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Automated Process Monitoring of Monoclonal Antibody Production

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Antifibronectin, monoclonal antibody was monitored through 52 h of production. Samples were automatically drawn from a bioreactor into the injection valve of an HPLC system without prior sample preparation. The hybridoma cell line was nonadherent, so whole cells were injected directly onto the perfusable protein A affinity column. There was only a modest column back pressure (ca. 1700 psi at a linear flow rate of 1.5 cm/s) after over 75 injections over the 52-h experiment. These experiments demonstrate the utility of high-speed chromatography for rapid process monitoring.

INTRODUCTION

Federal regulatory agencies require that the production and purification of therapeutic proteins be monitored to confirm that processes are functioning within established boundaries. Because process monitoring today is a slow, off-line procedure, it can limit the rate of protein production. Rapid process monitoring would make it possible to both validate and feedback control processes.

Progress is being made in rapid process monitoring[1] particularly in downstream processing. Procedures have recently been developed that measure specific proteins in the effluent from process-scale chromatography columns in less than 60 s.[2] The most useful of these would appear to be high-speed immunological assays. Using a system designed for high-speed immunosorbent assays, it has been possible to automatically control fraction collection and select the purity of antigen fractions that will be collected from chromatography columns.[3]

Rapid process monitoring in fermentors is equally important. When production is complete, the product from a bioreator should be harvested to prevent degradation. Because cell growth and biosynthetic activity vary from batch to batch, the point at which production is maximum must be monitored. Harvesting based on monitoring avoids losses of product from both premature harvesting and degradation in the fermentor. Unfortunately, monitoring biosynthetic activity in living cells is more difficult than analyses in downstream processing. Aseptic sampling, the occasional need to lyse cells, and removal of particulates are additional problems that must be solved in monitoring cell cultures.

Cation exchange chromatography with soft gel media was used to monitor the biosynthesis of monoclonal antibodies almost a decade ago.[4] The limitations of this approach were that it was slow, automation was difficult, and cation exchange chromatography is of low selectivity. More recently, an automated, on-line HPLC system for monitoring fermentation in *Escherichia coli* has been described.[5] Although vastly superior to a soft gel chromatography, HPLC systems are still of limited selectivity and generally too slow to allow rapid monitoring. Bioaffinity methods probably hold the most promise for monitoring cell cultures. In a study designed to simulate antibody production in a hybridoma culture, it has been shown that IgG added to a hybridoma supernatant at concentrations of 20 and 200 μg/mL can be quantitated with a standard deviation of 3% in 20 min by affinity chromatography on a protein A–Sepharose column.[6] The great advantage of the affinity approach is that the target protein is released from the column as a single peak that is either pure or highly purified. The limitations of this specific paper[6] are that it does not deal with the issue of aseptic sampling, removal of cells, and automation. Also, soft gels are not useful in rapid, high-throughput analytical systems. Immunological assays are another bioaffinity approach. Using a fluorescence-based immunoassay, it has been possible to monitor fermentation in animal cells within 30 min by use of a nonautomated microtiter plate format.[7] The principal disadvantage of such a microtiter plate based immunological assay system for monitoring a fermentor would be the difficulty of automation.

This paper describes an automated, high-speed analytical system with direct, aseptic sampling for monitoring the product of monoclonal antibody in a hybridoma cell culture.

EXPERIMENTAL SECTION

Hybridoma Cells. The nonadherent hybridoma cell line, CRL-1606, that produces a monoclonal antibody against fibronectin was originally obtained from the American Type Culture Collection (Rockville, MD). The hybridomas were split to 150 000 cells/mL into Dubelco's modified Eagles medium (DMEM) containing 4500 mg/L glucose, L-glutamine, 5% fetal calf serum, 1% antibiotics, and without sodium pyruvate. The total media volume was 70 mL in a 175-cm² T-flask which was placed in a 37 °C water bath with a cap that contained an inlet and an outlet.

