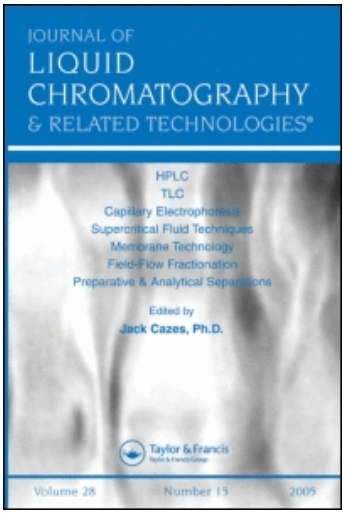


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# ANION-EXCHANGE HPLC OF FIBRINOGEN AND FIBRINOGEN DEGRADATION PRODUCTS USING pH GRADIENTS

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# ANION-EXCHANGE HPLC OF FIBRINOGEN AND FIBRINOGEN DEGRADATION PRODUCTS USING pH GRADIENTS

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## ABSTRACT

Factors were studied which affect the resolution of fibrinogen and fibrinogen degradation products (FDPs) in weak anion-exchange HPLC employing pH gradients. One factor studied was the effect of the buffer capacity of the mobile phase on resolution. A linear pH gradient generated by a gradient chromatofocusing technique, which has an approximately equal buffer capacity throughout the gradient pH range, was found to only marginally separate fragment D and fibrinogen. In contrast, a gradient of an elution buffer of diethanolamine/phosphate, which is strongly buffered in the pI range of fragment D but less so in the pI range of fibrinogen, yielded much better resolution. Another factor studied was the effect of pre-generating a pH gradient within the column prior to chromatography. A pre-generated column pH gradient was found to significantly improve resolution, as a single peak for native fibrinogen was resolved into two peaks. This work also showed different chromatographic profiles for fibrinogen from different commercial sources, indicating different proportions of native and denatured/degraded fibrinogen in the samples.

## INTRODUCTION

Presently there is increasing interest in the determination of fibrinogen-related proteins in assessing hemostatic status. Increased concentration of fibrinogen in plasma has been shown to be a strong independent risk factor in myocardial infarction and other cardiovascular diseases.[1,2] This increased risk is understandable, given the central role which fibrinogen plays in clot formation. Conversely, increased concentration of fibrinogen degradation products (FDPs) in plasma has been shown to be associated with increased risk of bleeding in different disorders.[3-6]

Fibrinogen has a trinodular structure, consisting of a central E domain connected by coiled coils to two identical terminal D domains.[7,8] Fibrinogen is degraded into a series of FDPs under the action of plasmin.[7,9] At the end-stage of the degradation, the largest components of the FDPs are fragments D and E, which consist principally of the D and E domains of fibrinogen, respectively.



Fibrinogen-related proteins are chromatographed in the present work, extending our previous work on the separation of these proteins by HPLC.[10-13] Employment of a mobile phase at constant pH with a linear gradient in salt concentration showed a domain-specific retention of fibrinogen on weak anion-exchange columns, precluding separation of fragments D and E from the corresponding domain-retained fibrinogen.[13] In the present work, factors were studied that affect the resolution of fibrinogen and FDPs in weak anion-exchange HPLC employing pH gradients.

## EXPERIMENTAL

### Materials

Fibrinogen (human plasma, plasminogen-free, >95% clottable proteins, Cat. No. 341578), fraction D (produced from human fibrinogen, $M_r$ 85,000, Cat. No. 341600) and fraction E (produced from human fibrinogen, $M_r$ 50,000, Cat. No. 341605) were purchased from Calbiochem (La Jolla, CA, USA). Fibrinogen from ICN (human plasma, 90% clottable proteins, Cat. No. 151123, Costa Mesa, CA, USA) was also used in this work. FDPs were produced from the digestion of fibrinogen with plasmin.[13]

