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Gradient chromatofocusing high-performance liquid chromatography I. Practical aspects

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Abstract

In this work, a versatile method for generating linear pH gradients using weak anion-exchange HPLC has been developed, which is termed gradient chromatofocusing high-performance liquid chromatography. This method utilizes a linear external pH gradient generated in the mobile phase entering the column (inlet pH gradient), superimposed on an internally-generated pH gradient within the column (column pH gradient), which results from the buffering action of the ion exchanger on the mobile phase and vice versa. The method shows significant advantages over conventional chromatofocusing, including: decreased expense due to the use of common buffer components, ease of adjusting the slope of the pH gradient produced at the outlet of the column (outlet pH gradient) through the manipulation of the inlet pH gradient and the ability of using high concentration buffers in the mobile phase. Chromatography of fibrinogen degradation products was done using gradient chromatofocusing. Bandwidths comparable to conventional chromatofocusing were obtained in the separation of fibrinogen degradation products.

Keywords: Chromatofocusing; Gradient elution; pH gradients; Mobile phase composition; Fibrinogen degradation products

1. Introduction

Presently, chromatofocusing is the only ion-exchange technique which can produce a linear pH gradient. Sluyterman et al. [1,2] developed chromatofocusing, which is referred to as an "internal" pH gradient technique. In addition to producing a linear pH gradient, chromatofocusing also focuses the protein band, producing high resolution separations. Reviews of chromatofocusing have been published [3,4].

The technique most commonly employs a weak anion-exchange column equilibrated with a high pH mobile phase (equilibrating buffer). With injection of sample, anionic species (i.e., proteins) are retained on the column, which are then eluted in the order of their p*I* upon a step change to a mobile phase consisting of a low pH buffer (focusing buffer). A linear pH gradient is generated by the progressive mutual titration of the stationary and mobile phases as each mobile phase section proceeds through the column, with the protein approximately following the movement of the $pH=pI_{protein}$ portion of the gradient [1,3,5]. Another mechanism proposed for chromatofocusing depicts the movement of a protein through the column in terms of it following the movement of buffer components having the same p*I* and charge characteristics [1,6]. Focusing of the protein band occurs because: (1) the velocity of the mobile phase is higher than the velocity at which any particular pH moves through the column and (2) an internal pH gradient is generated within the column causing a compression of the band.

There are many limitations, however, to chromato-

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focusing. Stringent requirements are placed on both the mobile and stationary phases. An even buffering capacity throughout the range of the pH gradient is required for both the mobile and stationary phases in order to produce a linear gradient [1,4,6]. Control of the slope of the pH gradient is limited and difficult. Low mobile phase buffer concentrations are required, in order to generate appropriate pH gradient slopes that are not too steep, as well as being important for optimal resolution [1,2,6,7]. However, there is a lower limit to the buffer concentration, as Polybuffer diluted more than 1:10 is shown to give erratic and unsatisfactory gradients [8]. The pH gradient slope can also be decreased by using ion-exchange packing materials having increased buffer capacity [6], as well as by increasing the column length (which also leads to improved separation capabilities) [2,5,6,9].

There are other limitations to the chromatofocusing technique. Linear pH gradients below pH 5 or 6 are difficult to generate on a high-performance PEI column [10]. The pH range for the gradients is also limited, as small pH gradient ranges (<1 pH) lead to erratic gradients [8], while large pH gradient ranges lead to difficulty in controlling gradient slope (too steep) [6] and linearity (a range of more than 3 pH units is difficult to generate) [3]. Most chromatofocusing is performed on low-performance ion exchangers, although high-performance procedures have been reported [7,8,10–19]. The advantage of high-performance chromatofocusing is the speed of analysis, reported to be 50 times greater than the low-performance technique [8]. In general, however, the quality of linear pH gradients is poorer on high-performance supports than on low-performance supports [10].

Another disadvantage of chromatofocusing is expense, as polymeric ampholytes are required for the focusing buffer. Although focusing buffers consisting of common buffers have been employed in chromatofocusing [2,6,8,10,19–21], the quality of the gradient generated is usually poor compared to that generated by polymeric ampholytes. Most linear pH gradients generated from common buffers are not smooth, giving cascade steps [6,19], spikes [20] and protein elution plateaus [8,20]. The cause for these irregularities has been attributed to unequal distribution of buffer components between the mobile and stationary phases, the use of anionic type buffers in the mobile phase, the use of counterions in the mobile phase that are subject to pH dependent dissociation changes, poorly defined contributions of the ion-exchange packing material to the pH gradient, changes in the mobile phase ionic strength and/or kinetic effects [19,20]. A mobile phase buffer consisting of common components has been reported which gives good linear characteristics for the pH gradient, however, its composition, which consists of many buffer components, is not specified [9,10,19]. It should be noted that these authors tested many common buffer combinations that do not give satisfactory linear gradients [19]. Irregularities in pH gradients also occur with polymeric ampholytes [2,10].