Reagents and Buffers. Tris base, tris hydrochloride, and sodium chloride were obtained from Mallinckrodt Chemical Co. (Paris, KY). Hydrochloric acid was purchased from Pierce Chemical Co. (Rockford, IL). Dubelco's modified Eagles medium was obtained from JRH Biosciences.

Instrumentation. A BioCAD perfusion chromatography workstation from PerSeptive Biosystems (Cambridge, MA) was used in all chromatographic separations. This instrument is equipped with a sampling valve and autoloader that allows one to four reaction vessels to be coupled to the instrument, and samples of 5 μL to several milliliters can be taken automatically. The computer in this instrument was loaded with the rapid process monitoring software from PerSeptive Biosystems. This software controlled sampling, chromatographic analysis, data processing, and generation of the final report.

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Chromatography Column. Affinity separations were carried out with a 2.1 × 30 mm POROS A/M, protein A affinity column from PerSeptive Biosystems.

Sampling Apparatus. A 30-cm length of 0.030 in. inner diameter PEEK tubing was inserted through the outlet of the cap. It extended from the back of the T-flask through the cap to the inlet of the sampling valve on the HPLC system. A 20-cm length of surgical tubing was also inserted in the cap to allow pressure equilibration and gas exchange. The system was kept sterile by a 0.2-μm filter at the end of the surgical tubing. The entire cap assembly (viz. the cap, the inlet tubing, and the outlet tubing) was autoclaved before attachment to the T-flask.

Samples were drawn in triplicate from the flask at 2-h intervals. Although the sample loop volume was only 100 μL, 500 μL was drawn for each sample in order to flush the loop as well as the inlet tubing that extended into the T-flask. The loading buffer was 20 mM tris + 150 mM NaCl pH 7.0, and the desorption buffer was 12 mM HCl + 150 mM NaCl. The flow rate was 3.0 mL/min, and detection was at 220 nm. A step gradient was used for antibody elution. The experiment was repeated three times and similar results were obtained.

RESULTS AND DISCUSSION

The function of this analytical system was to automatically monitor the production of immunoglobulin G (IgG) in a hybridoma culture. IgG is secreted from hybridoma cells where it accumulates in the growth medium. For this reason, cell disruption is not required for analysis. While the system was being designed, it was determined that the following features were critical: the capability to (1) aseptically sample cultures, (2) deal with cells in the analysis, (3) detect antibodies between the concentration limits of 1 and 100 μg/mL, (4) discriminate IgG from pH indicators and other proteins in the sample, (5) make analytical measurements within 1 min, (6) operate all aspects of the assay automatically, and (7) archive processed data. The design rationale for each of these features and the results of testing will be described below.

Aseptic Sampling. Fermentation campaigns in modern fermentors can range from a few hours in the case of bacteria to 6 months with immobilized mammalian cells. During the course of a long campaign, it is necessary to provide the cells with oxygen and growth medium in addition to withdrawing fermentation product(s). Contamination of the culture with other rapidly growing organisms during invasive procedures is a constant problem, particularly in the case of slow-growing mammalian cells. A single bacterium in a 100-L fermentor can totally contaminate the fermentor in less than 2 days.

Manual withdrawal of analytical samples carries the same risk of contamination. Attempts to construct an automated mechanical sampling system using pipets or syringes were unsuccessful (data not shown). The contamination problems experienced in preliminary studies were apparently due to an inability to maintain the sterility of pipets or syringes for long periods of time during repetitive sampling.

Fluid withdrawal through membranes has also been used to sample fermentors. Hollow fibers and tangential flow membranes have both been used successfully in large-volume sampling.[8] Although aspiration of samples through a 0.2-μm sterile membrane interface attached to the fermentor was successful, a membrane interface was not selected for the automated analytical system. Occasional plugging of the membrane interface, cavitation in transfer lines, and variability in the volume of aspirated samples reduced the reliability of the membrane interface.