### Solutions

All buffers were prepared with ACS certified reagents using HPLC grade water. Fibrinogen and FDPs solutions were prepared in a buffer consisting of 25

mM tris-HCl, 0.15 M NaCl, pH 7.4, being dialyzed and ultrafiltered, respectively.[13] The application mobile phase solutions were: A1 (15 mM bis-tris propane and 25 mM piperazine-HCl, pH 7.6); A2 (25 mM tris-HCl, pH 7.4 or 7.8); A3 (25 mM piperazine-HCl, pH 6.4); and A4 (25 mM bis-tris propane-HCl, pH 7.8). The elution mobile phase solutions were: E1 (acetic, lactic and chloroacetic acids, 25 mM each); E2 (2.0 M urea and 0.5% (v/v) diethanolamine, pH 2.4, with the pH being adjusted using concentrated phosphoric acid); E3 (2.0 M urea that was adjusted to pH 2.6 with 6 M HC1); and E4 (2.0 M urea, approximately 0.18 M acetic and approximately 0.018 M phosphoric acids).

### Chromatography

The same HPLC set-up, apparatus for pH measurements, and experimental procedures were used as described previously,[10] with the highlights and exceptions given below. A UVIS 203 detector from ThermoQuest (San Jose, CA, USA) set to 280 nm was used. The column ($50 \times 4.1$ mm i.d.) was packed as described previously.[10] A DEAE polymethacrylate packing material (Protein-Pak DEAE 15HR, 15 μm, 1000 Å) from Millipore Corporation (Milford, MA, USA) was used. The injection volume was 20 μL, usually containing 200 μg of protein (unless otherwise specified).

A valve system was employed to conveniently control the flow direction through the column, as shown in Figure 1. V1 was a Model 7030 valve and V2 was a Model 7060-071 valve, both from Rheodyne (Cotati, CA, USA). All chromatographic runs employed a forward flow after the pre-equilibration step.

In some experiments, a pH gradient was established within the column prior to the injection of the sample. This pre-generated pH gradient had a low pH at the column inlet and a high pH at the column outlet, with an assumed linear pH gradient through a certain portion of the column length. The pre-generated pH gradient was generated by first equilibrating the column with a low pH mobile phase and then performing a limited backward pumping of a high pH mobile phase through the column.

## RESULTS AND DISCUSSION

In a previous work,[13] chromatography of fibrinogen was done on a DEAE anion-exchange HPLC column employing a linear salt gradient. Three peaks were produced, the first peak being attributed to a native form of fibrinogen binding to the packing material via the D-domain, with the second peak being attributed to the denatured/degraded form of fibrinogen binding to the packing

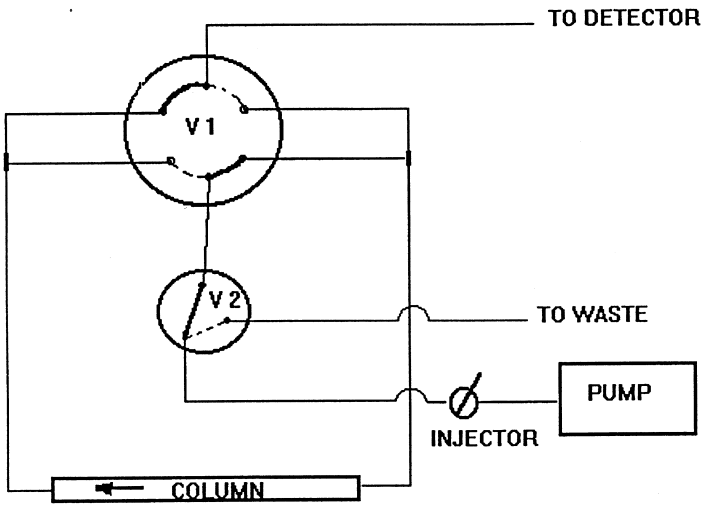


**Figure 1**. Schematic of valve system used in the HPLC design. The flow direction of mobile phase in the column was set using valve 1 (V1). Valve 2 (V2) was used to equilibrate the dead volume before the column with the desired composition of mobile phase.