A technique is developed in the present work with the proposed name gradient chromatofocusing HPLC, which is capable of generating linear pH gradients on a high-performance weak anion-exchange column. Gradient chromatofocusing HPLC greatly extends the capabilities of chromatofocusing, overcoming the shortcomings of the conventional technique. The technique generates linear pH gradients (outlet pH gradient) using only a few common buffer components in the mobile phase. The design of the technique combines an external gradient (inlet pH gradient), which is a pH gradient with respect to time, and an internally-generated pH gradient within the column (column pH gradient), which is a pH gradient with respect to distance. This design allows for ready control of the outlet pH gradient slope and use of higher concentration buffers in the mobile phase. These aspects, along with the proper choice of buffer components (which have minimal retention on the ion-exchange column), are important for generating smooth linear pH gradients. In addition, the technique has a focusing capability comparable to conventional chromatofocusing.

2. Experimental

2.1. Materials

Fibrinogen (human plasma, Cat. No. 151123) and streptokinase (Cat. No. 101114) were obtained from ICN (Costa Mesa, CA, USA). Bulk anion-exchange

packing material (Protein-Pak DEAE 15HR, DEAE-functionalized polymethacrylate, 15 μm particle and 1000 Å pore diameters) was purchased from Millipore Corporation (Milford, MA, USA). Fibrinogen degradation products were produced as before [22]. The protein solutions were prepared with a buffer solution containing 25 mM Tris–HCl, 0.15 *M* NaCl, pH 7.4 and stored frozen at −20°C until use.

Mobile phase buffer A (high pH) consisted of cationic buffer component(s) only. Mobile phase buffer B (low pH) consisted of various weak acids and in some cases a proportion of phosphoric acid. All buffer solutions were prepared with HPLC grade water, and were stored at 4°C, with buffer A and buffer B being discarded after one week and one month, respectively. The buffer components used in this work, with their pK_a values given in parentheses, were as follows:

1. Cationic type: tris (8.06), bis-tris propane (6.80) and piperazine (5.68).
2. Weak acid type: acetic (4.76), lactic (3.81) and chloroacetic (2.87) acids.

Phosphoric (2.15, 7.20) acid was also used. All buffer components were ACS certified reagents.

2.2. Chromatographic design and procedure

A WINner HPLC system from Thermo Separation Products (San Jose, CA, USA) consisting of an SP8800 ternary HPLC pump, an SP4270 integrator and an Epson Equity I+ personal computer was used. The proteins were detected at 280 nm using a flow-through Variable Wavelength detector from Dionex (Sunnyvale, CA, USA). A Rheodyne Model 7125 injection valve from Rainin (Woburn, MA, USA) was used, employing a 500 μl injection loop. The injection volume was 20 μl for the fibrinogen degradation product samples. The column (50×4.1 mm I.D.) was packed using a HPLC packer from Alltech (Deerfield, IL, USA). The dead volume of the system from the gradient valve to the column was approximately 2.7 ml. The column dead volume was 0.27 ml. Before the start of each run the dead volume, prior to the column, was equilibrated to the starting gradient composition through positioning a pre-column valve from Rainin (Rheodyne model 7060- 071, Woburn, MA, USA) to waste prior to the run.

2.3. Other procedures

The pH of 2.0 min fractions of the inlet and outlet mobile phase was determined immediately after collection using a model 915 pH meter and a standard Ag/AgCl Micro Probe Combination pH electrode from Fisher (Pittsburgh, PA, USA). The inlet fractions were collected immediately before the column while the outlet fractions were collected immediately after the detector.

A titration of the DEAE–polymethacrylate packing material was performed on 2.0 g of packing material, which was washed with the following solutions that were subsequently filtered off: 2×10 ml deionized water, 2×10 ml NaOH solution (0.0141 *M*) and 2×10 ml deionized water. The packing material was then suspended in 10 ml of 0.0141 *M* NaOH solution plus 10 ml of deionized water. The titration was performed with 0.0134 *M* HCl solution using magnetic stirring. Titration curves closely matched when pH readings were taken 4 min and 9 min after adding titrant.

