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Comparison of ampholytes used for slab gel and capillary isoelectric focusing of recombinant tissue-type plasminogen activator glycoforms.

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Abstract

Four commercial ampholytes: Ampholine and Pharmalyte (Pharmacia Biotech), Bio-Lyte (Bio-Rad) and Servalyt (Serva) were evaluated for their ability to resolve recombinant tissue-type plasminogen activator (rt-PA) glycoforms by isoelectric focusing (IEF) and capillary IEF (cIEF). Each brand of ampholytes focused rt-PA into 3–4 major and 5–6 minor bands on slab gel electrophoresis. Visually, focused bands stained with Coomassie Blue appeared to be similarly resolved by all the ampholytes except for Ampholines, where the bands were closely grouped and more intensely stained. When cIEF was performed, Pharmalytes and Ampholines resolved rt-PA glycoforms consistent with the slab gels. No discernible peaks were detected during cIEF of rt-PA using Servalyts or Bio-Lytes. UV spectrophotometric scans of the components used for cIEF showed that Servalyts absorbed intensely over a range which overlapped the detector bandpass. Bio-Lytes showed absorption over a narrower UV range but still overlapped the detector bandpass, thus preventing the discernment of protein peaks. For this cIEF system the best ampholytes were Ampholines and Pharmalytes.

Keywords: Isoelectric focusing; Ampholytes; Gels; Capillary columns; Tissue plasminogen activator; Proteins; Glycoproteins

1. Introduction

Traditional methods for the analysis of charged variants of proteins and glycoproteins have been by the techniques of ion-exchange chromatography and isoelectric focusing (IEF) [1]. Recently a rapid capillary IEF (cIEF) method (<10 min) for recombinant tissue-type plasminogen activator (rt-PA) glycoforms was developed [2], based on a one-step approach [3,4]. In one-step cIEF methods, focusing and mobilization occur simultaneously. 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. Using this method the charge heterogeneity exhibited by rt-PA could be detected as a series of approximately 8–10 peaks. A comparison of the cIEF electropherograms of intact rt-PA, Types I and II, and neuraminidase-digested rt-PA with IEF slab gels of the same samples indicated a good correlation between the two methods based on the number of species detected in each case [2]. Attempts were made to validate this method for routine use in a quality control environment [5] and as part of the

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validation, ampholytes from various manufacturers were tested. Although IEF could be performed using all four ampholyte brands, cIEF of rt-PA glycoforms could only be determined using two brands. Studies were performed to explain the differences observed.

2. Materials and methods

All chemicals were of analytical reagent grade. Hydroxypropylmethyl cellulose (HMPC) was obtained from Sigma (St. Louis, MO, USA). Pharmalytes and Ampholines, ranges pH 3–10 and pH 5–8, were obtained from Pharmacia Biotech (Piscataway, NJ, USA). N,N,N′,N′-tetramethylethylenediamine (TEMED), urea (electrophoresis grade) and Bio-Lytes were obtained from Bio-Rad (Hercules, CA, USA). Servalyts were obtained from Serva (Crescent Chemical Company, Hauppauge, NY, USA). Urea was deionized using a mixed bed resin prior to use. rt-PA was manufactured in-house.

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 mass 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 stained with Coomassie Brilliant Blue. Densitometric scans were performed using IS 1000 Digital Imaging System (Alpha Innotech Corp, San Leandro, CA, USA).

cIEF was performed using a Beckman P/ACE 5510 (Fullerton, CA, USA) or a Bio-Rad BioFocus

