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Preparative isolation of recombinant human insulin-like growth factor 1 by reversed-phase high-performance liquid chromatography

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Abstract

The isolation of recombinant human insulin-like growth factor 1 (rhIGF-1) is complicated by the presence of several rhIGF-1 variants which co-purify using conventional chromatographic media. These species consist primarily of a methionine-sulfoxide variant of the properly folded molecule and a misfolded form and its respective methionine-sulfoxide variant. An analytical reversed-phase high-performance liquid chromatography procedure using a 5-μm C_{18} column, an acetonitrile–trifluoroacetic acid (TFA) isocratic elution, and elevated temperature gives baseline resolution of the four species. Using this analytical method as a development tool, a process-scale chromatography step was established. The 5-μm analytical packing material was replaced with a larger-size particle to reduce back-pressure and cost. Since the TFA counter-ion binds tightly to proteins and is difficult to subsequently dissociate, a combination of acetic acid and NaCl was substituted. Isocratic separations are not good process options due to problems with reproducibility and control. A shallow gradient elution using premixed mobile phase buffers at the same linear velocity was found to give an equivalent separation at low load levels and minimized solvent degassing. However, at higher loading there was a loss of resolution. A matrix of various buffers was evaluated for their effects on separation. Elevated pH resulted in a significant shift in both the elution order and relative retention times of the principal rh-IGF-1 variants, resulting in a substantial increase in effective capacity. An increase in the ionic strength further improved resolution. Several different media were evaluated with regard to particle size, shape and pore diameter using the improved mobile phase. The new conditions were scaled up 1305-fold and resulted in superimposable chromatograms, 96% recovery and >99% purity. Thus, by optimizing the pH, ionic strength and temperature, a high-capacity preparative separation of rhIGF-1 from its related fermentation variants was obtained.

1. Introduction

Human insulin-like growth factor-1 (IGF-1) is a peptide of M_r 7649 with a pI of 8.4 [1,2] belonging to a family of somatomedins with insulin-like and mitogenic biological activities which modulate the action of growth hormone [3–6]. IGF-1 has hypoglycemic effects similar to

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insulin but also promotes positive nitrogen balance [7,8]. Due to this range of activities, IGF-1 is being tested in humans for uses ranging from wound healing to the reversal of whole-body catabolic states [9].

Genetically engineered biopharmaceuticals are typically purified from a supernatant containing a variety of diverse host cell contaminants. The recombinant human IGF-1 (rhIGF-1) peptide was isolated through a recovery process which utilized conventional low-pressure chromatography. However, the final process pool contained several variant species of rhIGF-1 which were difficult to separate. In this paper we describe a systematic optimization strategy for the purification of rhIGF-1 from its related fermentation variants using reversed-phase high-performance liquid chromatography (RP-HPLC).

2. Experimental

2.1. Reagents

The following chemicals were used: HPLC-grade acetonitrile (ACN; J.T. Baker, Phillipsburg, NJ, USA); HPLC-grade trifluoroacetic acid (TFA; Pierce, Rockford, IL, USA); analytical reagent-grade NaCl, dibasic sodium phosphate, hydrochloric acid, sodium hydroxide and acetic acid (HAc) (Mallinckrodt, Paris, KY, USA). All aqueous mobile phases were made using purified water. pH adjustment was done with HCl or NaOH. All buffers were 0.2-μm-filtered prior to use.

Analytical RP-HPLC was performed on a 25 × 0.46 cm stainless-steel column pre-packed with 5-μm, 300-Å, trifunctional, Vydac C_{18} spherical silica (Separations Group, Hesperia, CA, USA). The development of preparative RP-HPLC methods was carried out using a 30 × 0.39 cm stainless-steel column pre-packed with 15-μm, 300-Å, monofunctional, Waters C_4 spherical silica (Millipore, Waters Chromatography Division, Milford, MA, USA). The other preparative RP-HPLC columns which were evaluated include: Bakerbond C_4 (J.T. Baker); YMC-C_8 (Yamamura, Morris Plains, NJ, USA); Kromasil C_8 (Eka Nobel, Surte, Sweden); Amicon C_8 (Amicon, Danvers, MA, USA); Impaq C_4 (PQ Corp., Valley Forge, PA, USA); PLRP-S (Polymer Labs., Amherst, MA, USA); Eurosil-Bioselect (Paxxis, Belmont, CA, USA). Pilot- and process-scale RP-HPLC were performed using the Waters C_4 packed in 30 × 4.7 cm and 60 × 10 cm radial compression cartridges, respectively.

Additional chemicals purchased for electrophoresis include: premixed Tris–glycine and Tris–tricine sodium dodecyl sulfate (SDS) buffers (Novex, San Diego, CA, USA); methanol (J.T. Baker); Coomassie R-250 and Coomassie G-250 (Eastman Kodak, Rochester, NY, USA); sulfuric acid and glycerol (Mallinckrodt); trichloroacetic acid (Fisher Scientific, Fairlawn, NJ, USA).

2.2. Equipment

The Vydac C_{18} RP-HPLC analysis and Waters C_4 preparative RP-HPLC methods development were both carried out on a Hewlett-Packard 1090 HPLC system (Hewlett-Packard, North Hollywood, CA, USA), equipped with a ternary gradient system and diode-array detector. The pilot-scale RP-HPLC was done using a Waters DeltaPrep 600-E controller, LC-3000 pumping system fitted with 180 ml/min heads and a 30 × 4.7 cm PrepPak radial compression module (RCM). The preparative RP-HPLC was accomplished using a Biotage KiloPrep-250 system (Biotage, Charlottesville, VA, USA) and a 60 × 10 cm RCM.