material via the E-domain. Subsequent chromatography of fragment D and fragment E employing the same linear salt gradient showed fragment D and fragment E to elute at the same retention time as the first and second peaks of fibrinogen, respectively. Thus fragments D and E could not be separated from fibrinogen at the constant pH conditions used in this previous work (pH 7.4). The present work investigated the separation of fibrinogen and fragment D employing pH gradients. This would appear to be a reasonable separation strategy based on the reported pIs of fibrinogen (5.8-6.1)[14,15] and fragment D (6.05-6.45).[15]

## Chromatography Using a Linear pH Gradient

Figure 2 shows the separation of fibrinogen (chromatograms a and b for different commercial sources of fibrinogen) from FDPs (chromatogram c) obtained with a linear gradient in pH, which is attained by employing a newly-developed gradient chromatofocusing HPLC technique.[10,11] Similar to the salt gradient work,[13] fibrinogen was found to chromatograph into several peaks, consisting of one major early-eluting peak (22-23 min) and several late-eluting peaks (35-47 min).

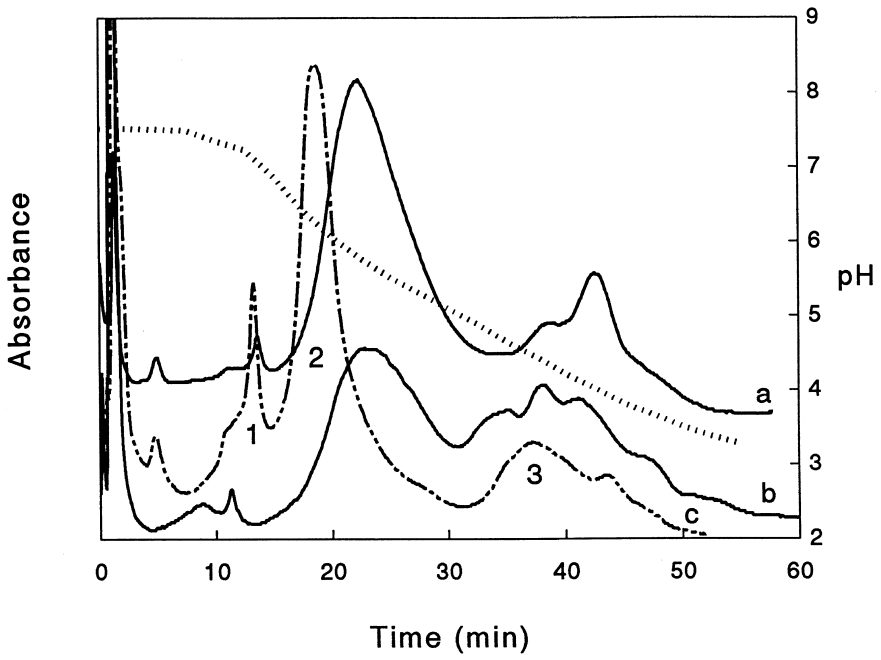


**Figure 2**. Chromatography of fibrinogen and FDPs using a linear pH gradient. The samples were commercial fibrinogen solutions from (a) Calbiochem, (b) ICN, and (c) FDPs [90 min fibrinogen (Calbiochem) digest]. In (c), peaks 1 and 2 correspond to fragment D, while peak 3 corresponds to fragment E. Mobile phases A1 and E1 were used. The column was pre-equilibrated with A1 for 18 min (forward flow). Linear gradients of 10% to 60% E1 over 50 min and then 60% to 75% E1 over 10 min were used. The flow rate was 0.5 mL/min. The outlet pH gradient curve is shown by the dotted line. AUFS is 0.0625 AU.

Conclusions from previous work[13] are applied here to identify the early-eluting peak as the native form of fibrinogen, while the later peaks are identified as the denatured/degraded forms of fibrinogen. Chromatography of FDPs using the same linear pH gradient conditions is shown in Figure 2c. Three peaks were produced.

Based on the chromatofocusing work done by Kalvaria, et al.,[16] peaks 1 and 2 are identified as fragment D while peak 3 is identified as fragment E. It is seen in Figure 2 that only marginal resolution of fragment D from native fibrinogen was obtained, while fragment E could not be separated from the late-eluting peaks of the denatured/degraded fibrinogen.

