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Non-flammable preparative reversed-phase liquid chromatography of recombinant human insulin-like growth factor-I

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Abstract

Acetonitrile is used as an eluent for reversed-phase chromatography. However, because it is a flammable solvent, using acetonitrile on a large scale requires expensive equipment and facilities specially designed for flammable solvents. Using a non-flammable solvent as an eluent eliminates this expense. A method was developed to purify recombinant human insulin-like growth factor I by reversed-phase high-performance liquid chromatography using gradient elution with hexylene glycol, a non-flammable replacement for acetonitrile. The separation produced equivalent yield, purity and throughput as reversed-phase chromatography using elution with acetonitrile.

Keywords: Growth factors; Mobile phase composition; Hexylene glycol

1. Introduction

Reversed-phase high-performance liquid chromatography (HPLC) is used for protein purification because it can separate closely related proteins. Acetonitrile is a common eluent for reversed-phase HPLC [1], and it is used at large scale for purifying recombinant proteins such as insulin [2]. However, acetonitrile is a flammable solvent and it is expensive to use on a large scale for bioprocess applications because it requires specialized equipment and facilities designed for flammable processes. Replacing acetonitrile with the non-flammable solvent hexylene glycol eliminates this expense.

Reversed-phase HPLC using elution with acetonitrile has been used to purify human insulin-like growth factor-I (IGF), a 70 amino acid protein [3] with a molecular mass of 7649 and three disulfide bonds [4,5]. IGF has several important biological functions [6] including growth regulation [7–9], and it may be useful for treating diseases such as Laron syndrome [10,11], diabetes mellitus [12–14], and neurodegenerative diseases [15] including amyotrophic lateral sclerosis [16]. Both natural IGF from plasma [17,18] and recombinant IGF from bacterial fermentation [19] have been purified by reversed-phase HPLC using elution with acetonitrile. Reversed-phase HPLC separates several variant forms of IGF, including: met^{59}O, which has a methionine sulfoxide at position 59 [20]; desGly^{1}Pro^{2}, which has an N-terminal glycine and proline missing; carbamylated [21]; misfolded, where disulfide bonds

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are improperly formed [22]; and aggregate, aggregated IGF, which includes dimers, trimers and multimers [23].

Recombinant IGF purification by reversed-phase HPLC was optimized [19] to use elution with acetonitrile, a buffer of 100 m*M* potassium phosphate at pH 7.0, and C_4 reversed-phase media. Later improvements included using 10 μm 150 Å Kromasil media run at a temperature of 50°C. Average recovery yield for this purification is approximately 80%, and throughput is 0.3 g/h/cm^2 (g IGF per h per cm^2 of column cross-sectional area).

This paper describes the development of a method to purify recombinant IGF by reversed-phase HPLC using elution with hexylene glycol, a non-flammable replacement for acetonitrile. In this method, hexylene glycol directly replaced acetonitrile in the separation, using the same buffer, temperature and chromatography media as HPLC using elution with acetonitrile. Hexylene glycol was chosen to replace acetonitrile for several reasons. It is non-flammable (flash point 93°C [24]), its has low viscosity (30%, v/v aqueous solution at 50°C has viscosity <1.5 cP), it does not absorb strongly at 280 nm, it is strongly eluotropic (in a buffer of 100 m*M* potassium phosphate, pH 7.0 on Kromasil 10 μm 150 Å C_4 media at 50°C, IGF elutes at 27% acetonitrile or 13% hexylene glycol), and it provides adequate separation. No other non-flammable solvents considered, including propylene glycol, neopentyl glycol and dipropylene glycol, met all of these criteria. The non-flammable HPLC process using elution with hexylene glycol was developed to be a robust, reliable process with equivalent purity, recovery yield and throughput as the HPLC process using elution with acetonitrile.

2. Experimental

2.1. Materials

Prepacked 1 cm diameter Kromasil columns and bulk Kromasil media were obtained from BTR Separations (Wilmington, DE, USA). Vydac columns were obtained from Phenomenex (Torrance, CA, USA). HPLC load material of partially purified recombinant IGF from bacterial fermentation, purified met^{59}O, and purified misfolded were obtained from Genentech (South San Francisco, CA, USA). Hexylene glycol was NF grade from Ashland (Newark, CA, USA). The BioCAD chromatography workstation was from PerSeptive Biosystems (Framingham, MA, USA), the HP1090 HPLC system was from Hewlett-Packard (Mountain View, CA, USA), the Delta-prep was from Waters (Milford, MA, USA), and the Prochrom DAC column and HPLC system were from Prochrom USA (Indianapolis, ID, USA).