2.3. Analysis

Purity was assessed by 12% SDS–polyacrylamide gel electrophoresis (PAGE) (Integrated Separation Systems, Hyde Park, MA, USA), pH 3.5–9.5 isoelectric focusing (IEF) gels (Pharmacia LKB Biotechnology, Piscataway, NJ, USA), and analytical RP-HPLC using a 25 × 0.46 cm Vydac C_{18} column and Hewlett-Packard 1090 HPLC system. The SDS gels were run with Tris–tricine buffers [10] and Coomassie-R250

stained [11]. The low-percentage-polyacrylamide IEF gels could not be stained with silver or Coomassie R-250 because the IGF-1 diffuses out during the staining procedure. The gels were therefore stained in the absence of alcohol with Coomassie G-250 [12].

3. Results and discussion

3.1. Analytical separation

A preparative-scale RP-HPLC step in the down-stream processing of recombinant proteins is typically implemented late in the recovery scheme. This strategy is utilized to help maximize the efficiency of the separation and the column lifetime by removing the majority of the contaminants during previous purification steps. The RP-HPLC step for rhIGF-1 was developed after several conventional low-pressure chromatographic steps had produced a partially purified process pool primarily containing rhIGF-1 and its variants. The pool appeared to be homogeneous by SDS-PAGE and IEF analysis (Fig. 1A and B). However, RP-HPLC analysis on a 25 × 0.46 cm Vydac C_{18} column using a 10–60% ACN/50 min gradient at 22°C revealed that other species were present (Fig. 1C). The expanded chromatogram (Fig. 1C, inset) shows two major peaks and some minor peaks. These minor later eluting peaks are immunoreactive to antibodies directed against IGF-1 and appear to be sequential multimeric species of IGF-1 by immuno-blot analysis (data not shown). Using the same column at elevated temperature (50°C), an isocratic separation baseline resolved the mixture into its four respective constituents (Fig. 1D) [13]. N-Terminal sequence, peptide mapping, RP-HPLC and mass spectrometry analysis (data not shown) were performed on the four collected peaks which identified them as rhIGF-1, a methionine-sulfoxide variant, a misfolded form, and the respective misfolded-methionine-sulfoxide form. These results are consistent with those previously reported [14–16].

3.2. Initial scale-up considerations

The two initial development goals were finding a suitable replacement buffer for TFA and translating the analytical separation of rhIGF-1 variants to preparative media. The TFA counter-ion used in the analytical separation appeared to form a tight ion pair with the product and was difficult to remove in subsequent process steps. This strong ion-pair interaction has been previously observed for synthetic peptides [17]. A similar separation to 0.1% TFA, 28.5% ACN was achieved on the Vydac C_{18} column by using a low-ionic-strength acetate–halide pH 3 buffer (20 m*M* HAc, 20 m*M* NaCl) in 27.5% ACN (Fig. 2). The beneficial ion-pairing effects of NaCl in RP-HPLC have been previously reported [18]. HAc or NaCl levels higher than 20 m*M* showed no further increase in resolution. The retention times were stabilized by changing the elution conditions from isocratic to a very shallow linear gradient, 27–28% ACN over 40 min.

The acetate buffer mobile phase optimized on the Vydac C_{18} column was adapted to a Waters C_4 preparative medium packed in a 30 × 0.39 cm column (Fig. 3A). The shorter C_4 alkyl chain substitution was chosen to maximize product recovery [19]. Irreversible binding of insulin and proinsulin to C_{18} stationary phases has recently been reported [20]. Using the acetate buffer at 50°C, four peak fractions were collected during a shallow gradient elution, 27–28% ACN over 40 min. Fractions 1–4 were analyzed on the Vydac C_{18} column as described above with the following modifications. To increase analytical throughput, a rapid, near-isocratic (27–28% ACN with 0.1% TFA) method was developed by increasing the flow-rate from 0.5 to 2 ml/min. The gradient volume was kept constant by shortening the duration from 40 to 10 min. Vydac C_{18} analysis of the four peak fractions collected during the Waters C_4 preparative chromatography confirmed that the elution order of the rhIGF-1 species using the acetate buffer was identical to the order observed initially with TFA (Fig. 3B).

