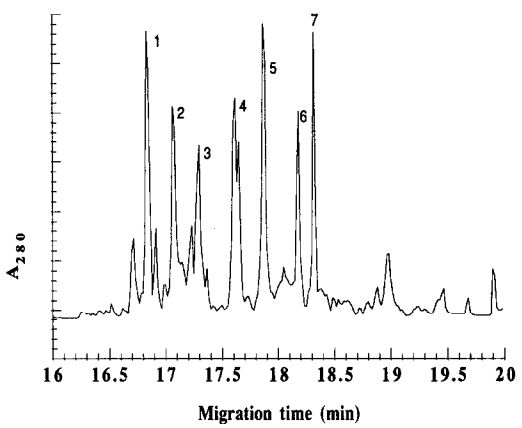

Fig. 1. IEF pattern of a mixture of p*I* markers in 1% (pH 3–10) ampholyte solution containing 0.4% TEMED. Peaks: 1 = lectin (8.6); 2 = lectin (8.4); 3 = lectin (8.2); 4 = horse myoglobin (7.3); 5 = horse myoglobin (6.8); 6 = human carbonic anhydrase (6.6); 7 = bovine carbonic anhydrase (5.8).

step, the sample is mixed with an ampholyte solution and introduced into a capillary by pressure loading. The second step is the focusing step. After the high voltage is applied, sample components are focused in a pH gradient formed by the carrier ampholytes according to their isoelectric points. The third step is the mobilization step where the separated zones are mobilized cathodically [7,8], by adding sodium chloride to the catholyte thus eluting to the focused species electrophoretically. As a result, focused bands with shorter migration times have higher p*I* and *vice versa*.

Fig. 2A and B are the cIEF patterns of Type I and Type II rtPA, respectively. It is observed that, despite the fact that rtPA is purified to homogeneity (with respect to the polypeptide chain), these samples can be resolved in a narrow pH range (6–8) into as many as 20 peaks. This microheterogeneity is expected on the basis of the known carbohydrate structures present in rtPA [4], illustrated in Figs. 3 and 4. If there is only one glycosylation site, the number of glycoforms is simply the number of different oligosaccharide structures at that site (site heterogeneity). Thus, a given subset of Type II rtPA glycoforms may have a biantennary oligosaccharides at Asn-448 with no sialic acid residue, while another subset may have a tetraantennary oligosaccharides with 4 sialic acids residues etc.. However, the number of glycoforms increases dramatically with the number of glycosylation sites due to the number of permuta-

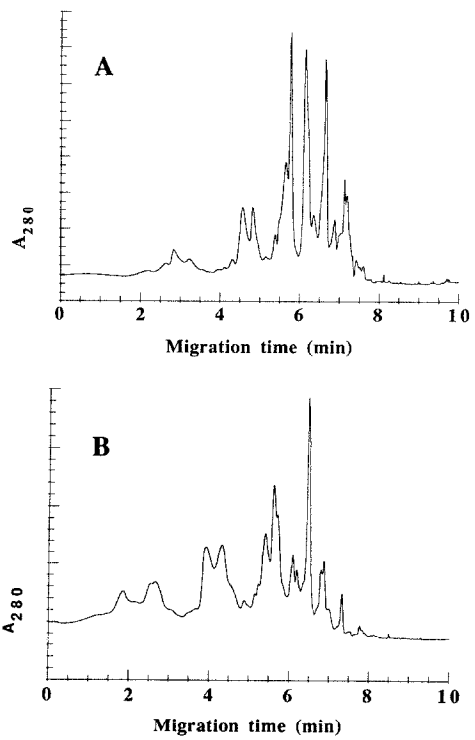

Fig. 2. IEF patters of (A) Type I rtAP and (B) Type II rtPA obtained at 280 nm using a Bio-Rad coated silica capillary (14 cm × 25 μm). The focusing voltage was 12 kV and mobilization voltage was 8 kV. The ampholyte solution contained 2% ampholyte (pH 6–8), 2% CHAPS and 6 *M* urea.

