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Short Communication

Fast protein monitoring in fermentation broth using non-porous micropellicular reversed-phase columns

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ABSTRACT

The use of a short non-porous polymeric reversed-phase column is described for the rapid monitoring of the formation of recombinant human granulocyte colony stimulating factor in fermentation broths. The described procedure is capable of completing the assay in less than 10 min. Validation of the method showed acceptable recovery, accuracy, precision and specificity.

INTRODUCTION

In recent years the use of high-performance liquid chromatography (HPLC) has had a major impact on the separation and isolation of proteins. As a result of the development of highly efficient columns and instrumentation, HPLC is now the preeminent analytical technique used for rapid separation and precision measurement of proteins. Of the various separation modes available, reversed-phase with covalently bonded non-polar functional groups, has been the most prominent system used. The widespread use of these bonded stationary phases arises from the simplicity, the high column resolution attainable, and the relative ease with which selectivity can be manipulated through the use of pH and organic solvents. Silica-based wide-pore, 300 Å or greater, bonded phases currently dominate the high-performance chromatographic field as stationary phase materials. Instability at high pH, >8.0, has long been considered a problem. In addition, low diffusivity and restricted mass transfer result in long analysis times for proteins. Recently, a number of micropellicular supports, both silica based and polymeric, have been introduced commercially. These supports have been shown to allow much faster mass transfer due to absence of intraparticle diffusional resistances, and as a result, very fast (less than 10 min) separations of peptides and proteins are possible [1–4]. Moreover, polymeric supports have been shown capable of being operated at elevated temperatures of up to 80°C and at pH 11 without any stability deleterious results [5,6].

The majority of reports published have been concerned with either peptide separations obtained from enzymatic digestion or with separation of mixtures of standard proteins. The present paper describes the use of a non-porous polymeric micropellicular column for monitoring the rate of production of recombinant human granulocyte colony stimulating factor (r-met-HuG-CSF) produced in *E. coli* cells. Analyses were carried out at room temperature with an HPLC system that has been designed to accommodate regular 25 cm × 4.6 mm

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I.D. stainless-steel columns. Total analyses times selected were 5-min gradient with a 3-min re-equilibration time between injections.

EXPERIMENTAL

Chemicals and reagents

Recombinant r-met-HuG-CSF (approximate molecular mass 18 000) was produced at Amgen (Thousand Oaks, CA, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Fairlawn, NJ, USA). Mercaptoethanol was obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad (Richmond, CA, USA) and sequenal-grade trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA).

Apparatus and chromatographic parameters

All chromatographic analyses were carried out using a Model 8800 pump (Spectra-Physics, San Jose, CA, USA), a SPD-6A variable-wavelength detector (Shimadzu, Cole Scientific, Calabasas, CA, USA) and a Chrom Jet Integrator (Spectra-Physics). A Model 7125 injector (Rheodyne, Cotati, CA, USA) with a 10- or 5-μl loop was used to introduce the sample.

Dead volume was reduced by using 10-μl injection loops, replacing any 0.020 in. tubing with 0.010 in., inserting a low-volume gradient solvent mixing tube between pump heads and injection loop and removing dynamic/static solvent mixers. These modifications resulted in a total dead volume of 0.52 ml.

The mobile phase consist of (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile–water (95:5). The gradients were generated from 40 to 85% B with a flow-rate of 1 ml/min. The analytical column used is commercially available from Toso Haas (Philadelphia, PA, USA). It has dimensions of 3.5 × 4.6 mm with a polymeric-based support, TSK-gel-Octadecyl-NPR (Octadecyl-NPR) covalently bonded to 2.5-μm beads.

Isolation of r-met HuG-CSF from fermentation media

A 10-ml aliquot of fermentation broth was centrifuged at 10 000 rpm for 15 min. The pellet was resuspended in water and a 100-μl aliquot was removed for preparation. The sample was reduced using 2% SDS and mercaptoethanol. After heating the resulting mixture in boiling water for 10 min, the sample was passed through a 0.22-μm cellulose acetate filter and diluted prior to analysis.

RESULTS AND DISCUSSION

Production time for r-met-HuG-CSFcompletion is relatively short, being in the order of 6–10 h. The utility of the micropellicular column for fast r-met-HuG-CSF analysis in fermentation broth extract is shown in Fig. 1. Fig. 1A shows the chromatogram obtained from fermentation media at time 0, and Fig. 1B shows the corresponding chromatogram obtained at 6 h. These chromatograms were obtained using a 5-min gradient with a 3-min solvent re-equilibration, the total assay time being 8 min. From Fig. 1A, it is seen that there are no peaks co-chromatographing with r-met-HuG-CSF or eluting nearby that could interfere with the determination of r-met-HuG-CSF using the described chromatographic conditions.