As increasing levels of protein are consecutive-

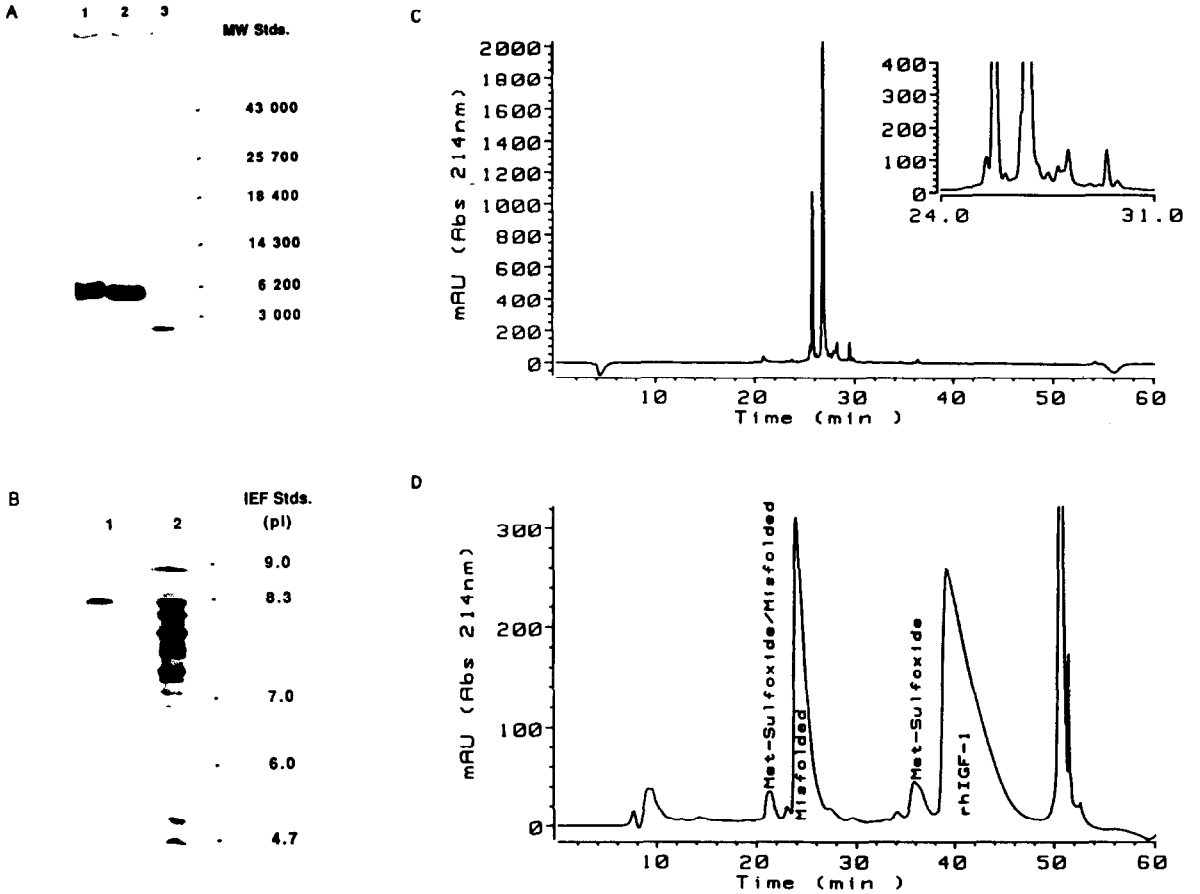

Fig. 1. Characterization of starting material. (A) Analysis by Tris–tricine SDS-PAGE. Samples of a partially purified rhIGF-1 process pool and molecular mass standards (MW Stds.) were loaded as follows: lanes: 1 = process pool, non-reduced, 2 μg rhIGF-1; 2 = process pool, reduced, 2 μg rhIGF-1; 3 = low-molecular-mass standards, reduced. The gel was stained with Coomassie-R250. (B) Analysis by IEF. Process pool sample and p*I* standards were loaded as follows: lanes: 1 = rhIGF-1, 10 μg; 2 = p*I* standards. The gel was stained with Coomassie-G250. (C) Gradient RP-HPLC analysis. The chromatography was performed on a 5-μm Vydac C_{18} column using a 10-60% ACN gradient over 50 min, 0.1% TFA, 1 ml/min, 22°C. The process pool sample was diluted 5-fold into the initial mobile phase and 250 μl were loaded. The inset shows an expanded view of the later-eluting peaks. (D) Isocratic RP-HPLC analysis. The chromatography was performed isocratically on a 5-μm Vydac C_{18} column at 28.5% ACN, 0.1% TFA, 0.5 ml/min, 50°C. The process pool sample was diluted 10-fold into the mobile phase and 25 μl were loaded. After 40 min, the column was washed with 60% ACN to remove the more hydrophobic species. The four predominant rhIGF-1 variants are labeled on the chromatogram.

ly loaded onto a RP-HPLC column, from an analytical load to a preparative mass overload condition, the non-linear elution peak profile takes the shape of a right triangle [21]. When a mixture of components is loaded to this level, the highly concentrated leading edge of a product peak can be displaced forward during the elution into the next less hydrophobic species while the dilute trailing edge continues to elute true. This observation is thought to be due to a combination of the various component equilibrium isotherms and the intrinsic column efficiency [22–24]. For most separations, the optimal condition is usually achieved by maximizing the difference in retention time between the product and any leading edge contaminants, thereby effectively augmenting the effective capacity of the separation. The effective capacity

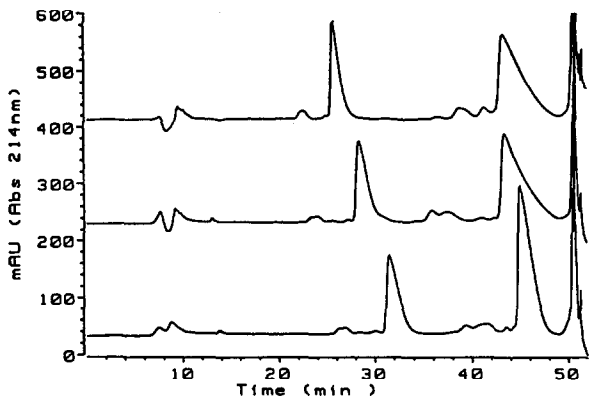

Fig. 2. Counter-ion substitution and gradient optimization. The chromatography was performed at 50°C on a 5-μm Vydac C_{18} column at 0.5 ml/min; isocratically at 28.5% ACN–0.1% TFA (upper trace); isocratically at 27.5% ACN, 20 m*M* HAc, 20 m*M* NaCl (middle trace); or with 20 m*M* HAc, 20 m*M* NaCl using a shallow 27–28% ACN/40 min gradient (lower trace).

is defined as the dynamic capacity (mg) of well resolved product per unit bed volume (ml). When the capacity of the C_4 column using the acetate buffer mobile phase was evaluated the non-linear profile became apparent. Even moderate loading (50 μg of rhIGF-1/ml bed volume) caused a loss in resolution between the product and the leading edge variants (Fig. 3C).