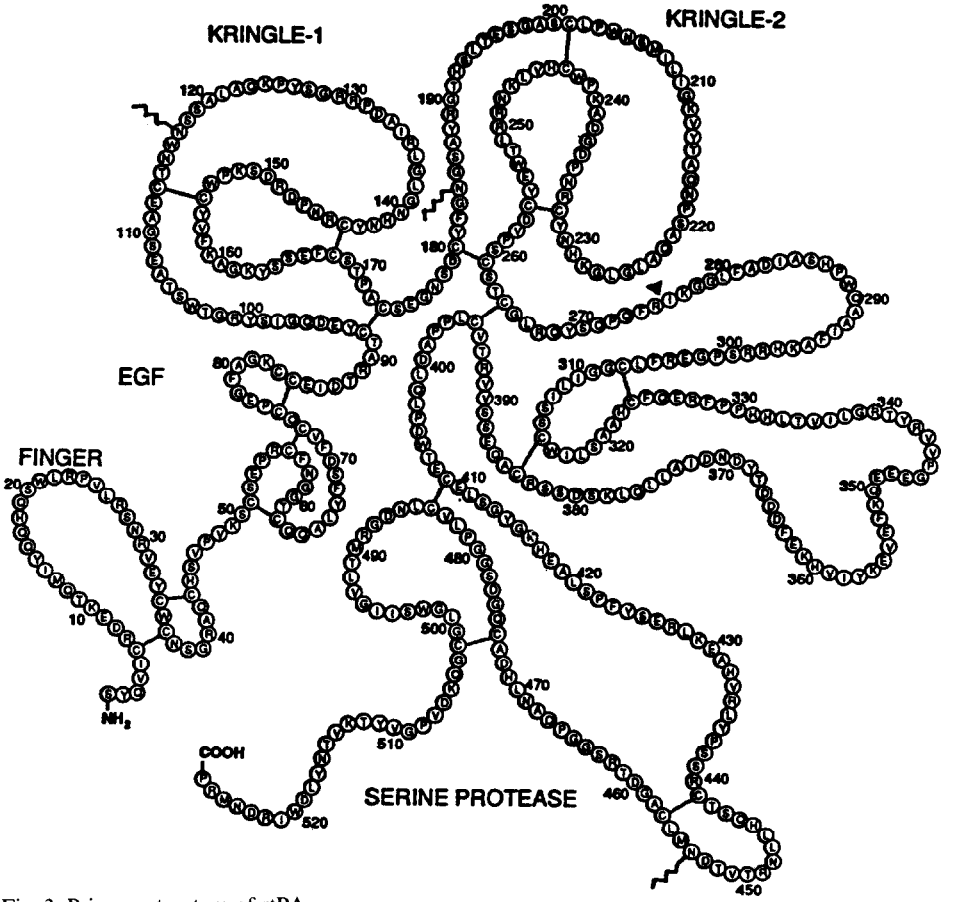

Fig. 3. Primary structure of rtPA.

tions of heterogeneity at each site. Each peak in the IEF pattern may represent a combination of glycoforms having the same p*I*, since there are probably more glycoforms than peaks. Type I and Type II rtPA are expected to contain different subsets of glycoforms; as a result, their IEF patterns are different, as observed. That cIEF can discriminate such subtle differences between two complex and yet similar mixtures (Type I and Type II rtPA) suggests that this technique could be very useful in studying subtle changes in protein molecules (*e.g.* deamidation, conformational changes etc.).

One might expect the IEF patterns to be additive, (*i.e.* the arithmetic sum of the IEF patterns of Type I and Type II rtPA should yield the IEF pattern of the mixture), if the run-to-run migration time reproducibility is good. However, this additivity is not observed. If one could scan the IEF pattern in the capillary without having to mobilize the focused bands, this additivity could very well have been observed. But since the current methodology requires a mobilization step to move the focused bands through the detector, an additional variable is introduced. The migration time of a