The most reliable sampling device was the simplest. The BioCAD instrument is equipped with a computer–controlled sampling valve and variable-volume syringe for sampling up to four reaction vessels (Figure 1). Sampling rate may be varied from once per minute to several hours while sampling volumes of 5-5000 μL may be selected. Although only one fermentor was monitored in these studies, this instrument could monitor four reactors simultaneously if they were immediately adjacent.

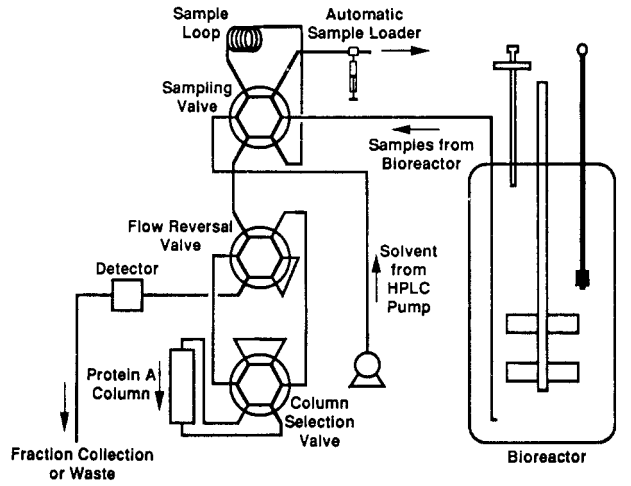

Figure 1. BioCAD HPLC system. A 175-cm² T-flask was connected directly to the sample valve of the system. A 0.2-μm filter was used for gas exchange and to filter out bacteria; 500 μL of media was flushed through the 100-μL sample loop for each sample during the time course.

The fermentor was connected directly to one of the bulk sample inlets with a 30-cm length of sterile 0.762 mm internal diameter tubing of ~137-μL internal volume. Samples (500 μL) were withdrawn in these studies to displace all of the liquid in the interface line and instrument. Stainless steel and Teflon tubing appeared to be of equal efficacy. No attempt was made to sterilize any of the sample-contacting components of the BioCAD. Because sampling occurred with a frequency of every 2 h or less and fluid flow toward the valve swept foreign organisms from the interface tubing, even motile organisms could not traverse the 30-cm length of sterile tubing between sampling events. Interface tubing was replaced after several weeks to preclude the possibility of bacteria attaching to the walls and eventually growing back into the fermentor. This could be a problem in 6-month fermentation campaigns where it is undesirable to break sterile connections to the fermentor by replacing the interface tube. An air filter allowed gases to displace the liquid removed during sampling while sterility was maintained.

Sampling events were programmed through the BioCAD computer. The time of fluid withdrawal from the fermentor and sampling volume were programmed as individual events in the sampling and gradient elution protocol for the chromatographic analysis. During most of the studies conducted, a linear set of commands was used to execute the analysis. This means that the instrument is only executing one operation at a time. When the rate of analysis is high, it is necessary to carry out operations in parallel; i.e., both sampling and column elution may be occurring simultaneously. Because separate commands are used to "sample" and "inject" in the instrument, it is possible to sample the fermentor while the chromatography column is being eluted and inject the sample on the next chromatographic run. Sample was generally withdrawn 30 s before it was used for analysis. The first sample was discarded, and for subsequent injections, it is assumed that the biosynthetic activity of cells in the sample loop would be nearly identical to those in the fermentor during this short time period and would still be representative of the bulk solution at the time of analysis.

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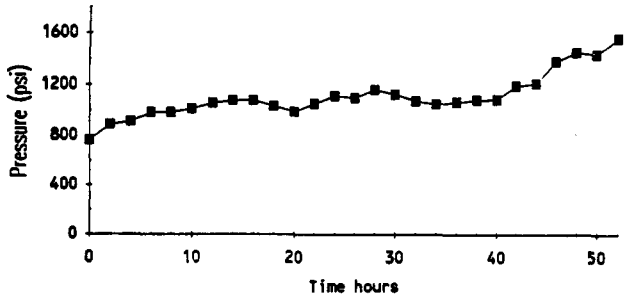

Figure 2. Pressure change over the sampling time course. The plot indicates a pressure increase of ~800 psi. During the time course, the column was neither cleaned nor back-flushed. Other experiments show that the pressure increase is reversible.

