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Quantitation of monoclonal antibodies by perfusion chromatography–immunodetection

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Abstract

The ImmunoDetection PG (protein G) cartridge which is based on perfusion chromatography has been evaluated for the quantitation of murine monoclonal antibody (IgG1) in biological fluids. The results indicate a low intra-day coefficient of variation (C.V.) ≤5% and a sample analysis time of 5.5 min. A standard curve generated using injection of known amounts of monomeric antibody against the eluted peak area shows linearity ($r^2 = 0.99$) over the 0.1–100 μg range. The minimum detectable concentration (MDC) at 280 nm is about 2 μg/ml which can be decreased to 200 ng/ml if the antibody elution is monitored at 214 nm. Under these conditions the C.V. is ≤5%. Accuracy of the quantitation is independent of operator, solution composition, sample pH and the ionic strength, all of which are factors to consider in performing immunoassays. Antibody samples containing aggregated and fragmented F_C regions of the antibody will interfere with this method of quantitation. An optimized validated method for the quantitation of antibody is described. This mode of perfusion chromatography could be applied in the biopharmaceutical field due to its speed, efficiency and sensitivity.

1. Introduction

The rapid quantitation of monoclonal antibodies in complex biological fluids is a requirement in quality control laboratories dealing with biologies in the presence of excipients, salts, stabilizers and buffers. It is also a requisite in other biopharmaceutical settings such as hybridoma laboratory, fermentation, purification and determination of process recovery. Typically these needs are met by the use of enzyme-linked immunosorbent assay (ELISA) and radio immunoassay (RIA) techniques. These techniques are sensitive in the pg/ng range but they are complex, time-consuming and operator and technique dependent and give high coefficients of variation (C.V.s). In addition, they require either radiolabeling of or conjugation of fluorescent labels/tags or enzymes to the primary or secondary antibody.

High-performance liquid affinity chromatography (HPLAC) utilizing protein G offers a rapid, precise and specific technique for the quantitation of antibodies [1–3]. An antibody to human growth hormone (hGH) has been quantitated using HPLAC in conjunction with fluorescence detection with a minimum detection limit of 250 ng/ml [4]. Combination of a protein G column and reversed-phase column chromatography has been successfully used for the quantitation of an antibody and contaminants and to checking the purity of the antigen [5]. The use of

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ImmunoDetection (ID) protein G (PG) cartridges for immunoassays is a novel technique which combines the resolving power of perfusion chromatography with the precision and speed of HPLC. This immunoassay setup offers many advantages for the quantitation of antibodies in terms of its adaptability to conventional HPLC systems, its efficiency, and its operator independence [6]. Tru-Scint AD is a monoclonal antibody used for the in vivo radio-imaging of adenocarcinomas. It is a murine antibody (IgG1) raised against synthetic asialo-Tn antigen [7]. The exact nature of the epitope recognized by this monoclonal antibody is still under investigation. The antibody is formulated as a lyophilized powder containing sucrose as an excipient and stabilizer. It is reconstituted at the clinic using USP grade sodium pertechnetate Tc-99m injection solution. We have investigated the utility of perfusion chromatography for the quantitation of this monoclonal antibody. We studied various parameters which affect the reproducibility of this method. A method is described for the quantitation of the Tru-Scint AD antibody at various stages of the processing, product development and manufacturing.