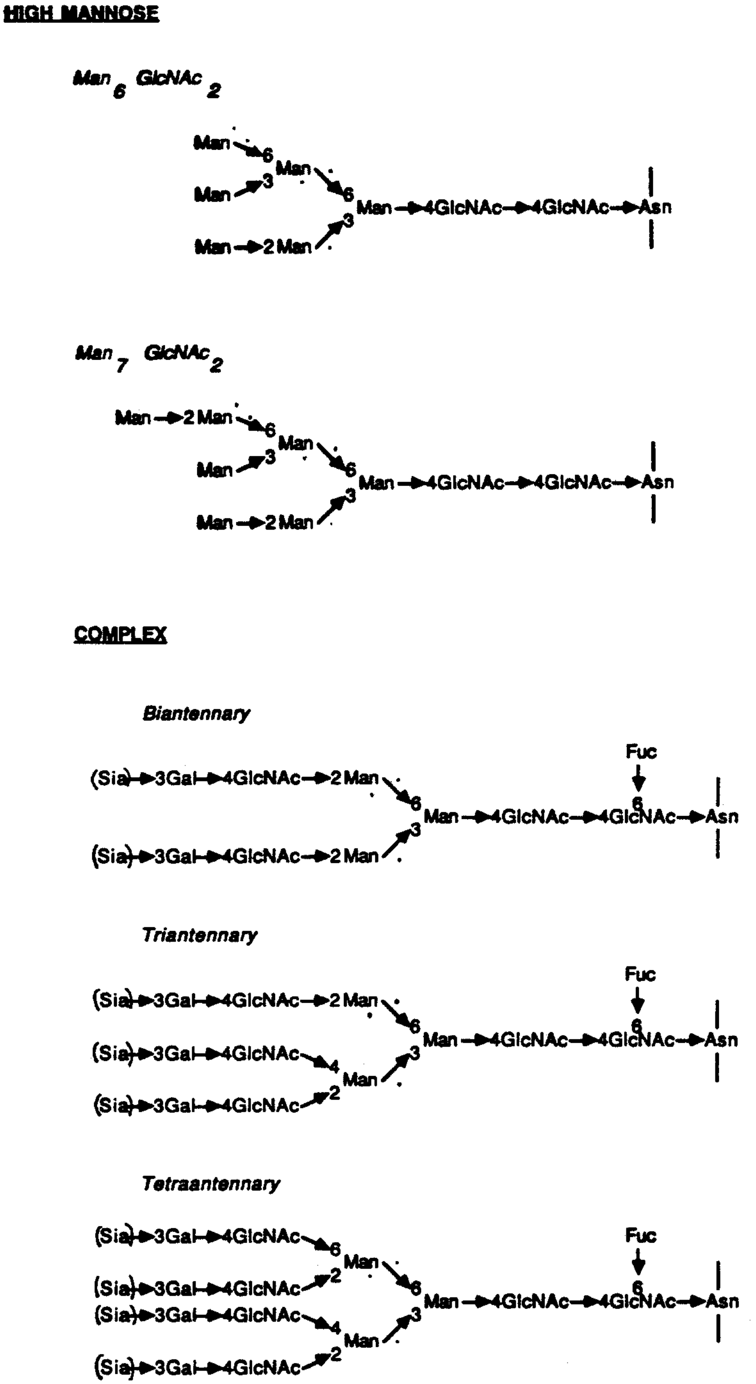

Fig. 4. Schematic structures of some of the Asn-linked oligosaccharides present in rtPA (see ref. 2 for details).

focused protein band appears to depend not only on the p*I* but also on the nature of the protein, its concentration and the amount of time it has been focused and possibly the viscosity of the band and the mobilization conditions, all of which affect the mobilization step. In short the absolute migration time of a focused band is quite variable. This variability in migration time makes correlation of p*I* *vs.* migration time very difficult to assess. However, the reproducibility of the IEF pattern of rtPA between runs was quite good, except that the migration times were shifted. So if one could correct for this variability (*e.g.* by use of a internal standard p*I* marker), then the "corrected" IEF pattern would be much more reproducible. The ideal situation would be to bracket the analyte sample between two internal standard p*I* markers, not unlike the use of p*I* markers in conventional IEF gels. This approach is being investigated currently.

When one overlays the IEF patterns of Type I and Type II rtPA over that of the mixture, one can see that the patterns overlap quite well but only after some shifting in migration times. That is, if the IEF pattern of Type II rtPA was shifted −0.86 min and added to the IEF pattern of Type I rtPA, the resultant pattern compares favorably with the IEF pattern of a mixture of Type I and Type II rtPA after it shifted 3.3 min. Fig. 5 shows the overlay of the time-shifted IEF patterns. While this is only a simulation exercise, it suggests that cIEF patterns may be compared from run to run if the time shift problem can be corrected for by the use of internal standards.