Dealing with Cells. It has been a long-standing practice in liquid chromatography not to put particulate matter into columns. Samples containing cells are generally filtered or centrifuged before analysis. This is the second reason that membrane interfaces have been used to obtain samples for analysis. While the sampling interface was being developed it was recognized that (i) mammalian cells generally grow at low density, (ii) only 10–20% of the solution withdrawn from the fermentor is used in the analysis, and (iii) the sensitivity of modern instruments allows analyses to be achieved with 10-1000 ng of analyte. Analyte samples of this size can frequently be obtained with only a small number of accompanying cells. These facts led to a reexamination of the question of adding samples to a chromatography column without first removing cells.

Studies were carried out in which cell cultures were injected directly onto 2.1 × 30 mm chromatography columns fitted with a 2-μm inlet frit and operated at a flow rate of 3 mL/min (1.5 cm/s linear velocity). Accumulation of debris at the column inlet caused the operating pressure of the system to increase as expected. However, the problem was far smaller than anticipated. Approximately 10^6 cells could be applied to a column before measures had to be taken to deal with cell accumulation. In a typical case, 75 sample injections during 52 h of operation caused the pressure to increase 800 psi without loss of resolution (Figure 2). This is well within the operating limits of HPLC instrumentation.

Quantification of the relationship between cell mass applied to the column and increasing operating pressure was achieved by repeated 50-μL injections of hybridoma culture (9.7×10^5 cells/mL) into the column. This is near the maximum cell density experienced in the growth of hybridoma cells. The cell suspension was continuously stirred and samples were analyzed immediately to preclude sampling errors from cell settling. Operating pressure was recorded and averaged over 2 min following each injection. Even at the very high mobile-phase velocity of 1.5 cm/s, the column operating pressure did not exceed 2000 psi after 22 injections (Figure 3). A linear regression analysis shows that there is a ~1 psi increase in operating pressure for every 1000 cells injected. A plot of the residuals from the regression analysis shows that the pressure increase is not truly linear. With increasing cell mass injected into the column, the incremental increase in pressure becomes smaller. The exact reason for this phenomenon is not known. After this experiment, the column was back-flushed with 100 mL of mobile phase for 1 h. This treatment reduced the operating pressure accumulated from the injection of cell by 80% (Figure 3). Replacement of the column frit restored the column to the original operating pressure, showing that only the frit had been plugged. Although it is likely that some of these 10–50-μm mammalian cells were disrupted on the frit and passed into the column, the fragments were apparently too small to block passages in the column.

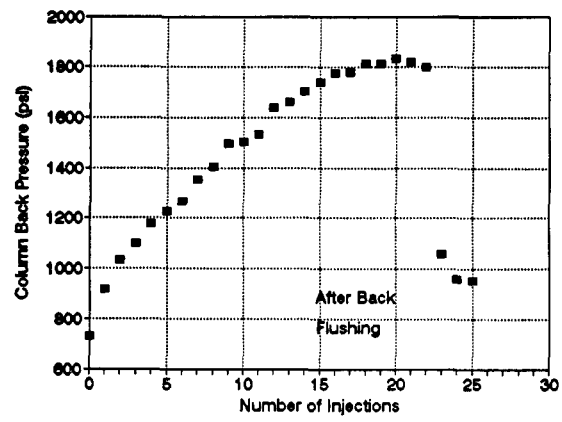

Figure 3. Column operating pressure vs number of injections. Sample volumes of 50 μL of 9.7×10^5 cells/mL were injected onto the head of a 1.2 × 30 mm protein A column at 3.0 mL/min. The column back-pressure was then monitored for 2 min following each injection. At a linear velocity of 1.5 cm/s, the column back pressure never exceeded 2000 psi. The pressure increase was also reversible by simply back-flushing the column.

