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Analytical method optimization for protein determination by fast high-performance liquid chromatography

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Abstract

A method based on fast HPLC allowing the rapid and efficient determination of proteins in mammalian cell cultures is proposed, involving the use of two chromatographic modes, RP-HPLC and high-performance liquid affinity chromatography. These two sequential chromatographic analyses separate the proteins most often used as supplements in serum-free media such as bovine serum albumin, insulin and transferrin, and also monoclonal antibodies secreted by hybridoma in cultures. Rapidity, reliability and flexibility are the main characteristics of the method. The monitoring of proteins in the course of a discontinuous hybridoma culture is presented.

1. Introduction

HPLC has made major advances over the past 10 years, especially in applications to protein determination and purification. The technological improvements to and the theoretical knowledge of this technique have widened its area of application. As the analytical level, HPLC ensures qualitative and quantitative control of protein molecules [1]. Among HPLC techniques, fast HPLC with a non-porous stationary phase has permitted the analytical separation of various proteins [2–6].

The need to associate efficient and reliable analytical methods with production and purification bioprocesses is a major concern of recent research in biotechnology [7]. Efficient analytical control of a given production process contributes to improving the productivity and the understanding of physiological and metabolic mechanisms. Moreover, once the molecule of interest has been produced, analytical control has to be performed in each step of its purification. These requirements are principally encountered when high-value molecules are produced by bioprocesses.

Qualitative analysis of a protein secreted by recombinant microorganism may be checked by HPLC based on the detection of undesirable protein variants [8–10]. Peptide mapping may be considered as the most efficient method for determining the biochemical quality of proteins to be used in medecine [11–13]. This technique is widely employed for rt-PA and hGH determinations [1].

The use of HPLC as a quantitative tool is becoming more widespread. Notably, HPLC allows the establishment of the evolution of

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protein concentrations. The integration of HPLC in an automated system interfaced with a bioreactor in order to monitor on-line the evolution of protein concentrations is of current interest [14–16].

Concerning hybridoma cultures performed to produce monoclonal antibodies, studies dealing with the monitoring of proteins in the medium are scarce. However, the use of serum-free media characterized by an accurately defined formulation makes easier the specific determination of each protein added as a supplement [17–19]. Some examples of chromatographic modes accurately applied to determine secreted IgG may be noted [20–24]. Nevertheless, this field of application does not fully take advantage of the overall potential of HPLC.

The aim of this study was to develop a rapid and accurate chromatographic method for measuring proteins encountered in hybridoma cultures. The method involves the use of two chromatographic modes, RP-HPLC and high-performance liquid affinity chromatography (HPLAC). Optimization of each chromatographic mode and the parameters that influence them are presented. The use of the optimized method to determine proteins during a hybridoma culture is described to illustrate its potential in this area of application.

2. Experimental

2.1. HPLC method

Instrumentation

Chromatography was performed with an HP 1090 liquid chromatography (Hewlett-Packard) with a ternary DR5 solvent-delivery system, a diode-array detector (400–600 nm) and an autosampler. The chromatographic system and data evaluation were controlled by an HP 9000 Chem Station computer.

The reversed-phase column used was a Hy-Tach C_{18} micropellicular reversed-phase column provided by Professor Cs. Horváth (Yale University). This column (30 × 4.6 mm I.D.) consists of non-porous silica microspheres ($d_p = 2$ μm) coated with C_{18}-alkyl chains. The affinity column used was a Perstorp Biolytica (Lund, Sweden). ProAnaMabs column. This column (50 × 5.6 mm I.D.) is packed with 10-μm Selectispher silica particles covalently linked to a proprietary Fc receptor (protein A + protein G).

Reagents

RP-HPLC mobile phase and standard proteins. The mobile phase consisted of an aqueous phase (A) and an organic (B) phases with the following compositions: A = 0.1% trifluoroacetic acid (TFA) (Sigma) in water and B = 0.1% TFA + 95% acetonitrile (Prolabo). The aqueous and organic phases were filtered through a 0.45-μm filter (Interchim) on a Millipore filtration system and degassed by sparging with helium before use. Standard proteins to be measured by RP-HPLC (albumin, insulin, transferrin) were purchased from Sigma.

HPLAC mobile phase and standard IgG. The binding solution consisted of 100 m*M* citrate buffer (pH 5), obtained by mixing equimolar solutions of citric acid and sodium citrate and adjusting the pH by the addition of an acidic solution. The eluent solution was 100 m*M* citric acid adjusted to pH 1.6 with concentrated HCl. Both solutions were filtered through a 0.2-μm filter (Sartorius) and degassed by sparging with helium before use. Both solutions are stable for over 1 week at 4°C. Two standard monoclonal antibodies were used for calibration: mouse IgG_1 with 80% purity (Sigma) and mouse IgG_1 with 100% purity (Zymed).

