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Journal of Chromatography B, 695 (1997) 49–58

JOURNAL OF CHROMATOGRAPHY B

Method of analysis of recombinant acidic fibroblast growth factor by capillary electrophoresis

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Abstract

Fibroblast growth factors are a series of well characterized proteins that have intriguing pharmacological properties. Acidic fibroblast growth factor (aFGF) recently appeared in the literature for its efficacy in spinal cord repair in rats. The protein has proven difficult to analyze by capillary electrophoresis, because it has a tendency to unfold, aggregate and precipitate, especially near and above physiological temperatures. By studying the turbidity of capillary electrophoresis running buffers and aFGF at 50°C, conditions were found that stabilize the aFGF solution, thereby allowing the capillary electrophoretic separation of the protein from its recombinant production impurities. The buffer system employs 50 m*M* phosphate buffer at pH 2.5 with 0.25% hydroxypropylmethylcellulose (HPMC) additive. This system provided the best efficiency and selectivity of the systems studied and was developed for pharmaceutical purity analysis. ©1997 Elsevier Science B.V. © 1997 Elsevier Science B.V.

Keywords: Acidic fibroblast growth factor; Fibroblast growth factor

1. Introduction

Acidic fibroblast growth factor (aFGF) has recently received national media attention for its role in spinal cord repair in adult paraplegic rats [1]. Furthermore, recombinant production of human aFGF yields enough protein for pharmaceutical research. Although its biological functions have been extensively characterized [2], a method of purity analysis by capillary electrophoresis has not been published to the best of our knowledge. Typically, the protein is analyzed by slab gel techniques that do not have the speed, reproducibility and automation of most instruments. Capillary electrophoresis was chosen as a complement to HPLC for purity analysis, because it has demonstrated a marked ability to separate proteins and peptides that differ by as little as one amino acid [3,4]. However, issues of wall adsorption and protein stability must be addressed when developing a method of analysis for capillary electrophoresis.

Adsorption occurs through electrostatic interaction, hydrogen bonding, hydrophobic interaction, or any combination thereof depending on the structure of the protein [5]. Fused-silica capillaries, which are widely employed for CE, are particularly problematic. The wall of a fused-silica capillary contains silanol groups which have pK_a values between 4 and 8. Electrostatic interactions between polycationic species and ionized silanols have been shown to be the driving force of adsorption and can have a large impact on peak shape and efficiency [6]. Much work has been done to reduce these interactions since capillary electrophoresis was applied to protein

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PII S0378-4347(97)00100-X

analysis. Approaches used to reduce this problem include (i) utilizing buffer additives to prevent protein–wall interactions, (ii) changing the protein's effective charge by varying pH, (iii) denaturing the protein with chaotropic agents and (iv) reducing wall interactions by using coated capillaries.

Ideally, the protein should be separated from impurities while the protein is in its native conformation so that the pure native protein can be collected for further investigation. Moreover, analysis of the native protein allows detection of different conformations that may not be observed with traditional denaturing methods, such as SDS–PAGE [7].

There are many buffer and additive systems used to improve the separation of proteins, however the protein *must* be stable and soluble in the running buffer during the separation. It has been shown that stabilization of human aFGF is necessary, as it has a tendency to unfold at physiological temperatures [7]. In this study, the physical stability of aFGF in buffer solutions was studied to ensure that the protein would not precipitate during capillary electrophoresis. An investigation of aFGF stability in capillary electrophoresis buffer solutions was modeled after a turbidity study used by Eberlein et al. [8] to determine bFGF (basic fibroblast growth factor) solution stability. Subsequently, the acceptable buffer systems were screened for effectiveness in separating the protein from the impurities using bare fused-silica and other functionalized fused-silica capillaries. The capillary electrophoresis method was developed for the purity analysis of aFGF solutions.

