Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1041 (2004) 87–93
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Preparative and analytical chromatography of pegylated myelopoietin using monolithic media

Troii Hall*, David C. Wood, Christine E. Smith

Pfizer Inc., 700 Chesterfield Parkway West, Chesterfield, MO 63017, USA

Received 30 January 2004; received in revised form 19 April 2004; accepted 21 April 2004

Abstract

Monolithic media were compared with Q- and SP-Sepharose high performance chromatography for preparative purification and with Q- and SP-5PW chromatography for analysis of a pegylated form of myelopoietin (MPO), an engineered hematopoietic growth factor. The use of either monolithic or Sepharose based supports for preparative chromatography produced highly purified pegylated MPO with the monolithic media demonstrating peak resolution and repeatability at flow rates of 1 and 5 ml/min resulting in run times as much as five-fold shorter compared to Sepharose separations. The monolithic disks also resulted in 10-fold shorter run times for the analytical chromatography, however, their chromatographic profiles and peak symmetry were not as sharp compared to their Q-5PW and SP-5PW counterparts.

Keywords: Monolithic media; Myelopoietin, pegylated

1. Introduction

The advent of protein therapeutics, such as anti-TNF and alpha interferon, has lead to the successful treatment of several debilitating diseases including arthritis and cancer [1,2]. One shortcoming associated with many protein therapeutics is the necessity for frequent dosing as a result of the rapid renal clearance of the therapeutic molecules. The conjugation of polyethylene glycols (PEGs) to these therapeutic agents has proven effective for limiting renal clearance by improving pharmacokinetic parameters such as protein solubility and circulatory half-life [3–6].

Myelopoietin (MPO), a member of a family of novel cytokine receptor agonists, is one example where pegylation with a 30,000 molecular weight PEG was able to reduce the frequency of administration in animal models while maintaining its pharmocodynamic or potency effect, in this case hematopoietic recovery [7,8]. An SP-Sepharose high performance column was used in a one-step purification for the successful isolation of the monopegylated MPO [9]. Although successful, the agarose based matrix of the Sepharose column is prone to mechanical instability and thus lower performance at higher flow rates ultimately limiting the ability to reduce processing times and improve throughput.

High throughput sample purification and rapid processing of samples while maintaining column performance would provide significant processing advantages. The introduction of monolithic chromatography media, of which Convective Interaction Media (CIM^{TM}) is one example, has shown promise for high throughput applications [10–12]. These and other monolithic supports are made of a highly cross-linked porous monolithic polymer that, according to theory, maximizes mass transport through a convection based flow [13–15]. These properties allow for faster flow rates and shorter processing times while providing sufficient surface area for analyte interactions in order to maintain resolution typical of standard chromatographic methods [16].

Monolithic columns have been used for the synthesis of peptides [17], purification of oligonucleotides [18] and proteins [19,20] and production of antibody affinity columns [21,22]. However, no reported studies have examined the ability of monolithic columns to purify and analyze pegylated proteins. We compared the performance of QA-CIM^{TM} and SO_3-CIM^{TM} disks to Q-Sepharose HP and

* Corresponding author. Tel.: +1-636-247-5694; fax: +1-636-247-6232.
E-mail address: troii.hall@pfizer.com (T. Hall).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.04.037

SP-Sepharose HP HiTrap™ columns for the preparative purification of PEG MPO. The same CIM™ disks were also compared to traditional analytical columns (Q-5PW and SP-5PW) for analysis of the PEG MPO reaction mixture. We demonstrate the potential of ion-exchange monolithic media for the purification and analysis of pegylated MPO and suggest that this technology may be applied to other pegylated proteins.

2. Materials and methods

2.1. Instruments

An AKTA™ FPLC system from Amersham Pharmacia Biotech (Piscataway, NJ) with P-920 pumps, UPC-900 detector and a Frac-900 fraction collector were used for the preparative purification of PEG MPO and UNICORN 3.10 software for the collection and analysis of data. A high performance liquid chromatography (HPLC) system from Waters (Milford, MA) was used for the analytical separations. The system consists of Waters Alliance 2695 pumps with a model 2487 dual absorbance detector and a Waters 717 Plus autosampler using Millenium 32 software for data collection and analysis. A Kodak image station 440 using Kodak 1D Image analysis software purchased from Perkin-Elmer Life Sciences (Gaithersburg, Maryland) was used for the densitometry analysis.

