Short communication

Flow injection analysis for simultaneous quantification of prolactin concentration and glycosylation macroheterogeneity in cell culture samples

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Abstract

A flow injection analysis (FIA) system is presented for a two step immunoassay-based determination of the total human prolactin (hPRL) concentration along with its degree of glycosylation. Separate measurement of total hPRL and non glycosylated human prolactin (nG-hPRL) were made using two flow-through cartridges each containing immobilized antibodies of different specificity. The antibodies are immobilized on the surface of a carrier. Glycosylated hPRL (G-hPRL) and, thus, the degree of glycosylation were calculated by the difference between the two specific determinations. Enhanced specificity for the determination of nG-hPRL was obtained using unfavorable binding conditions through incorporation of alkaline pH and chaotropic agents into the carrier/dispersion buffer. The assay for total hPRL and nG-hPRL were each found to be linear within the relevant concentration range. The results of the two-step FIA method were found to agree with those obtained by the standard methods of ELISA and western blotting while offering the advantage of minimal analysis time (10 min) and elimination of manual manipulations.

Introduction

Animal cell culture is often preferable for the production of recombinant therapeutic proteins due to the ability of mammalian cells to perform important post-translational modifications, such as glycosylation. Glycosylation is an important factor of product quality due to its role on biological activity, immunogenicity, stability, and pharmacokinetics of many therapeutic proteins (Jenkins and Curling, 1994). As glycosylation heterogeneity of recombinant glycoproteins can be sensitive to cell culture conditions (Goochee and Monica, 1990), it is essential to monitor the glycosylation patterns of such products at frequent intervals.

Analytical methodologies for the rapid assessment of recombinant protein quality are an important re-

quirement. Several approaches have been performed using automated immunoassays (Afeyan et al., 1992; Liapis and McCoy 1992; Price et al., 1998; Reinecke and Scheper, 1997; Hsieh et al., 1997; Goldman et al., 1998). Human prolactin (hPRL) is a pituitary hormone with a wide range of biological functions. Since the amino acid sequence of hPRL exhibits a single N-linked glycosylation site, the proteins exist as two macroheterogeneous glycoforms: glycosylated (G-hPRL, NW 25000–27000) and non glycosylated (nG-hPRL, MW 23000) (Heffner et al., 1995). The ratio of G-hPRL relative to nG-hPRL form varies between 0.13 and 0.25. The two macroheterogeneous glycoforms can be readily separated by lectin affinity chromatography using a lectin-Sepharose column, as G-hPRL exhibits a greater binding affinity to the immobilized lectin than nG-hPRL (Löhrke et al., 1993;

Lewis et al., 1989; Cole et al., 1991; Price et al., 1995). Cleaved forms of the hormone (MW 19000 and 15000) have also been reported, but they typically account for less than 5% of the total protein in humans (Pellegrini et al., 1988; Claustrat et al., 1994). G-hPRL and nG-hPRL have been reported to exhibit different biological activities. The glycosylated form demonstrates twice the ability to stimulate growth of a lymphoma cell line, while different microheterogeneous forms of G-hPRL can also represent varying biological activity (Lewis et al., 1985).

Numerous assays have been reported for the determination of hPRL, primarily for measurement of the hormone concentration in serum. Immunorecognition has been the basis of most methods, including enzyme-linked immunosorbent assay (ELISA), enzymimmunoassay (EIA) and radioimmunoassay (RIA) (Sinha et al., 1973). The assays for prolactin originating from different organisms generally differ only on the use of different specific antibodies. ELISA and EIA based determinations have been reported for prolactin of the following origin: mouse, buffalo, xenopus, rat, bovine and human (Tomita et al., 1992; Khurana and Muralidhar, 1997; Yamamoto et al., 1995; Hoier and Jensen, 1996; Ezan et al., 1994; Tavernier et al., 1995; Secchi et al., 1991; Lewis et al., 1992; Campier et al., 1997).