2. Experimental

The ID sensor PG cartridge (55 × 12 mm I.D.) (Persceptive Biosystem, Cambridge, MA, USA) was connected to a Beckman HPLC equipped with a Model 166 detector module, a Model 126 programmable solvent delivery module and System Gold software (Beckman, CA, USA). Analytical grade sodium mono- and diphosphate and hydrochloric acid were from Fisher Scientific (Ottawa, Ont., Canada) and Curtin Matheson Scientific (Houston, TX, USA), respectively. All samples and buffers were filtered through a 0.2-μm filter before loading on the cartridge. The standard curve of monoclonal antibody over the ranges 100 ng–5 μg and 5–100 μg per 50 μl, was developed using the following gradient system. The standards and samples were injected on a pre-equilibrated cartridge at a flow-rate of 0.5 ml/min followed by washing with equilibration buffer (10 m*M* phosphate buffer pH 7.2 containing 150 m*M* NaCl) at 2 ml/min for 1.5 min. The monoclonal antibody was eluted with elution buffer (2.5 m*M* HCl pH 2.6 containing 150 m*M* NaCl) at a flow-rate of 2 ml/min and monitored at 280 nm or 214 nm as appropriate. The integrated peak areas of antibody standards were plotted against the injected amounts using the Curve fit option of the System Gold software and the unknown samples were extrapolated from this standard curve.

The antibody concentration was determined using an extinction coefficient of $\epsilon_{280}^{1\%} = 1.59$ with the aid of a Beckman DU 640 spectrophotometer (Beckman, Fullerton, CA, USA).

The aggregated dimeric antibody was generated by irradiating the monomeric antibody with UV light, followed by purification of the aggregated antibodies on a size-exclusion column TSK G3000 (600 × 7.7 mm I.D.) (Beckman). The protein concentration of the aggregated antibody was determined by the Bradford method [8] using a Bio-Rad kit.

3. Results and discussion

A typical standard chromatogram of 10 μg of eluted monoclonal antibody on this PG cartridge is shown in Fig 1. The elution time of the antibody was quite reproducible at 2.7 ± 0.1 min ($n = 20$). The turn-around time for sample analysis is only 5.5 min. The standard curve is generated by loading monomeric monoclonal antibody on the PG cartridge and integrating the peak areas of the eluted antibody detected at 280 nm (curve not shown). The maximum and minimum capacities of the cartridge as recommended by the manufacturer are 100 μg and 100 ng, respectively. We found the standard curve to be linear ($r^2 = 0.99$) over this range.

Using this standard curve we quantitated monomeric monoclonal antibodies from several complex mixtures such as cell supernatants, lyophilized antibodies with excipients, salts, buffers etc. While validating this technique for the quality control of antibodies, we investigated various parameters which could affect this quan-

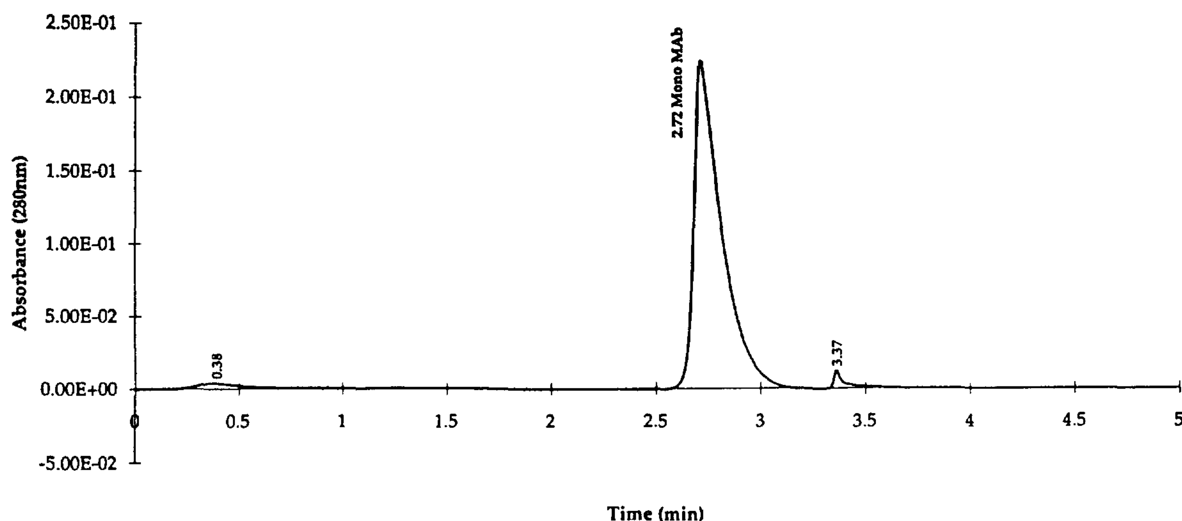

Fig. 1. Elution profile of Tru-Scint AD antibody on protein G cartridge: 10 μg of antibody were injected as described in Experimental.

titative method and the concentration of the antibodies.