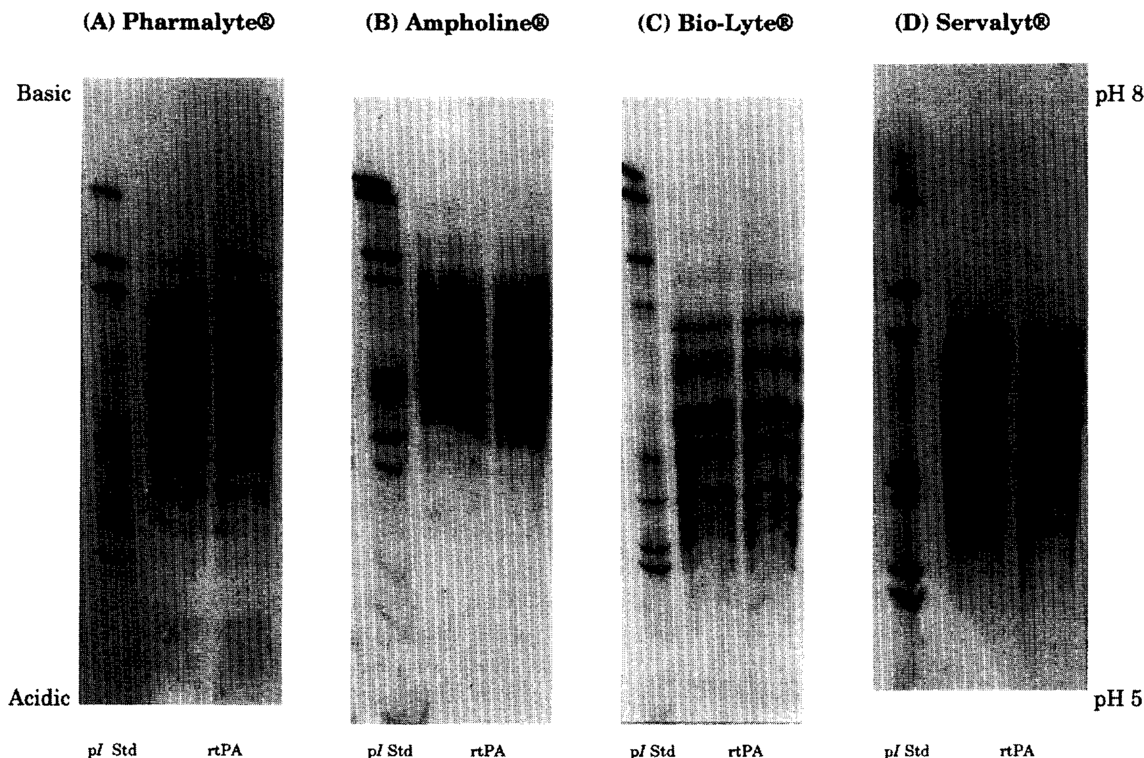

Fig. 1. Coomassie Blue stained slab gel IEF profiles obtained for rt-PA using: (A) Pharmalytes; (B) Ampholines; (C) Bio-Lytes and (D) Servalyts. p*I* standards (p*I* range 5.20 to 10.25) were obtained from Pharmacia.

3000 as described previously [4], with minor modifications. Briefly, rt-PA samples were prepared at final concentrations of 125 μg protein/ml, 4 *M* urea, 0.1% (w/v) HPMC, 0.75% (v/v) TEMED and 3% (w/v) pH 3–10 and pH 5–8 Pharmalytes in the ratio 50:50. The final salt concentration of samples was 50 m*M* or less. These solutions were used to fill the capillary, an eCAP neutral capillary, 27 cm×50 μm internal diameter (Beckman) with a 2 min rinse. The neutral capillary has a much reduced, but not zero, EOF value. Focusing was carried out using reverse polarity for 10 min at 500 V/cm. The capillary temperature was 20°C, detection was at 280 nm. The anolyte was 10 m*M* phosphoric acid, the catholyte 20 m*M* NaOH. Prior to each injection the capillary was rinsed for 1 min with water. After each analysis the capillary was rinsed with 10 m*M* phosphoric acid for 2 min and with water for one minute. A single 1 min HCl wash was performed at the beginning and at the end of each set of experiments. After the final HCl rinse the capillary was rinsed with water and stored wet. Reagents were replaced with fresh solutions on a daily basis.

Ultraviolet (UV) spectrophotometric scans were performed using HP 8452A diode array spectrophotometer

3. Results

Isoelectric focusing, Coomassie stained gels of rt-PA performed under identical experimental conditions using the various ampholytes demonstrate resolution of rt-PA into its constituent glycoforms (Fig. 1). The patterns vary somewhat, perhaps related to their composite ampholytes. Pharmalytes and Bio-Lytes spread the glycoforms to a greater extent than the Ampholines or Servalyts. The Ampholines appeared to concentrate the glycoforms into several closely spaced, sharp bands; the bands with Servalyts were more spread apart but did not show the larger number of bands seen with Pharmalytes and Bio-Lytes. The cIEF electropherograms of rt-PA using the same brands of ampholytes and performed on the P/ACE are shown in Fig. 2. Similar to the slab gel profile, Pharmalytes produced 8–10 peaks repre-