3. Results and discussion

3.1. Packing material and mobile phase considerations for generating linear pH gradients

As expected, an attempt to produce a linear pH gradient (7.4–3.5) by simply increasing the content of hydrogen ion in the mobile phase failed, as shown in Fig. 1. The lack of buffering ability of the mobile phase in the intermediate pH range caused the pH to suddenly drop after the buffer capacity of the anion-exchange packing material had been consumed. No separation of the fibrinogen degradation products could be obtained with this sudden drop of pH. In Fig. 2 the titration curve of the DEAE–polymethacrylate packing material revealed that the packing material had its strongest buffer capacity below pH 8, having an even buffer capacity from pH 7.5 to 3.6 at low ionic strength. This is different from other DEAE anion exchangers, such as DEAE–

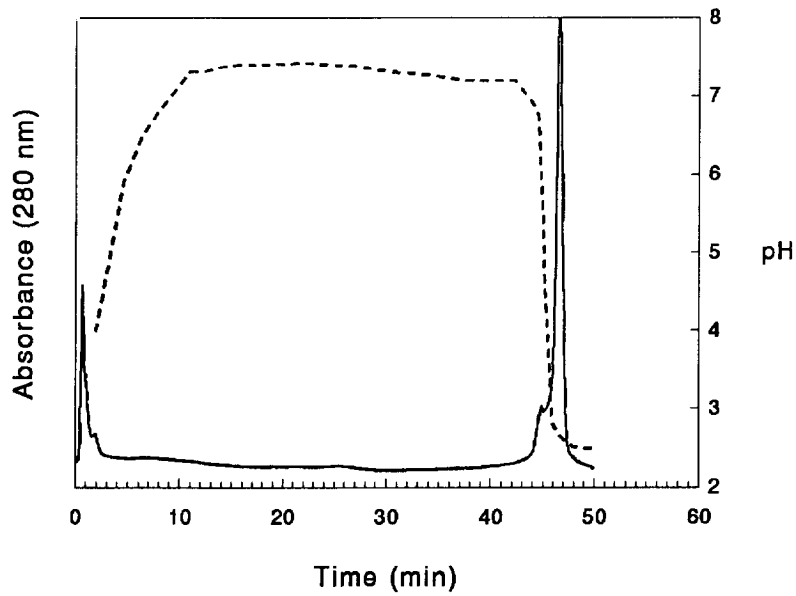

Fig. 1. Chromatogram of fibrinogen degradation products (40 min plasmin digest of fibrinogen, 200 μg total proteins). Buffer A consisted of 25 m*M* Tris–HCl, pH 7.4. Buffer B consisted of 2.0 *M* urea–HCl, pH 2.6. Pre-equilibration conditions were 100% A, 50 min, 0.5 ml/min. The external gradient was 0% B to 100% B over 50 min, 0.5 ml/min. The outlet pH gradient is shown by the dashed line. AUFS is 0.15 AU.

Sepharose, which shows its strongest buffer capacity from pH 7.0–9.0 [20]. This unexpectedly wide pH range for the buffer capacity of the packing material is a critical factor for generating a wide range for the linear pH gradient.

With the discovery of a suitable ion-exchange

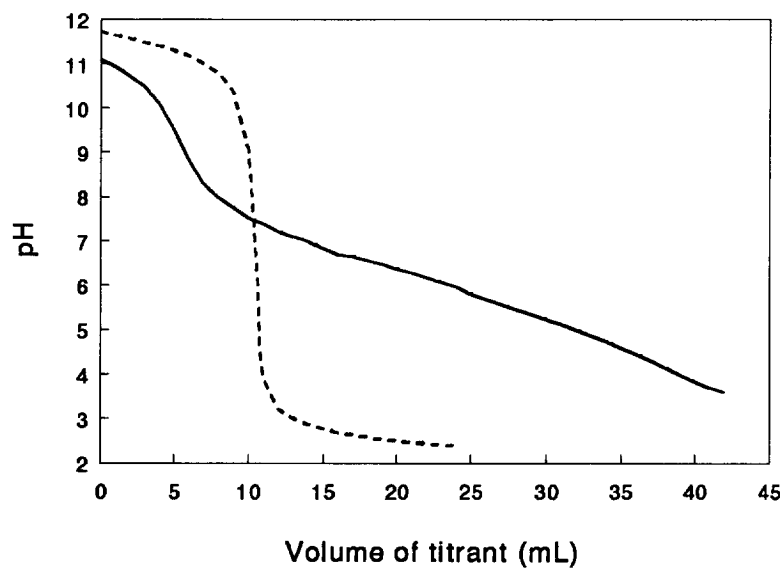

Fig. 2. The titration curves of 2.0 g DEAE packing material plus 20 ml of $7.05 \cdot 10^{-3}$ *M* NaOH (solid line) and 10 ml of 0.0141 *M* NaOH solution (dashed line). Titrant was 0.0134 *M* HCl.

