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Purification and quantitation of tumor necrosis factor receptor immunoadhesin using a combination of immunoaffinity and reversed-phase chromatography

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Abstract

The development of an automated, dual column assay to quantitate and recover the glycoprotein, tumor necrosis factor receptor immunoadhesin (TNFr-IgG) from monkey plasma, human serum, cell culture fluid and buffer samples is described. A combination of immunoaffinity and reversed-phase chromatographies are used. The targeted protein was captured using an anti-TNFr-1 monoclonal antibody immobilized on POROS resin. After non-specific adsorption had been reduced, the affinity column was placed in-line with a reversed-phase column and eluted with dilute acid. The reversed-phase column was subsequently eluted with an acetonitrile gradient and the TNFr-IgG collected and quantitated by comparison with peak areas of similarly treated standards. Detection was performed by measurement of absorbance at 214 nm. The dynamic range is from 0.5–15 μg total sample. Samples were quantitated and recovered from monkey and human pharmacokinetics samples, as well as from cell culture fluid and buffers. The lowest concentrations assayed were 100 ng ml^{-1}. Quantitation is reproducible, with a coefficient of variation of 2%. The procedure was used to develop a pharmacokinetic profile for the clearance of TNFr-IgG in humans and cynomolgus monkeys. Sufficient material was recovered such that the glycoforms could be identified. Additionally it has been used for process monitoring. The results compared favorably with data generated by ELISA. Optimization of the method and results are presented.

Keywords: Glycoproteins; Tumor necrosis factor receptor immunoglobulin G

1. Introduction

Immunoaffinity chromatography is widely used as a powerful preparative method in the purification of proteins, [1]. It has also been incorporated into analytical methods where the goal has been to recover the protein for characterization purposes [2] or as an alternative to immunoassays [3]. Depending on the purpose, such as high sensitivity, high throughput or specific information sought, these analytical methods have been automated to different extents and can incorporate a wide range of steps and detection systems [4,5]. The initial goal of the work presented here was to recover a glycoprotein from serum samples from pharmacokinetic (PK) studies to evaluate the clearance of the different glycoforms.

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The glycoprotein studied was an immunoadhesin [6], comprising the extracellular domain of the TNF receptor 1 and the Fc domain of human IgG1, referred to as TNFr-IgG. This molecule resembles an immunoglobulin lacking the light chains in which the VH1 and CH1 domains have been replaced with the receptor domain [7]. It has a dimeric structure with 8 N-linked glycosylation sites per dimer, two in the Fc domain and 6 in the receptor domains. It has been evaluated in human clinical trials for rheumatoid arthritis [8] and sepsis [9]. During the development of the commercial manufacturing process, it was found that different batches displayed different PK clearance parameters [10] and that the variability was attributed to subtle differences in glycosylation patterns unrelated to sialic acid or exposed galactose content [10,11]. Many studies have been performed to assess the relationship of glycosylation to clearance, [12], but these have always studied the injected material and clearance parameters for the whole population of molecules. The method presented in this paper allowed the recovery of TNFr-IgG from serum samples in amounts of 0.5–10 μg, and purity, sufficient for extensive characterization of the glycans at timepoints of up to 10 days [13]. During the development and optimization of this method, it became clear that it was capable not only of providing highly purified samples of recovered TNFr-IgG but also resulted in a method with high precision capable of quantitating concentrations of the drug in serum samples at levels down to 100 ng/ml. The procedure employs an immunoaffinity column (with an immobilized monoclonal anti-TNFr antibody) followed by a reversed-phase HPLC separation. The whole procedure is automated. This paper presents the development, optimization and several applications of the method.

2. Experimental

2.1. Instrumentation and equipment

Samples were analyzed using an Integral Workstation (PerSeptive Biosytems, Cambridge, MA, USA) configured in the dual column gradient configuration [14]. This instrument in most regards is similar to any modern dual pump HPLC instrument, but has the additional capabilities of being able to select from 6 solvent reservoirs as well as directing solvent flow via 3×10 port switching valves. These additional capabilities allow multiple solvent pathways and multiple column use. Detection was performed using a built in dual wavelength UV–Vis detector.

Reversed-phase column temperatures were controlled using a control unit obtained from Cera Inc., (Baldwin Park, California).