2.2. Buffers

Buffer A: 50 mM Tris–HCl, pH 9.0; buffer B: 50 mM Tris–HCl, pH 9.0, 0.2 M NaCl; buffer C: 10 mM Na acetate, pH 4.5; buffer D: 10 mM Na acetate, pH 4.5, 1.0 M NaCl.

2.3. Materials

Water used for the preparation of solutions was purified using a Milli-Q A10 system from Millipore (Bedford, MA). Purified MPO was provided by Pharmacia Discovery Research, St. Louis, MO and methoxy–polyethylene glycol–aldehyde (30,000 MW) was from Shearwater Polymers Inc. (Huntsville, AL). Sodium cyanoborohydride, sodium acetate and Tris–HCl were from Sigma Chemical Co., St. Louis, MO. The QA-CIM™ and SO_3-CIM™ disks (16 mm diameter × 3.0 mm length) were from Advanced Separations Technologies Inc. (Whippany, NJ) and the Q-and SP-Sepharose HP HiTrap columns (25 mm length × 7.0 mm diameter) were from Amersham Pharmacia Biotech (Piscataway, NJ). The Q-5PW and SP-5PW analytical columns (75 mm length × 7.5 mm diameter, 10 μm) were from Tosoh Biosep (Montgomeryville, PA). Simply Blue SafeStain, Tris-Glycine SDS Sample Buffer (2×), SEE BLUE molecular weight standards, 4–12% Bis–Tris Gels and MES buffer were from Invitrogen (Carlsbad, CA).

2.4. Conjugation of 30 K linear PEG to MPO

Purified recombinant MPO (lyophilized powder) was solubilized in 25 mM MES buffer, pH 5.0 at 1.5 mg/ml. A 30 K linear methoxy PEG aldehyde powder was reacted with the MPO solution at a 6:1 molar ratio via aldehyde chemistry of available amine groups such as the amino terminus and lysine groups. Sodium cyanoborohydride was added to make a final concentration of 20 mM and the reaction was allowed to proceed for 24 h at 4 °C resulting in approximately 25% monopegylation of the MPO. These reaction conditions were performed to quickly obtain repeatable pegylation of the MPO and were not optimized for the complete conversion of MPO to PEG MPO.

2.5. Preparative purification of PEG MPO reaction mixture

The PEG MPO reaction pool was diluted three-fold with buffer A (anionic) or buffer C (cationic) and the pH adjusted to 9.5 using NaOH or 4.0 using acetic acid, respectively. Separations were performed at 4 °C loading 2 ml of sample (0.5 mg/ml) onto the QA-CIM™ (three disks at 0.34 ml per disk), SO_3-CIM™ (three disks at 0.34 ml per disk), 1 ml Q-Sepharose HiTrap and 1 ml SP-Sepharose HiTrap columns. Each column was washed with ten column volumes of respective starting buffer and the protein eluted using a linear NaCl gradient. Flow rates were between 1 and 10 ml/min for the CIM™ separations and 1 ml/min for the Sepharose separations. Each gradient was formed over 20 column volumes collecting 1 ml fractions and monitored at 280 nm.

2.6. Analytical chromatography of PEG MPO reaction mixture

Samples (100 μl) were loaded onto a QA-CIM™ (1 × 0.34 ml disk), SO_3-CIM™ (1 × 0.34 ml disk), Q-5PW Tosoh Biosep and SP-5PW Tosoh Biosep column and separations performed at room temperature. A NaCl linear gradient run at 6.0 ml/min carried out between 1.5 and 2.0 min for the CIM™ columns and at 1 ml/min over 20 min for the Sepharose columns was used to elute the proteins. All separations were monitored at 280 nm.

2.7. SDS PAGE analysis

The purity of the isolated proteins was determined by SDS PAGE. A Novex PowerEase 500 power source was used to run the 4–12% Bis–Tris NuPAGE gels using MES buffer. The samples were mixed with sample buffer under reducing conditions loading 1–8 μg per lane. The proteins were detected using a Simply Blue SafeStain and purity determined by densitometry using a Kodak Image Station 440.

3. Results and discussion

3.1. Purification of PEG MPO using QA-CIM™ and Q-Sepharose HP chromatography

The monopegylated MPO was purified using both the Q-Sepharose high performance HiTrap column (Fig. 1A) and QA-CIM™ disks (Fig. 1B) under identical gradient conditions. For both columns the minor component, PEG MPO, eluted first at 0.06 M NaCl followed by the major component, unreacted MPO, at 0.10 M NaCl. A minor peak eluted after MPO but SDS PAGE analysis did not identify any unusual protein bands suggesting a non-covalent aggregate of MPO (data not shown).