packing material, development of a mobile phase scheme was pursued toward the goals of producing smooth linear pH gradients and developing a technique with demonstrated advantages over conventional chromatofocusing. The overriding consideration was to define buffer components which would give a composite buffering capacity throughout the pH range of interest, and thus avoid the results shown in Fig. 1. In addition, several strategies were incorporated, including: selection of buffer components with minimal retention on the anion-exchange packing material, allowance for use of a large range of buffer concentrations and external mixing for greater operator control of the gradient.

Common buffers were employed in this study to circumvent the expense associated with commercial ampholytes. The charge characteristics of the buffers were of critical importance. The buffers were divided into two types: cationic (e.g., amines) and weak acid. Building on previous work [20], cationic buffers were used whenever possible. These buffer components are neutrally (or positively) charged at high pHs and are thus not retained on the anion-exchange packing material, precluding the appearance in the gradient of pH spikes resulting from the elution of retained buffer components. Anionic (or weak acid) buffers, on the other hand, are negatively charged at high pH and are thus retained on the anion exchanger. Cationic components, however, could not be found for the lower pH range. Thus, weak acids were required to regulate the buffer capacity of the mobile phase in the low pH range, in addition to being necessary for reaching the more acidic pHs of the linear gradient. The use of an external gradient minimized the potential adverse effects of these weak acids on gradient quality, since these components were only present in high concentration at the low pH portion of the pH gradient.

3.2. Representative examples of linear pH gradients

Fig. 3a and Fig. 3b give representative examples of linear pH gradients generated; with the outlet pH gradient given in the upper plot of each figure and the inlet pH gradient given in the lower plot of each figure. In each case there was mixing of buffer A, consisting of cationic buffers (bis-tris propane and/or piperazine), with progressively greater proportions of buffer B, consisting of weak acid components (different combinations of acetic, lactic and/or chloroacetic acid) and in some cases a proportion of phosphoric acid. Buffer B should only consist of weak acids and not their conjugate base forms. The gradient chromatofocusing method is a significant improvement over the conventional chromatofocusing technique, which has been largely unsuccessful in employing common buffer systems for generating linear pH gradients with time.

3.3. External control of the outlet pH gradient

The most significant feature in gradient chromatofocusing for controlling the slope of the outlet pH gradient is the external gradient design. Flexibility in controlling the slope and shape of the gradient is shown in Fig. 4. The slope of the outlet pH gradient is a function of: (1) the upper and lower limits of the pH gradient established by buffers A and B and (2) the rate of increase of the proportion of buffer B (i.e. the programmed external gradient). The ready control of the outlet pH gradient slope by varying the external gradient is shown in Fig. 4a. Fig. 4b shows that concave and convex gradient shapes are also possible with this technique.

3.4. Other advantages

The concentration of the mobile phase buffer components used in this work is higher than that allowed for conventional chromatofocusing. Fig. 5 shows conventional chromatofocusing results for the same column and mobile phase components used for the gradient chromatofocusing technique of Fig. 3a. The outlet pH gradient for this conventional chromatofocusing run is seen to be too steep to be useful. The only way to generate shallow gradient slopes in conventional chromatofocusing is to limit the pH range of the gradient or to reduce the concentration of the mobile phase buffer components [6]. The use of high concentration buffer components in gradient chromatofocusing is possible because of the overriding effect of the external gradient in controlling the slope of the outlet pH gradient.

Another advantage of gradient chromatofocusing is that the column length is not critical to the slope of