2.2. Analytical chromatography

Purity and yield were determined by a reversed-phase HPLC assay (Fig. 1) which is similar to an assay previously characterized [25] and previously used for analyzing results from preparative IGF purification [26]. Purity and variant levels are expressed as a percent of total peak area. Aggregate is the sum of peaks integrated from approximately 11.5 min to approximately 14 min. The HPLC assay used a Vydac 250×4.6 mm C_{18} 5 μm 300 Å column at a flow-rate of 2 ml/min, a 10 μg injection and detection at 214 nm. Buffer A was 0.12% trifluoroacetic acid (TFA) in water, and buffer B was 0.1% TFA in acetonitrile. The method was: 27.5–28.5% B over 9 min, 28.5–40% B over 4 min, 40–90% B over 2 min, hold 90% B for 1 min, then 27.5% B for 4 min. Analytical chromatography was run on an HP1090 HPLC system.

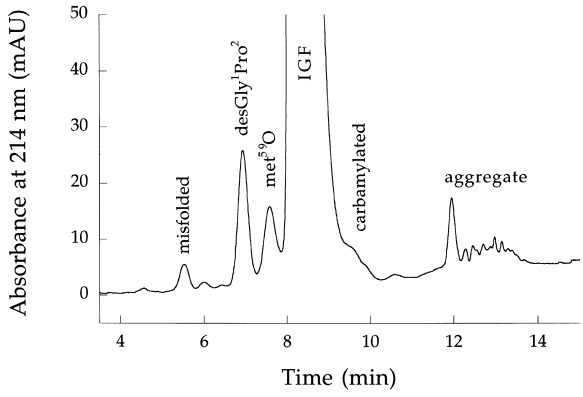

Fig. 1. Chromatogram from the HPLC assay, analyzing typical IGF load material for the HPLC process.

2.3. Preparative chromatography

Loads are stated in g IGF per l column volume. Flow-rates are stated in cm/h, calculated as the volumetric flow-rate divided by the column cross-sectional area. All preparative experiments used Kromasil 10 μm 150 Å C_4 media.

Experiments to characterize the preparative separation all used 100 m*M* K_2HPO_4, pH 7.0 and 1 cm diameter columns. Columns were equilibrated with approximately 10% hexylene glycol (HG) for at least three column volumes (CVs), and were regenerated with at least 25% HG for at least two CVs. Fractions were collected throughout the elution and analyzed by the HPLC assay. Load material contained 10% met^{59}O (determined by the HPLC assay), and approximately 10% HG. This preparative chromatography was performed on a BioCAD instrument.

Flow-rate experiments used a 25 cm length column at 30°C loaded to 3 g/l with a gradient from 14.25–15.75% HG over 15 CVs at flow-rates of 50, 75, 150, 225, 300 and 400 cm/h. Load experiments used a 25 cm length column at 30°C at a flow-rate of 255 cm/h with a gradient from 12.5–15.5% HG over 15 CVs at loads of 3, 9, 17 and 26 g/l. Temperature experiments used a 25 cm length column at a flow-rate of 255 cm/h loaded to 3 g/l with a gradient from 12.5–17.5% HG over 15 CVs at temperatures of 30, 50, 65 and 80°C. Column length experiments used a temperature of 50°C with a gradient of 10–15% HG/22 CVs and column lengths of 10, 15, 25, 40 and 50 cm at flow-rates of 520, 420, 400, 300 and 200 cm/h, respectively. Gradient slope experiments used a 25 cm length column at 30°C with a flow-rate of 255 cm/h loaded to 3 g/l with a gradients from 12.5–22.5, 12.5–17.5, 14–17, 14.25–15.75% HG over 15 CVs.