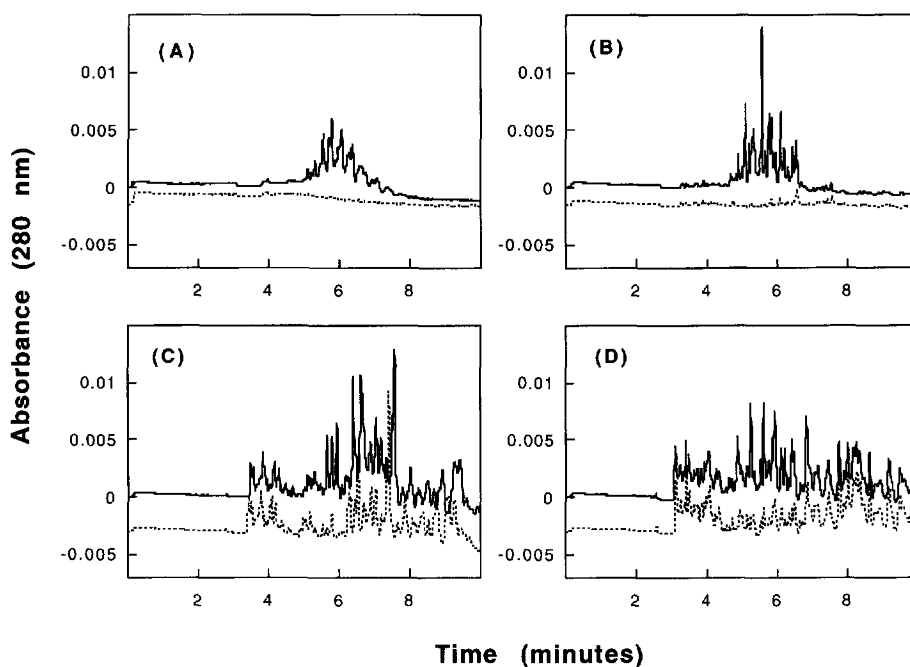

Fig. 2. cIEF profiles (solid lines) of rt-PA with the Beckman P/ACE using: (A) Pharmalytes; (B) Ampholines; (C) Bio-Lytes and (D) Servalyts. The cIEF profiles without rt-PA are shown in dashed lines.

senting different rt-PA glycoforms. The background electropherogram without rt-PA showed only minor peaks in comparison to the rt-PA profile. Ampholines produced sharp peaks which eluted over a narrow time span. One of these peaks exhibited the highest absorbance observed. Overall, the profile is similar to what was observed on the slab gel. The background electropherogram showed some minor peaks but the rt-PA profile was easily detected.

Although many peaks were observed with Bio-Lytes and Servalyts, they could not be interpreted as arising from rt-PA since they occurred throughout the p*I* range and were also present in the blank sample. These data were quite reproducible as each electropherogram is representative of at least 12 separate injections performed four months apart by two analysts using two lots of each ampholyte.

UV spectrophotometric scans of individual non-ampholyte components which were used in the cIEF mixtures are shown in Fig. 3A. Only rt-PA showed significant absorption at 280 nm (0.27 AU). For the ampholyte–rt-PA mixtures prior to cIEF, absorption at 280 nm was observed with all the ampholytes (Fig. 3B). The lowest absorption was seen with Pharmalytes and Ampholines, 0.48 and 0.34 AU respectively. Servalyts, however, demonstrated far greater absorption at 280 nm (1.49 AU) and over a wide UV range. The cIEF peaks observed with Servalyts were most likely due to focused ampholytes masking the absorption due to focused rt-PA glycoforms.

However, it was still uncertain why rt-PA glycoforms were not discernable with Bio-Lytes, since the absorption at 280 nm (0.64 AU) was close to that for Ampholines. Fig. 4 shows the UV spectrophotometric scan of the filter used in the Beckman P/ACE. It is a 10 nm bandpass filter allowing transmittance of a considerable amount of light at 275 nm and lower wavelengths. This lot of Bio-Lytes showed considerable absorbance at 270–275 nm, whereas Pharmalytes and Ampholines did not (Fig. 3B). Thus focused Bio-Lyte peaks will be detected and contribute to the background in the Beckman P/ACE.