Fig. 6 is the IEF of a neuraminidase treated rtPA sample. The microheterogeneity is considerably reduced and the resulting peaks have migration times which are shorter. This observation is consistent with the fact that when all the sialic acid residues are removed (data not shown), the p*I* of the desialylated rtPA is increased. The simplification of the pattern indicates that the large cause of the microheterogeneity in the p*I*s of the glycoforms of untreated rtPA are due to different levels of sialylation.

Fig. 7A and B are the capillary zone electropherograms of Type I and Type II rtPA, respectively. It can been seen that Type II rtPA dominates the front part of the

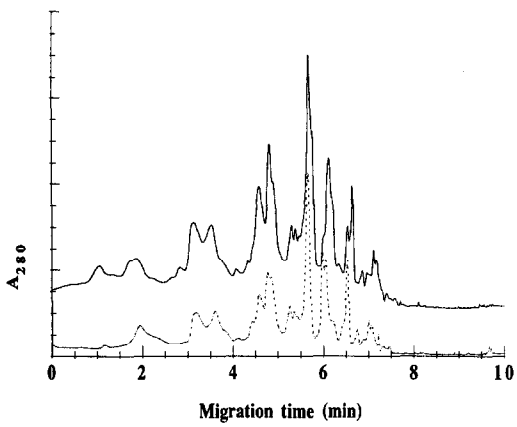

Fig. 5. Comparison of the IEF of a mixture of Type I and Type II rtPA (- - -) with the arithmetic sum (——) of the IEF patterns of Type I and Type III rtPA.

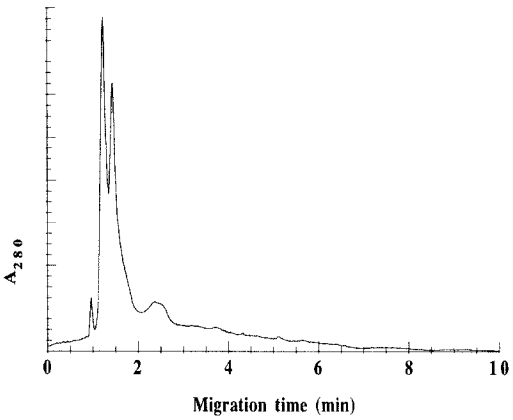

Fig. 6. IEF of a neuraminidase-treated rtPA sample. A Bio-Rad coated silica capillary (14 cm × 25 μm) was used, the focusing voltage was 12 kV and mobilization voltage was 8 kV, the ampholyte solution contained 2% ampholyte (pH 6–8), 2% CHAPS and 6 *M* urea.

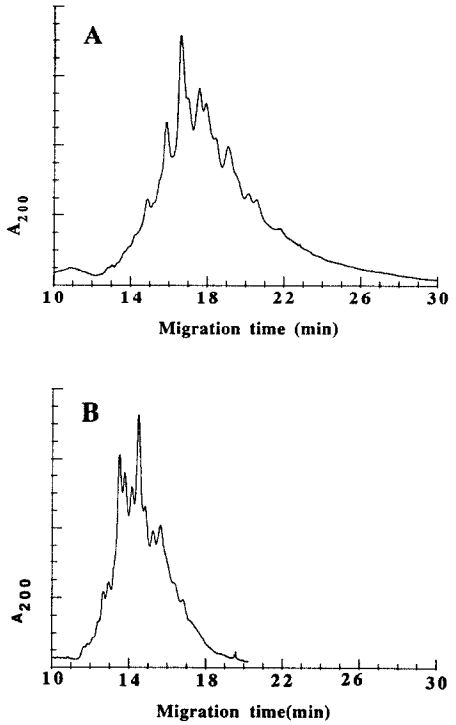

Fig. 7. CZE of (A) Type I rtPA and (B) Type II rtPA obtained at 200 nm. Samples were introduced electrophoretically at 8 kV for 8 s. The run voltage was set at 6 kV. The rtPA sample was the same as described above in the IEF section. The running buffer consisted of 0.1 *M* ammonium phophate pH 4.6 with 0.01% Triton X-100 (chemically reduced to minimize UV absorbance) and 0.2 *M* EACA.

rtPA profile and Type I dominates the back part of the rtPA profile. The resolution of the glycoforms was not was good as in the IEF mode, but still about 15 peaks could be recognized. It should be noted that the separation was done on a very short capillary (20 cm); a longer capillary (*e.g.* 50 cm) should give better separations. A publication [9] reported that an earlier attempt to separate the glycoforms was not successful. It was observed that rtPA solubility is too low in many common buffers and hence successful CZE could not be accomplished in them. The addition of 0.2 *M* EACA solubilizes rtPA and led to the success of the CZE experiment. It appears the choice of buffer components is crucial to the success of CZE of proteins.