2. Experimental

2.1. Chemicals

Sodium phosphate monobasic, sodium tetraborate, ethylene glycol, poly(ethylene glycol) 400 and hexane sulfonic acid (HSA) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sodium hydroxide solution was obtained from VWR (Bridgeport, NJ, USA). Methanol, *o*-phosphoric acid (85%), urea and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from EM Science (Gibbstown, NJ, USA). Mercaptoethanol was obtained from BioRad (Hercules, CA, USA). Sodium dodecyl sulfate (SDS) and 1,4-butanediamine dihydrochloride (putrescine) were obtained from Kodak (New Haven, CT, USA). 4-Morpholinoethanesulfonic acid (MES) was obtained from ICN (Costa Mesa, CA, USA). Hydroxypropylmethylcellulose (HPMC, viscosity of 2% aqueous solution (25°C): approx. 4000 cP), pyrophosphate, cetyltrimethylammonium bromide (CTAB), Brij 35 and cyclohexylaminoethane sulfonic acid (CHES) were obtained from Sigma (St. Louis, MO, USA). A sample from a batch of recombinant acidic fibroblast growth factor (aFGF) was provided by Rhône-Poulenc Rorer Pharmaceuticals (Collegeville, PA, USA). The impurities in the sample are unknown and were not characterized prior to this work. Another sample from a different batch that degraded during repeated freezing and thawing of the samples was also analyzed to investigate method selectivity. These samples will be referred to as the "original" sample and the "degraded" sample. Due to changes in processing the protein, the storage buffer of the original sample was different than that of the degraded sample buffer. Milli-Q purified water or Fisher Scientific HPLC grade water was used for all experiments. All buffer and sample solutions for CE were filtered through Millipore (Bedford, MA, USA) 0.45 μm syringe filter devices and degassed before analysis.

2.2. Instrumentation

Turbidity measurements were performed on a Beckman DU640 spectrophotometer (Fullerton, CA, USA). Unless otherwise noted, separations were performed on a Hewlett-Packard HP^{3D} CE system with HP^{3D} CE Chemstation data acquisition (Waldbronn, Germany). A Spectra-Physics SpectraPhoresis 500 (San Jose, CA, USA) with Waters ExpertEase software also was used to screen buffer systems and test ruggedness between instruments during method development. The data was collected at 1250 data points per min on the Hewlett-Packard instrument and at 60 data points per min on the Spectra-Physics instrument.

2.3. Materials

Beckman quartz cuvettes with a 1.0 cm pathlength were employed in the turbidity studies. Fused-silica

capillaries of 75 μm I.D. obtained from Polymicro Technologies (Phoenix, AZ, USA) were used for all separations unless otherwise noted. The capillaries were pretreated for 20 min with 1.0 *M* NaOH at 60°C (or maximum temperature of the instrument if less than 60°C) followed by 10 min each with 0.1 *M* NaOH, water and running buffer at running temperature. Coated capillaries, the BioRad LPA capillary and the Supelco (Bellefonte, PA, USA) P-150 and H-150 capillaries, were prepared according to the manufacturer's recommendations.

2.4. Turbidity studies

To monitor aFGF precipitation, solutions of buffer, additive and 0.1 mg/ml aFGF were quickly mixed in cuvettes and placed into the spectrophotometer. The temperature was ramped to 50°C over 1 min to accelerate any interactions and monitored at 50°C for 20 min. Since aFGF has no chromophore above 315 nm, the spectrophotometer monitored turbidity (apparent absorbance) at 350 nm as an indication of light scattering from precipitate formation.

2.5. Capillary electrophoresis

Unless otherwise noted, a conditioned 40 cm fused-silica capillary with 75 μm I.D. was used on the Hewlett-Packard instrument with each buffer system. Running voltages were varied from 10 kV to 30 kV to achieve the fastest separations possible with acceptable Joule heating (≤2.0 W/m). As noted by Tsuda [9], EOF is reversed in the presence of CTAB, so the running voltage was reversed for the separations employing CTAB additive. Along with the HP instrument, the Spectra-Physics instrument was used to screen several buffer systems. On the Spectra-Physics instrument, 1.0 mg/ml of aFGF solution was vacuum injected for 2 s. When the HP instrument was used, the 1.0 mg/ml solution of aFGF was pressure injected at 2.00 kPa (20.0 mbar on the HP instrument) for 5 s. Injection times and running voltages were varied as needed to obtain reasonable peak heights and migration times. Absorbance was measured at 200 nm and 350 nm to allow distinction of protein peaks and precipitate peaks. A spectrum at each peak apex was collected to confirm the presence of protein matter.

Ohm's law plots were generated for the different buffer systems by flushing the capillary with the running buffer for 10 min and applying voltages between 0 and 30 kV in 2 kV increments. The current was recorded at each voltage, and the resulting plots revealed a significant deviation from linearity above a certain voltage indicating excessive Joule heating [10].

