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Size exclusion HPLC method for the determination of acidic fibroblast growth factor in viscous formulations

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Abstract

A size exclusion HPLC method has been developed to determine the protein concentration of pharmaceutical formulations of recombinant acidic fibroblast growth factor (aFGF). These topical aFGF formulations not only contain low levels of protein mass (50 μg ml^{-1}), but also include buffer ions, polysaccharide polyanions to conformationally stabilize aFGF and 1% hydroxyethylcellulose to increase the solution's viscosity. A cesium chloride mobile phase is utilized during SEC-HPLC to dissociate aFGF from the pharmaceutical excipients and to minimize nonspecific interaction of the protein with the column matrix. The protein content of a viscous aFGF formulation is determined by comparison of aFGF peak areas to standards of known concentration. Fluorescence spectroscopy was utilized to directly demonstrate that the protein remains in its native conformation during sample preparation and analysis. © 1997 Elsevier Science B.V.

Keywords: Acidic fibroblast growth factor; Hydroxyethylcellulose; SEC-HPLC; Fluorescence spectroscopy

1. Introduction

Acidic fibroblast growth factor (aFGF; FGF-1), a well characterized member of a structurally related family of fibroblast growth factor proteins, stimulates mitogenic, angiogenic and chemotactic responses in a variety of cell types [1]. Due to this wide range of biological activities, fibroblast growth factors have been considered for a wide variety of clinical applications including the acceleration of wound healing [2]. The development of stable, efficacious formulations of aFGF for topical application to wounds requires both an understanding of the causes and mechanisms of the protein's inactivation during storage and the preparation of drug dosage forms appropriate for clinical use [3]. It has been demonstrated that although unliganded aFGF exhibits intrinsic conformational lability, a wide variety of polyanions bind and stabilize aFGF against thermal, pH and proteolytic degradation [3–5]. In addition, these ligands protect the protein's chemically labile cys-

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teine residues from oxidation [3,6,7]. Formulation of aFGF as a wound healing agent requires application and retention of biologically active protein at the wound site in a clinical setting. Topical application of aFGF solutions to a wound site can best be accomplished if the formulation has sufficient viscosity to prevent beading and run-off. To this end, viscous solutions of aFGF-polyanions have been prepared by including hydroxyethylcellulose [3].

Based on these considerations, it has been possible to prepare conformationally stabilized formulations of the growth factor for clinical use. These preparations, however, presented unusual analytical challenges. Methodologies are required to determine the protein content after formulation and to monitor for any changes in protein mass during storage. Although analytical HPLC methods for analogous protein growth factors such as basic fibroblast growth factor have been described, these HPLC methods have been primarily used to analyze bulk drug substance (unformulated) or to determine pathways of inactivation during storage by identifying various degradants [8,9]. The SEC-HPLC (size exclusion high performance liquid chromatography) method described in this work allows for the quantitation of aFGF concentration at low protein content (50 μg ml^{-1} or 100 ng per injection) in the presence of pharmaceutical excipients and stabilizers.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Cesium chloride (optical grade) was purchased from Gibco-BRL (Grand Island, NY, USA). Sodium phosphate monobasic and dibasic were acquired from Fisher Scientific (Pittsburgh, PA, USA). Heparin of approximately 16 kDa molecular weight was purchased from Hepar (Ohio, USA) and other polyanions were purchased from Sigma (St. Louis, USA). Recombinant acidic fibroblast growth factor (aFGF), an amino-truncated 141 amino acid form with a molecular weight of 15.9 kDa, was expressed in transformed *Eschericia coli* cells as previously described [6,7] and purified by a combination of ion-exchange and affinity chromatography [4,5]. Purified aFGF was stored at −70°C. Syringe filters (25 mm, 0.22 μm) for sample prefiltration were Millex GV from Millipore (Bedford, MA, USA).