Scale-up experiments were done on a 25 cm length column in a 6 cm diameter Prochrom column at 50°C. Chromatography was run on a Waters Delta-prep or Prochrom HPLC system at 400 cm/h. Buffer A was 55 m*M* K_2HPO_4, 45 m*M* KH_2PO_4, pH 7.0 and buffer B was 55 m*M* K_2HPO_4, 45 m*M* KH_2PO_4, pH 7.0 with 30% HG. The method was: equilibration for three CVs at 30% B, load 10 g/l (9% HG in load), wash for one CV at 30% B, gradient 40–50% B over 10 CVs, regenerate for one CV at 80% B.

3. Results and discussion

During reversed-phase HPLC of IGF, all variants should be removed to 1% or less as measured by the HPLC assay. However, misfolded and desGly^{1}Pro^2 are already less than 1% in the HPLC load material, and aggregate and carbamylated are typically well resolved from IGF. Therefore, the development focused on the separation of met^{59}O from IGF.

Scale-up optimization was enabled by studying the effect of several process variables on separation performance. With preparative loads, met^{59}O is not separated to baseline from IGF, so for this study separation performance was calculated as yield at a constant purity of 1% met^{59}O. Fractions of the preparative HPLC peak were analyzed by the HPLC assay to determine the concentration of IGF and met^{59}O in each fraction. The results from individual fractions were summed and yield was calculated as IGF in a pool with 1% met^{59}O divided by the total IGF eluted. This yield calculation takes into account only eluted IGF, as opposed to loaded IGF, and corrects for effects (such as IGF aggregation on the column, discussed later) that may reduce the total IGF recovered but do not affect the separation of IGF from met^{59}O.

This study determined that separation performance is unaffected by flow-rate or column length, is slightly affected by temperature and gradient slope, and is highly affected by load (Fig. 2). Because preparative reversed-phase HPLC of proteins is based primarily on adsorption–desorption [27], especially for C_4 columns [28], flow-rate and column length do not strongly affect chromatographic separations [29,30]. Because temperature increases protein diffusivity and decreases mobile phase viscosity, improving the protein's kinetic and transport properties [31–33], temperature slightly increased separation performance. Gradient slope affects separation performance by changing the relative retention values for each peak [34]. Gradient slope had a small effect on IGF yield, with lower yield as gradient slope increased.

During the column length study, as the column length increased, the residence time of the protein on the column also increased. As the residence time increased, the amount of misfolded and aggregate increased (Fig. 3). Residence time was calculated as

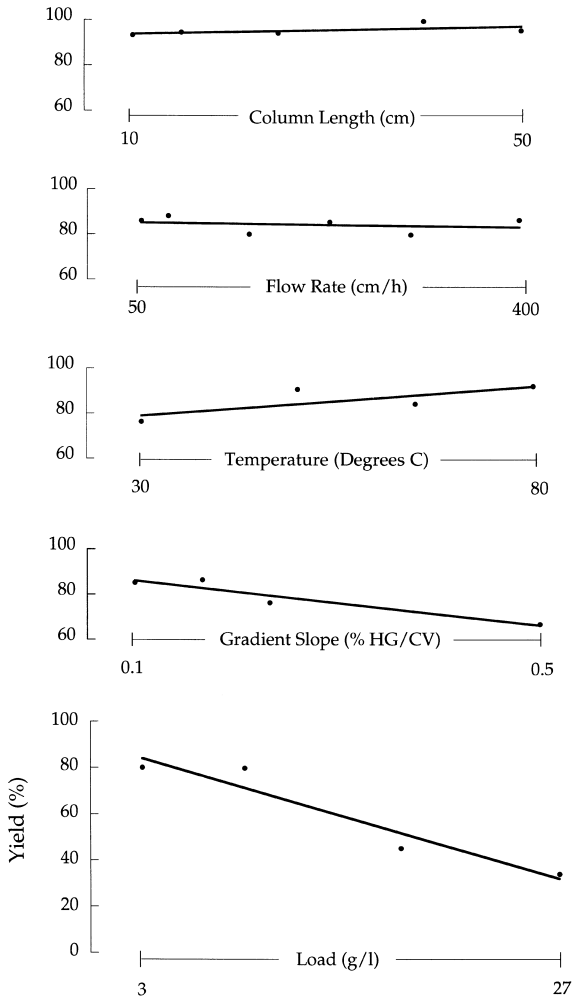

Fig. 2. Quantitative evaluation of the effect of column length, flow-rate, temperature, gradient slope and load on separation performance using 1 cm diameter columns. *y*-Axis on all graphs is yield at constant purity calculated as IGF in a pool with 1% met^{59}O divided by the total IGF eluted (described in Section 3). Linear fit is shown for reference only. The slope of the line indicates the proportionate effect of each process condition. Gradient slope is percent hexylene glycol (HG) per column volume (CV) of gradient volume.