Because the width at half-height ($W_{0.5}$) was provided automatically by the data acquisition software, the half height equation (Eq. (1)) was used for convenience to calculate the efficiency of all peaks. Efficiencies quoted in Figs. 5 and 6 were taken as an average of aFGF efficiencies from three replicate injections.

$$N = 5.545\left(\frac{t_R}{W_{0.5}}\right)^2 \quad (1)$$

3. Results and discussion

3.1. Buffer selection

Buffers and additive candidates were chosen with the following criteria: demonstrated ability to improve protein peak efficiency and recovery, UV cutoff and availability. Candidates must have published results on improved peak efficiency, peak shape or protein recovery. aFGF lacks a strong chromophore above 220 nm, so detection was performed at 200 nm. For best sensitivity, the buffer or additive should not absorb significantly at the wavelength of detection. For convenience, the reagent should be readily available from chemical suppliers and require no difficult preparations. Since there is an abundance of additives reported in the literature, only a few that represent most classes of additives were studied [10].

As demonstrated in Fig. 1, solutions which stabilized aFGF showed no change in absorbance and remained clear. Conversely, the solutions which did not stabilize the protein solution became turbid due to protein precipitation, and the apparent absorbance of the solution increased.

Table 1 lists the additives used in the turbidity

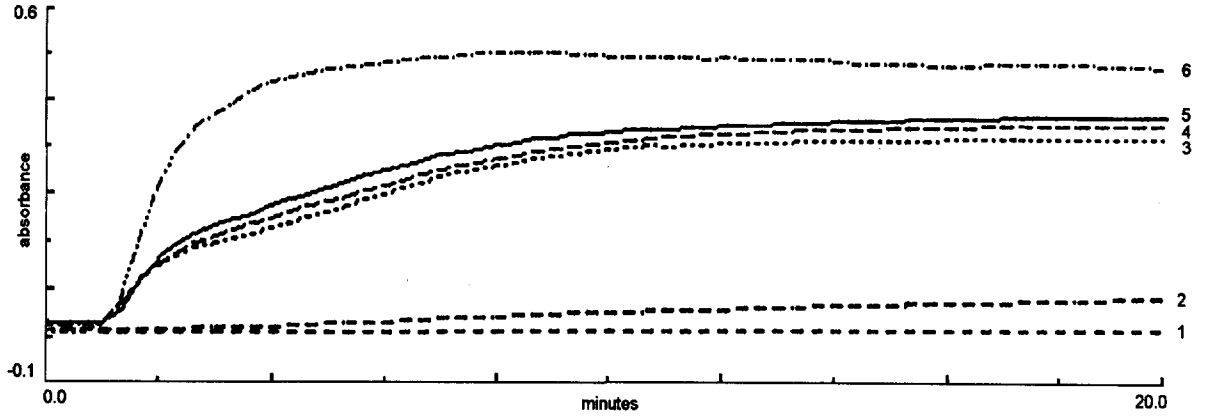

Fig. 1. Apparent absorbance of buffered 0.1 mg/ml aFGF pH 7.5 phosphate (10 m*M*), 350 nm, 50°C. Additives: (1) 0.5 m*M* CTAB; (2) 1 m*M* pyrophosphate; (3) 0.02% HPMC; (4) no additive; (5) 0.05% Brij 35; (6) 5 m*M* putrescine.

Table 1
The appearance of 0.1 mg/ml aFGF solutions in pH 7.5 phosphate (10 m*M*) with the additive indicated, measured after 20 min at 50°C

Additive	Class	pH 2.0	pH 2.5	pH 3.0	pH 6.5	pH 7.0	pH 7.5
None	None	clear	clear	clear	**turbid**	turbid	**turbid**
1 m*M* Pyrophosphate	Polyanion	clear	clear	**turbid**	**turbid**	**turbid**	clear
5 m*M* Putrescine	Amine modifier	clear	clear	clear	**turbid**	**turbid**	**turbid**
0.5 m*M* CTAB	Cationic surfactant	clear	clear	clear	clear	clear	clear
0.05% Brij 35	Nonionic surfactant	clear	clear	clear	**turbid**	**turbid**	**turbid**
0.02% HPMC	Cellulose polymer	clear	clear	clear	**turbid**	**turbid**	**turbid**

study with phosphate buffer at several pHs, the class of the additive and the effect on the protein solution. Table 2 lists other buffer/additive systems tested in the turbidity study. When examining Tables 1 and 2, several trends were observed. The protein has a p*I* of approximately 6.5, and the solutions were generally stable at pH values far above or below the p*I*. This is characteristic of proteins having a greater tendency to precipitate at a pH near the p*I*, because their charge density is not high enough to induce electrostatic repulsion [11]. CTAB stabilized aFGF solutions over the entire pH range studied, and urea solubilized aFGF around its p*I*. However, these effects may have been accompanied by protein denaturation.