3.3. Parameter evaluation

After transferring the analytical method to a preparative medium, the second goal was to optimize the selectivity of the mobile phase for enhanced resolution which would ultimately translate to higher effective capacity. However, the evaluation of even a limited number of variables increases as a power function of the combinations. Therefore, to efficiently optimize this system, a limited three-dimensional set of conditions was established within the framework of the following parameters: pH (3, 5 and 7), buffer concentration (20 and 100 m*M*) and temperature (22 and 50°C). For this screen the solvent was limited to ACN and the counter-ions to sodium, chloride, phosphate and acetate. These conditions were chosen following considerations of buffer–solvent compatibility, instru-

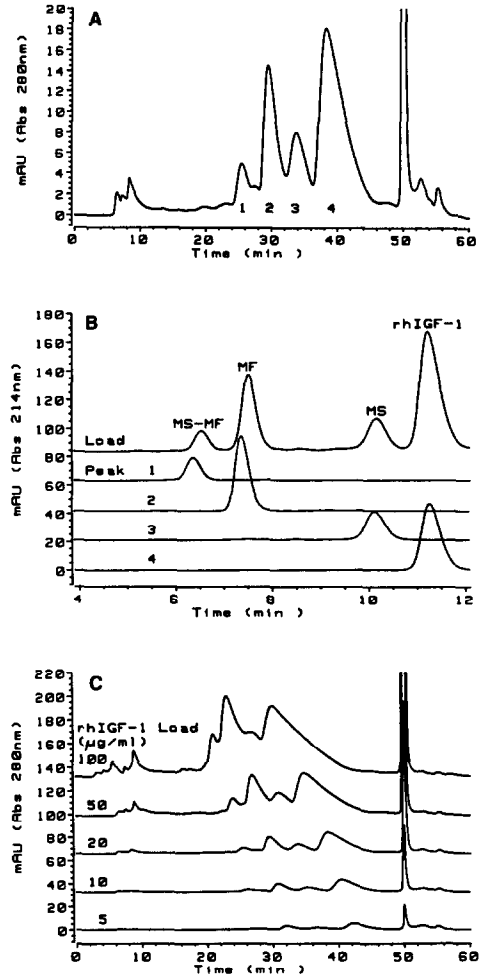

Fig. 3. Preparative evaluation of acetate buffer pH 3 mobile phase. (A) Analytical separation on preparative media. The preparative chromatography was performed on an analytical-size, 30 × 0.39 cm, 15-μm Waters C_4 column: 27–28% ACN/40 min, 20 m*M* HAc, 20 m*M* NaCl, pH 3, 0.5 ml/min, 50°C. rhIGF-1 (20 μg) was loaded onto the column and four peak fractions were collected. (B) Analysis of peak fractions. The rapid analytical chromatography was performed on a 5-μm Vydac C_{18} column using a modified version of the initial analysis (Fig. 1D). The flow-rate was increased to 2 ml/min at 50°C. A 28–29% ACN/10 min gradient, with 0.1% TFA, maintained a constant gradient volume. The fractions collected from (A) were diluted two-fold with water and 100 μl were injected. The four predominant rhIGF-1 species are labeled on the chromatogram: 1 = methionine-sulfoxide/misfolded (MS-MF); 2 = misfolded (MF); 3 = methionine-sulfoxide (MS); 4 = rhIGF-1. (C) Effective loading capacity. The chromatography was performed as in (A). Increasing levels of rhIGF-1 were sequentially loaded onto the column from 5 to 100 μg rhIGHF-1/ml bed volume.

mentation limits and potential molecular instability.

A Waters C_4 column was equilibrated and loaded at 10% ACN for each mobile phase counter-ion condition, then ramped over 1 min to the initial gradient condition. The solvent level was adjusted such that the rhIGF-1 would elute during a 1% ACN gradient over 40 min. Low load levels (20 μg rhIGF-1/ml bed volume) were used throughout the evaluation to allow easy peak identification. Comparing the acetate buffer mobile phase Waters C_4 chromatography at 22 and 50°C resulted in a dramatic peak sharpening (Fig. 4A and B). This effect is