2.2. Instrumentation

HPLC analysis of aFGF was performed using a Rainin Rabbit HP/HPX solvent delivery system (Rainin, CA, USA) connected to a refrigerated 237 autosampler (Gilson, WI, USA) fitted with a 20-μl loop. The UV detector used was either a Gilson 116 UV or a Spectrafocus forward optical scanning unit (Spectra Physics, CA, USA) operated at 215 nm. All chromatographic control and data collection were performed using Dynamax method manager software (Rainin). Chromatographic separations were performed at ambient temperature using a TOSOHASS TSK—3000SWxl size exclusion column (TOSOHASS, PA, USA), (300 × 7.5 i.d., 5 μm particle, 250 Å pore). A minimum of 2500 theoretical plates were required to meet system suitability requirements.

2.3. Fluorescence spectroscopy

Fluorescence emission spectra were measured with a Hitachi (Connecticut, USA) F-4500 fluorometer. Excitation of aFGF was at 270 nm and its emission spectrum recorded from 285 to 395 nm in a 2 × 10 mm pathlength cuvette. Viscous formulations of aFGF at 800 μg ml^{-1} protein were diluted 1:10 in either SEC-HPLC mobile phase or phosphate buffered saline, mixed for 30 min on a rotator and then analyzed at 15°C.

2.4. Chromatographic conditions and sample preparation

The mobile phase consisted of a sodium phosphate buffer (0.1 M, pH 6.8) containing cesium chloride (0.5 M). The mobile phase was filtered and degassed through a 0.45 μm filter (Corning). The flow rate was set at 1.0 ml min^{-1}. Formula-

tions of recombinant acidic fibroblast growth factor (aFGF) contained 0.05 mg ml^{-1} protein, 0.15 mg ml^{-1} heparin, 1% hydroxyethylcellulose in a 6 mM phosphate buffer with 120 mM sodium chloride (pH 7) filled into polypropylene tubes, unless otherwise stated. In order to decrease the viscosity of the formulation prior to analysis by HPLC, exactly 0.1 ml of the viscous formulation is transferred to a polyethylene tube containing 0.9 ml of mobile phase buffer. The diluted sample is rotated on a tube rotator for 30 min followed by filtration through a Millex GV filter (25 mm, 0.22 μm) of which 20 μl is injected for HPLC analysis (or the entire 1.0 ml used for fluorescence analysis).

2.5. Preparation of standards

Standard aFGF containing heparin was freshly prepared each day by diluting to the necessary concentration in mobile phase and filtered. A aFGF standard of known concentration was run before and after test samples thus allowing for concentration determination of aFGF in the viscous formulation by comparison of peak areas. All standards and formulations were assayed in duplicate and area counts were averaged.

2.6. Linearity and injection precision

Linearity was determined by making six stock formulations over the range of 800–25 μg ml^{-1} aFGF, 1% hydroxyethyl cellulose and 3X heparin (compared to aFGF by weight). Each stock was then diluted one to ten, as described in the sample preparation section, to yield six aFGF concentrations from 80 to 2.5 μg ml^{-1}; duplicate injections were performed and averaged. Linear regression was performed in order to determine the slope, intercept, standard error of the slope, standard error of the intercept and the correlation coefficient.

Injection precision was assessed using a 50 μg ml^{-1} aFGF formulation (1% hydroxyethyl cellulose, 3X heparin by weight), prepared as described above, and injecting this sample 17 times over approximately a 12-h period to determine the % R.S.D. Typically, a chromatography run containing both aFGF formulations and aFGF standards resulted in some 15–17 injections. Samples were stored refrigerated in the system autosampler until tested.

2.7. Analytical recovery and assay precision

Recovery was determined by comparing the peak area of formulated drug at various concentrations to a standard aFGF preparation of known concentration. Thus, quantitation of protein mass by comparison to an aFGF standard under the identical chromatographic conditions compensates for any potential irreversible binding of the protein to the column packing.

Inter and intra-assay precision were obtained by replicate assays of viscous formulation at 50 μg ml^{-1}. The intra-assay precision was determined from duplicate injections of three different 50 μg ml^{-1} aFGF viscous formulations. Inter-assay precision was determined from five separate time points of the same formulation over a time period in which aFGF is stable. In each case the experimental concentrations were determined and used to calculate % R.S.D., standard deviations and deviations from % theoretical values.