Each buffer system that inhibited precipitation of aFGF was investigated with capillary electrophoresis. Neutral pH phosphate buffer with CTAB or urea added and various high and low pH buffers with each of the additives were examined. Several parameters, such as concentration, pH, running voltage and capillary pretreatment, were systematically altered

Table 2
The appearance of 0.1 mg/ml aFGF solutions in other buffer systems measured after 20 min at 50°C

Buffer/Additive	Class	pH	Result
90 m*M* MES	Zwitterionic buffer	6.1	**turbid**
50 m*M* HSA in 12.5 m*M* phos.	Sulfonic acid	7.1	**turbid**
4 *M* urea in 12.5 m*M* phos.	Denaturant	7.1	clear
90 m*M* HEPES	Zwitterionic buffer	7.5	**turbid**
90 m*M* borate	High pH	9.0	clear
90 m*M* CHES	High pH, zwitterionic buffer	9.6	clear

for each system in an attempt to find suitable conditions.

Low pH phosphate buffer and CTAB at any pH provided solution stability, but poor peak efficiencies (Figs. 2 and 3). Finally, the addition of HPMC to phosphate buffer improved efficiency over phosphate alone, and increased selectivity (Fig. 4). The added selectivity is a result of a sieving mechanism provided by the polymer network of the HPMC [12].

3.2. Method optimization

Based on the electropherograms obtained from each system, the low pH phosphate and HPMC method was chosen for validation. Ohm's law plots of different concentrations of pH 2.5 phosphate buffer with and without HPMC additive were prepared to investigate Joule heating. For the Hewlett-Packard instrument, a deviation from linearity occurred around 2.0 W/m for each buffer system studied. This was used as the criterion for the highest running voltage as each buffer system was examined.

The method was tested for ruggedness, precision and selectivity on the Hewlett-Packard CE. The type and length of capillary, pH, phosphate concentration, HPMC concentration and temperature were systematically investigated for method ruggedness. The method was then performed on the Spectra-Physics instrument.

Neither capillary length nor the use of coated capillaries (see Section 2.5) significantly changed the separation, so 40 cm bare fused-silica capillaries were selected for convenience and economy.

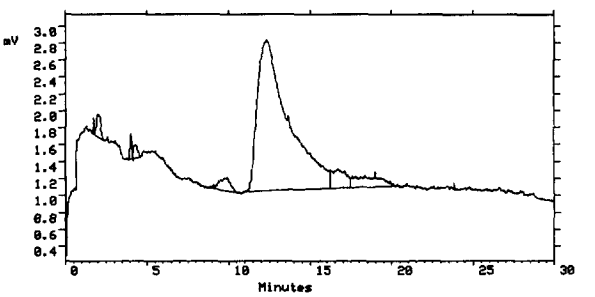

Fig. 3. Low pH phosphate and CTAB with reversed voltage also provided solution stability, but poor peak efficiencies. Conditions: pH 2.5 m*M* phosphate (10 m*M*) running buffer, 0.5 m*M* CTAB, −10 kV running voltage, 5 s injection of 0.2 mg/ml aFGF solution, 38 cm capillary on the Spectra-Physics instrument, ambient temperature.

To determine the effects of buffer composition on the separation, each component of the buffer was individually altered. The pH of an 87 m*M* phosphate buffer with 0.25% HPMC was changed from pH 2.3 to 2.9 in 0.1 pH increments. The efficiency exhibited no trends, however the first two peaks of the electropherogram (Fig. 4) were not resolved above pH 2.5.