Samples were filtered using Acrodisc PF 0.8 μm/0.2 μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA)

The following equipment was also used: Pharmacia PhastGel System, obtained from Pharmacia-LKB (Uppsala, Sweden)

2.2. Samples and reagents

Phosphate buffered saline, pH 7.2 (PBS) containing 9.4 m*M* sodium phosphate, 136.9 m*M* sodium chloride and 2.7 m*M* potassium chloride is referred to as loading buffer throughout this manuscript. The same buffer, titrated to pH 2.0 with HCl, is referred to as elution buffer. Non-specific wash buffer was 1 *M* NaCl in 80% loading buffer, pH 7.2. Tween 20 was purchased from Sigma Chemical Company (St.Louis, MO, USA). Trifluoroacetic acid (TFA), HPLC/spectro grade, was obtained from Pierce Chemical Company (Rockford, IL, USA). Acetonitrile, HPLC grade, was purchased from Burdick and Jackson (Muskegon, MI, USA). Sodium cyanoborohydride was purchased from Aldrich, (Milwaukee, WI, USA). Milli-Q water was produced by a Millipore water purification system. Anti-TNF-receptor-1 monoclonal antibodies were obtained from Genentech's hybridoma group. Activated aldehyde immunoaffinity resin (AL-50), reversed-phase POROS resins (R210 and R220) and column packing devices were obtained from PerSeptive Biosytems, (Cambridge, MA, USA). Empty PEEK columns, 30×2.1 mm (100 μl) and 20×1 mm (16 μl) were purchased from Upchurch Scientific (Oak Harbor, WA, USA).

2.3. Antibody immobilization

Monoclonal antibodies were covalently attached to POROS AL-50 resin using the general guidelines as in PerSeptive Biosytems published Application Guide [15]. When using this procedure binding

occurs mainly through primary amines on the protein and aldehyde groups on the support. The resulting imine formed is then stabilized by reduction.

Briefly, the activated support (POROS AL-50) was packed into a PEEK column (30×2.1 mm) using a PerSeptive Biosytems packing device. The antibody (3.3 mg) was exchanged into loading buffer (1.5 ml) using a Centricon 10 concentrator (Amicon). Sodium cyanoborohydride (30 mg/0.5 ml loading buffer) and 1 ml sodium sulfate (1.5 *M*) was added. The liquid chromatograph, i.e. Integral Workstation, was plumbed to allow recycling. The protein, reducing agent and salt solutions were recycled through the column for 5 min at a flow-rate of 1 ml.min^{-1}. At 10 min intervals, 1 ml sodium sulfate (1.5 *M*) was added, until the sodium sulfate concentration reached 0.7 *M*. At the end of the reaction, residual aldehyde functionality was quenched in a similar manner by reacting with 0.2 *M* Tris in the presence of reducing agent (sodium cyanoborohydride, 5 mg/ml). All reactions were carried out at room temperature.

2.4. Stability and capacity of affinity column

We investigated the stability of the affinity column to the potentially denaturing acid elution conditions used. Simultaneously we obtained an estimate of column capacity, (when loaded at 1 ml min^{-1}) as well as loading characteristics of the column as it bound increasing amounts of TNFr-IgG. Four antibodies were immobilized as described in Section 2.3 and tested. These were packed into 30×2.1 mm columns and each affinity column was equilibrated with loading buffer. TNFr-IgG (20 μg/10 μl) was loaded every 2 min for a total of 10 loads. The flow-rate was 1 ml min^{-1}. When loading was completed, the column was eluted with elution buffer (0.5 ml). After washing with loading buffer (5 ml) a second elution was performed (0.5 ml). The column eluate was monitored at 214 and 280 nm. Following re-equilibration with loading buffer (10 ml) the multiple loading and elution sequence was repeated again. This complete analysis cycle of multiple loading, elution, and re-equilibration was repeated five times. A comparison of peak areas for the flow-through of each load (i.e. non-binding material) as well as the bound and then eluted material, was made between analyses, to determine column stability. Quantitation was determined by comparing the peak area with the peak area produced by known standards. Recoveries were determined by comparing peak area of eluted material with detector response (i.e. with no column) obtained for known standards.