The PEG MPO reaction mixture was also separated at 5 ml/min using the QA-CIM™ disks (Fig. 1C) that required 4 min to complete the gradient—five-fold faster than the Q-Sepharose HP run at 1 ml/min. The resulting chromatographic profile was similar to the QA-CIM™ and Q-Sepharose HP separations performed at 1 ml/min.

A relationship between an increase in backpressure and increased flow rate was observed and is represented in Table 1. Flow rates of 5 ml/min or less were within system and disk operating conditions as recommended by the manufacturer. However, flow rates of 10 ml/min when using the QA-CIM™ disks quickly exceeded the manufacturers recommended backpressure (5 MPa) resulting in an aborted run. A flow rate of 8 ml/min also ultimately exceeded the backpressure limit.

3.2. Purification of PEG MPO using SO_3-CIM™ and SP-Sepharose HP chromatography

The SP-Sepharose HP HiTrap separations (Fig. 2A) eluted two components with similar peak ratios to those observed using the anionic supports. The smaller peak, PEG MPO, eluted first at approximately 0.21 M NaCl followed by MPO eluting at 0.24 M NaCl.

Separations using the SO_3-CIM™ disks (Fig. 2B–D) resulted in run times as much as 10-fold faster than the Q- and SP-Sepharose HP columns performed at 1 ml/min. The three methods consistently eluted two peaks, PEG MPO and MPO, at NaCl concentrations of 0.75 and 0.88 M NaCl, respectively. However, unlike the SP-Sepharose separations, visual differences were apparent with the peak shapes and ratios among the three different flow rates.

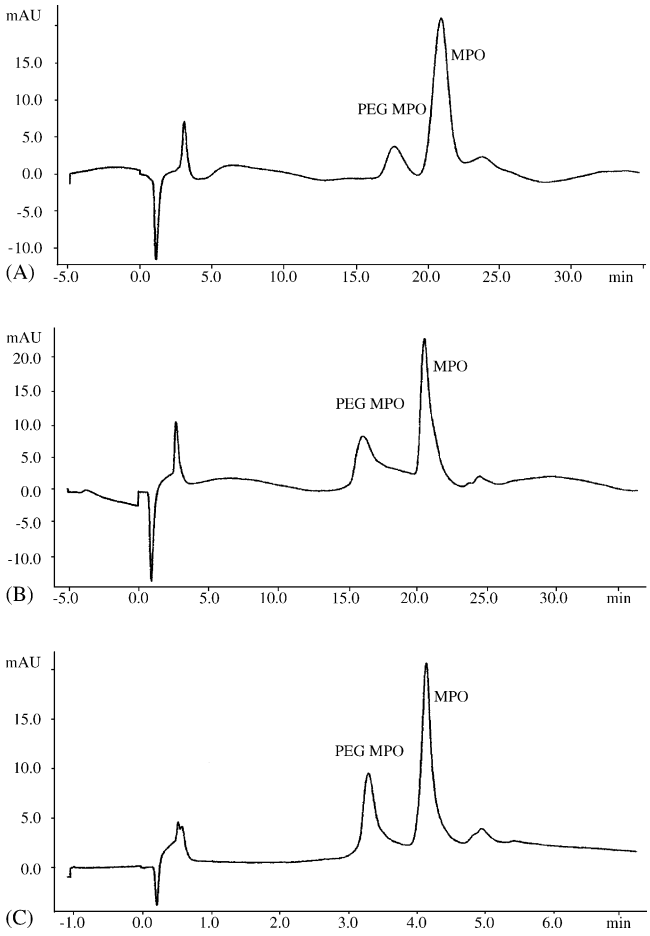

Fig. 1. Preparative separation of the PEG MPO reaction mixture using anionic media (A) Q-Sepharose HP HiTrap (25 mm × 7.0 mm i.d.) run at 1 ml/min and QA-CIM™ (9.0 mm × 16 mm i.d.) run at 1 ml/min (B) and 5 ml/min (C) over 20 column volumes from 0 to 100% buffer B with UV detection at 280 nm.