The inter-day and intra-day coefficients of variation are $\leqslant 9\%$ and $\leqslant 5\%$, respectively, while monitoring the eluted peak at 280 nm. As expected the C.V. increases with a decrease of the amount of antibody injected (data not shown). By switching the monitoring wavelength to 214 nm the inter-day C.V. can be lowered to $< 4\%$ and the sensitivity and limit of detection to 200 ng/ml (data not shown). This is comparable to the 250 ng/ml found for fluorescence-based quantitation of human growth hormone antibody [4]. The advantage of protein G based HPLC is that no labeling of the antibodies with the fluorescent dye Texas red is required.

The non-retained peak (0.38 min), i.e. the flow-through, was checked for the presence of the antibody by collecting and re-injecting the solution. No antibody could be found (data not shown). This suggests that, in this concentration range and with the injection flow-rate used, the total amount of antibody loaded is bound to the cartridge matrix. We did not observe the 50% unbound fraction seen by other investigators [10], who used an ImmunoDetection CO cartridge where the antigen was immobilized on the cartridge. This inconsistency may be due to differences in the immobilization mechanism between the PG and CO cartridges.

The accuracy of the present technique was established by comparing the determined antibody content of a purified antibody sample with the results obtained using another method such as absorbance at 280 nm using the specific extinction coefficient and ELISA. When compared with the absorbance method the present method showed an antibody recovery of $\sim 96\%$. The method was also compared with an ELISA using few antibody samples obtained from a cell culture and at various steps of purification. It appears that both methods are comparable for each set of samples. With ELISA all samples showed amounts ca. 20–25% higher. The differences could be explained by the specificity of the ID PG method, which quantitates only native antibody–either in monomeric or aggregated form–whereas the ELISA may also score interfering substances and degraded epitopes.

The effect of excipients such as sucrose, buffers and the presence of salts on the quantitation of Tru-Scint AD MAb-L4 antibody was evaluated. The lyophilized antibody containing 3% excipient sucrose was reconstituted in saline and about 27 μg of the antibody was injected on the ID PG cartridge. On elution it showed a re-

covery of ~96%. There is no significant difference in the determination of the antibody in the presence of excipients, salts and stabilizers.

The effect of dimer aggregates was investigated in terms of their ability to bind to the protein G and their effect on the determination of monomeric IgG concentration, as they present a potential intrinsic contaminant in our product on prolonged storage. To evaluate the effect we purified the dimers using HPLC size-exclusion chromatography (data not shown). The dimers were injected and quantitated using the Immunodetection PG cartridge. The purified dimer bound to the protein G and showed a broad elution profile (Fig 2). On extrapolation of its concentration from a standard curve generated using monomeric antibody, we recovered a higher (~157%) amount of antibody than was loaded onto the cartridge as determined by the Bio-Rad kit. This was not surprising, as this method is based on the absolute mass which is proportional to the peak area. This confirms that dimer-containing samples will interfere with the determination of monomeric antibody concentration.

Results from samples containing aggregates should be interpreted with caution. One can establish the effect of various percentages of dimer on the determination of monomeric antibody concentration by spiking the samples with appropriate amounts of dimer.

We investigated the effect of the pH and the ionic strength of the antibody sample. Sample pHs of 4.0 and 11.0 did not have any effect on the ability of the antibody to bind to the PG cartridge. Similarly, increasing the salt concentration in the sample up to 0.3 *M* NaCl did not show any effect on binding and retention of antibody (data not shown). This versatility is important in downstream processing involving various antibody samples of high and low pH and ionic strengths. We have used this method to directly analyze such samples without any processing.