As the concentration of phosphate was increased from 22 to 87 m*M* (constant pH of 2.5 with 0.25% HPMC), the efficiency increased from 31 200 plates

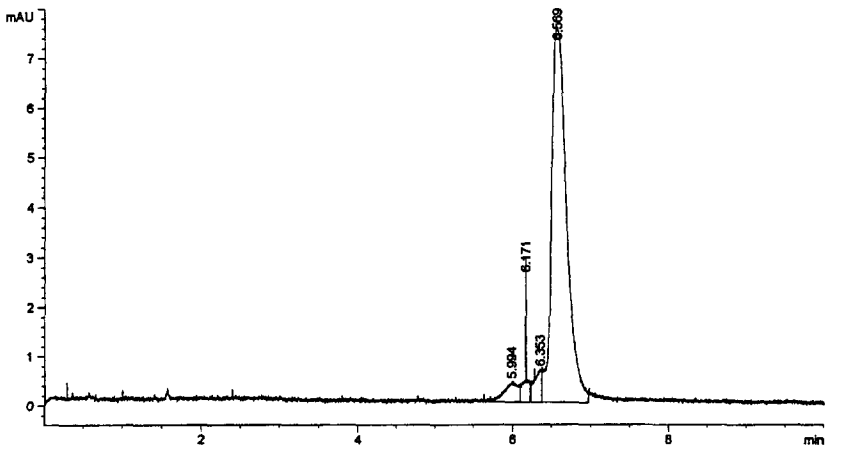

Fig. 2. Low pH phosphate buffer provided solution stability, but poor peak efficiencies. Conditions: 10 m*M* phosphate running buffer (pH 3.0), 20 kV running voltage, 5.00 kPa injection of 1.0 mg/ml aFGF solution for 4 s.

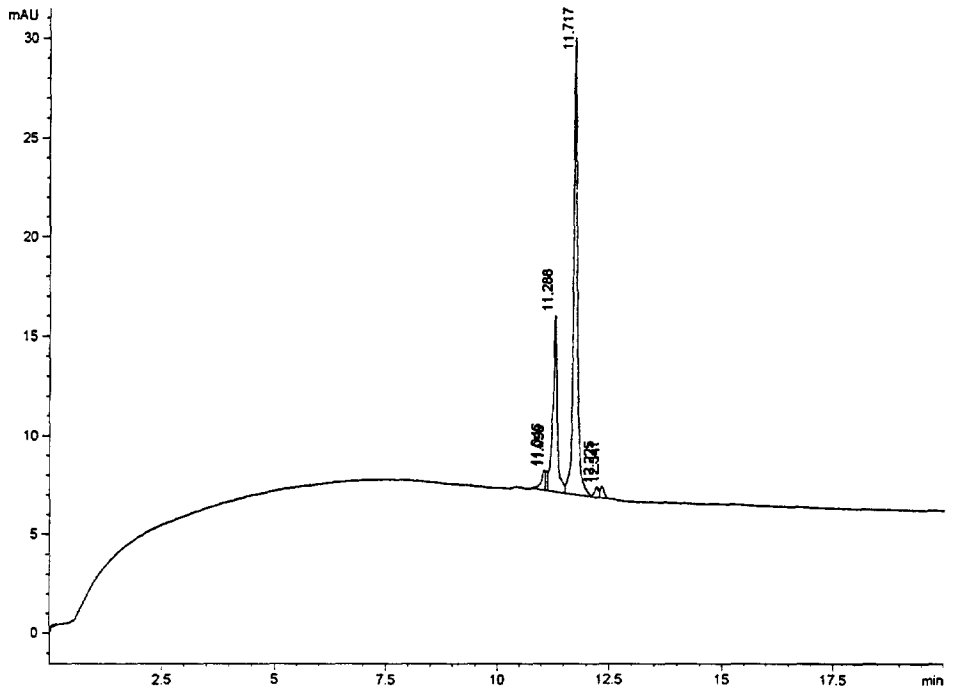

Fig. 4. Phosphate with HPMC additive improved efficiency over phosphate alone. Conditions: pH 2.5 phosphate (64 m*M*) 0.25% HPMC running buffer, 10 kV running voltage, 5.00 kPa injection of 1.0 mg/ml degraded aFGF solution for 2 s.

to over 80 000 plates with 64 m*M* and 87 m*M* phosphate (Fig. 5). The increase in peak efficiency may be a result of the higher ionic strength running buffer supplying more competing charged species to reduce the interaction of the protein with the capillary wall [13]. When Joule heating is not excessive, the efficiency increases with phosphate concentration. According to the Ohm's law plots (data not shown), Joule heating is not significant at 10 kV for up to 100 m*M* phosphate at pH 2.5 with 0.25% HPMC. Still, to allow for reasonable analysis times, there should be a balance between (i) higher peak efficiency with high concentration buffers and low running voltage and (ii) faster analysis times from lower ionic strength buffers and higher running voltage.