the time from the beginning of load to the end of elution, with five residence times corresponding to five different column lengths. The amount of misfolded was measured in the peak fraction (the fraction of the preparative IGF peak where absorbance is highest) by the HPLC assay. Aggregate was measured by integrating the preparative chromatograms and assumed that all aggregate eluted in the regeneration peak. Both misfolded and aggregate increased linearly with residence time. Misfolded forms on the column at a rate of 0.002% per min, and aggregate forms at a rate of 0.02% per min (Fig. 3). This rate is about 150-times slower in the purified pool at 50°C, suggesting that misfolded and aggregate formation is accelerated when bound to the column. The formation of aggregate will not affect purity because aggregate is well-separated, but it can affect recovery yield when the residence time is long. The formation of misfolded will not significantly affect recovery yield because its formation rate is so small, but it could affect purity.

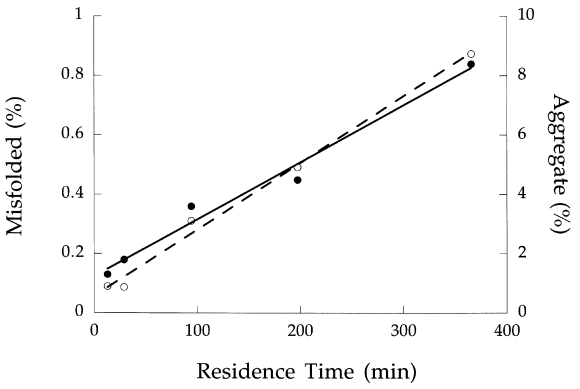

Fig. 3. The effect of residence time on misfolded formation (closed circles, solid line) and aggregate formation (open circles, dashed line). Data is from the column length study (five column lengths for five different residence times) in Fig. 2. Misfolded: $y=0.12+0.0019x$, $R^2=0.98$. Aggregate: $y=0.57+0.022x$, $R^2=0.99$.

When optimizing the process for scale-up, there are several important considerations. The HPLC process should not generate much misfolded or aggregate, so protein residence time is an important consideration when choosing column length, flow-rate, temperature and gradient slope. A temperature of 50°C (the temperature used for the HPLC separation with acetonitrile) produces low viscosity and backpressure but still ensures the stability of IGF, which could be compromised at a higher temperature. The column temperature for the 1 cm diameter column was maintained by a column oven. The 6 cm diameter column was insulated, but a temperature-controlled jacket was not used, and the temperature of the mobile phase was maintained by in-line

mobile phase heating using a heat exchange coil. These methods of temperature control could induce temperature gradients inside the column [35,36], but no significant change in the separation was observed between the 1 cm diameter column and the 6 cm diameter column. While the inlet and outlet temperatures of the 1 cm diameter column remained the same, a temperature drop of approximately 5°C was observed between the column inlet and outlet on the 6 cm diameter column.

At a flow-rate of 400 cm/h the pressure drop across a 25 cm length column at equilibration is 700 p.s.i. at 50°C; this pressure is significantly lower than the 1000 p.s.i. maximum for the Prochrom column (1 p.s.i.=6894.76 Pa). Although a column shorter than 25 cm will produce less protein residence time, 25 cm length columns provided robust separations during development, and the residence time on a 25 cm length column is short enough that misfolded formation is very small. A gradient from 12–15% hexylene glycol over 10 CVs provides a shallow gradient slope while ensuring that small changes in buffer composition will not affect the process. A load of 10 g/l permits adequate yield while maintaining a high throughput.