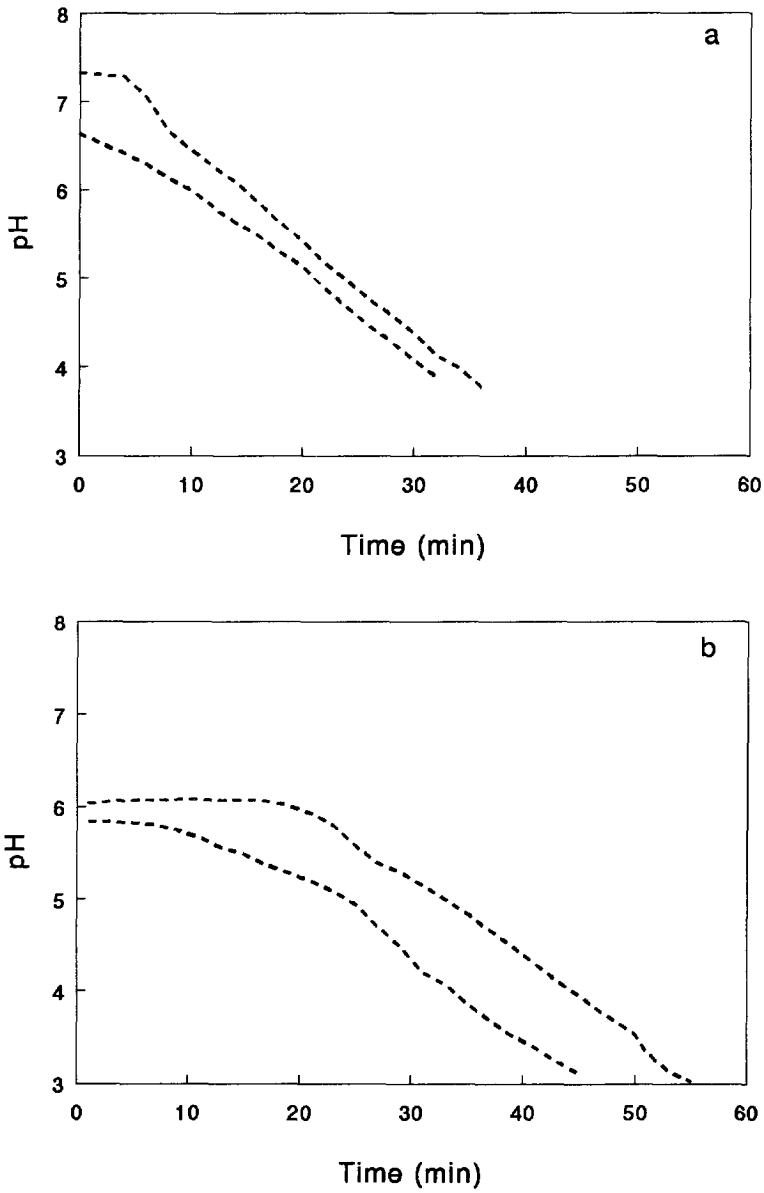

Fig. 3. Two examples of inlet and outlet pH gradients generated. Time zero is the start of the gradient at the gradient valve (applies for gradients plotted in all figures). For each figure, plots are the pH gradients generated after the column (outlet, top) and before the column (inlet, bottom). (a) Buffer A consisted of 15 m*M* bis-tris propane and 25 m*M* piperazine–HCl, pH 7.5. Buffer B consisted of 100 m*M* acetic acid, 30 m*M* phosphoric acid and 2.0 *M* urea. The column was pre-equilibrated with 100% A for 20 min and then 4% B to 8% B over 8 min. The external gradient was 9% B to 19% B for the first 10 min and then 19% B to 79% B over 30 min. The flow-rate was 0.5 ml/min. The pH window (see Part II for definition [23]) was approximately 0.6 pH units. (b) The column was pre-equilibrated with buffer A: 10 m*M* piperazine–HCl, pH 6.3. Buffer B consisted of acetic, lactic and chloroacetic acids, 5.0 m*M* each. The external gradient was 10% B to 30% B over 20 min and then 30% B to 90% B over 30 min. The flow-rate was 0.6 ml/min. The pH window was approximately 0.2 pH units.