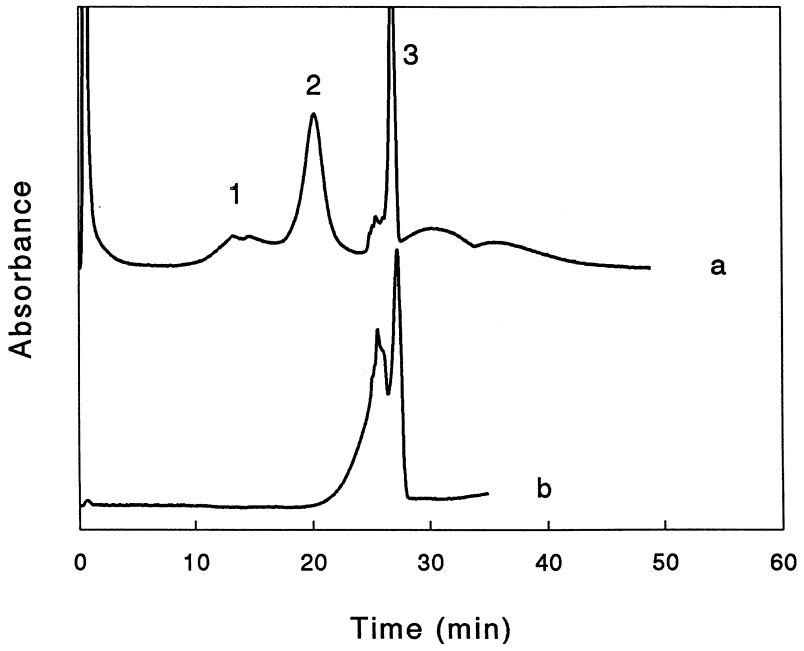


**Figure 3**. Chromatography of fibrinogen and FDPs. Mobile phases A2 (pH 7.8) and E2 were used. The column was pre-equilibrated with A2 (pH 7.8) prior to injection. The linear gradient was 0% to 100% E2 over 50 min at a flow rate of 0.5 mL/min. The samples were (a) FDPs [300 min fibrinogen (Calbiochem) digest (200 μg total protein)] and (b) 100 μg fibrinogen (Calbiochem). In (a), peaks 1 and 2 correspond to fragment D and peak 3 corresponds to fragment E, as determined from the injection of commercial fraction D and E samples. AUFS is 0.125 AU.

Use of a very shallow pH gradient in gradient chromatofocusing did not significantly improve the separation of fragment D from native fibrinogen (data not shown). In fact, use of a very shallow pH gradient greatly reduced the peak area of the native fibrinogen peak, most likely due to an extended denaturing effect on the fibrinogen.[11,13]

### Chromatography Using a Diethanolamine/Phosphate Elution Buffer

Figure 3 shows improved resolution of fragment D from fibrinogen using a diethanolamine/phosphate buffer mobile phase. This is the first work reporting the separation of fragment D from fibrinogen using ion-exchange chromatography.