Using the conditions described above, the opti-

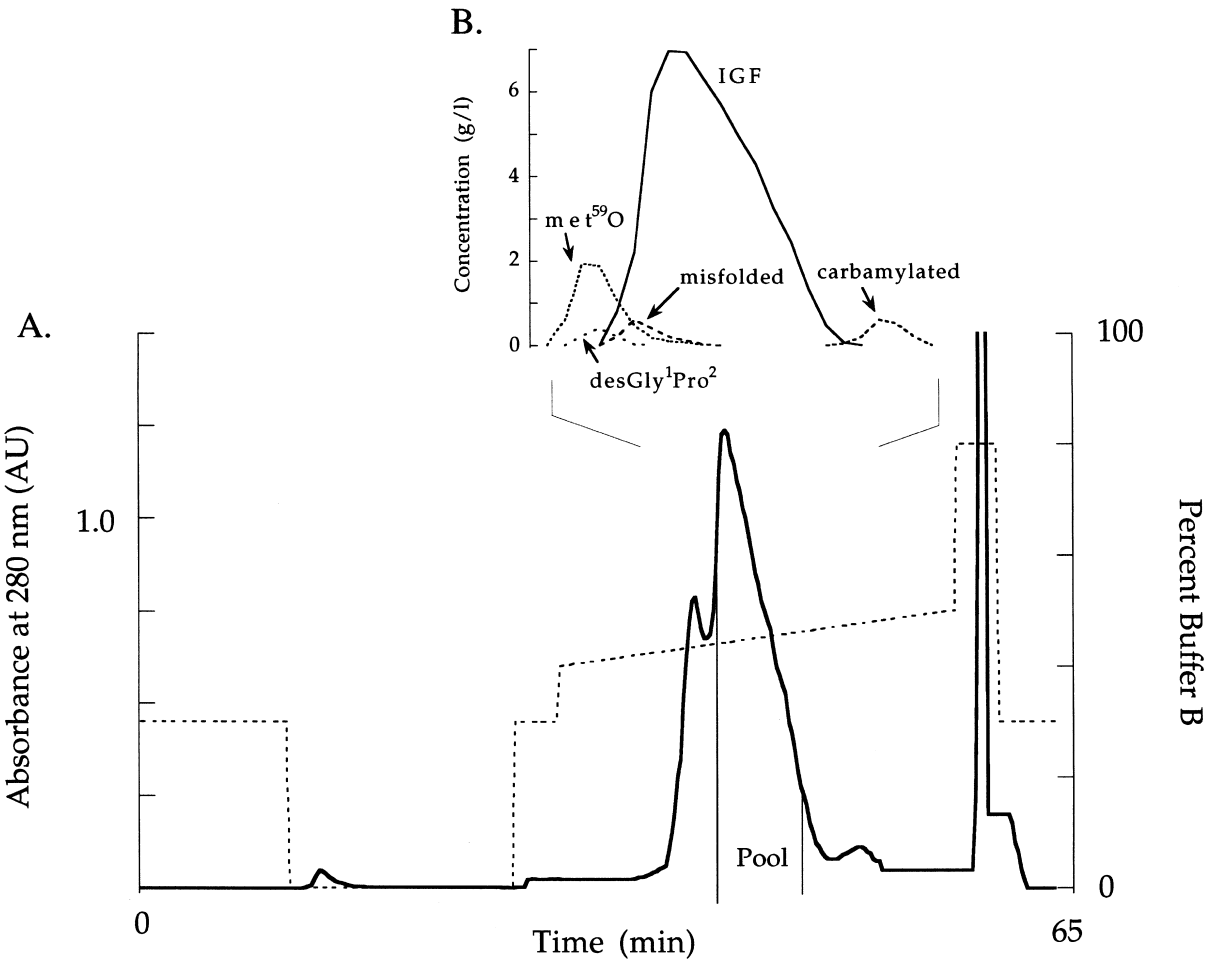

Fig. 4. (A) Chromatogram from the optimzized HPLC process using hexylene glycol elution on the 6 cm diameter column. Pool lines indicate a pool with 1% met^{59}O. (B) Inset is an analysis of fractions from the preparative peak in (A) with IGF and variants shown as determined by the HPLC assay. Aggregate elutes in the 80% B wash.

mized, scaled-up non-flammable separation is 65 min long (Fig. 4A), but this time can be reduced to 50 min by beginning regeneration immediately after pooling has stopped. The separation does not resolve met^{59}O to baseline from IGF, but by pooling only the center portion of the preparative peak, met^{59}O can be removed to 1% or less while maintaining an average recovery yield greater than 80%. Carbamylated is resolved to baseline after the IGF peak (Fig. 4B), and aggregate elutes in the regeneration (80% B wash) at the end of the separation. The protein residence time is approximately 25 min, which results in an aggregate formation of 0.5% and a misfolded formation of 0.05%. However, because misfolded is slightly resolved from IGF (Fig. 4B), the separation reduces misfolded by approximately 15%.