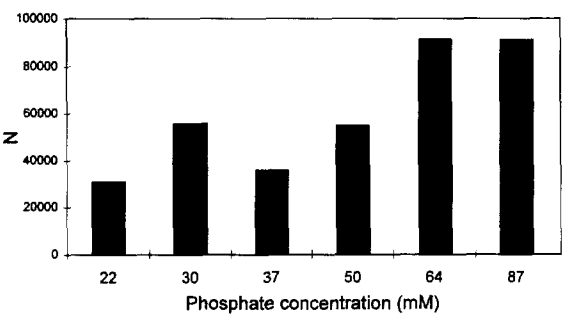

Fig. 5. Effect of buffer concentration on aFGF peak efficiency. Conditions: pH 2.5 phosphate, 0.25% HPMC, 10 kV, 5.00 kPa injection of 1.0 mg/ml degraded protein sample for 2 s.

The effect of HPMC concentration on peak efficiency was examined with 87 m*M* phosphate buffer (pH 2.5) at 10 kV and is reported in Fig. 6. The efficiency of the aFGF peak increased with HPMC concentration up to 0.35% HPMC, but was unexpectedly low at 0.50% HPMC. These higher efficiencies are the result of one or more of the following: (i) better separation of the peaks originally underneath the aFGF peak, (ii) decreased longitudinal diffusion (band-broadening) due to an increase in viscosity and (iii) reduction of protein–wall interactions because coating the capillary wall may reduce some protein–wall interactions that may exist between the protein and the neutral silanols at a pH of 2.5. The

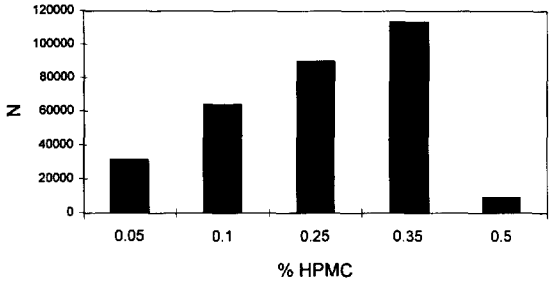

Fig. 6. Effect of HPMC concentration on aFGF peak efficiency. Conditions: pH 2.5 phosphate (87 m*M*), 10 kV, 5.00 kPa injection of 1.0 mg/ml degraded protein sample for 2 s.

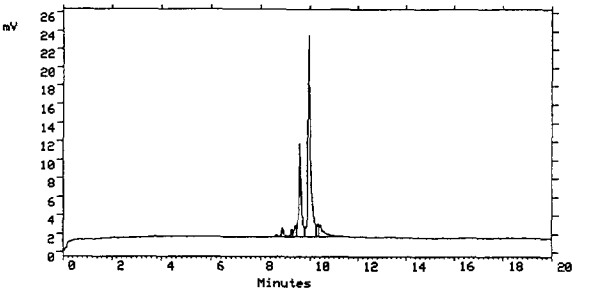

Fig. 7. Separation of degraded aFGF on the Spectra-Physics instrument. Conditions: pH 2.5 phosphate (50 m*M*) 0.25% HPMC running buffer, 10 kV running voltage, 1 s vacuum injection of 1 mg/ml degraded protein sample, 38 cm, ambient temperature.

0.50% solution was too viscous to filter with the 0.45 μm filter tips, so particulates may have impaired the separation. Other more concentrated HPMC solutions (0.25, 0.35%) were viscous, difficult to filter and required longer injection times and rinsing times to obtain reasonable peak heights and reproducibility. They also exhibited slightly longer migration times than the lower concentration solutions due to higher viscosity and increased molecular sieving. However, the first two peaks (Fig. 4) were not resolved with concentrations of HPMC less than 0.25%.

All of the above factors – pH, buffer concentration and polymer concentration – were considered when the buffer system was chosen. Proteins can undergo acidic cleavage, and pH 2.5 phosphate was the least acidic buffer that provided resolution of the first two impurity peaks. Ohm's law plots have shown that 50 m*M* is the highest concentration of phosphate that allowed relatively fast separations at 15 kV without excessive Joule heating ($\geq$2.0 W/m). Finally, very viscous solutions are difficult to prepare and use, and 0.25% HPMC was the least viscous solution that provided sufficient resolution of the first two impurity peaks. Thus, a 50 m*M* phosphate buffer (pH 2.5) containing 0.25% HPMC was selected as the running buffer for the separation of aFGF.