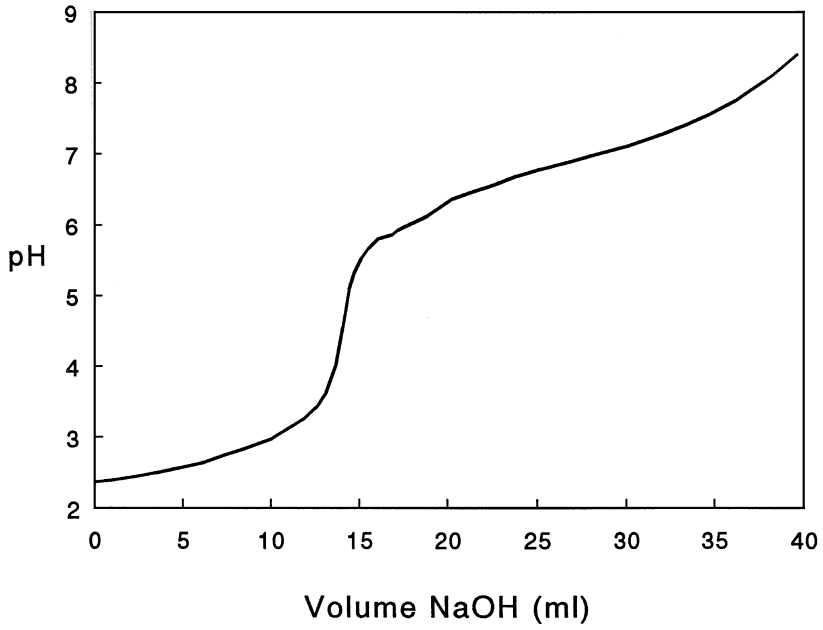


**Figure 4**. Titration curve of the diethanolamine/phosphate elution mobile phase (E2). The plot was obtained by titrating 25.0 mL E2 with 0.134 M NaOH.



A plausible explanation for the improved separation of fragment D from fibrinogen using the diethanolamine/phosphate buffer system is given below. It was thought that the linear pH gradient employed in Figure 2 was not optimal for separating fragment D from fibrinogen because of the strong buffer capacity in the mobile phase in the pH range encompassing the pIs of both fragment D and fibrinogen. Contrastingly, a strategy of employing a buffer that was strong in the pH range of the pI of fragment D, but weaker in the pI range of fibrinogen was used in Figure 3. Figure 4 shows titration data for the diethanolamine/phosphate system, which shows a strong buffer capacity in the fragment D pI range (6.05-6.45), with a drop off in capacity occurring at pH ~5.8, which is the lower portion of the fibrinogen pI range. Separation for the two proteins may thus be enhanced because of the lesser buffer capacity in the pI region of fibrinogen, leading to its delayed elution compared to that seen in Figure 2.

Another unique aspect of the experimental design which could explain the enhanced resolution of fibrinogen and fragment D was the employment of a protonated base (i.e., a basic buffer that is adjusted to low pH) as an elution buffer titrant.