Fig. 8 is the overlay of the CZE of rtPA sample with that of a desialylated rtPA sample. The CZE of the desialylated rtPA sample is much simpler than that of rtPA and the migration time is also much shortened. This is the expected result: the desialylated rtPA becomes more positively charged and hence has higher mobility. The loss of the sialic acid residues again reduces the charge heterogeneity and results in the simpler profile. The removal of sialic acid only reduces that charge heterogeneity but does not alter the other causes of carbohydrate heterogeneity. The appearance of two to three peaks in the desialylated rtPA coud possibly be attributed to the heterogeneity of molecular mass of these glycoforms resulting in differences in mobility. Fig. 9 is an overlay of the CZE of a mixture of rtPA and desialylated rtPA with the arithmetic sum of the CZE patterns of the two run separately. The fact that the two profiles are very similar indicates that CZE of proteins can be performed more reproducibly and data analysis using CZE is consequently much more straightforward.

CONCLUSIONS

This paper reports the successful fractionation of rtPA by capillary zone electrophoresis and cIEF. The results confirm the existence of different glycoforms and are consistent with the known carbohydrate structures of rtPA. A key to the success-

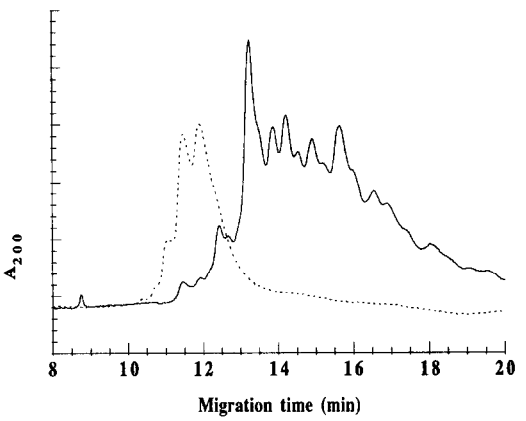

Fig. 8. Overlay of CZE patterns of rtPA (——) and desialylated rtPA (- - -) obtained at 200 nm. Samples were introduced electrophoretically at 8 kV for 8 sec. The run voltage was set at 6 kV. The rtPA samples were the same as described above in the IEF section. The running buffer consisted of 0.1 *M* ammonium phosphate pH 4.6 with 0.01% Triton X-100 (chemically reduced to minimize UV absorbance) and 0.2 *M* EACA.

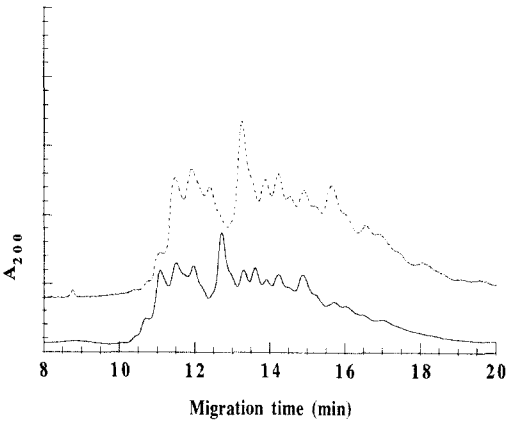

Fig. 9. Comparision of the CZE of a mixture of rtPA and desialyated rtPA (——) with the arithmetic sum of the CZE patterns of rtPA and desialylated rtPA (- - -).

ful separation of these glycoforms was to increase the solubility properties of rtPA in order to obtain high enough sample concentration for the electrophoretic sample loading method. rtPA solubility is too low in many common buffers and hence successful CZE could not be accomplished in them. The addition of 0.2 *M* EACA solubilizes rtPA and led to the success of the CZE experiment. After the optimum buffer was found, both techniques worked well as expected. In summary, we have investigated the glycoforms of rtPA using capillary zone electrophoresis and capillary IEF. The information obained provides a rapid method for evaluating the complexity of rtPA and at the same time validates the usefulness of these two techniques in protein characterization.

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