Calibration procedure

Sample preparation. The require concentration range of each protein was obtained by dilution of a 1 mg/ml solution. This concentrated solution and all dilutions were made with the appropriate phase: water for BSA, insulin and transferrin and binding solution for IgG. Both BSA and transferrin concentrated solutions (1 mg/ml) are stable for over 1 month at 4°C but IgG concentrated solutions are stable for over only 1 week

at 4°C. Insulin concentrated solutions used for dilutions have to be freshly prepared. Calibration graphs were obtained according to the concentration of each protein in the serum-free medium: 10–50 μg/ml for transferrin, 5–50 μg/ml for insulin, 10–300 μg/ml for BSA and several levels for IgG and BSA–IgG mixture.

Precision determination. The reliability of each calibration graph was established by a statistical study. The calculation of each standard deviation relative to one protein concentration permits the determination of the precision of quantitative measurements by HPLC. Each standard sample corresponding to a defined protein concentration was injected ten times. By pooling the results obtained over several analyses, standard deviations were determined. A typical calibration graph for each protein assay was established. The reproducibility of the measurements and the influence of various parameters were tested by comparison with the calibration graphs.

Analytical conditions

The conditions for RP-HPLC and HPLAC were optimized in order to achieve a rapid and reliable determination of the proteins albumin, transferrin, insulin and IgG. Consequently, several parameters were studied such as flow-rate and gradient shape for RP-HPLC and time of the adsorption phase for HPLAC. This optimization investigation resulted in the following operating conditions.

RP-HPLC. The temperature is maintained at 80°C during the stabilization phase, the analysis phase and the washing phase. The volume taken is 25 μl and is injected automatically into the chromatographic system. Absorbance at 215 nm was chosen. Protein separation was performed with an organic solvent gradient in 5 min with a flow-rate optimized at 1.5 m/min. Further details of the conditions for RP-HPLC are discussed under Results.

HPLAC. The temperature is controlled at 25°C. The volume of each sample injected is 250 μl and IgG is detected at 280 nm. An equilibration phase is proposed in which binding solution is run for 45 min. Determination of IgG is achieved by changing the mobile phase pH: after 2 min of running 100% binding solution, IgG desorption is induced by injecting 100% eluent solution for 2 min. In both binding and elution phases the flow-rate is 3 ml/min.

2.2. Hybridoma cultures

Cell line and medium

Mouse hybridoma cells VO 208 secreting IgG_1 of unknown specificity were provided by Bertin. The line results from the fusion of mouse BALB/C spleen cells with myeloma cells. The serum-free medium used was optimized in our laboratory [17]. The basal medium consists of 50% IMDM (Iscove Modified Dubelco Medium) + 50% Ham F12 with 18 m*M* glucose, 3 m*M* glutamine (Intermed); the supplements were 4 mg/l insulin, 25 mg/l transferrin saturated with iron, 0.2% (w/v) polyethylene glycol, 20 m*M* aminoethanol, 50 m*M* β mercaptoethanol, 12 n*M* sodium selenite, 20 mg/l ascorbic acid, 18 ml/l liposomes containing cholesterol, oleic acid, dipalmitoyl-L-α-phosphatidylcholine and 180 mg/l BSA (Sigma).

Culture operating conditions

For batch operation, a 250-ml spinner flask (Techne) was used, containing 200 ml of medium with an inoculum cell density of $2 \cdot 10^5$ cells/ml. Twice a day, 8-ml aliquots are collected from the spinner. Cells count and viability were determined by the Trypan Blue dye exclusion method using a haemocytometer. The remaining sample was centrifuged and the supernatant was divided into aliquots and stored at −20°C until protein analyses. Proteins were determined by RP-HPLC and HPLAC.

3. Results

3.1. Method optimization

The principal of the proposed method is based on the complementary performances of two

Table 1
Optimum RP-HPLC conditions for the determination of insulin, transferrin and BSA + IgG mixture

Aqueous phase (A)	0.1% TFA in water
Organic phase (A)	95% acetonitrile in water containing 0.1% TFA
Gradient	From 0 to 60% B in 5 min
Flow rate	1.5 ml/min
Temperature	80°C
Volume injected	25 μl

analytical columns, RP-HPLC and HPLAC. This sequential analysis allows the four proteins of interest to be accurately measured: proteins added to the serum-free medium (insulin, transferrin, BSA) and monoclonal antibodies secreted by hybidoma.