The column capacity was determined by the following procedure. The affinity column was equilibrated in load buffer. An initial baseline was obtained and then by valve switching the column effluent was diverted through the pumps and recycled. An estimated column capacity excess of TNFr-IgG was added to the system and recycled through the column and detector approximately seven times at 1 ml min^{-1}. After 20 min the excess TNFr-IgG was flushed out and the column washed with loading buffer again until the baseline had returned to the initial level. Bound TNFr-IgG was then eluted using elution buffer (0.5 ml) and quantitated as previously described.

2.5. Effect of loading flow-rate on efficiency of capture

The effect of the loading flow-rate on the efficiency of capture (of TNFr-IgG) was investigated at a high and low percentage of total column capacity. The total column capacity was determined to be approximately 200 μg (see previous section), therefore, 200 μg of TNFr-IgG was taken for binding by injecting 20 μg loads every 2 min until all 200 mg had been loaded. After capture the bound protein was eluted and quantitated as described in the previous section. This was repeated with loading flow-rates of 0.1, 0.2, 0.5, and 1.0 ml min^{-1}.

Similarly, to determine the effect of the loading flow-rate on the efficiency of capture of TNFr-IgG but at a low percentage of total capacity, the experiment was repeated by injecting 10×0.5 μg loads (total load of 5 μg).

2.6. Recovery and quantitation of TNFr-IgG from cynomolgus monkey samples

The Integral Workstation was configured in the dual column mode. The solvent reservoirs were: Solvent 1A, affinity loading buffer; Solvent 1B, reversed-phase aqueous buffer, 0.1% TFA in water; Solvent 1C, affinity elution buffer; Solvent 2A, reversed-phase organic elution buffer, 0.1% TFA/

acetonitrile; Solvent 2B, non-specific wash buffer, 1 *M* NaCl in 80% load buffer, pH 7.2 and Solvent 2C, water.

The first column was the affinity column containing the immobilized anti-TNFr-1 antibody (30× 2.1 mm), at ambient temperature. The second column was the reversed-phase column containing the polymer based POROS R220 packing material (20×1 mm). The reversed-phase column temperature was maintained at 45°C.

Plasma samples were thawed and centrifuged for 10 min at 10 000 rpm. Supernatant (100 μl) was removed and diluted with an equal volume of loading buffer containing 5 m*M* EDTA for loading onto the affinity column. The affinity column was equilibrated in loading buffer and a 200 μl sample was loaded at a flow-rate of 0.5 ml min^{-1}. The flow-through was directed to waste. After sample loading the affinity column was washed with loading buffer (6 ml), followed by the non-specific wash buffer (10 ml). These washes were also directed to waste. The affinity column was now connected to the reversed-phase column and eluted with 2 ml elution buffer. Re-equilibration of the affinity column was subsequently performed with loading buffer (4 ml) after removing its connection to the reversed-phase column.

The loaded reversed-phase column was washed with 0.1% TFA in water (2 ml). The flow-rate was set to 0.5 ml min^{-1} and a rapid (2 min) gradient was run to 30% Solvent 2A (0.1% TFA/acetonitrile) followed by a gradient to 40% Solvent 2A, over 5 min. Fractions (0.5 ml) were collected into 1 *M* Tris, pH 7.2 (50 μl) and stored at −70°C. After elution the column was returned to initial conditions over 1 min and re-equilibrated with 0.1% TFA (3 min at 2 ml min^{-1}). The column eluate was monitored at 280 and 214 nm. Quantitation was performed by comparison of the integrated peak areas with TNFr-IgG standards of known concentrations.

2.7. Recovery and quantitation of TNFr-IgG from human samples

To recover TNFr-IgG from human clinical samples the same general procedure was used as for the analysis of TNFr-IgG in cynomolgus monkey samples, but with modifications. Briefly these were; changes in sample diluent, changes in sample volume, changes in non-specific washes and changes in the RP gradient.

Human plasma samples obtained from TNFr-IgG clinical trials were thawed and diluted with 3 volumes of loading buffer containing 5 m*M* EDTA, 0.5 *M* NaCl and 0.05% Tween 20. The diluted samples were filtered using Acrodisc PF 0.8 μm/0.2 μm syringe filters and loaded onto the affinity column in 2×1700 μl loads using a 2 ml sample loop.