It may be concluded that mammalian cell suspensions can be analyzed in one of the following ways. One is to back-flush either the column or an in-line filter after each injection of cells. This procedure precludes accumulation of cells on frits but also takes more time. A second is to back-flush the column after every 50–100 analyses. This treatment was adequate in these studies. The third way is to replace the column inlet frit when the operating pressure reaches unacceptable levels. There is also the possibility that a step in which the column is eluted with 1 N KOH would reduce the accumulation of cells. Strong base disrupts cells and solubilizes many cellular components. However, strong base will destroy carbohydrate- or silica-based chromatographic supports. For this reason, a polystyrene-based support was used in this study.

Selective Detection. Chromatographic systems are often required when available detection methods do not allow direct discrimination between an analyte and other interfering species in a sample. In the case of excreted proteins in a complex growth medium, their absorption spectrum is so similar to that of the contaminants they they may be neither identified nor quantitated directly by absorbance measurements. It is necessary that the chromatographic system eliminate impurities from the analyte before it is delivered to the detector. The highest degree of purification for a specific protein analyte is almost always obtained with some type of bioaffinity chromatography.

Immunoglobulin G produced by a hybridoma culture could be purified chromatographically either by an anti-mouse IgG immunoaffinity column or by one of the columns prepared with an immobilized IgG binding protein. The most well-known of these IgG binding proteins are protein A from *Staphylococcus aureus* and protein G from *Streptococcus*. These proteins bind IgG by the Fc region, maximizing the ability of the Fab domain to bind antigen.[9] Studies have shown that protein A and G columns can also differentiate between some classes of IgG from different animals. An immobilized protein A column was selected for the following reasons. First, it is easier to elute IgG from a protein A column than an immunosorbent. Recovery is probably higher as a consequence. Second, it has been shown that protein A columns may be used in the quantitation of IgG.[10] Third,

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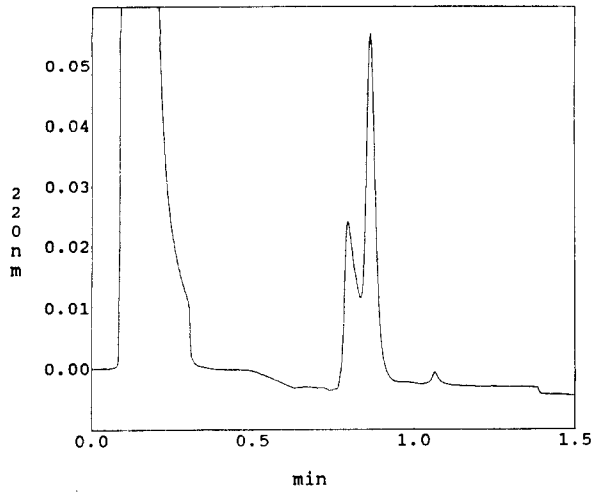

Figure 4. Chromatogram from the sample at 46 h, showing that interfering substances in peak 1 account for 98.1% for the total absorbance. There are two IgG peaks because at least two forms of the antibody are being produced in this particular cell line.

protein A columns are commercially available with sufficiently large pores that operation in the convective transport mode is possible. It has been shown that support particles with large through-pores allow liquid to flow through the support during operation at high mobile-phase velocity. This perfusion of liquid through supports enhances intraparticle transport of analytes more than 1 order of magnitude. The net result is that columns may be operated at 10 times higher mobile-phase velocity than conventional HPLC columns. Chromatographic separations in 1 min are common with perfusion chromatography.[11,12]

IgG concentrations in hydridoma culture media can range from 1.0 to 50 μg/mL. The IgG binding capacity of the 2.1 × 30 mm protein A column used in these studies was 2 mg as determined by frontal loading with bovine IgG. Although the binding capacity of the column could be slightly different with mouse IgG, this column has 10–100 times the capacity necessary for assays of IgG in hybridoma cultures. Sample volume in the studies described here was 100 μL. It is desirable to use the smallest possible sample to minimize the load of cells applied to the column. For this reason, detector absorbance measurements were made at 220 nm to increase detection sensitivity and allow smaller samples to be used. The extinction coefficient of proteins is higher at 220 nm than either 254 or 280 nm.