Table 1
Summary of preparative purifications of PEG MPO reaction mixture

Support	Flow rate (ml/min)	Flow rate (cm/min)[a]	Run time (min)[b]	Pressure (MPa)	Column dimensions: length × diameter (mm)
Q-Sepharose HP	1.0	2.6	20	Negligible	25 × 7.0
QA-CIM™	1.0	0.5	20	0.45	9.0 × 16
QA-CIM™	5.0	2.5	4.0	2.60	9.0 × 16
QA-CIM™	10	5.0	2.0	Overpressure	9.0 × 16
SP Sepharose HP	1.0	2.6	20	Negligible	25 × 7.0
SO_3-CIM™	1.0	0.5	20	0.13	9.0 × 16
SO_3-CIM™	5.0	2.5	4.0	2.05	9.0 × 16
SO_3-CIM™	10	5.0	2.0	3.90	9.0 × 16

[a] Linear flow rate (ml/min): $1/p r^2$; where r is the column radius (cm).
[b] Run time does not include sample loading and column washing time.

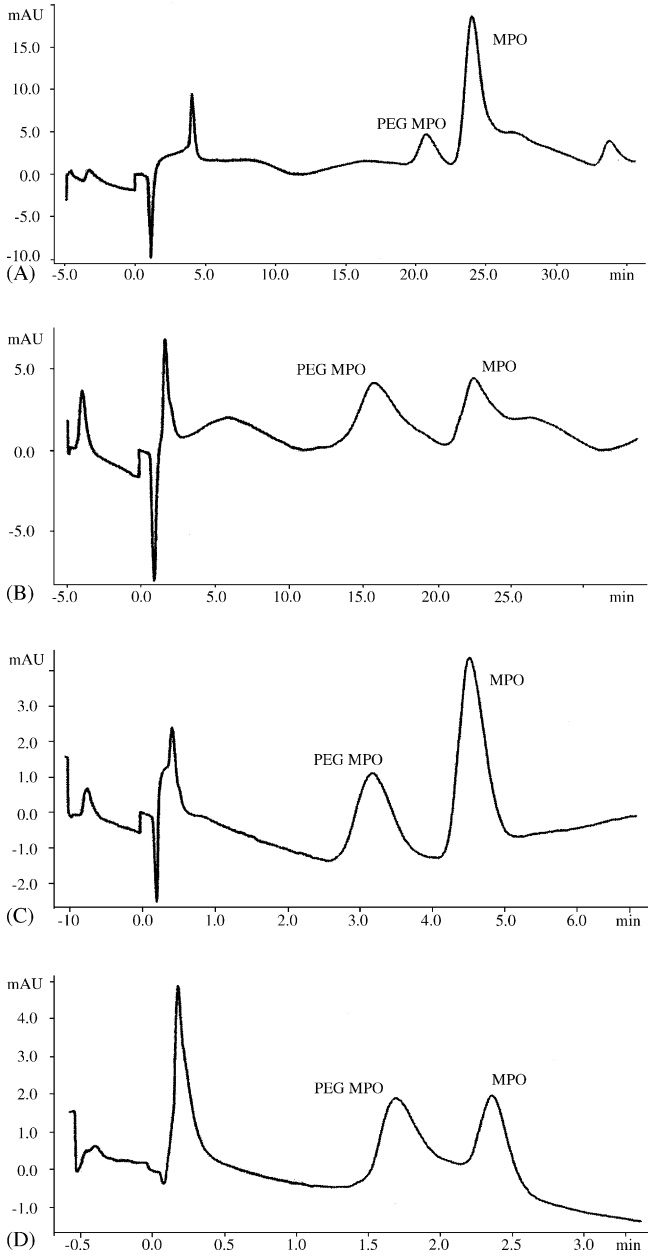

Fig. 2. Preparative separation of the PEG MPO reaction mixture using cationic media (A) SP-Sepharose HP HiTrap (25 mm × 7.0 mm i.d.) run at 1 ml/min over 20 column volumes from 0 to 30% buffer D detecting at 280 nm. SO_3-CIM™ (9.0 mm × 16 mm i.d.) run at 1 ml/min (B), 5 ml/min (C) and 10 ml/min (D) over 20 column volumes from 40 to 100% buffer D with UV detection at 280 nm.

Both PEG MPO and MPO displayed a significantly higher affinity for the SO_3-CIM™ media than for the SP-Sepharose HP column requiring a three to four-fold higher NaCl concentration for their elution. The higher affinity may be a consequence of non-specific interactions with the CIM™ disk that is not present with the Sepharose matrix. However, one might anticipate similar non-specific effects to occur when using the anionic columns and these were not observed. It is also possible that the functional group density for the CIM™ disk is higher than for the SP-Sepharose HP, allowing more opportunity for interactions. Further experiments will be required to decipher this issue.