The non-specific washes were changed from 1 *M* NaCl/80% loading buffer to a water wash (5 ml) then loading buffer (2.5 ml), followed by a gradient from loading buffer to 1 *M* NaCl/80% PBS, pH 3.0 over 25 ml. The non-specific washes were completed with a 5 ml wash at these final buffer conditions. As in the case of recovery of TNFr-IgG from cynomolgus serum, all the non-specific washes were directed to waste.

The reversed-phase gradient was modified to increase the resolution of TNFr-IgG from co-purifying plasma proteins. Accordingly, the loaded reversed-phase column was washed with 0.1% TFA (2 ml). The flow-rate was set to 0.5 ml min^{-1} and a rapid (1 min) gradient was run to 38% solvent 2A (0.09% TFA/80% acetonitrile) followed by a gradient to 50% solvent 2A, over 10 min. To strip the column of any remaining components, a gradient was run to 90% solvent 2A over 2 min with a flow-rate of 1 ml min^{-1}. All other conditions were as described in Section 2.6.

2.8. Stability of TNFr-IgG in cynomolgus monkey plasma

As a control for chemical stability of the PK samples, the stability of TNFr-IgG in plasma was investigated. TNFr-IgG (100 μg) was spiked into a 50% solution of cynomolgus monkey plasma in loading buffer (2 ml). Thimerosal was added to 0.01% and the sample incubated at 37°C. At time zero and then every 24 h for 20 days, a sample was removed and stored at −70°C until analysis.

2.9. Process samples

Culture supernatant samples from an experimental fermentation of TNFr-IgG were taken daily and

stored frozen at −70°C until analysis. The samples were thawed and filtered using Acrodisc PF 0.8 μm/0.2 μm syringe filters. TNFr-IgG concentrations were determined using the dual column immunoaffinity assay as described in Section 2.6 and compared to those obtained using an in-house, validated LC Protein A assay (assay not described).

3. Results and discussion

3.1. Stability and capacity of affinity column

After preparing the affinity column it is necessary to determine various parameters related to capacity, loading, and elution. In general the protein being immobilized is one of a pair chosen because of its desirable specificity and affinity constant with the other. However the immobilization process can dramatically affect these properties causing some proteins (those that are not stable to the immobilization conditions) to lose their binding ability completely. In the immobilization procedure used here (see Section 2.3), binding occurs mainly through primary amines on the protein and aldehyde groups on the support. The resulting imine formed is then stabilized by reduction. Therefore, one criterion necessary to produce a usable column is that the protein must be stable to these conditions. Additionally the binding site(s) must remain easily accessible and not be involved in the covalent attachment. Even with these criteria met, column capacity can be affected by the ligand density and resulting steric hindrance [16].

Assuming that a column is able to bind the other half of the pair, it is then necessary to find suitable elution conditions. The ideal eluting agent disrupts binding quickly and completely (resulting in sharp elution peaks) while not causing any significant denaturation of the immobilized protein and subsequent decrease in column capacity. These conditions are best determined experimentally [15,17].

The profiles for loading 10 sequential injections of 20 μg TNFr-IgG followed by two consecutive elutions are shown in Fig. 1. Five traces from separate replicate analyses are shown overlaid. The