The separation was also performed on the Spectra-Physics instrument as shown in Fig. 7. The electropherogram was comparable to those obtained on the Hewlett-Packard instrument (Fig. 4), and the relative standard deviation (R.S.D.) of the migration time of aFGF was 0.12% for five sequential injections.

The separation was also performed at 15 and 40°C with 15 kV running voltage on the Hewlett-Packard instrument. The better separation was obtained at 15°C (Fig. 8). The precision of the migration time on this system was 0.23% R.S.D. for five consecutive injections. The buffer system was also tested at 10 and 20°C, and there were no significant differences from the 15°C plot. Based on these results, the remaining studies were conducted at 15°C.

The method was used to analyze an original sample and a degraded aFGF sample. The efficiencies of the aFGF peak in the original sample had a mean (three consecutive injections) of 72 000 plates with 6.7% R.S.D., and the efficiencies of the aFGF peak in the degraded sample had a mean (first three injections) of 201 000 plates with 0.35% R.S.D.. The differences in efficiencies and reproducibility of efficiencies are probably due to a difference in the sample matrix. The original sample matrix had a higher ionic strength than the running buffer, whereas the degraded sample matrix had a lower ionic strength because of the way it was prepared. A high ionic strength sample may result in band broadening and affect reproducibility. With low ionic strength samples, stacking of the analytes may occur when voltage is applied resulting in higher peak efficiencies.

The precision (%R.S.D.) of area and area percent for the original sample and for the degraded sample is reported in Tables 3 and 4. The calculations were based on Fig. 8 for the original sample and Fig. 9 for the degraded aFGF sample. Area percents were calculated as the quotient of individual peak areas

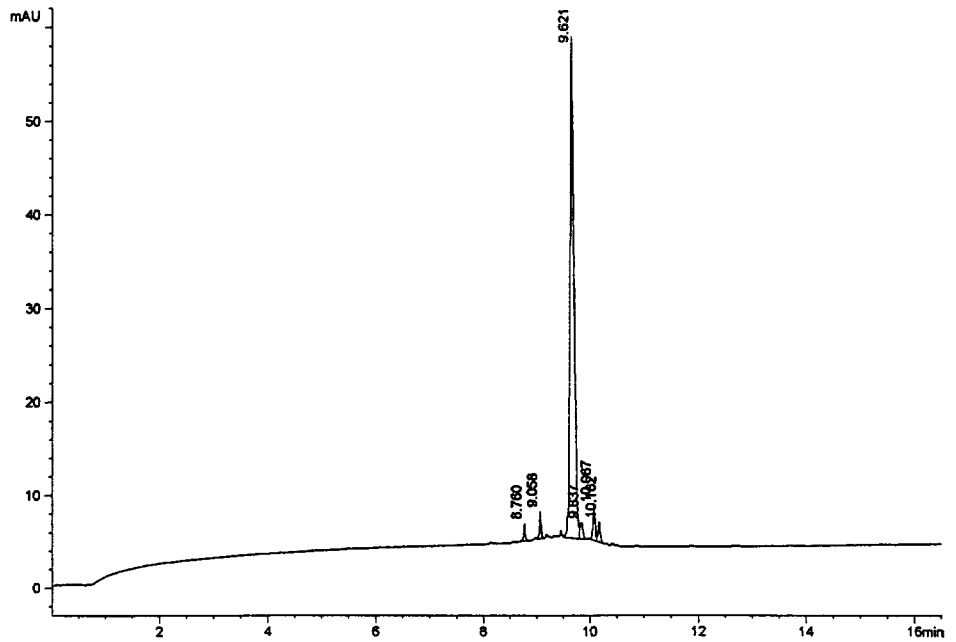

Fig. 8. Optimized separation for the purity analysis of original aFGF. Conditions: pH 2.5 phosphate (50 m*M*) 0.25% HPMC running buffer, 15 kV running voltage, 5.00 kPa injection of 1.0 mg/ml aFGF solution for 6 s.

Table 3
Precision of absolute and normalized areas in the analysis of the original aFGF sample

Peak	Mean absolute area ($n=3$)	%R.S.D.	Mean area percent ($n=3$)	%R.S.D.
1	3.49	2.17	1.22	6.91
2	5.36	0.61	1.87	9.61
3 (aFGF)	*257.19*	*9.24*	*89.39*	*0.31*
4	7.19	5.93	2.51	5.23
5	9.48	13.19	3.28	4.29
6	4.97	7.73	1.73	5.03

Results for aFGF, the major component, are italicized.