Assays for human prolactin can also be performed with a heterogeneous (two phase) sandwich assay based on anti prolactin antibodies covalently bound to microparticles (Bodner et al., 1991) or with antibodies bound on glass fiber filter paper (Mahmood et al., 1989). Additionally RIA and microbioassays for human PRL are used (Maddox et al., 1992). Haro et al. (1990) have observed that RIA determination of hPRL can be affected by the degree of glycosylation of the protein due to glycosylation-dependent variability in binding efficiency. This study suggests that antibodies exhibiting varying specificities for the two macroheterogeneous glycoforms could be utilized to determine the degree of glycosylation. Brue and coworkers (1992) have reported a two-step RIA for the determination of the degree of glycosylation of hPRL by employing two antibodies of varying specificity. An antibody which binds both nG-hPRL and G-hPRL yields the total hPRL concentration, while an antibody specific for nG-hPRL provides the concentration of the non glycosylated form. nG-hPRL and the degree of glycosylation are calculated by differential measurement. A monoclonal antibody specific for nG-hPRL is required for this twostep immunoassay

methodology. Such an antibody must be directed to an epitope in the region of the glycosylated site that is being obscured in the presence of a glycan. The use of an antibody specific for the glycosylated form is more difficult due to the oligosaccharide microheterogeneity typically observed for glycoproteins.

Although the two step immunoassay methodology described by Brue and coworkers offers sufficient accuracy, precision and detection limits for the analysis of serum samples, the analysis time and manual manipulation required make it hardly acceptable for routine process monitoring of recombinant animal cell cultures producing hPRL. The purpose of this study was to apply the principles of flow injection analysis (FIA) to the two-step immunoassay methodology for determination of the total hPRL concentration and its degree of glycosylation and to demonstrate its utility for process monitoring of mouse adeno carcinoma cell cultures producing hPRL.

Materials and methods

Flow injection analysis system

The FIA system used in these experiments is shown in Figure 1. It consists of two peristaltic pumps (Alitea XV, Alitea, Sweden), an injection valve (Type AA, peek, A 0624, Knauer, Germany) and a selector (Type AA, peek, A 0627, Knauer, Germany). An autosampler was utilized to load the sample into a sample loop (Isis AutoSampler 1290026; Isco, Lincoln, NB). The injection volume was based on the size of the sample loop and can be varied within a wide range (20 μ l to >1 ml). For the flow system Teflon tubing with an ID of 0.5 mm was used. The valves, pumps, and autosampler were controlled by a 486 PC (AD card: PCL 818; relais card: PCL 725; Advantech, CA). Data acquisition, peak area calculation, calibration and equipment control was performed by a FIA software package (CAFCA, Vers. 1.1; Anasyscon, Germany). Analytes eluting from the cartridge were detected at 2 Hz by a fluorimeter (Spectrofluorimeter F 1050; Hitachi, Japan) using a 40 μ l flow cell.

Assay principle

The described FIA was based upon specific binding of the hPRL to antiprolactin antibodies covalently immobilized (110 mM phosphate, 4 °C, pH = 7.4 over night) to Sepharose (Cyanogen Bromide Activated Sepharose 4B fast flow, C9142 Sigma) within a

Figure 1. Measurement setup for the FIA assay.

1 ml flow-through cartridge (Mobicols M1002; Mobitec, Germany; filter: radius 3.4 mm, 35 μm pore size, Mobitec). After injection of a sample, from a murine C 127 cell expression system producing a recombinant human prolactin, into the carrier buffer (typically 40 μl), flowing at constant rate of 1 ml min^{-1} , only the antigens are bound in the cartridge. Subsequently the carrier buffer rinses out the impurities and the target antigens were eluted by pH shift (0.1 mM phosphate, 55 mM NaCl, pH 12.3). The antigens were detected by native protein fluorescence (excitation wavelength 280 nm, emission wavelength 240 nm). For prolactin monitoring quantification was based upon peak area.