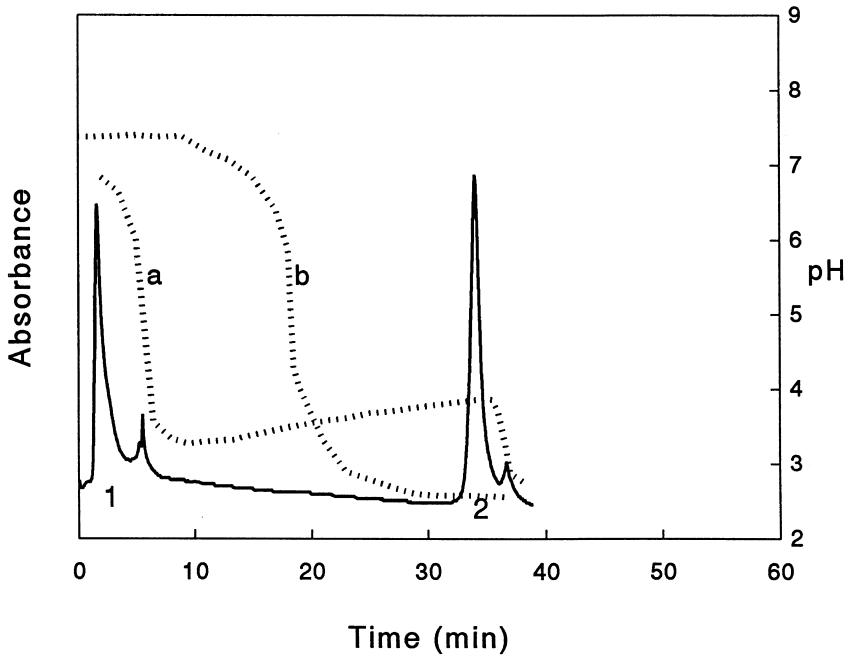


**Figure 5**. Chromatography of FDPs (300 min digest of fibrinogen from Calbiochem, 100 μg total protein) using a HCl elution buffer mobile phase. Mobile phases A2 (pH 7.4) and E3 were used. After being equilibrated with mobile phase E3, the column was conditioned with mobile phase A2 (6 mL), using a backward flow. The linear gradient was 0% to 80% mobile phase E3 over 40 min, 0.5 mL/min. Curve a is the pH of the mobile phase emerging from the column and curve b is the pH of the mobile phase fed to the column. Peak 2 is the elutable FDP molecules, while peak 1 is the void volume peak noted when FDPs are chromatographed (see Figure 2c). AUFS is 0.125 AU.

In the gradient chromatofocusing approach (Figure 2) acidic buffers were used to adjust the pH downward, which means that shallow buffer gradients are more difficult to attain, since the addition of the acidic buffer pulls the buffer capacity of the mobile phase to lower pHs. Use of a protonated base to adjust the mobile phase pH means that the basic buffer components were increasingly added to the column at the same time that hydrogen ions are added, allowing for a shallow gradient in the basic pH range.

### Chromatography Using HCl Elution Buffer

Further evidence for the importance of buffer capacity controlling elution is seen in Figure 5. In this experiment, a step pH gradient was established

within the column by equilibrating the column with the elution mobile phase [E3: pH 2.6 HCl (2M urea)] prior to a limited backflush of the column with the application mobile phase (A2: tris-HCl, pH 7.4). Curve a (outlet pH gradient) indicates that the application mobile phase titrated only a small portion of the column to a high pH, since the outlet mobile phase drops to a low pH early within the pH gradient run. It is expected that the binding of FDPs occur in the high pH zone near the column outlet, which should readily elute upon the sharp decrease in pH documented in Figure 5. However, elution was delayed subsequent to the drop in the outlet pH (< 4) by approximately 30 min. This is unexpected, as Figure 2c indicates that all FDPs elute at a mobile phase pH slightly less than 4. Consistent with the previous discussion is the fact that HCl has very little buffer capacity at the elution pH, which could be the reason for the delayed elution.



**Chromatography of Fibrinogen from Different Commercial Sources**

Chromatography of fibrinogen with a linear pH gradient can be used to determine the proportion of the native and denatured/degraded fibrinogen forms in a sample. Figures 2a and 2b show different chromatographic profiles for fibrinogen from different commercial sources indicating different proportions of the forms of fibrinogen in the two samples. Based on our previous work[13] the early-eluting peak (22-23 min) is the native fibrinogen form and the late-eluting peaks (35-47 min) are the denatured/degraded forms of fibrinogen.

**The Effect of a Pre-Generated Column pH Gradient on Resolution**

The effect of a pre-generated column pH gradient on the separation of heterogeneous fibrinogen forms was examined, as shown in Figure 6. Both chromatograms in Figure 6 were obtained using identical elution methods, however different methods were used to condition the column prior to the injection of a fibrinogen sample. In Figure 6a, the column was conventionally conditioned by pre-equilibrating with a high pH buffer. This resulted in a constant pH throughout the column at the beginning of the chromatographic run.

The chromatographic pattern obtained for this pretreatment procedure was similar to the results of Figure 2b, which also employed a conventional pre-equilibration step; showing a major early-eluting peak (11 min) and several late-eluting peaks. In Figure 6b, the column was conditioned differently. In this case, a linear column pH gradient was pre-generated down a portion of the column length, with the inlet and outlet of the column at a low and high pH, respectively.