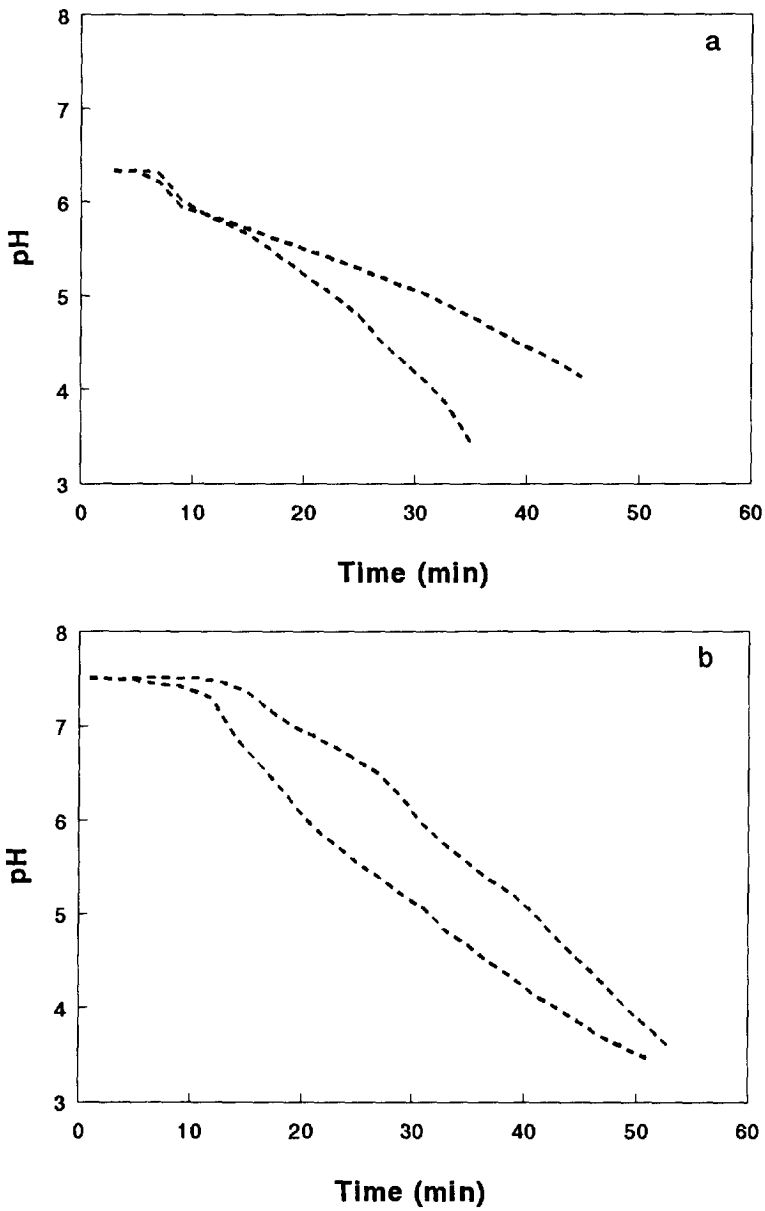

Fig. 4. Control of outlet pH gradient by the external gradient in gradient chromatofocusing. (a) A decrease in the slope of the outlet pH gradient was accomplished by a decrease in the slope of the inlet pH gradient. The column was pre-equilibrated with buffer A: 25 m*M* piperazine–HCl, pH 6.4. Buffer B consisted of acetic, lactic and chloroacetic acids, 12.5 m*M* each. The flow-rate was 0.6 ml/min. The upper plot resulted from an external gradient that was 10% B to 70% B over 60 min, yielding an average slope of 0.045 pH unit/min. The lower plot resulted from an external gradient that was 10% B to 15% B over 5 min and then 15% B to 75% B over 30 min, yielding an average slope of 0.099 pH unit/min. (b) Examples of convex and concave outlet pH gradients. The column was pre-equilibrated with buffer A: 15 m*M* bis-tris propane and 25 m*M* piperazine–HCl, pH 7.6. Buffer B consisted of acetic, lactic and chloroacetic acids, 25 m*M* each. The external gradient was 10 to 60% B over 60 min for the bottom (concave) plot. The external gradient was 5 to 20% B over 30 min and then 20 to 70% B over 25 min for the top (convex) plot. The flow-rate was 0.5 ml/min.