The test of several operating parameters (flow-rate, gradient shape, temperature) on the improvement of the resolution between BSA and IgG by RP-HPLC did not give satisfactory results. Consequently, to overcome these limitations, the RP-HPLC conditions were optimized in order to obtain the determination of insulin, transferrin and the mixture of BSA and IgG, whereas IgGs were determined by HPLAC. The albumin concentration was deducted from these two types of measurement; the BSA concentration resulted from subtraction from the total BSA + IgG concentration obtained by using the RP column of the IgG concentration determined with the affinity column.

Protein determination by RP-HPLC

The optimized conditions allowing the simultaneous determination of insulin, transferrin and BSA + IgG are reported in Table 1. The optimization criteria were essentially rapidity and resolution. Protein determinations were realized in less than 6 min with optimum resolutions between each protein pool considered (insulin, transferrin and BSA + IgG). Under these conditions BSA and IgG have the same retention time and their total peak allows the concentration of their mixture to be obtained. Fig. 1 illustrates chromatograms obtained for proteins determined by RP-HPLC under the optimized operating conditions.

Three calibration graphs were established with the appropriate range of concentration of each protein, insulin, transferrin and BSA + IgG (Figs. 2 and 3).

To assess the precision of the method, standard deviations (S.D.) of the protein concentrations were determined. The error range of BSA and transferrin measurements increased from 1.5% at the higher concentrations (200 and 50 μg/ml, respectively) to 6% at lower concentrations (10 μg/ml). The precision of insulin determination is not so good, especially for low concentrations which make the integration of the peaks difficult (the S.D. Varies from 30 to 10% for the concentration range 5–50 μg/ml). Consequently, the threshold of sensitivity for insulin determination was estimated to be 10 μg/ml.

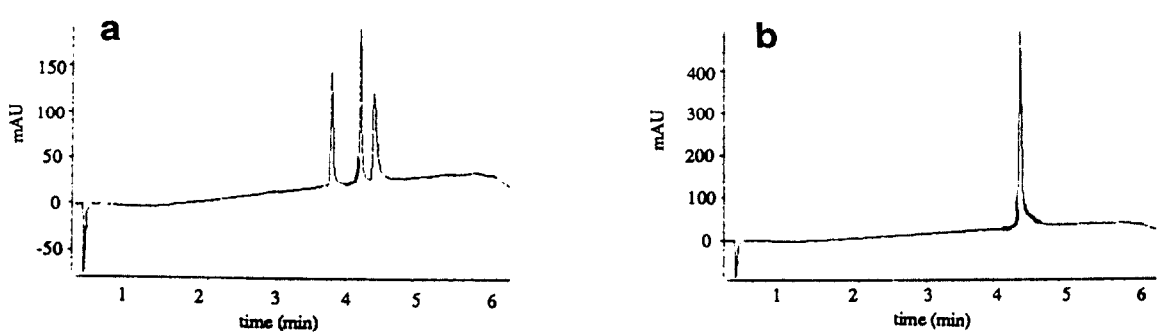

Fig. 1. Chromatograms for a protein mixture obtained by RP-HPLC. Column, 30 × 4.6 mm I.D. packed with 2-μm spherical silica particles having covently bound octadecyl functions at the surface. Analytical conditions as in Table 1. Sample components: (a) insulin 50 μg/ml, transferrin 50 μg/ml, BSA 50 μg/ml; (b) BSA 200 μg/ml, IgG 40 μg/ml.

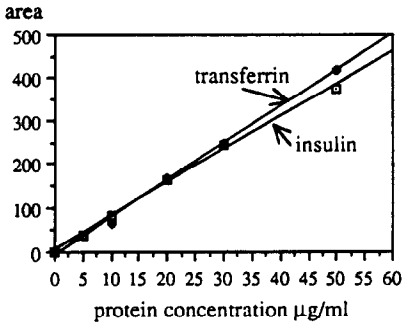

Fig. 2. Calibration graphs for transferrin and insulin. Calibration by RP-HPLC under the operating conditions given in Table 1.

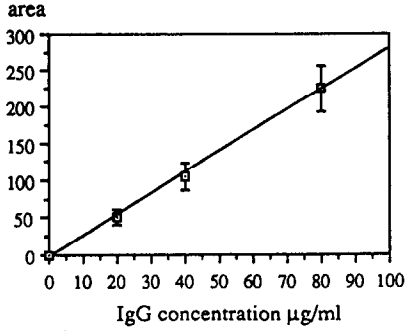

Fig. 4. IgG calibration with the affinity column under the optimized conditions given in Table 2. Vertical bars indicate confidence intervals.