In contrast to the QA-CIM™ separations, the runs performed at 10 ml/min using the SO_3-CIM™ disks did not exceed the backpressure limit of 5 MPa. The highest recorded backpressure was 3.9 MPa when run at 10 ml/min, well below the 5 MPa limit. Both the QA- and SO_3-CIM™ column chromatography developed at 1 ml/min resulted in backpressures of 0.45–0.13 MPa, respectively, where no detectable pressure was measured for either the Q-Sepharose HP nor SP-Sepharose HP separations.

The reaction mixture and fractions collected from the preparative purifications were analyzed by SDS PAGE. With the exception of the SO_3-CIM™ separation at 10 ml/min, the protein purity of the pegylated MPO fractions from the Q/SP-Sepharose HP and QA/SO_3-CIM™ columns was greater than 98%. The SO_3-CIM™ column at 10 ml/min resulted in lower protein purity due to significant overlap of the MPO and PEG MPO peaks (data not shown).

3.3. Evaluation of preparative separations using QA/SO_3-CIM™ and Q/SP-Sepharose HP media

Several quantitative parameters including retention time, resolution, peak width at half height ($W_{1/2h}$) and integrated peak area for PEG MPO were employed to evaluate the consistency of separation and peak shape achieved using the different chromatographic supports. The calculated mean values and respective standard deviations were calculated from repetitive experiments ($n = 3$) for each parameter and summarized in Table 2.

Retention times for the Q-Sepharose HP and QA-CIM™ columns were highly repeatable showing a coefficient of variation (CV) of 1.0% or less. Also, with the exception of the SO_3-CIM™ run at 1 ml/min, which exhibited a CV of 21%, values of 2.0% or less were observed for both the SP-Sepharose HP and SO_3-CIM™ columns.

The peak shapes for PEG MPO, described by the width at half the peak height, were statistically the same for the Q-Sepharose HP, SP-Sepharose HP and QA-CIM™ separations performed at 1 ml/min. In contrast, separations using the SO_3-CIM™ disks resulted in peak width values greater than two-fold compared to the other medias performed at the same flow rate. The longer NaCl gradient used for the SO_3-CIM™ separations or interaction with column support matrix may be responsible in part for the peak broadening. Further method development of the SO_3-CIM™ separation conditions may lead to chromatograms with sharper peaks.

Resolution, R, values (Eq. (1)), where a value of greater than 1.5 indicates baseline separation, were above 1.5 for all separations except for the SO_3-CIM™ separation performed at 10 ml/min [23].

$$R = \frac{(V_{R2} - V_{R1}) \times 1.177}{W_{h1} + W_{h2}} \quad (1)$$

Table 2
Evaluation of PEG MPO separations from preparative runs

Support	$W_{1/2h}$ (min)	Resolution[a]	Integrated peak area (%)	Retention time (min)
Q-Sepharose HP: 1 ml/min	1.31 ± 0.08	1.51 ± 0.03	19.0 ± 3.42	17.6 ± 0.04
QA CIM^{TM}: 1 ml/min	1.36 ± 0.31	2.41 ± 0.03	31.6 ± 11.7	16.2 ± 0.16
QA CIM^{TM}: 5 ml/min	0.22 ± 0.03	2.82 ± 0.19	35.4 ± 1.63	3.26 ± 0.03
SP-Sepharose HP: 1 ml/min	1.38 ± 0.06	1.48 ± 0.02	17.5 ± 6.47	20.8 ± 0.04
SO_3 CIM^{TM}: 1 ml/min	3.46 ± 0.79	1.40 ± 0.16	53.4 ± 13.9	18.2 ± 3.82
SO_3 CIM^{TM}: 5 ml/min	0.58 ± 0.04	1.62 ± 0.11	38.7 ± 0.77	3.20 ± 0.03
SO_3 CIM^{TM}: 10 ml/min	0.35 ± 0.01	1.34 ± 0.02	56.1 ± 1.18	1.69 ± 0.02

[a] Resolution of PEG MPO from MPO was quantitatively defined using Eq. (1) where a value of 1.5 or greater is indicative of baseline separation.

where $V_{R2} > V_{R1}$ and V_{R1} is the retention (elution) volume for peak 1; V_{R2} the retention (elution) volume for peak 2; W_{h1} the peak width at half height for peak 1 (for Gaussian peaks); W_{h2} the peak width at half height for peak 2 (for Gausssian peaks).