3. Results and discussion

3.1. Selectivity

Size exclusion HPLC was demonstrated to be able to distinguish the individual formulation components (Fig. 1A and B). For example, comparison of a formulation lacking aFGF (Fig. 1A) with a formulation containing 50 μg ml^{-1} aFGF demonstrates that no interfering excipients co-elute at the same retention time as aFGF (Fig. 1B). HEC and heparin are seen to elute significantly before and after the protein. The elution sequence, retention time and capacity factor for this SEC-HPLC method are summarized in Table 1. The growth factor elutes at approximately 10.5 min thereby allowing quantitation of protein mass within the viscous formulation by comparison to aFGF standards of known concentration (see below).

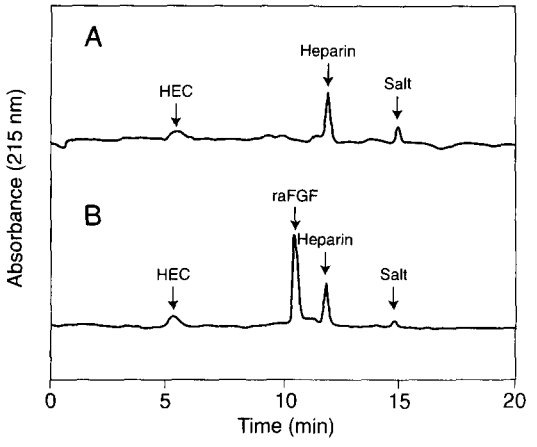

Fig. 1. Representative size exclusion HPLC chromatograms of excipient only formulation (A) and aFGF formulation, 50 μg ml^{-1} protein (B). Formulations consist of the following excipients: 1% hydroxyethylcellulose and 150 μg ml^{-1} heparin in a phosphate buffered saline solution (pH 7.2). SEC-HPLC was performed in a 0.5 M cesium chloride containing mobile phase with UV detection at 215 nm as described in the methods section.

3.2. Linearity and injection precision

The peak area counts of aFGF compared to internal standards varied linearly ($n = 6$) with concentration over the range from 2.5 to 80 μg ml^{-1} (Table 2A), with a correlation coefficient of 0.999 (the remaining statistical tests performed are listed in Table 2A). Acceptable linearity is observed at concentrations as low as 2.5 μg ml^{-1}. Thus, viscous formulations containing 50 μg ml^{-1} aFGF (5 μg ml^{-1} after dilution into the mobile phase) could be detected and losses of at least 50% during storage could be quantified. Since the y-intercept is slightly negative, the assay quantitation limit is 2.5 μg ml^{-1} aFGF, although lower quantities such as one μg ml^{-1} protein could be detected.

Table 1
Retention times and capacity factors of aFGF formulation components in an SEC-HPLC assay

Component	Retention time (min)	Capacity factor
HEC	5.5	Void volume
aFGF	10.5	0.9
Heparin	12.0	1.2

Table 2
Statistical analysis of assay linearity and injection precision of the SEC-HPLC method with aFGF at 2.5–80 μg ml^{-1} (A) and at 50 μg ml^{-1} (B) formulated with 3X heparin by weight, 1% hydroxyethylcellulose in a PBS buffer (pH 7.2)

A. Assay linearity—aFGF 2.5–80 μg ml^{-1}

Correlation coefficient	0.999
Slope ($\times 10^5$)	0.45
Intercept ($\times 10^5$)	−0.973
$\sigma_{(m)}$ ($\times 10^5$)	0.00995
$\sigma^{(a)}$ ($\times 10^5$)	0.375

$\sigma_{(m)}$ = Standard deviation of the slope
$\sigma^{(a)}$ = Standard deviation of the intercept on the y-axis

B. Injection precision—aFGF at 50 μg ml^{-1}

n	Mean (area counts) ± S.D.	% RSD
17	46188 (±4247)	9.2%

UV absorption detection of formulated aFGF at 280 nm (final injection concentration of 5 μg ml^{-1} or 100 ng of protein per injection) is very difficult since aFGF contains only a single tryptophan residue per molecule. UV detection at 215 nm (from absorbance by the peptide backbone) was thus required to detect aFGF at these low protein concentrations. By examining multiple injections ($n = 17$) of the viscous formulation of aFGF, an injection precision with a 9.2% R.S.D. was determined (Table 2B). Similar aFGF viscous formulations containing higher protein concentrations such as 500 μg ml^{-1} showed about a 2.5% R.S.D. (data not shown).