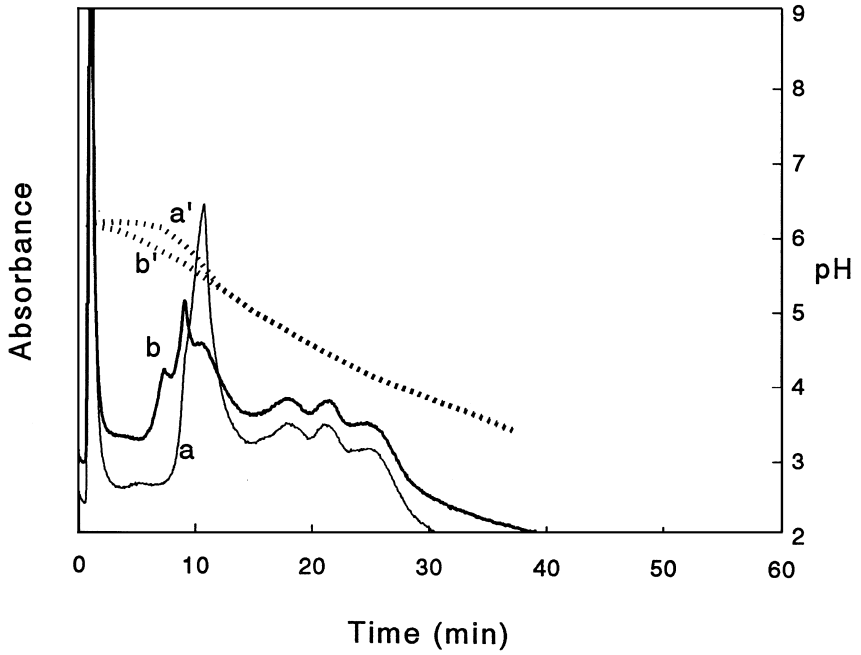


**Figure 6**. Results for different initial column conditions in the chromatography of fibrinogen (ICN): (a) initial conditions of a constant pH throughout the column, accomplished through pre-equilibration of the column with mobile phase A3 using forward flow for 15 min, and (b) initial conditions of a pre-generated pH gradient on the column, accomplished by conditioning the column after the chromatographic run (described below) with 4.0 mL A3 using a backward flow and then applying 1.5 mL of the starting mobile phase (96% A3, 4% E4) using a forward flow. Upon injection of the sample, the following gradient (mixed with A3) was done for both (a) and (b): 4% to 14% E4 (linear gradient) over 10 min and then 14% to 74% E4 (linear gradient) over 30 min. The outlet pH gradients for chromatograms (a) and (b) are given by (a') and (b'), respectively. The flow rate was 0.5 mL/min. AUFS is 0.0400 AU.

This pre-generated column gradient significantly improved resolution, as the major early-eluting peak in Figure 6a was separated into a triplet in Figure 6b. The earliest component in the triplet was a minor component and could be well separated from the other two major components when a higher starting pH was employed, as was the case in Figure 2b (noting the peak at 9 min). The existence of at least two components in the major peak in Figure 2b could thus be shown with the pre-generated column pH gradient method. The formation of a column pH gradient prior to injection is designed to distribute the different forms of fibrinogen along the axial distance of the column at the initial stage of chromatography. When the column is conditioned to only one pH, as was the

case in Figure 6a, all injected analyte components are bound into a narrow zone of packing material at the inlet of the column. However, with the establishment of a column pH gradient prior to injection (Figure 6b), analyte components are bound in different pH zones on the column. Improved resolution for the pre-generated column gradient runs can be explained by: 1) a smaller elution volume for those analyte components initially retained in the interior of the column (instead of being retained at the inlet of the column) and/or 2) a decrease in interaction between the analyte components during chromatography, resulting from the preliminary separation of the separation of the different forms on the column.

An alternate explanation for the improved resolution of the early-eluting peaks in Figure 6b needs to be considered;decreased outlet pH gradient slope for b' compared to a'. This explanation, however, was ruled out by experiments which showed no improvement in the resolution when shallow pH gradients were used (data not shown). In fact, a combination of a pre-existing column pH gradient prior to injection, along with a steeper outlet pH gradient during the chromatography, improved the resolution of the native fibrinogen peaks. This is seen by comparing Figure 7 with Figures 2 and 6. The predominate early-eluting peak in Figures 2a, 2b, and 6a was resolved into two sharp peaks in Figure 7, through use of a pre-generated column pH gradient and a steep outlet pH gradient. The two early-eluting peaks were sharper in Figure 7 (peaks 1 and 2) compared to Figure 6b, presumably explained by the steeper pH gradient in Figure 7.