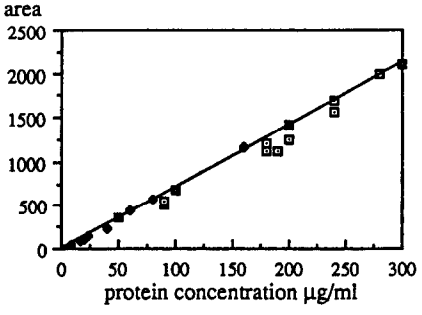

Fig. 3. Calibration graph for BSA + IgG established by RP-HPLC under the operating conditions given in table 1. Three ranges of dilution were used: ■ = calibration with BSA; ◆ = calibration with IgG; ⊡ = calibration with BSA + IgG mixtures.

IgG determination by HPLAC

The affinity column used for IgG measurement consists of a protein A and protein G stationary phase. Under the optimized operating conditions presented in Table 2, this column allowed the efficient determination of IgG.

The calibration of the method was defined within confidence intervals related to IgG concentrations (Fig. 4). The standard deviation varied from 7 to 15% for concentrations between 80 and 10 μg/ml, respectively. This calibration graph established with the precision lines was considered as a reference plot and, consequently, allowed the study of the influence of various parameters on the performance of the method. For testing the flexibility of the calibration method, the influence of various parameters involved in the protocol was investigated, vis., the nature of the dilution buffer, the source of standard IgG and the methods of conservation of standard solutions.

The results of these experiments compared

Table 2
Optimum conditions for the determination of IgG by HPLAC

Binding solution	100 m*M* citric acid (pH 5)
Eluent solution	100 m*M* citric acid (pH 1.6, adjusted with HCl)
Flow-rate	3 ml/min
Time of adsorption phase	2 min
Time of desorption phase	2 min
Temperature	Ambient (25°C)
Volume injected	250 μl
IgG standard	IgG_1, 80% purity
IgG diluted in	Binding solution

with the standard calibration graph showed that: serum-free medium, phosphate-buffered saline (PBS) and water could be used as dilution solutions for standard IgG; IgG_1 80% pure and IgG_1 100% pure could equally be chosen as standard monoclonal antibodies; fresh and unfrozen concentrated solutions used to prepare the range of dilutions could equally be adopted.

Further, the reproductibility of the measurements with repeated injections of one supernatant with unknown IgG concentrations was checked (Fig. 5).

Overall, the studies show that this method for IgG determination can be considered to be reproductible and flexible with regard to the analytical conditions.

3.2. Method application

This method was used as an efficient and fast means of monitoring the evolution of protein concentrations during cultures. A batch culture of hybridoma VO 208 was carried out with a serum-free medium in a spinner flask and with initial concentrations of protein supplements of insulin 4, transferrin 25 and BSA 180 μg/ml. Direct analysis of cell culture supernatants were performed on the RP-HPLC and HPLAC columns. No problem with column fouling was noticed.

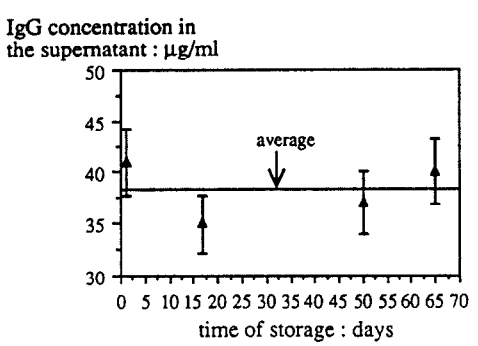

Fig. 5. Reproducibility of IgG determination tested by HPLAC. An aliquot of supernatant stored at −20°C was analysed four times over a period of 65 days. Vertical bars indicate the confidence interval relative to the measured concentrations (▲) (S.D. = 8%). Operating conditions are given in Table 3.

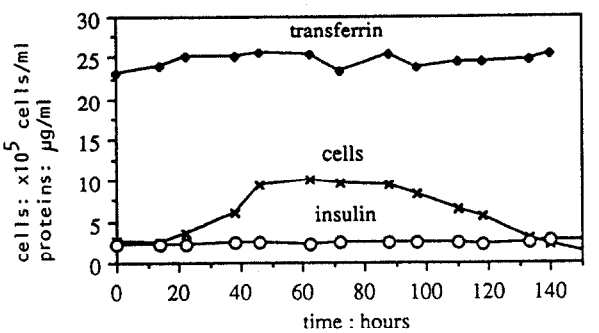

Fig. 6. Evolution with time of (×) viable cell density, (○) insulin concentration and (◆) transferrin concentration during a discontinuous hybridoma (VO 208) culture. Proteins were determined by RP-HPLC under the operating conditions given in Table 1.