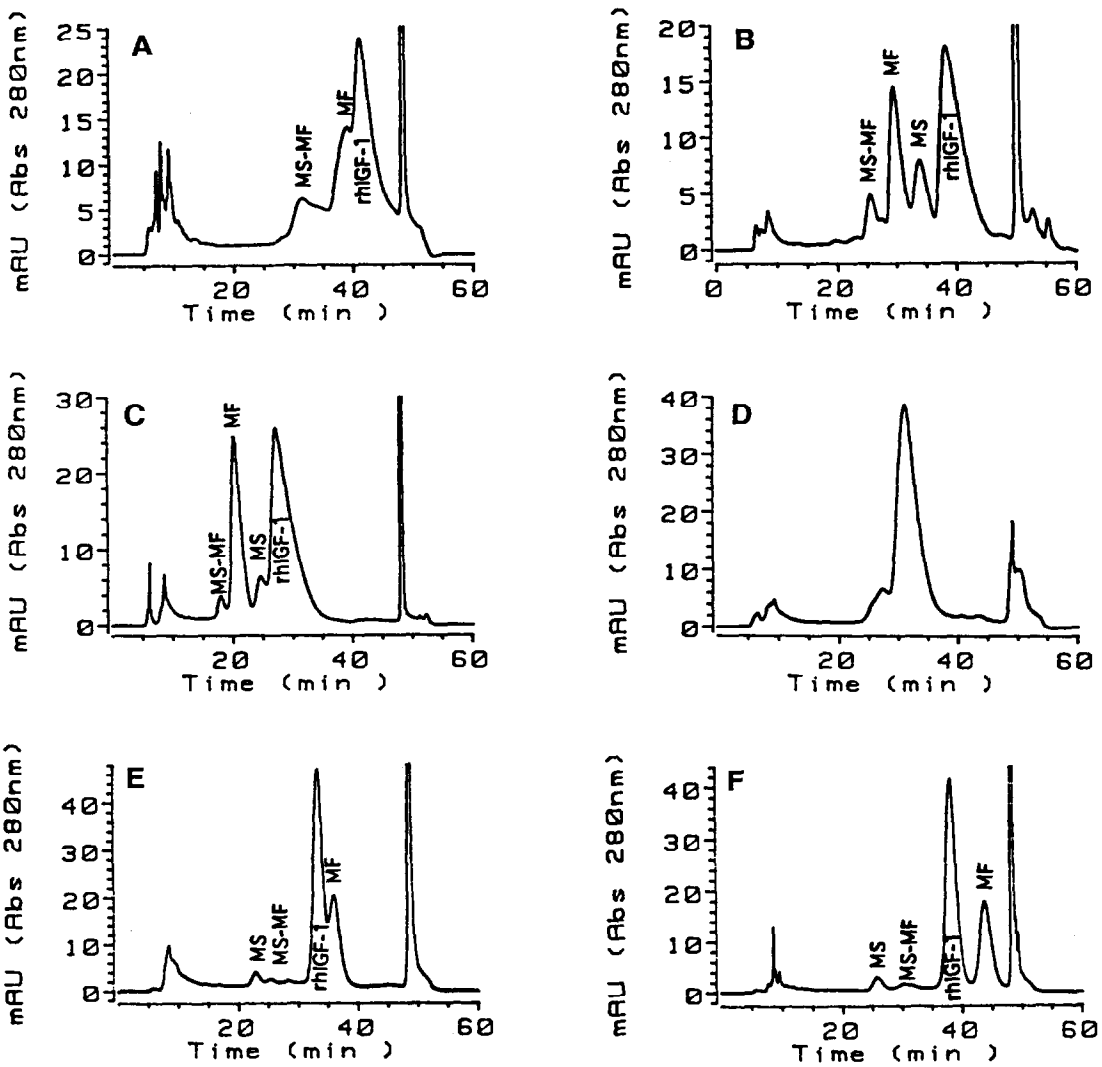

Fig. 4. Optimization of pH and ionic strength. The chromatography was performed on an analytical-size, 30 × 0.39 cm, 15-μm Waters C_4 column equilibrated in 10% ACN and the respective counter-ion. The % ACN was ramped in 1 min to the initial gradient condition. The solvent strength was independently modified such that the rhIGF-1 would elute during a near-isocratic 1% ACN/40 min gradient at 0.7 ml/min. The load level was maintained at 20 μg rhIGF-1/ml bed volume. The four predominant rhIGF-1 species are labeled on the chromatogram: methionine-sulfoxide/misfolded (MS-MF), misfolded (MF), methionine-sulfoxide (MS) and rhIGF-1. (A) 20 m*M* HAc, 20 m*M* NaCl, pH 3, 22°C, 26–27% ACN; (B) 20 m*M* HAc, 20 m*M* NaCl, pH 3, 50°C, 27–28% ACN; (C) 20 m*M* H_3PO_4, 20 m*M* NaCl, pH 3, 50°C, 27–28% ACN; (D) 20 m*M* HAc, 20 m*M* NaCl, pH 5, 50°C, 26–27% ACN; (E) 20 m*M* Na_2HPO_4, pH 7, 50°C, 23–24% ACN; (F) 100 m*M* Na2HPO4, pH 7, 50°C, 23–24% ACN.

presumably due to increased diffusivity through a viscosity mediated decrease in the resistance to mass transfer [25]. Since the higher-temperature effects appeared generally beneficial, the 50°C condition was maintained in all subsequent experiments. Concerning product stability, material generated at the higher temperature was biologically active. However, activity may be compromised at temperatures higher than 50°C. Changing the counter-ion from 20 m*M* acetate buffer (HAc–NaCl) to 20 m*M* phosphate buffer (20 m*M* H_3PO_4 + 20 m*M* NaCl, pH adjusted to 3 with NaOH) while maintaining the pH at 3 had no major effect on resolution (Fig. 4C). Increasing the pH from 3 to 5 using acetate buffer caused all four species to co-elute (Fig. 4D). However, a significant change in selectivity resulting in a further shift in both relative retention time and elution order of the four rhIGF-1 variants occurred with an increase in the pH using 20 m*M* phosphate buffer, pH 7 (20 m*M* Na_2HPO_4, pH adjusted to 7 with HCl, Fig. 4E). Based on peak area, it was shown that the misfolded peak eluted after the main rhIGF-1 peak and the methionine-sulfoxide peak shifted from the leading edge of the product to the beginning of the gradient. The gradient conditions necessary for elution changed from 26–27% (pH 3) to 23–24% ACN (pH 7). The optimum conditions were achieved by raising the buffer concentration from 20 to 100 m*M* phosphate (Fig. 4F). Enhanced resolution correlated with an increase in peak symmetry (Table 1). This change in selectivity with increased pH could be caused by an ion-pair suppression of hydrophilic or non-specific ionic interactions of rhIGF-1 with the base matrix [26], an observation also made for other basic molecules [27]. Alternatively, if the solubility of rhIGF-1 is enhanced in the 100 m*M* phosphate buffer pH 7 mobile phase, it could potentiate rapid partitioning and a concomitantly sharper peak shape [28].