Since the two-step immunoassay methodology for the determination of hPRL concentration and the degree of glycosylation requires two different antibody-antigen interactions, the assay was adapted to a FIA format by covalently immobilizing, an antibody specific for nG-hPRL and a second binding both macroheterogeneous glycoforms of hPRL in separate cartridges. The times for sample loading (including rinse out time for other media and sample proteins), elution and equilibration (with carrier buffer) were 6, 3 and 1 min, respectively, thus yielding a total analysis time for the two-step assay of 20 min.

Results

Assay optimization

The specificities of the two antibodies were critical to the success of the two-step immunoassay methodology for the determination of total hPRL concentration

Table 1. Peak area of non glycosylated and glycosylated prolactin and antibody selectivity (peak area non glycosylated prolactin/peak area glycosylated prolactin)

Effect of pH (without SCN^-)					
pH	7.4	8	8.5	9	9.5
Selectivity	1,408	1,93	2,38	4,24	4,66
nG-Peak	3794	2561	2605	121	732
G-Peak	2693	1326	1090	286	164
Effect of chaotropic ions (pH 7.4)					
SCN^{-1}	0 M	0.05 M	0.1 M	0.2 M	
Selectivity	1,75	1,65	2,28	3,39	
nG-Peak	7611	3307	3346	2408	
G-Peak	4349	2005	1471	710	

and degree of glycosylation. In a cartridge with immobilized antibodies against nG-hPRL, 40 μl samples of standard G-hPRL and nG-hPRL of the same concentration (20 $\mu\text{g ml}^{-1}$) were injected, washed by carrier/dispersion buffer, and eluted at high pH. Binding affinity against the two glycoforms were estimated by comparing the eluted peak areas. Although the non-specific antibody showed similar binding affinity for the two macroheterogeneous glycoforms at physiological binding conditions pH 7.4 (100 mM phosphate, 55 mM NaCl and without chaotropic agents), the antibody directed towards the non glycosylated form demonstrated a 2-fold greater binding affinity for nG-hPRL than G-hPRL. This difference in elution peak area was insufficient to reliably quantify the two glycoforms.

However, the binding affinity of the antibody cartridge against nG-PRL was found to be highly sensitive to binding conditions. As shown in Table 1, the use of alkaline pH and chaotropic agents in the carrier/dispersion buffer diminished the binding of G-hPRL more rapidly than that of nG-hPRL towards the antibody specific for the non glycosylated form. Thus, the difference between the two glycoforms was enhanced by performing the assay at conditions less favorable to antibody-antigen interactions. A disadvantage of performing the assay at less favorable binding conditions was a loss in sensitivity (elution peak area) due to decreased antigen recovery. However, the loss in sensitivity could be counteracted by concurrently increasing the injection volume.

The antibody against nG-hPRL was found to be less sensitive to binding conditions. SCN^- concentration up to 0.4 M did not greatly impact the binding for either macroheterogeneous glycoform and, thus, it retained its lack of specificity for nG-hPRL or G-hPRL. However, using conditions for which the individual cartridges exhibited different binding affinity required that separate calibration curves be prepared for each portion of the two-step assay.

As shown in Table 1, although the use of SCN^- as a chaotropic agent in the carrier/dispersion buffer also yielded enhanced specificity (nG peak/G peak) for the antibody directed against nG-hPRL, the binding to nG-hPRL was greatly impacted. As a result, the sensitivity of assays using the chaotropic agent was reduced relatively to those of similar specificity employing alkaline pH. A pH 9 carrier/dispersion buffer without SCN^- was found to offer good specificity for the antibody directed against nG-hPRL while retaining sufficient binding to assure assay sensitivity for the quantification of total hPRL and nG-hPRL and, thus, was utilized for subsequent experiments.