Peak resolution using the SO_3-CIM^{TM} and QA-CIM^{TM} disks was greater at flow rates of 5 ml/min than at 1 ml/min. Some loss in resolution was observed when increasing the flow rates from 5 to 10 ml/min using the SO_3-CIM^{TM} disks but the chromatographic profiles were similar. Repeatability, as indicated by lower coefficient of variation and standard deviation values, also tended to improve as flow rates increased.

The greatest parameter variability, notably integrated peak area percent, was observed for the CIM^{TM} media at the lowest flow rate of 1 ml/min. Comparison of chromatograms from triplicate injections of the same sample suggests no carryover of protein occurred (data not shown). Further inspection of the chromatograms indicated a drifting baseline was the cause of this variability. The peak shape improved at higher flow rates for the CIM^{TM} media that led to the production of better baselines and thus more accurate peak integration as shown in Figs. 1 and 2. These data not only suggest that performance is equal to the HiTrap columns when performed at the same flow rate but also that improved performance occurs at the higher flow rates for the monolithic media. This observation has been previously observed where the increased performance was attributed to the improved convective flow and thus mass transfer for the monolith rather than diffusion that dominates standard chromatographic methods [24].

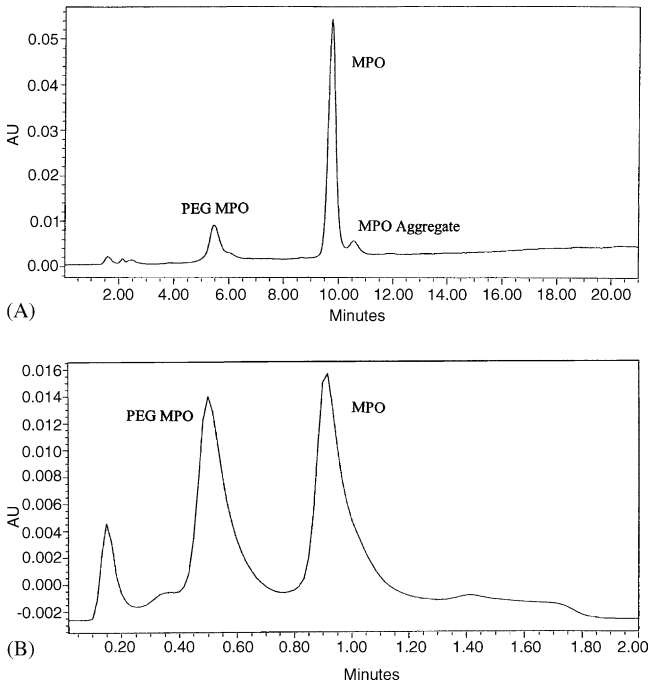

Fig. 3. Analytical separation of the PEG MPO reaction mixture using a Tosoh Biosep analytical Q-5PW column and QA-CIM^{TM} disk (A) Q-5PW column (75 mm × 7.5 mm i.d., 10 μm) run at 1 ml/min over 20 min from 0 to 100% buffer B with UV detection at 280 nm. (B) QA-CIM^{TM} disk (3.0 mm × 16 mm i.d.) run at 6.0 ml/min over 1.5 min from 0 to 100% buffer B with UV detection at 280 nm.

In general, the monolithic columns demonstrated improved recovery of PEG MPO relative to the bead based preparative columns, as indicated by the percent recovery parameter in Table 2. This improvement in the PEG MPO recovery may be due to the differences in chemical composition of the stationary phase or to a difference in the effective pore volume. It will be of interest to determine whether this observation holds for other pegylated proteins.

3.4. Analytical chromatography of PEG MPO reaction mixture using QA-CIM^{TM} and Q-5PW media

A flow rate of 1 ml/min effectively separated the MPO components using the Q-5PW analytical column (Fig. 3A). The QA-CIM^{TM} disk completed the gradient in 1.5 min when run at 6 ml/min, greater than 10-fold less time with respect to the Q-5PW column (Fig. 3B). Clear baseline separation of two peaks was seen with the Q-5PW column and although two distinct peaks were visible when using the QA-CIM^{TM} column the separation was not as great under the conditions used. Attempts at improving peak separation by manipulating the flow rate were not successful for the QA-CIM^{TM} separations.