3.3. Assay precision and recovery

To determine the concentration of aFGF in a formulated sample, the peak areas from duplicate injections were averaged and then compared to the averaged peak areas of aFGF standards of known concentration that were injected before and after the test sample. Intra-assay precision for 50 μg ml^{-1} aFGF with 3X heparin formulated in 1% hydroxyethylcellulose in phosphate buffered

saline was typically observed to be 2–3% R.S.D. (average of two injections each for duplicate samples). Inter-assay analysis of the same 50 μg ml^{-1} aFGF formulation over a time period in which the aFGF is stable (five timepoints) revealed a 4.7% R.S.D. These results demonstrate the acceptability of this SEC-HPLC assay as a means to follow the protein mass content of aFGF viscous formulations over time as required by many stability protocols. Absolute recovery of aFGF from the analytical column via collection and reanalysis was not possible at these low protein concentrations (100 ng per injection). Throughout the use of this SEC-HPLC method, however, no 'carry over' from sample to sample was observed, as routinely determined by injection of placebo formulations chromatographed immediately after aFGF containing formulations, suggesting complete recovery of the sample from the column.

3.4. Detection of aFGF in alternative low dose, viscous formulations

Stabilization of formulated aFGF can be achieved by the addition of lower molecular weight, more chemically well defined sulfated polyanions than heparin [3]. In addition, chelation of trace metal ions with EDTA has also been shown to stabilize the protein by minimizing the oxidation of the protein's free cysteine residue [3–5]. This SEC-HPLC method was successfully utilized to analyze alternative aFGF formulations which contained both 0.5 mM EDTA plus various alternative stabilizing polyanionic ligands. Complete separation of aFGF from EDTA and polyanions such as sucrose octasulfate, pentosan polysulfate and sulfated β-cyclodextrin was achieved (Fig. 2B). In fact, SEC-HPLC analysis of each of these aFGF-polyanion formulations resulted in the same chromatographic profile with each component eluting at the same retention time as those observed in the heparin formulated aFGF (Fig. 2A). These results suggest that highly charged polyanionic compounds such as sulfated polysaccharides and EDTA perhaps interact with the column matrix and are thus separated from aFGF not solely by molecular weight differences.

3.5. SEC-HPLC method and the conformational stability of aFGF

By comparison to protein standards of known molecular weight, aFGF complexed to heparin or other sulfated polyanions (as described above) elutes from the sizing column (with a cesium chloride containing mobile phase) at a time expected for a molecule of molecular weight approximately 16 kDa. When the column is operated with a mobile phase lacking cesium chloride, aFGF complexed to heparin or heparin analogs such as sulfated β-cyclodextrin elutes in a heterogeneous fashion at times corresponding to higher molecular weights, presumably due to multiple sites on the polyanion in which aFGF can bind [3,10]. The cesium chloride mobile phase presumably results in the dissociation of aFGF from heparin during sample preparation and/or chromatography by shielding the electrostatic interaction responsible for complex formation [5,12].

To determine if the dissociation of heparin by cesium chloride causes a drastic change in the conformation of unliganded aFGF during sample preparation, a high dose formulation of aFGF (800 μg ml^{-1}) was examined by fluorescence spec-

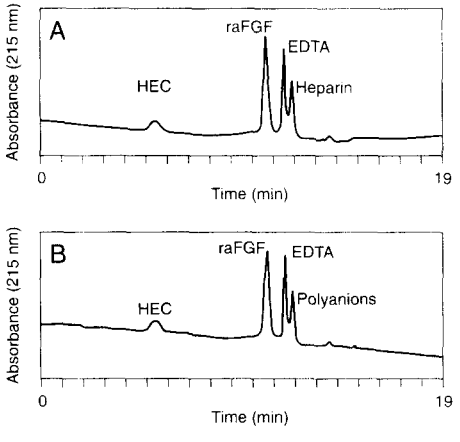

Fig. 2. SEC-HPLC chromatograms of a 50 μg ml^{-1} aFGF formulation containing 3X heparin (Fig. 2A) or sucrose octasulfate (Fig. 2B) by weight in a phosphate buffered saline solution with 1% hydroxyethylcellulose and 0.5 mM EDTA. SEC-HPLC was performed in a cesium chloride containing mobile phase with UV detection at 215 nm as described in the methods section.