Four peak fractions were isolated from the optimized (100 m*M* phosphate–ACN, pH 7, 50°C) Waters C_4 preparative chromatography conditions (Fig. 5A). Analysis on the Vydac C_{18} column confirmed the relative mobility of the rhIGF-1 variants (Fig. 5B). A fifth variant which was apparently co-migrating with the main rhIGF-1 peak using the acetate buffer is now well resolved (peak 2 analysis) and was identified as a hydroxamate species [13]. By starting with the protein load level used during the methods development and then increasing the load in subsequent runs by a factor of two, the optimized chromatography appears to have an effective capacity >1000 μg rhIGF-1 product/ml of bed volume (Fig. 5C). This 100-fold enhancement in the effective capacity between the initial acetate and optimized phosphate mobile phases is primarily due to the misfolded rhIGF-1 peak shift and the 5-fold increase in the difference in relative retention times between rhIGF-1 and the less hydrophobic variant species, and is especially pronounced during non-linear elution due to mass overloading, as described earlier.

Additional analytical size columns packed with a variety of preparative RP-HPLC media from

Table 1
Effects of pH and buffer concentration on resolution and peak symmetry

Counter-ion	pH	Buffer concentration(m*M*)	Temperature (°C)	Main, A_s	Main/Met, R_s	Main/Mis, R_s
HAc + NaCl	3	20	22	0.64	*	0.28
HAc + NaCl	3	20	50	0.37	0.65	1.51
H_3PO_4 + NaCl	3	20	50	0.29	0.47	1.39
HAc + NaCl	5	20	50	*	*	*
Na_2HPO_4	7	20	50	0.70	2.97	0.74
Na_2HPO_4	7	100	50	0.78	3.48	1.51

* = Peaks overlap. Main peak symmetry [19], $A_s = B/A$, where A, B = half peak width from vertical line from peak apex. Resolution, $R_s = 1.18(t_{R,2} - t_{R,1})/(w_2 + w_1)$, where t_R = retention time and w = peak width at half peak height. Main/Met = R_s between main IGF-1 peak and met-sulfoxide peak; Main/Mis = R_s between main IGF-1 peak and misfolded peak.

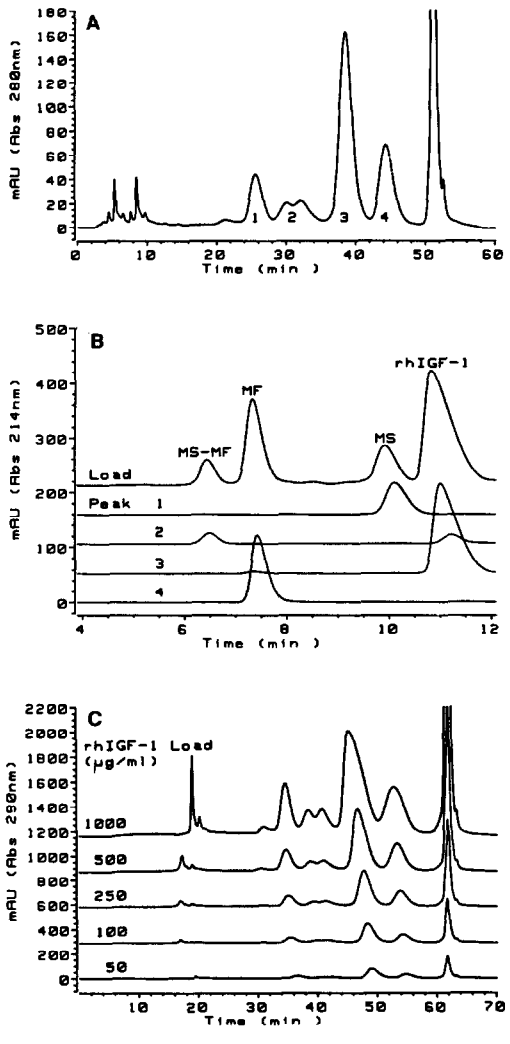

Fig. 5. Preparative evaluation of 100 m*M* phosphate buffer pH 7 mobile phase. (A) Differential selectivity at pH 7. The preparative chromatography was performed on a 15-μm Waters C_4 column using the optimized conditions (Fig. 4F), 23–24% ACN/40 min, 100 m*M* Na_2HPO_4, pH 7, 0.7 ml/min, 50°C. rhIGF-1 (100 μg) was loaded onto the column and four peak fractions were collected. (B) Analysis confirms shift in retention times. The rapid analytical chromatography was performed on a 5-μm Vydac C_{18} column as in Fig. 3B. The fractions collected from (A) were diluted two-fold with water and 100 μl were injected. The order of elution of the species was altered as labeled on the chromatogram: 1 = methionine-sulfoxide (MS); 2 = methionine-sulfoxide/misfolded (MS-MF) and a second rhIGF-1 co-eluting peak; 3 = rhIGF-1; 4 = misfolded (MF). (C) Effective loading capacity. The chromatography was performed as in (A). Increasing levels of rhIGF-1 were sequentially loaded onto the column from 50 to 1000 μg rhIGF-1/ml bed volume.

other suppliers were evaluated using the optimized 100 m*M* phosphate buffer, pH 7, ACN mobile phase (Fig. 6A–F). The conditions were adjusted as necessary for each individual column in order to have the product elute within a similar gradient slope (1% ACN gradient per 40 min). Chromatography on the various media resulted in similar profiles, independent of base matrix (silica or polymer), alkyl chain length (C_4 or C_8) or pore diameter (200–300 Å). Differences in particle size (7–20 μm) and pore diameter can significantly alter surface area and appear to affect resolution. The Amicon medium was unable to resolve the different variants under these conditions, although it was not independently optimized further to improve the separation.