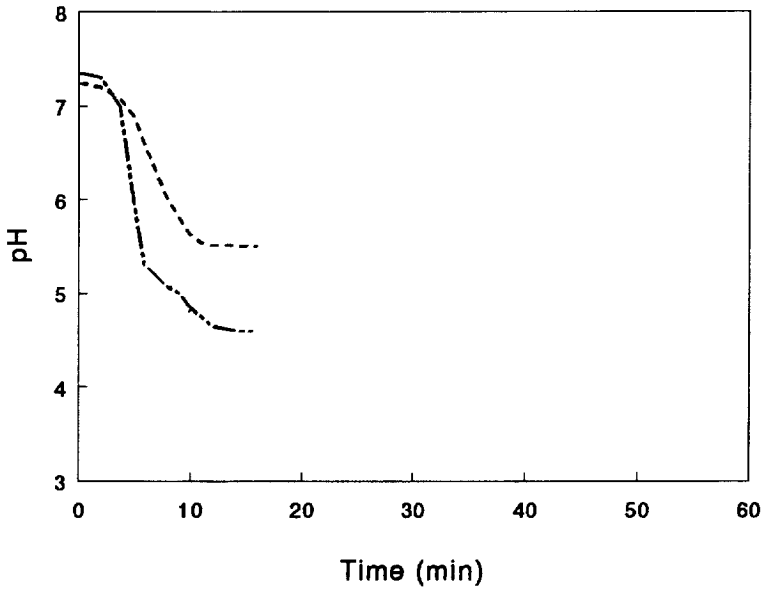

Fig. 5. Two outlet pH gradients obtained by conventional chromatofocusing. The buffers were the same as that in Fig. 3a. Equilibrating buffer was buffer A for both gradients. Focusing buffers were 85% A and 15% B (pH 5.5) for the top plot and 70% A and 30% B (pH 4.6) for the bottom plot. The flow-rate was 0.5 ml/min.

the outlet pH gradient. A column size of 50×4.1 mm was used in the present work, as opposed to the 250×4.1 mm column size commonly employed for high-performance chromatofocusing techniques. Longer columns are required in conventional chromatofocusing to ensure a reasonable slope in the pH gradient (not too steep).

3.5. Focusing capability

The column pH gradient is an important parameter in conventional chromatofocusing for focusing the protein bands on the column [1]. The following analysis illustrates the relative magnitudes of the column pH gradient compared to the inlet and outlet pH gradients. In Fig. 3b, the slopes of both the inlet and outlet pH gradients are approximately 0.17 pH/ml (after the plateau region). The slope of the column pH gradient for Fig. 3b (normally in units of pH per distance) can be calculated in units comparable to the inlet/outlet pH gradient by dividing the difference in pH between the column inlet and outlet (approximately 1 pH unit) by the dead volume of the column (0.27 ml), yielding an approximate value of 3.7 pH/ml. This gradient slope is significantly greater than the inlet or outlet pH gradient slopes, underscoring the magnitude of the focusing effect generated by the column pH gradient.

Experiments by Sluyterman and Wijdenes [2] were done to demonstrate the focusing effect in conventional chromatofocusing. Protein was injected onto the column and eluted with a focusing buffer. This was followed by another injection of the protein well into the chromatographic run, after the previously injected protein band had begun to move down the column. In this experiment, the protein band from the second injected sample was shown to be focused into the first injected protein band, demonstrating the focusing effect. A similar experiment was done with the present gradient chromatofocusing HPLC technique. In Fig. 6 the peak width of an injected streptokinase sample is compared with the peak width of a double injection of streptokinase (same total amount of streptokinase), the second injection of the double injection taking place after the first injected band had begun to move down the column. The half-height peak widths were 0.10 pH units for both the single and double injections.

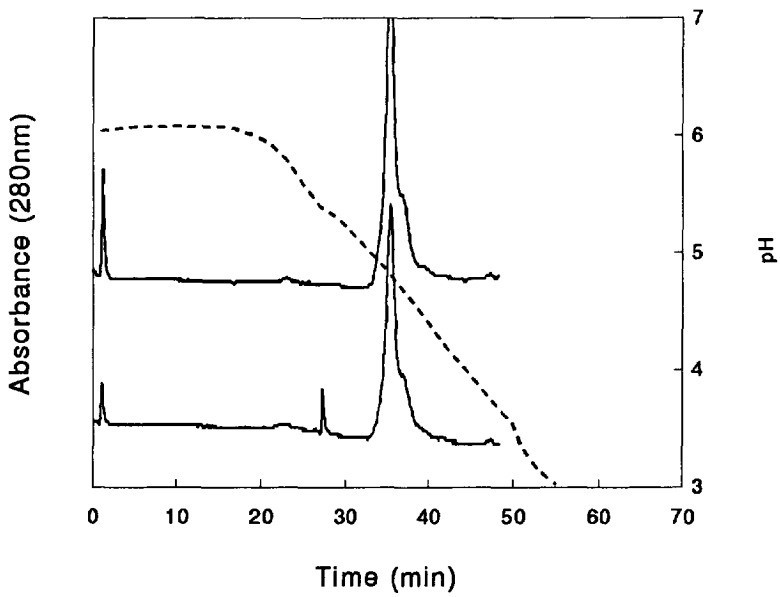

Fig. 6. Demonstration of the focusing effect of the gradient chromatofocusing method. Chromatogram (top) obtained with one 50-μl of streptokinase solution (0.2 mg/ml) injected at 0 min, compared to chromatogram (bottom) obtained with two 25-μl of the same solution injected separately at the start of the gradient and at 26 min after the start of the gradient (the second injection was made 1.5 min after the first band began to move down the column, as determined from the column travel time given in Fig. 3 in Part II [23]). See Fig. 3b for the buffer system and method of generation of the pH gradient. The outlet pH gradient is shown by the dashed line. AUFS is 0.0375 AU.