Since the detector on the Bio-Rad BioFocus system uses a monochromator with a 6 nm bandpass, we evaluated the resolution of rt-PA glycoforms on this capillary electrophoresis (CE) system. The distance from the end of the capillary to the detector is

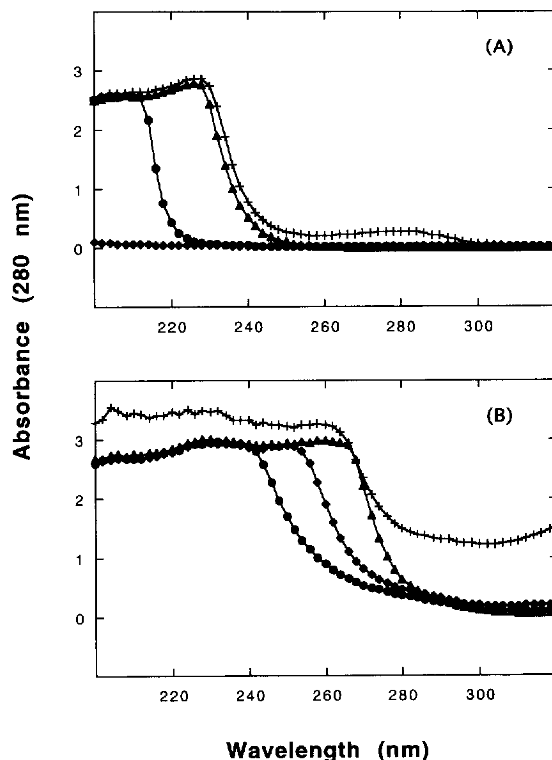

Fig. 3. UV spectrophotometric scans. (A), each component of cIEF mixture: +—+, rt-PA; ●—●, 4 *M* urea; ▲—▲, 0.75% TEMED; ◆—◆, 0.1% HPMC. (B), each sample prior to cIEF: ◆-◆, Pharmalytes; ●-●, Ampholines; ▲-▲, Bio-Lytes; +—-+, Servalyts.

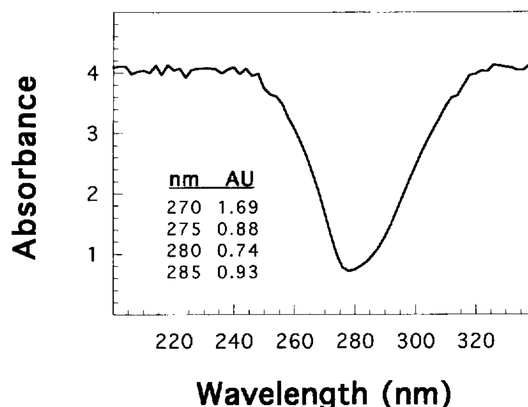

Fig. 4. UV spectrophotometric scan of the 280 nm filter used in the Beckman P/ACE.