3.5. Analytical chromatography of PEG MPO reaction mixture using SO_3-CIM^{TM} and SP-5PW media

Separations using the SP-5PW column (Fig. 4A) resulted in the separation of the two major species (PEG MPO and

Table 3
Evaluation of PEG MPO separation from analytical runs

Support	$W_{1/2h}$ (min)	Resolution	Integrated peak area (%)	Retention time (min)
Q-5PW	0.45 ± 0.01	6.24 ± 0.58	28.5 ± 0.62	4.56 ± 0.10
QA-CIM™	0.12 ± 0.01	2.61 ± 0.24	41.5 ± 1.74	0.43 ± 0.01
SP-5PW	0.71 ± 0.04	3.22 ± 0.36	18.4 ± 1.25	10.9 ± 0.11
SO_3-CIM™	0.17 ± 0.01	1.36 ± 0.14	36.7 ± 5.70	0.96 ± 0.01

MPO) with a third smaller contaminating peak eluting between the two. The contaminant may be a partially unfolded form of the PEG MPO or possibly MPO with a PEG conjugated at a different site that was not separated using the other methods. Further analysis of this specie will be needed to confirm its identity. Peaks using the SO_3-CIM™ disk (Fig. 4B) appeared less symmetrical with noticeably more peak tailing. The lack of a noticeable third peak with the SO_3-CIM™ separations may be due to the drifting baseline and wider peaks that may be masking it.

3.6. Evaluation of analytical separation using QA/SO_3-CIM™ and Q-5PW/SP-5PW media

Some of the same parameters that were used to describe peak shape and separation for the preparative runs were also employed for evaluating the analytical runs. A summary of the calculated values from repetitive runs ($n = 3$) is shown in Table 3.

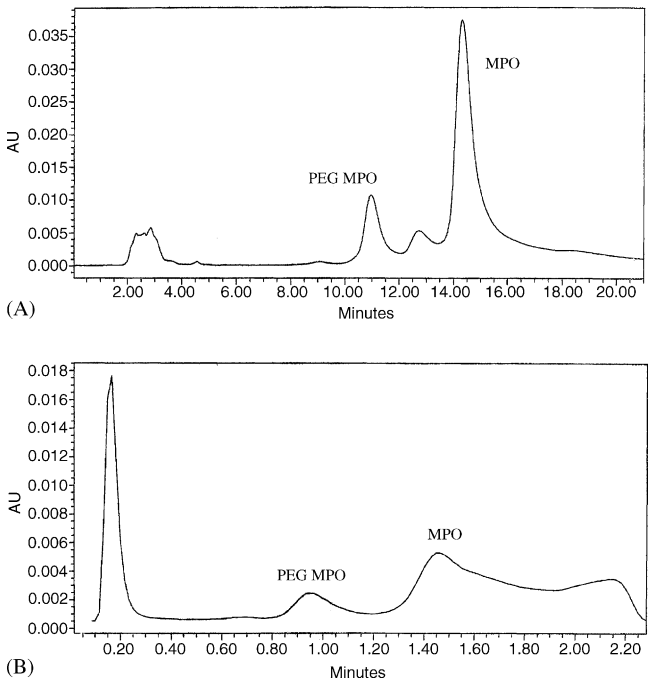

Fig. 4. Analytical separation of the PEG MPO reaction mixture using a Tosoh Biosep analytical SP-5PW column and SO_3-CIM™ disk (A) SP-5PW (75 mm × 7.5 mm i.d., 10 μm) column run at 1 ml/min over 20 min from 0 to 30% buffer D with UV detection at 280 nm. (B) SO_3-CIM™ disk (3.0 mm × 16 mm i.d.) run at 6.0 ml/min over 2.0 min from 40 to 100% buffer D with UV detection at 280 nm.

Retention times for the PEG MPO peaks were highly repeatable for all chromatographic supports with the largest CV of 2.3% occurring when using the QA-CIM™ disk. The integrated peak area values for PEG MPO from the different media also proved repeatable with CV values ranging from 2.2 to 15%. Although the reason remains unclear, the monolithic media consistently demonstrated a 1.5 to 2.0-fold greater peak area compared to their particle based counterparts. This observation was consistent with that seen with the preparative separations. Peak shapes for PEG MPO and MPO described by width at half height, demonstrate that the QA-CIM™ disks produced sharper peaks by approximately 1.5-fold compared to the SO_3-CIM™ disk run at the same flow rate. The Q-5PW and SP-5PW methods consistently produced symmetrical peaks while the Q-5PW produced slightly sharper peaks than the SP-5PW column. Resolution was achieved for each of the medias except the SO_3-CIM™ separations with the Q-5PW and SP-5PW columns showing superior resolution compared to the QA-CIM™ media.