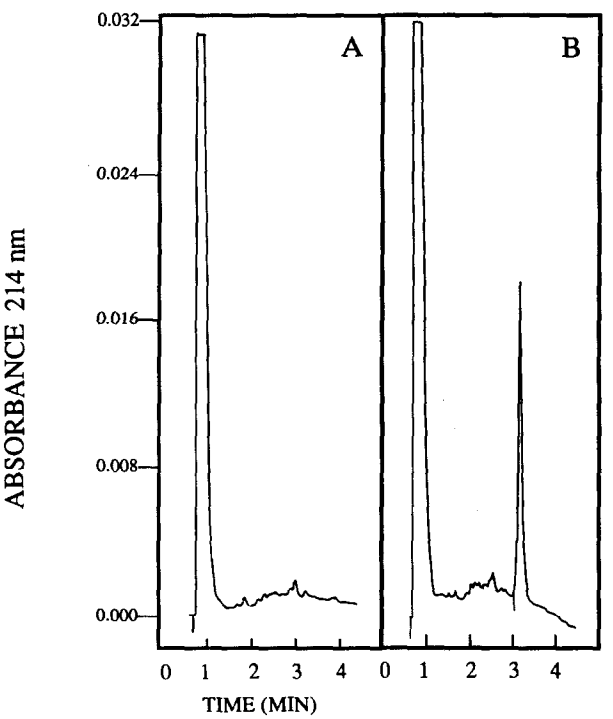

Fig. 1. (A) Chromatogram obtained from fermentation broth at time 0. (B) Chromatogram showing the presence of r-met-HuG-CSF at 0.34 μg in fermentation broth after 6 h.

The linearity of the assay was established by injecting various amounts of r-met-HuG-CSF in the range 0.1–1.0 μg and plotting area *versus* amount injected. Linearity was evident from 0.1–0.5 μg with correlation coefficients of 0.998 being obtained. From 0.5–1.0 μg, the curve showed some non-linearity but could still provide quantitative data. These results indicate that the loading capacity of the column is low and that it cannot be used for any preparative chromatography. Since the intent here is to provide fast protein determination of r-met-HuG-CSF in fermentation media, there is no absolute requirement of work within the linear range, and provided a suitable response curve is determined, a non-linear curve can be used to obtain reliable, reproducible results. Reproducibility of retention times (3.69 min ± 2.3%, $n = 7$) and peak areas (±3.0%) was determined by the repeated injection of 0.34 μg r-met-HuG-CSF.

Trifluoroacetic acid is used almost always in reversed-phase gradient elution schemes. Several other acids have been used including phosphoric and perchloric acids. The overall effect on chromatographic performance of trifluoroacetic, perchloric and phosphoric acids was compared. Phosphoric acid being the most hydrophilic caused r-met-HuG-CSF to elute the earliest while perchloric acid caused the slowest elution. Trifluoroacetic acid resulted in the sharpest peak shapes and was used in all subsequent studies for this reason.

Ghosting or hysteresis is a problem quite common to silica-based reversed-phase supports and its effect is most evident in the re-emergence of peaks from previous runs during a subsequent blank run. Frequently a blank run is required before a protein can be analyzed. Since r-met-HuG-CSF is a hydrophobic protein, the extent of ghosting was evaluated. The carry-over peak was determined to be *ca.* 0.2% and this level tended to increase as the column deteriorated. In one column that had been used quite extensively, a ghosting value of 2% was found. When this occurs, the column is cleaned with sodium hydroxide and if ghosting persists, the column is discarded.

Several publications describing the use of non-porous columns have stressed the absolute need for specialized HPLC equipment and the use of column heating ovens [7,8]. In the present study, non-porous columns were evaluated using a commercially available HPLC system that had been designed for use with conventional column dimensions, *i.e.*, 25 × 0.46 cm. The only changes made involved minimizing dead volumes.

The proposed method for the rapid determination of r-met-HuG-CSF produced in fermentation broth possesses the important characteristics of precision and specificity. In practical terms, the method has proven useful in monitoring r-met-HuG-CSF levels both in fermentation media and in the various steps involved in purification.

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