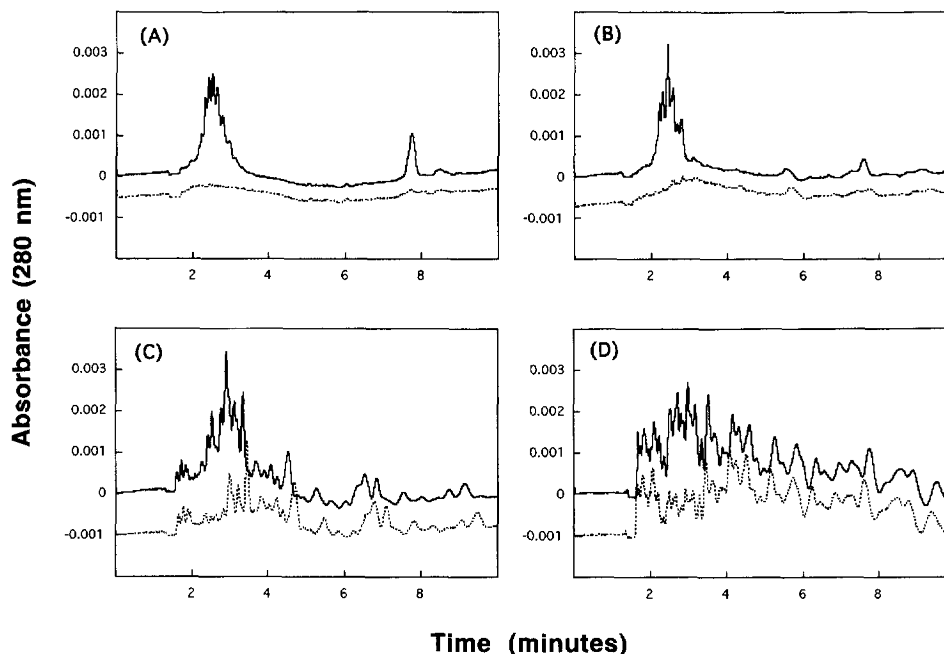

Fig. 5. cIEF profiles (solid lines) of rt-PA with the BioFocus 3000 using: (A) Pharmalytes; (B) Ampholines; (C) Bio-Lytes and (D) Servalyts. The cIEF profiles without rt-PA are shown in dashed lines.

4.5 cm compared to 7 cm on the P/ACE. Therefore it is expected that bands would pass the detector window earlier as they are focusing, and the bands may not be as focused as in the P/ACE. As shown in Fig. 5A–D, rt-PA glycoforms could clearly be detected using Pharmalytes and Ampholines. Background peaks were too intense with Servalyts to discern rt-PA glycoforms. By comparison to Pharmalytes and Ampholines, peaks could be detected with Bio-Lytes where rt-PA was expected to migrate, but there were again many major background peaks. This suggests that the absorption spectra of the Bio-Lytes overlap the bandpass of the Biofocus detector.

4. Discussion

During initial optimization of the cIEF method, ampholytes (Pharmalytes) covering a wide pH range (pH 3–10) were used. The best separation of rt-PA glycoforms was obtained when the wide range ampholytes (pH 3–10) was mixed with ampholytes covering a narrower pH range (pH 5–8) [2]. Using these conditions, ampholytes from other sources were tested as part of validation studies [5] and Ampholines were qualified as an alternate source. It was puzzling that IEF, but not cIEF, could be performed with the other ampholytes. UV spectra of the ampholytes and examination of the Beckman filter suggested that the lot of Servalyts tested absorbed a greater amount of light at and around 280 nm than did the other three lots of ampholytes. The focused Servalyts were probably the main contributors to the background peaks observed. The spectrum of Bio-Lytes overlapped the P/ACE filter bandpass and BioFocus detector bandpass considerably more than did the spectra of Pharmalytes and Ampholines. Thus these Bio-Lytes produced background peaks and prevented proper detection of rt-PA glycoforms.

Two groups have reported cIEF of rt-PA using Servalyts. In the first instance, Thorne et al. [6] used a Beckman P/ACE, a two step approach for performing cIEF, twice the protein concentration as in this study and a lower percentage of ampholytes, 1.3% as compared to 3%. The latter two changes could have increased the signal to background ratio enabling the

peaks to be detected over the background of Servalyts peaks. Alternatively, the lot of Servalyts used by these workers could have had a different UV spectrum. These authors also concluded that Ampholines were the best source of ampholytes for cIEF of rt-PA. The second group, Kubach et al. [7], used a Hewlett-Packard CE instrument which employs a diode array for detection. This group also used a two step cIEF method and Servalyts. Thus a lower % ampholytes, higher protein concentration, a two step approach and a screened batch of ampholytes may be necessary to enable cIEF of rt-PA with Servalyts.

Zhu et al. [8] presented a UV spectrum of Bio-Lytes (pH 3–10, final concentration 2%) showing zero absorbance at 280 nm. This contradicts our data showing significant absorbance at this wavelength (Fig. 3B) again suggesting lot to lot variability in batches of ampholytes. Evaluating the different ampholytes it appeared that Ampholines focused the rt-PA glycoforms into very sharp bands on the slab gel and in cIEF, whereas the glycoforms were more spread apart with Pharmalytes. This may be due to the fact that the separation was optimized for Pharmalytes and an optimized mixture of Ampholines could have resulted in a similar separation. Thus in performing validation of cIEF methods, it may not be feasible to simply substitute one source of ampholytes for another and additional optimization may be necessary. Alternatively, manufacturers could be requested to produce cIEF grade ampholytes which have no or negligible overlap with the detectors used. This would greatly improve the sensitivity of the method for detecting minor charged variants.

Acknowledgments

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