Table 4
Precision of absolute and normalized areas in the analysis of the degraded aFGF sample

Peak	Mean absolute area ($n=6$)	%R.S.D.	Mean area percent ($n=6$)	%R.S.D.
1	1.63	10.75	1.59	12.10
2	32.22	4.34	31.42	1.21
3	0.89	13.92	0.87	12.42
4	2.13	13.21	2.07	12.08
5 (aFGF)	*61.26*	*2.85*	*59.75*	*1.20*
6	1.94	8.21	1.90	5.70
7	2.47	11.94	2.40	9.32

Results for aFGF, the major component, are italicized.

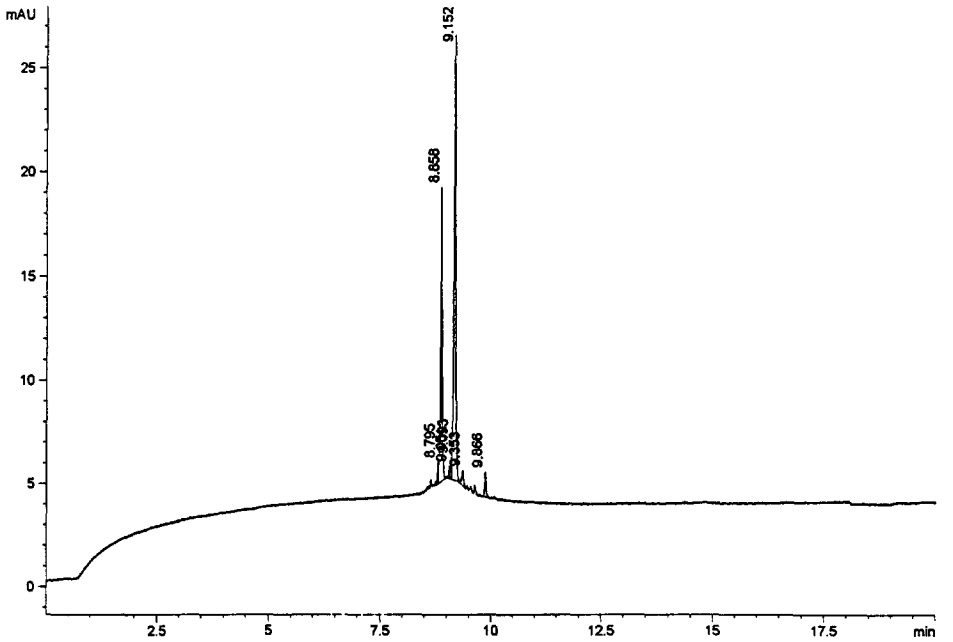

Fig. 9. Separation of degraded sample using optimized separation conditions. Conditions: pH 2.5 phosphate (50 m*M*) 0.25% HPMC running buffer, 15 kV running voltage, 5.00 kPa injection of 1.0 mg/ml degraded aFGF solution for 6 s.

divided by the total area of the peaks in the electropherogram.

The precision of absolute areas is generally poor, but the precision of the area percents is acceptable, especially for the aFGF peaks and the major impurity peak of the degraded sample. Peaks 1, 3 and 4 of the original sample (Fig. 8) have limited area reproducibility because the peaks are not baseline resolved. The integration method for these peaks greatly influences the calculated peak areas and lowers the precision of absolute peak areas. As a result, the method could be used to measure the percent composition of each component in the solution when the response factors are measured for aFGF and each of the impurities. The poor reproducibility of absolute areas may be due to the difficulty in hydrodynamically injecting viscous solutions and may improve when using electrokinetic injection. However, injection bias becomes an issue to resolve when using electrokinetic injection. To avoid this, an internal standard could be used to determine concentrations of the components of the protein solution when using hydrodynamic injection.

4. Conclusion

aFGF has become an important protein for pharmaceutical study. Its tendency to precipitate has limited the number of different analyses possible for purity analysis. A systematic procedure of testing protein stability and buffer system efficacy was used to find an appropriate CE method for aFGF purity assays. One method, which employs a simple low pH phosphate buffer with HPMC, provided higher efficiencies and greater selectivity for aFGF and its impurities than the other methods studied. It was further developed to provide a relatively rapid, convenient and rugged method of purity analysis of aFGF by capillary electrophoresis.

Acknowledgments

The authors wish to thank Chong M. Won of Rhône-Poulenc Rorer for assistance and discussions on the turbidity study and aFGF stabilization.

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