Serum proteins, pH indicators, and small UV-absorbing nutrients and metabolites contained in a sample taken from the fermentor after 46 h of fermentation were eluted from the column as a single peak in 0.25 min (Figure 4). IgG was subsequently desorbed with 5 mL of 12 mM HCl, eluting as a split peak. This indicates that at least two forms of IgG are being produced by the CRL-1606 cell line which the protein A column was able to resolve. It has been shown that CRL-1606 cells can produce multiple forms of IgG in which there is a mixing of heavy and light chains.[13] Both these peaks were collected from the protein A column and subjected to SDS gel separation. This showed the presence of both heavy and light chains, validating that these peaks were IgG peaks. An overlay of selected chromatograms from the fermentation

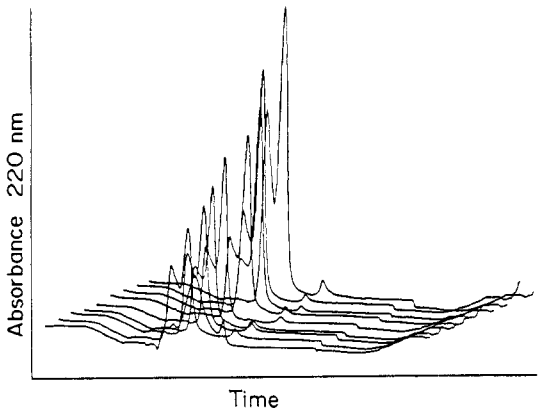

Figure 5. Tracing of stacked chromatograms, indicating that both IgG peaks rise together over time. The chromatograms shown were taken at 0, 10, 16, 26, 36, 40, 44, 48, and 52 h from front to back.

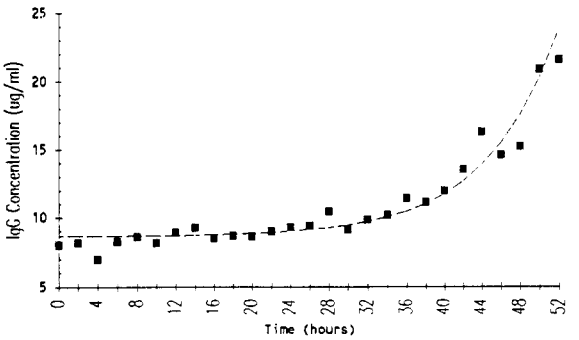

Figure 6. IgG concentration vs fermentation time. The amount or IgG in the media is determined from the peak areas of the individual chromatograms and plotted as a function of time. The solid squares represent the actual concentration, and the dashed line is an exponential function fit to the data.

shows that both forms are produced throughout the time course of the reaction (Figure 5). Because the selectivity of the protein A column was beyond the requirements of the assay, the data system was instructed to sum the area under the two peaks when computing IgG concentration. (It was assumed that the two forms of IgG have the same extinction coefficient.) By use of the sum of the two IgG forms, a production curve was produced for a fermentation campaign (Figure 6). Assuming that the biosynthetic rate of cells is constant during the fermentation process, the increase in IgG synthesis would indicate an exponential increase in cell density, as expected.

Although protein A affinity columns were used in these studies, it may be concluded that other binding proteins could be used to monitor protein synthesis and purification. Through careful selection of lectins it is probable that specific glycoproteins can be monitored. Protein L is another case. This new recombinant protein binds to other classes of immunoglobulins and may extend immunoglobulin analysis.[14] Finally, there is the case of immunosorbents. Studies on the quantitation of antigens in cellular extracts and serum all show the ability of antibodies to bind specific proteins in the presence of a large number of other species. A recent review on tandem liquid chromatographic–immunological assays (LCIA) shows that even variant forms of the same protein may be quantitated in a few minutes.[15] In the LCIA approach, immunological recognition of specific epitopes is coupled with the broad structure discrimination provided by liquid chromatography columns.