## CONCLUSIONS

Various pH gradient strategies were employed to optimize separation of fibrinogen-related proteins in anion-exchange HPLC. It was found that optimal separation of fibrinogen from fragment D could not be obtained with a linear pH gradient, even with shallow gradient slopes. Optimal separation was obtained by employing a strategy using an elution buffer with high buffer capacity in the pI region of fragment D and lesser buffer capacity in the pI region of fibrinogen.

These results point to an important principal in ion-exchange chromatography which is identified in the present work; namely the importance of buffer capacity of the mobile phase in the elution of proteins from an ion-exchange column. The mobile phase must have sufficient buffer capacity in the pH range surrounding the pI of the protein in order to effectively elute the protein. Selective manipulation of buffer capacity, as shown in present work, may be an effective tool in optimizing protein separation in ion-exchange HPLC.

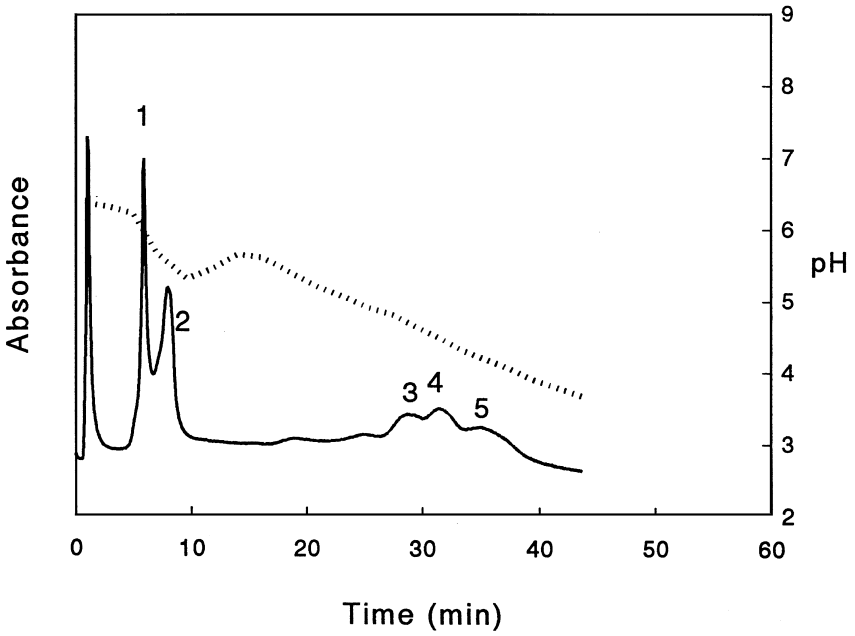


**Figure 7**. Chromatography of fibrinogen (ICN) using initial conditions of a modified pre-generated column pH gradient. The column, at a low pH after a run described below, was first conditioned with 3.5 mL A4, using a backward flow. Then, 1.5 mL of mobile phase (95% A3, 5% E4) was applied to the column using a forward flow before the injection of the sample. The gradient (a combination of mobile phases A3 and E4) was started immediately after the injection, consisting of linear gradients of 5% to 0% E4 over the first 5 min, then 0% to 5% E4 over 5 min, then 5% to 10% E4 over 10 min, and then 10% to 70% E4 over 30 min. The flow rate was 0.5 mL/min. The dotted line shows the pH of mobile phase at the column outlet. Peak numbers are used for ease of discussion in the text. AUFS is 0.0850 AU.

Another important design innovation introduced in the present work is the initial formation of a pH gradient in the column prior to the injection of the sample. An improved separation of fibrinogen components was observed using this design, as indicated in Figures 6 and 7.

Controlling pH gradients in ion-exchange chromatography is inherently more challenging than controlling salt gradients. However, pH gradient chromatography appears to offer more variables which can be manipulated to improve the separation of proteins. This has been observed in our studies of fibrinogen-related proteins. Further studies of other model proteins are warranted to more thoroughly characterize these effects.

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