The ImmunoDetection method using the perfusion chromatography PG matrix described

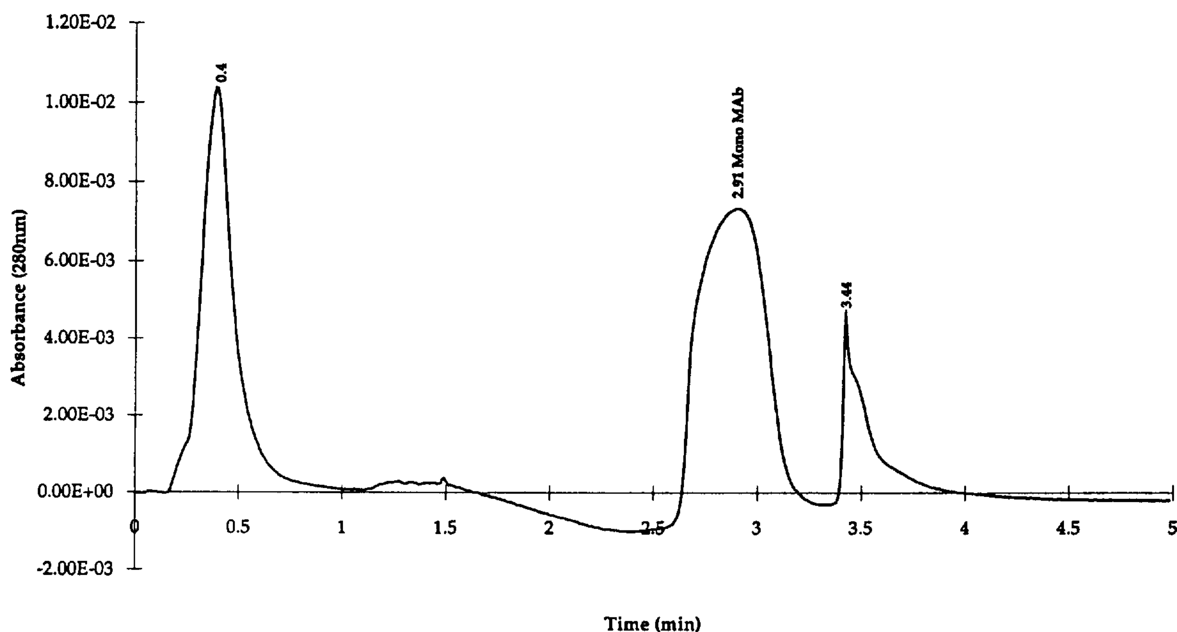

Fig. 2. Elution profile of aggregated antibody on protein G cartridge: ca. 3.1 μg of aggregated antibody at 62.2 μg/ml (as determined by the Bio-Rad protein assay) was injected, eluted and quantitated using the standard protocol detailed in Experimental.

here has multiple applications in quantifying various subtypes of antibody, such as IgG1, IgG2, IgG3 and IgG4, due to its broad specificity [9], from complex biological fluids in a more consistent and rapid way. This type of immunoassay shows a low C.V., broad dynamic range and high reproducibility due to operator independence. As the method is based on the integration of total peak area–which is proportional to the mass–one needs to be aware of the presence of aggregates and other F_C-binding fragments of antibody in the sample which might interfere with the determination of monomeric antibody concentration. One should be aware of differences in the glycosylation pattern of the antibodies when comparing one monomeric antibody standard curve against differently glycosylated monomeric antibodies, as differences result in a change of the peak area per unit of mass. To quantitate any unknown antibody sample, one needs to establish that the differences in the glycosylation patterns are minimal between the unknown sample and the standard antibody which is used to generate the curve. Compared to ELISA and RIA this method offers higher analytical precision, faster sample throughput and lower C.V.s. In addition, this type of chromatography can be performed using conventional HPLC equipment, which makes it an attractive technique in biopharmaceutical settings such as quality control, bioprocess development and downstream purification where it can be used to determine the recovery and mass balance.

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