IGF is not baseline resolved from impurities, which would suggest that collecting fractions across the preparative peak is necessary to ensure that only material with less that 1% met^{59}O is pooled. However, collecting fractions is undesirable for production, so the separation needs a method to accurately pool pure IGF regardless of the level of met^{59}O, which can vary in each batch of load material. An optimized pooling strategy for routine manufacturing should depend only on UV absorbance.

To find a method that could accurately pool IGF containing less than 1% met^{59}O using only UV absorbance, increasing amounts of purified met^{59}O were spiked into load pools and this load material was purified on the 6 cm diameter column (Fig. 5). As the amount of met^{59}O in the load increased, the met^{59}O peak moved further out in front of the IGF peak, which means that in order to remove increased amounts of met^{59}O more of the preparative peak must be cut away. By 75% peak height, most of the met^{59}O has eluted. A pool that begins at 75% peak height contains less than 1% met^{59}O at all met^{59}O levels, indicating that at 75% peak height pooling can begin regardless of the variant content. At 10 g/l load the height of the IGF peak is reproducibly 7 g/l, so the peak height at 280 nm can be predicted in advance for any UV monitor by detector calibration.

To demonstrate process robustness and reliability, the 6 cm diameter column was cycled using four different batches of load material. The preparative peak was cut from 75% peak height on the leading edge to 25% peak height on the trailing edge, and the

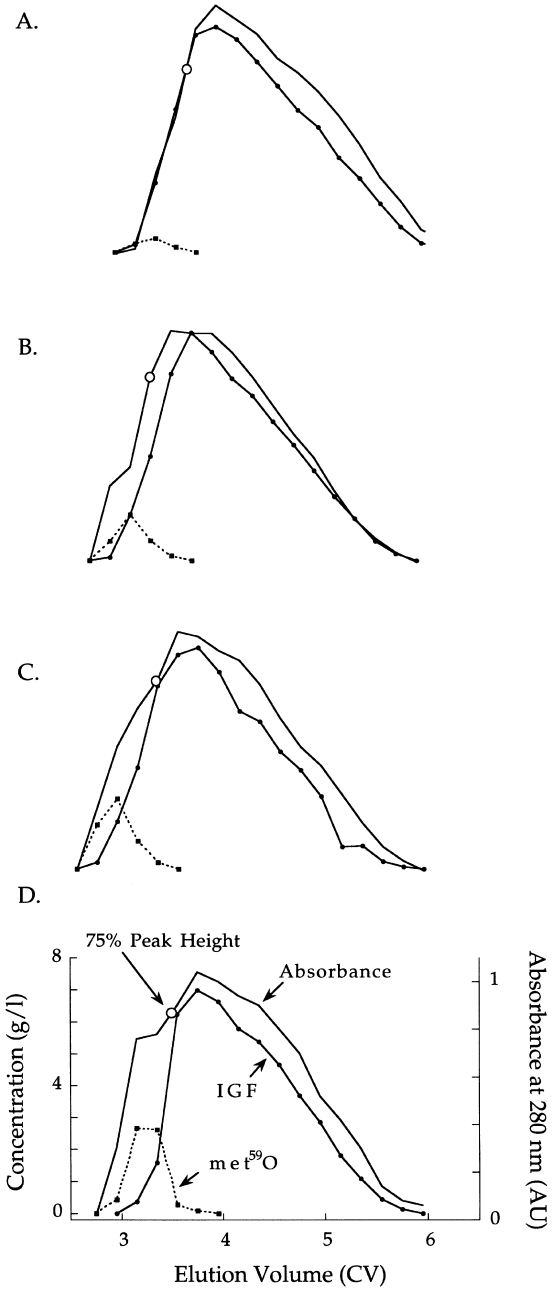

Fig. 5. Peak cutting using the 6 cm diameter column. Elution volume is volume after gradient start. Absorbance at 280 nm is solid line, with 75% peak height indicated by open circles. Fraction analysis by the HPLC assay is shown with solid and dashed lines as indicated. (A) 3% met^{59}O in load, (B) 6% met^{59}O in load, (C) 9% met^{59}O in load, and (D) 13% met^{59}O in load.