Automation. It was noted above that sequencing, timing, and setting the duration of individual events in an individual

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analysis were all controlled from standard software within the BioCAD. These events included sampling time and volume, timing and volume of sample injected, purging the sample lines and sample loop, solvent composition and gradient during gradient elution, column washes and recycling, and data collection. Because the analysis cycle was repeated automatically at the end of each run, a time hold was written into the end of the program to control the time between analytical runs. In this way, the time interval between analyses was the same but could be easily adjusted between different fermentation campaigns.

Another case was examined in which the time interval between analyses was long at the beginning of fermentation but became more rapid at the end of fermentation when product was accumulating quickly. This was achieved by using a time template that allowed the length of the "hold step" to be varied systematically between runs. Hold times were varied from 0 to 2 h.

Intermittent operations are also necessary. Back-flushing columns and cleaning the columns by elution with strong base were two cases that were examined. The BioCAD software allows several different analytical campaigns to be coupled into a single large, master program. Through this process, a program was written in which the analytical column was back-flushed and washed with 1 N KOH after sets of 50 analytical runs.

Data Management. The purpose of performing an analysis and recording data is to use the data to make an informed decision. Cell cultures are monitored to determine the optimum point for harvest. When purification systems are monitored, the objective is to obtain product that meets a defined purity or concentration.

The standards of purity and concentration were established before the process was started. The concentration limit was set at 23 μg/mL. Since, the minimum requirements were already determined, one need only decide whether these conditions were met. The process monitoring software on the BioCAD is able to make decisions based on defined concentration and purity limits and act on the results of the analysis by switching valves, activating remote equipment, or initiating any other feedback control event that can be activated by the computer. After the concentration limit of 23 μg/mL was reached, the computer automatically switched a valve that allowed the bioreactor to be harvested. The computer was able to make these decisions because each chromatogram is automatically integrated at the end of each method. The integration data are then used to calculate product concentration and purity, which are then compared to the established limits. External events are triggered on the basis of the outcome of these comparisons. Further, each method can have different predefined limits. Each method can be made to run a defined number of cycles, or several different methods may be run sequentially in a master method. The master method then allows a great deal of flexibility in controlling the timing of each method. This master method was then used to increase the rate of sampling toward the end of the process.

Another use of process monitoring is for process validation. Records must be kept in order to show that the process did indeed proceed as planned. The BioCAD software stores the chromatograms as well as the system pressure, pH, and conductivity. Of course, the time, date, column, solvent composition, and other chromatographic parameters are recorded as well with each chromatogram. Information from these files was used to monitor the data on column back pressure. This suggested that the column back pressure increased only marginally at the end of process. Such stored information was also used to schedule service for the columns. It was possible to follow the history of a column and to know how much solvent had passed through it and how much the operating pressure had increased. These stored data were also used to monitor peak drifting and peak skewing.

CONCLUSIONS

The objectives of these experiments were to monitor production of IgG over time and to demonstrate the robustness of the assay. The assay is sensitive because UV absorbance at 220 nm is used for detection. The assay is also highly selective since protein A affinity chromatography is used, and the assay is relatively fast, requiring only ~3 min. The back pressure on the column increased only modestly after over 75 injections of whole cells. Furthermore, no culture contamination was detected due to periodic sampling.

Future work with this system may include monitoring multiple reactors that are a considerable distance from the instrument. Since the assay is very fast relative to the culture time, the limiting factor is the ability to obtain remote samples. Peristaltic pumps close to the bioreactor may be needed to provide sufficient pressure to drive samples to the analytical instrumentation. Efforts should also be undertaken to develop special interfaces between bioreactors and process monitors.

ACKNOWLEDGMENT

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