The descriptive parameters suggest that all four columns produce repeatable and symmetrical peaks. However, visual inspection of the chromatographic profiles generated for both CIM™ columns show peaks with less symmetry and less baseline separation compared to their Q-5PW and SP-5PW counterparts.

4. Conclusions

We conclude that PEG MPO can be separated from MPO with baseline resolution under the conditions described for both the QA-CIM™ and SO_3-CIM™ media. Separations performed using the monolithic media for preparative purposes resulted in resolution of PEG MPO from MPO in 5 to 10-fold less time compared to traditional Q- and SP-Sepharose HP HiTrap columns yet maintained peak symmetry typical of traditional chromatography. Analytical chromatography of the PEG MPO and MPO mixture using the monolithic media also resulted in a 10-fold reduction of processing times when compared to the more traditional Q-5PW and SP-5PW analytical columns while maintaining baseline separation of the components.

Acknowledgements

Thanks to Rory F. Finn for his technical assistance with this paper.

References

[1] S. Santhanam, M. Decatris, K. O'Byrne, Biodrugs 16 (2002) 349.
[2] D.J. Shealy, P.H. Wooley, E. Emmell, A. Volk, A. Rosenberg, G. Treacy, C.L. Wagner, L. Maylon, D.E. Griswold, X.Y. Song, Arthritis Res. 4 (2002) R7.
[3] J.M. Harris, N.E. Martin, M. Modi, Clin. Pharacokinet. 40 (2001) 539.
[4] M.L. Nucci, R. Shorr, A. Abuchowski, Adv. Drug Deliv. Rev. 6 (1991) 133.
[5] P. Bailon, W. Berthold, Pharm. Sci. Technol. Today 1 (1998) 352.
[6] C. Delgado, G.E. Francis, D. Fisher, Crit. Rev. Ther. Drug Carrier Syst. 9 (1992) 249.
[7] T.J. MacVittie, A.M. Farese, W.G. Smith, C.M. Baum, E. Burton, J.P. McKearn, Blood 95 (2000) 837.
[8] A.M. Farese, D.B. Casey, W.G. Smith, R.M. Vigneulle, J.P. McKearn, T.J. MacVittie, Stem Cells 6 (2001) 522.
[9] A.M. Farese, D.B. Casey, R.M. Vigneulle, N.R. Siegel, R.F. Finn, J.A. Klover, W.G. Smith, J.P. McKearn, T.J. MacVittie, Stem Cells 19 (2001) 514.
[10] A. Strancar, P. Koselj, H. Schwinn, Dj. Josic, Anal. Chem. 68 (1996) 3483.
[11] T.B. Tennikova, B.G. Belenkii, F. Svec, J. Liq. Chromatogr. 13 (1990) 63.
[12] Dj. Josic, H. Schwinn, A. Strancar, A. Podgornik, M. Barut, Y.P. Lim, M. Vodopivec, J. Chromatogr. A 803 (1998) 61.
[13] A.I. Liapis, Math. Modelling Sci. Comput. 1 (1993) 397.
[14] A.E. Rodrigues, Z.P. Lu, J.M. Loureiro, G. Carta, J. Chromatogr. A 653 (1993) 189.
[15] A.I. Liapis, M.A. McCoy, J. Chromatogr. A 660 (1994) 85.
[16] Q.C. Wang, F. Svec, J.M.J. Frechet, Anal. Chem. 65 (1993) 2243.
[17] K. Pflegerl, A. Podgornik, E. Berger, A. Jungbauer, J. Comb. Chem. 4 (2002) 33.
[18] D. Sykora, F. Svec, J.M.J. Frechet, J. Chromatogr. A 852 (1999) 297.
[19] H. Podgornik, A. Podgornik, A. Perdih, Anal. Biochem. 272 (1999) 43.
[20] C. Kasper, L. Meringova, R. Freitag, T. Tennikova, J. Chromatogr. A 798 (1998) 65.
[21] L.G. Berruex, R. Freitag, T.B. Tennikova, J. Pharm. Biomed. Anal. 24 (2000) 95.
[22] N.D. Ostryanina, G.P. Vlasov, T.B. Tennikova, J. Chromatogr. A 949 (2002) 163.
[23] Unicorn Version 3.10 User Manual, Amersham Pharmacia Biotech, Piscataway, NJ, 1999, p. 10.
[24] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.