3.4. Scale-up

The optimized preparative chromatography was scaled in two stages. The Hewlett-Packard 1090 HPLC system used for method development delivers precise solvent blending by utilizing separate dual-syringe metering pumps for each reservoir to determine composition and flow, and is accurate even when using neat aqueous and organic phases. When neat solvents were tested with either the pilot-scale Waters DeltaPrep or preparative-scale Biotage KiloPrep instruments, neither one had the absolute accuracy to deliver the 1% gradient needed for the separation. These chromatographs use low-pressure mixing with solenoid-based gradient formation. This problem was overcome by premixing the 100 m*M* phosphate buffer with 20% ACN (A buffer) and 40% ACN (B buffer). In addition, these premixed buffers required no further degassing to prevent cavitation. The Waters RCM and cartridge format of column scale-up was chosen for its ability to easily and economically utilize the same column hardware for different products, in addition to the radial compression technology, *per se*. Of the columns tested, the Waters C_4 medium appeared to have comparable resolution and was readily available in the cartridge format. A counter-current heat exchanger

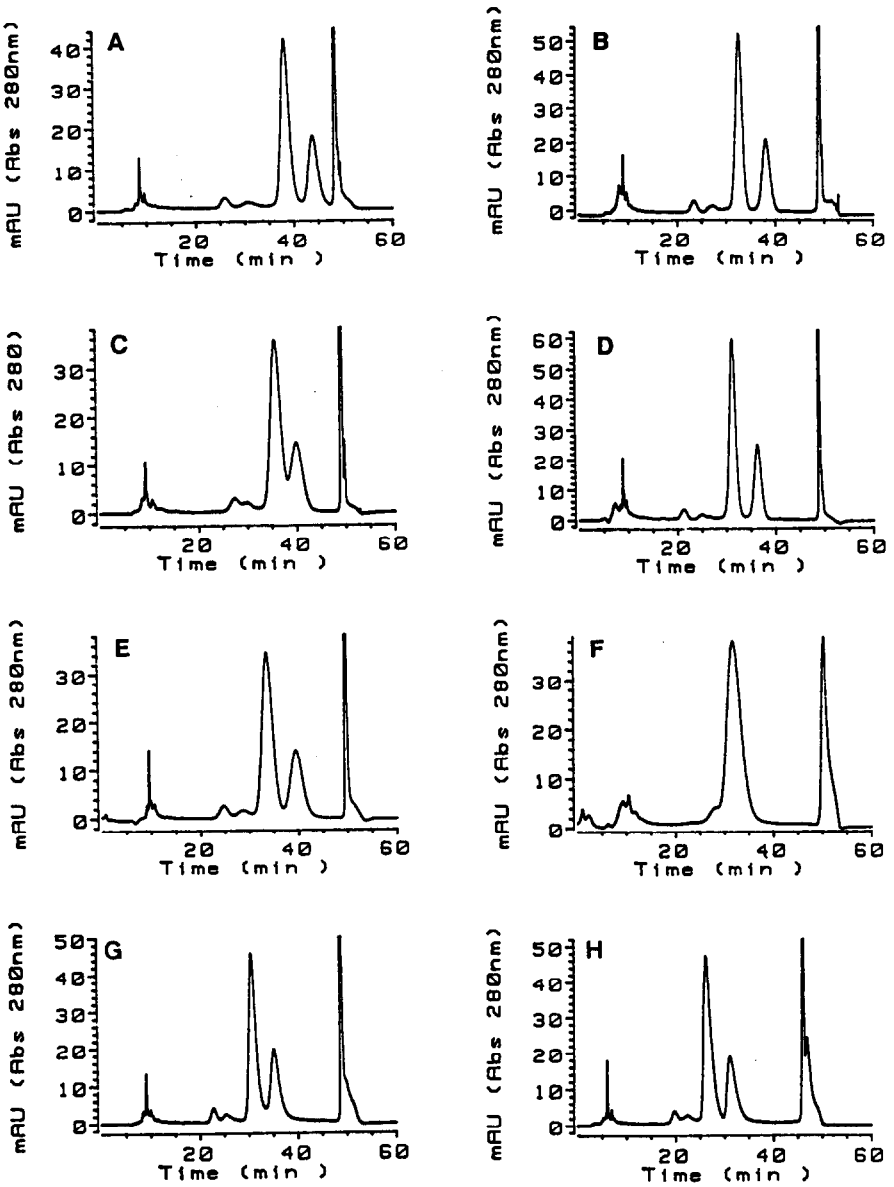

Fig. 6. Comparison of different preparative media. The chromatography in each case was performed on columns with similar geometry, using the optimized 100 m*M* phosphate buffer pH 7 mobile phase, 0.7 ml/min, 50°C. The load level was maintained at 20 μg rhIGF-1/ml bed volume. (A) Waters C_4, 15 μm, 300 Å, 23–24% ACN; (B) YMC-C_8, 15 μm, 300 Å, 23.5–24.5% ACN; (C) Baker C_4, 15 μm, 275 Å, 22.5–23.5% ACN; (D) Kromasil C_8, 10 μm, 200 Å, 25–26% ACN; (E) Impaq C_4, 20 μm, 200 Å, 25–26% ACN; (F) Amicon C_8, 20 μm, 250 Å, 26–27% ACN; (G) PLRP-S, 8 μm, 300 Å, 23–24% ACN; (H) Eurosil C_4, 7 μm, 300 Å, 21.5–22.5% ACN.

and recirculating water bath was placed in-line with the column and the temperature of the column inlet and outlet monitored with thermocouples to maintain a 50°C mobile phase throughout the separation.