The value for the half-height width obtained for the streptokinase peak with the gradient chromatofocusing technique is similar to the values obtained for other proteins using conventional chromatofocusing. For example, a half-height width of 0.099 pH units for myoglobin was found using conventional chromatofocusing [2]. In fact a three day isoelectric focusing method gave 0.098 pH units for the half-height width for myoglobin [2]. In a direct comparison, fibrinogen degradation products were chromatographed by a conventional chromatofocusing technique in the literature [24] and by the gradient chromatofocusing technique, as shown in Fig. 7. The results gave essentially the same profiles, with the gradient chromatofocusing technique giving narrower peaks [0.35 pH units for the half-height peak-width for the present work (peak at 17 min), compared to 0.45 pH units for conventional chromatofocusing]. This demonstrates that gradient chromatofocusing HPLC has the same capabilities as conventional chromatofocusing in terms of focusing the bands.

4. Conclusions

The newly-developed gradient chromatofocusing HPLC technique described in this paper shows considerable promise for replacing conventional chromatofocusing as the chromatographic technique which produces both linear pH gradients and focused separations. Gradient chromatofocusing HPLC has across-the-board operational advantages over conventional chromatofocusing, while having comparable (or better) focusing capabilities. One major advantage of this technique is that a linear outlet pH gradient can be produced using only a few common buffer components. This is in contrast to previous reports using common buffer components in conventional chromatofocusing, which showed marked gradient irregularities and had a requirement for a large number of buffer components. Another substantial advantage of gradient chromatofocusing is the flexibility offered, as the slope of the outlet pH gradient is easily adjusted externally through a gradient controller. In fact, generation of convex and concave

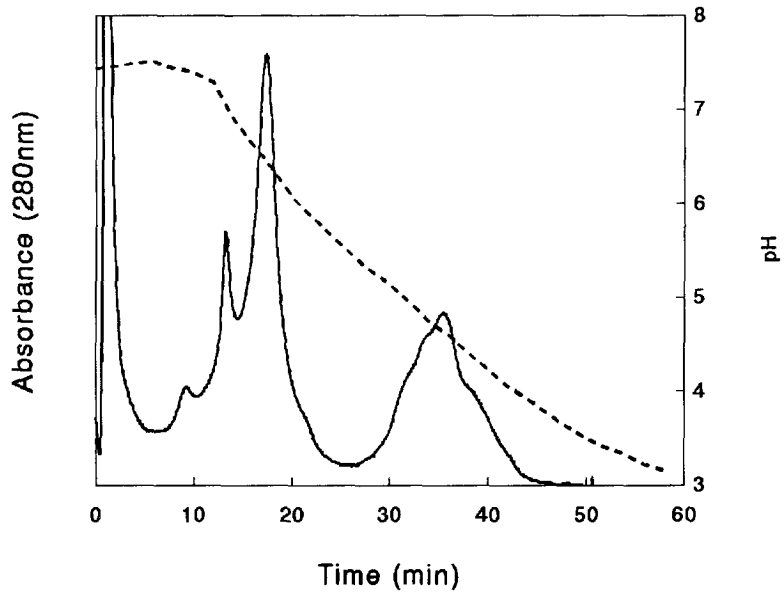

Fig. 7. Gradient chromatofocusing result of fibrinogen degradation products (40 min plasmin digest of fibrinogen, 200 μg total protein). The column was pre-equilibrated with buffer A: 15 m*M* bis-tris propane and 25 m*M* piperazine–HCl, pH 7.6. Buffer B consisted of acetic, lactic and chloroacetic acids, 25 m*M* each. The external gradient was 10 to 60% B over 50 min and 60 to 90% B over 20 min, 0.5 ml/min. The outlet pH gradient is shown by the dashed line, having an average slope of 0.093 pH unit/min from 15 to 45 min. AUFS is 0.05 AU.

outlet pH gradients is possible with gradient chromatofocusing. In contrast, control of the slope of the outlet pH gradient is very difficult and inconvenient in conventional chromatofocusing. Gradient chromatofocusing HPLC also has the flexibility of being able to use a wide range of buffer concentrations and column sizes.

Acknowledgments

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