Monitoring prolactin and the ratio of glycosylated and non glycosylated prolactin

Figure 2 depicts a typical calibration curve for total hPRL using the non specific antibody cartridge. Samples have been prepared from a murine C 127 cell line expressing recombinant human prolactin. Each data point represents the mean of three replicate determinations. Linear regression of the data yielded $y = 3.1x + 1.4$ with a correlation coefficient of 0.999. The linear range extended throughout the samples range 0 to 30 $\mu\text{g ml}^{-1}$. This concentration range represents values typically observed during the course of a re-

Figure 2. Calibration curve for total prolactin concentration with a non specific antibody cartridge that does not differentiate between glycosylated and non-glycosylated prolactin. Samples were obtained from a murine C 127 cell culture.

Figure 3. Calibration curve between eluted peak area and fraction of non glycosylated prolactin (nG-hPRL) using a nG-hPRL specific antibody in the FIA cartridge.

combinant mouse adeno carcinoma cell culture, thus indicating that hPRL can be quantified during culture by the FIA method. The near-zero value of the y-intercept indicates minimal nonspecific binding of matrix components from the cultivation media.

Figure 3 shows the effect of the degree of glycosylation on the eluted peak area for the FIA cartridge containing the antibody specific for nG-hPRL. 70 μl samples containing constant total hPRL concentrations of 20 $\mu\text{g ml}^{-1}$ but varying proportions of the two macroheterogeneous glycoforms were assayed. The eluted peak area increased linearly with the fraction of nG-hPRL. The peak area resulting from injection of pure nG-hPRL was eight times as great than the one obtained for pure G-hPRL. This indicates that the nG-

Figure 4. Comparison of total prolactin concentrations measured by ELISA and the proposed FIA method. Samples were obtained from murine C 127 cell culture.

hPRL has an eightfold greater binding to the antibody than the glycosylated form. The positive value of the intercept of the x axes is mainly due to some binding of the glycosylated prolactin to the antibodies immobilized in the cartridge. Nonspecific binding as a cause for that effect can be ruled out, because, as shown in Figure 2, other proteins get eluted by the carrier buffer.

Measurement of prolactin concentration of cell culture samples

Following optimization of assay conditions and determination of calibration curves, the FIA system was utilized to measure prolactin concentration in cell culture supernatant using 100 μ l cell free sample. Figure 4 shows a comparison of the values obtained by the FIA system and the ELISA test, currently the most common method. A comparison of the values from both methods by xy plot results by linear regression gives $Y = 0.934 X - 0.09$. The correlation coefficient is 0.958. The difference between the two techniques is on the average less than 6%. Considering the inaccuracy of the ELISA Test of 10 to 15% (Hanning et al., 1991), the values of both methods agree satisfactorily.

The ratio of glycosylated and non-glycosylated prolactin was measured with the two step heterogeneous FIA method described previously and compared to results obtained by western blotting, the standard technique for assessing the ratio of the two prolactin forms. Figure 5 offers a comparison of the two methods. These results indicate that the two-step FIA immunoassay did not yield significantly different results compared to the standard method of western

Figure 5. Comparison of the degree of glycosylation of prolactin in murine C 127 cultures as measured by an ELISA assay and the proposed FIA method.

blotting. The measured degree of glycosylation varies only as little as 5%. Additionally the 10 min analysis time of the two step FIA immunoassay offered a significant advantage with respect to the time required to perform western blotting analysis (typically 16 h).

Discussion

In order to monitor the secretion of prolactin in cell culture a fast immunoassay based on flow injection analysis (FIA) has been developed. The presented results show that the two step immunoassay yielded results for the total hPRL concentrations and degree of glycosylation comparable to those obtained by the standard methods of ELISA and western blotting, respectively. Additionally, the 10 min analysis time of the two step FIA immunoassay offers a significant advantage compared to the time required to perform ELISA and western blotting (typically 6 and 16 h, respectively). The main application are in areas where single samples containing undefined impurities have to be analyzed which is typical for cultivation samples.

The highly specific monoclonal antibodies lead to a very selective measurement in the complex and uncharacterized cell culture media with varying concentrations of other proteins. This leads to reliable data within the relevant concentration range. The FIA immunoassay methodology is applicable to the analysis of other cell culture products provided that appropriate antibodies are available.

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