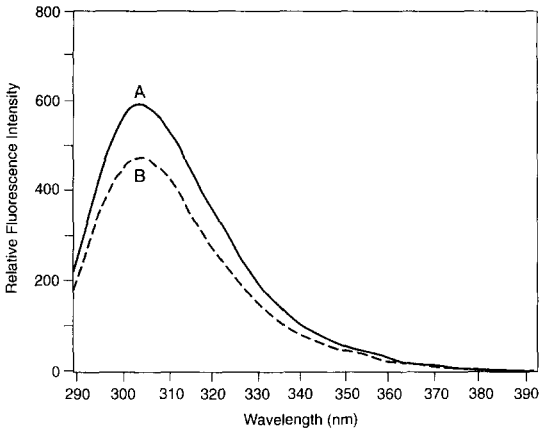

Fig. 3. Fluorescence emission spectra of formulated aFGF (800 μg ml^{-1}) protein with 3X heparin by weight in a PBS buffer containing 1% hydroxyethylcellulose) diluted (1:10) into either PBS (A) or a cesium chloride containing SEC-HPLC mobile phase (B). The excitation wavelength is 270 nm.

troscopy (Fig. 3). This optical method is a very sensitive technique to monitor the conformational integrity of aFGF [11]. The native, folded conformation of aFGF has a tyrosine dominated fluorescence emission spectrum containing a maximum at 305 nm. The unfolding of aFGF results in the unquenching of the single tryptophan residue resulting in a tryptophan dominated fluorescence emission spectrum with a maximum at 350 nm [11]. Freshly prepared viscous formulations of aFGF (diluted 10-fold in either phosphate buffered saline or the SEC-HPLC mobile phase and mixed for 30 min) showed no significant change in conformation as evidenced by a fluorescence emission wavelength maximum at approximately 305 nm (Fig. 3; the difference seen in fluorescence intensity is predominantly due to the partial quenching of the protein's fluorescence signal in the presence of high concentrations of cesium chloride; data not shown). Although aFGF is conformationally unstable in the absence of polyanions (leading to aggregation of the protein [3]), the protein appears to be sufficiently stable under these SEC-HPLC sample preparation conditions since no major conformational alteration of the protein is observed. This is probably due to either some remaining complexion of the heparin polymer or the conformational stabilization of unliganded aFGF by high concentration of chloride anion [12].

Due to its free cysteine residue, aFGF is susceptible to intermolecular disulfide formation resulting in soluble and insoluble aggregate formation as detected by SDS-PAGE [3]. This SEC-HPLC method could potentially detect these soluble, covalent aFGF aggregates if appearing during storage. Soluble aFGF aggregates would theoretically elute between the HEC and monomeric aFGF peaks while larger, insoluble aggregates would be removed by 0.22 μm filtration of the sample prior to SEC-HPLC analysis. In practice, however, it was not possible to quantify the amount of aFGF aggregate formation directly by this SEC-HPLC method perhaps due to either detection sensitivity limitations at these low protein concentrations or loss of higher molecular weight aggregates on the column matrix. Protein mass was therefore determined by monitoring the native protein peak area and the aggregation of the protein (or adsorption to the container) during storage was followed indirectly by monitoring loss of the native protein peak area.

4. Conclusions

Development of a rapid non-denaturing HPLC method is important in the analysis of total mass of active drug in formulated protein based pharmaceutical products. The method described in this work permits the accurate quantitation of formulated aFGF at low protein concentrations (50 μg ml^{-1}) in the presence of both a viscous delivery system (hydroxyethylcellulose) and various stabilizers such as sulfated polyanions and EDTA. This SEC-HPLC procedure shows good sensitivity, reproducibility and assay precision and has been used extensively to monitor the in vitro stability of viscous formulations of aFGF during storage by quantitatively measuring changes in protein mass over time.

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