The first scale increase from the Hewlett-Pac-

kard 1090 to the Waters Delta-Prep System involved a 145-fold increase in cross-sectional area at constant column length, from the analytical size 30 × 0.39 cm (3.58 ml bed volume) stainless-steel column to a 30 × 4.7 cm (520 ml) radial compression cartridge. By using the same gradient slope of 1% ACN over 40 min and maintaining the residence time at approximately 5 min (5.76 cm/min), the flow-rate was increased to 100 ml/min with identical chromatography. To initiate a sequence of cycles, a single blank run through the system fully equilibrated the column from room temperature to 50°C (as measured at the column outlet) with a 1°C temperature drop across the column.

The second scale increase from the Waters DeltaPrep to the Biotage KiloPrep System was accomplished in both column dimensions. The cross-sectional area was increased 4.5-fold and the length 2-fold to a 60 × 10 cm (4.71 l) cartridge for an additional 9-fold, or an overall total of 1305-fold scale-up relative to the analytical column. Since the bed geometry had changed, the flow-rate was adjusted based on a linear velocity to 450 ml/min, using a proportionately larger heat-exchanger. The cycle time was increased to account for the slower flow-rate, maintaining the same relative gradient volume. The chromatogram was similar to those from the analytical and pilot scale runs. The purity of the collected product peak was >99% rhIGF-1, as assessed by the Vydac C_{18} analysis. At load levels of 1000 μg rhIGF-1/ml of bed volume, 4.5 g of product can be processed per cycle in the large RCM. The mass balance of product across the column at the various scales is identical (Table 2).

4. Conclusions

Because of its stable structure, small size, and relatively hydrophobic character, the selectivity of RP-HPLC was utilized to isolate rhIGF-1 from its fermentation variants. Procedures utilizing RP-HPLC have been published for many molecules [29], and are included in the purification of native, synthetic and rhIGF-1 [14,30–34], However, few papers have shown a systematic optimization of a preparative RP-HPLC isolation step of a recombinant protein resulting in pharmaceutical purity [35]. Preparative chromatography differs from analytical chromatography in three principal ways which dictate the approach of the respective methods development. (a) Purity—when isolating a single species from a complex mixture, only the resolution of the product from its closest eluting neighbors is of interest. In addition, the use of mobile phase solvents and modifying agents whose pharmacological safety is questionable, especially if they interact tightly with the product and whose removal necessitates validation, should be restricted. (b) Effective capacity—high yield, recovery and throughput should be developed in parallel with an optimized separation to address questions relating to process economics and feasibility. (c) Molecular integrity—bioactivity of the product must be maintained.

The practical limitations of process-scale RP-HPLC include the high cost of small-particle high-efficiency packing material and the high-pressure equipment needed to generate moderate flow-rates [36]. Assuming that the integrity of the molecule is not compromised by the potentially denaturing conditions of reversed-phase

Table 2
Recovery of rhIGF-1 during RP-HPLC scale-up

Column	Dimensions (cm)	Bed volume	Scale-up	Purity	Recovery	
					Mass	%
Analytical	30 × 0.39	3.58 ml	1-fold	>99	3.5 mg	97
Pilot RCM	30 × 4.7	520 ml	145-fold	>99	0.5 g	96
Prep RCM	60 × 10	4.71 L	1305-fold	>99	4.5 g	96

chromatography, the approach of large-scale HPLC process development has typically had three stages [21,37]. First, develop a high resolution separation using 2–5-μm media. Second, transfer that method to a 15–40-μm particle with similar surface chemistry. Third, modify the method as needed for optimal process considerations. Each change in medium can result in another cycle of optimization.

For this RP-HPLC isolation of rhIGF-1, a conventional low-pressure development methodology was utilized. Initially, a sensitive, rapid, high-resolution analytical separation was developed which could be used to both identify closely related species of rhIGF-1 and quantitate the level of product. The selectivity of the initial analytical and preparative methods was similar. However, as the components in the methods diverged the selectivity was significantly altered. Then the RP-HPLC analysis using the analytical method became justifiable. Using preparative-size media packed in a small column with easily scalable geometry, parameters based on various pH and ionic strength buffers, counter-ions, temperatures, and media were evaluated. As the other analytical techniques of SDS-PAGE and IEF utilized during process development were unable to adequately quantitate the level of rhIGF-1 variants, the rapid RP-HPLC analytical chromatography became the principal technique to monitor the progress of the development effort, analyze for product purity and determine yield and effective capacity. When an acceptable set of preparative conditions was elucidated, a 1000-fold scale-up of the separation was achieved based on the chromatographic principles of constant linear velocity or residence time.

The bulk of the literature describing protein purification via preparative RP-HPLC typically describes a multi-peak, high-resolution analytical procedure being directly transferred to a larger bed volume. The columns are typically 1–2.5 cm in diameter, contain 5-μm media and the method is scaled 5–10-fold using analytical instrumentation. This approach is satisfactory from a research perspective for isolating micrograms or even milligrams of protein. However, for pharmaceutical manufacturing where grams or kilograms of ultrapure product are required, a different developmental format should be applied. As described in this paper, a systematic evaluation of practical process parameters using medium-particle preparative media will facilitate translating an analytical RP-HPLC method into a high-capacity preparative recovery step. Chromatographic optimization should center on factors which influence selectivity, focusing on maximizing the band spacing between the product and its closest eluting neighbors. Since non-linear elution profiles occur during mass overload conditions, the first less hydrophobic contaminant to the product peak will be of particular interest. Special attention should be given to buffer conditions known to enhance product solubility. Subsequently, scaling-up the chromatography 10–1000-fold or more using preparative equipment is straightforward.

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