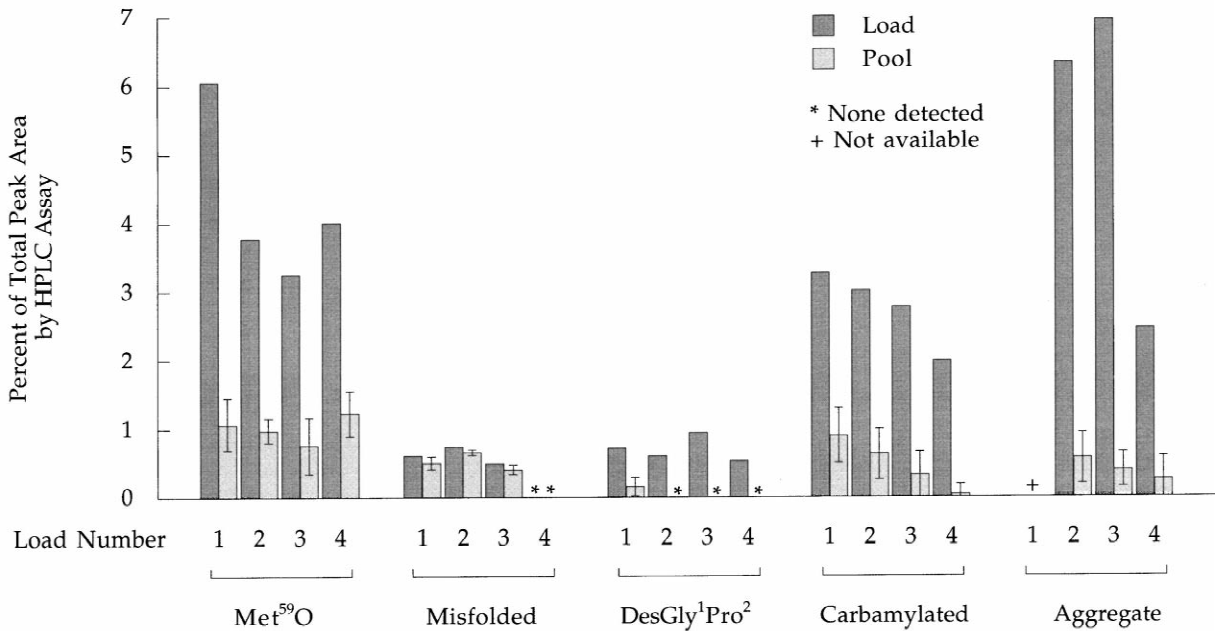

Fig. 6. Column cycling using the 6 cm diameter column. Percent is percent of total peak area by the HPLC assay. Pools were collected from 75% to 25% peak height based on height of first cycle. Pool values are averages, and error bars are one standard deviation. Four different load materials were used, with 11 cycles of load 1, five cycles of load 2, 19 cycles of load 3, and 32 cycles of load 4.

pools were analyzed by the HPLC assay to determine purity (Fig. 6). On average, all variants were removed to 1% or less, despite fluctuations of the variants in the load material. As expected, misfolded was reduced by 15%. A total of 67 cycles were run, purifying 465 g of IGF (410 g after purification) in 56 h.

Throughput for this non-flammable process was 0.3 $g/h/cm^2$, average recovery yield was 88% by the HPLC assay, and all variants were 1% or less in the purified pools. The process was robust to varying load purity, and pure IGF was reliably and reproducibly pooled. Thus, the non-flammable HPLC process using elution with hexylene glycol is robust and reliable, and produces purity, yield and throughput equivalent to that of the flammable HPLC process using elution with acetonitrile.

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