Figs. 6 and 7 show the kinetics of viable cell density and of each protein encountered in samples taken during the overall culture and analysed by the proposed method.

The transferrin concentration obtained by RP-HPLC does not change during the course of the culture, the measured concentration being equal to the initial concentration added to the medium (25 μg/ml). The insulin concentration seems to be stable during the culture and to remain lower than the initial concentration. However, this result has to be treated with caution because of the poor precision of the method at low concentrations of insulin. The albumin concentra-

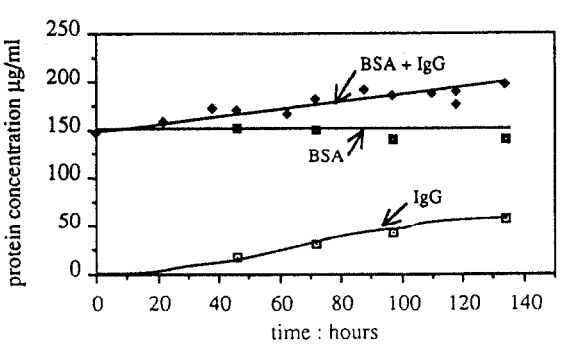

Fig. 7. Evolution with time of the BSA + IgG mixture and BSA and IgG concentrations during the discontinuous hybridoma culture. The BSA + IgG mixture (◆) was determined by RP-HPLC under the operating conditions given in Table 1 and the IgG concentration (□) by HPLAC under the operating conditions given in Table 2. ■ = BSA kinetic results from the first two analyses.

tion is deducted from the two consecutive chromatographic measurements, BSA + IgG determination by RP-HPLC and IgG determination by HPLAC. The determination albumin concentration in the supernatants indicated its kinetic stability at 155 μg/ml. The measured concentration is 10% below the initial concentration (180 μg/ml). Finally, the kinetics of monoclonal antibodies measured by HPLAC indicated a final concentration of ca. 50 μg/ml.

4. Discussion

The proposed method using two sequential chromatographic columns is considered to be suitable for the determination of the four proteins encountered in hybridoma cultures performed with a serum-free medium. Experiments were carried out in order to optimize the operation conditions and to investigate the characteristics of the method. The relative flexibility and rapidity of this technique can be emphasized: each analysis is completed in less than 6 min and the preparation of standards and samples is easy.

Concerning the determination of monoclonal antibodies, the specificity of the affinity column and the reproducibility of the measurements were checked. TEDESCO et al. [24] also investigated these interesting column characteristics. The precision of the determination of monoclonal antibodies varied from 7 to 15% for concentrations between 80 and 10 μg/ml, respectively. For comparison the enzyme-linked immunosorbent assay (ELISA) used for measuring monoclonal antibodies in cultures has a ca. 10–20% error. Other immunoassay methods have been proposed to overcome the limitations of ELISA [25,26].

The precision of the measurements of proteins added to the medium was studied. The standards deviation of transferrin and BSA measurements were in the range 2–6%. The precision for insulin was in the range 10–30% for concentrations varying from 50 to 5 μg/ml. Consequently, the results for insulin should be treated with caution.

The application of the method to discontinuous culture monitoring allowed us to establish the kinetics of the proteins and to obtain interesting data. According to the culture analysed, the transferrin concentration remained stable. Martial et al. [27] also showed the stability of transferrin by employing a nephelometric technique. However, they observed some consumption of insulin for several cultures of hybridoma cells. This difference may be explained by the limit of validity of the chromatographic method for measuring insulin accurately at low concentrations (<10 μg/ml). Concerning the determination of albumin, the stability of its concentration was noted. The difference between the measured and the theoretical concentration (180 μg/ml) may be explained by the fact that albumin is employed in the preparation of liposomes. This observation indicates that ca. 10% of albumin is incorporated into the lipidic vesicules. The measurement of IgG concentrations by using the chromatographic method allowed the production kinetics to be established with rapidity and reliability.

This study has demonstrated the interest and potential of the method in the context of hybridoma cultures, especially concerning IgG kinetics. Its applications could be widened to other cultures such as continuous cultures, cultures with serum or cultures with other hybridoma cell lines. Moreover, a completely automated bioreactor–HPLC interface with a sampling device could be considered for the on-line monitoring of IgG concentrations during hybridoma cultures.

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