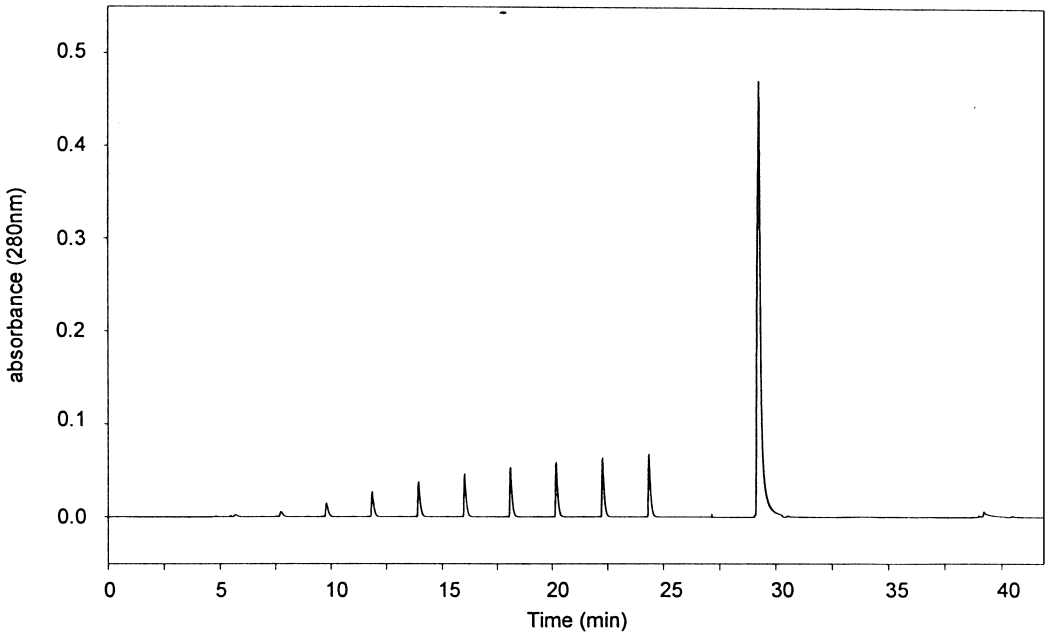

Fig. 1. Chromatogram showing 10×20 μg loads (5.5–24 min) followed by 2 elutions (29 and 39 min). The peak observed with each load is the non-binding fraction. Five analyses (overlaid) show no decrease in binding capacity with successive analyses, indicating column stability.

peak observed with each of the 10 loads (5.5–24 min) is the flow-through (i.e. non-binding) fraction of each load. The fact that all five analyses overlay precisely indicates the stability of this affinity column to the acid elution conditions. The first of the ten loads occurs at approximately 5.5 min. The area of this non-binding portion of the sample corresponds to 1 μg; therefore, by difference, 19 μg or 95% of this load binds to the affinity column. Subsequent loads result in an increase in the proportion of non-binding material as the total amount bound to the column increases. By the tenth load (24 min) approximately 65% of each load is now not binding to the column. The column has not yet reached its total capacity, but at this flow-rate (1 ml min^{-1}) only partial capture is occurring. If the amounts in the non-binding peaks are totaled, then 76 μg did not bind. Since 200 μg was presented for loading, we can calculate (by difference) that 124 μg must have bound.

The first elution occurs at 29 min, the second at 39 min (Fig. 1). Integration of these peaks indicates that 115 μg and 5 μg were eluted in each peak respectively. The first peak contained 96% of the total eluted material. The total eluted (120 μg) is in excellent agreement with the predicted amount bound (124 μg).

The significance of these results, besides indicating the stability of the affinity column to the acid elution conditions, is that although the column capacity is above 120 μg, less than 20 μg can be loaded (at the flow-rate used here of 1 ml min^{-1}) if 95% capture efficiency is required. When repeated at different flow-rates, different efficiencies of capture were observed (see Section 3.2).

Of the four antibodies immobilized, all bound TNFr-IgG initially but one was found to be unstable. The instability of this affinity column was evident by the increasing amounts of non-binding protein that occurred with each successive exposure to the acidic elution conditions as well as by the decreasing amounts observed in the elution peaks with each successive exposure (data not shown). It is presumed that the instability of this antibody is due to irreversible denaturation during exposure to the acid elution conditions. It is our experience that if an antibody is sensitive to acid denaturation then loss of binding ability occurs relatively rapidly and the test used here is an effective and rapid screening procedure for acid stability. It also provides a means to determine the relationship between the efficiency of capture and the partially loaded column.

3.2. Effect of loading flow-rate on efficiency of capture

Another parameter that needs to be determined on the newly made column is the effect of flow-rate on the efficiency of capture. Accordingly, the experiment used to determine antibody stability (Section 3.1) was repeated but at various flow-rates. Ten 20 μg loads were made before eluting. Loading flow-rates were 0.1, 0.2, and 0.5 ml min^{-1}. The results showed that the amounts bound and subsequently eluted were 200, 183 and 150 μg respectively, i.e. a decrease in binding with increasing flow-rate. In Section 3.1 it was determined that 120 μg bound at 1 ml min^{-1}. This decrease in binding with increasing flow-rate was also evident in the efficiency of capture of the initial loads. At 0.1 ml min^{-1} all of the ten 20 μg loads were captured whereas at 0.5 ml/min only the first two loads were completely captured (data not shown).

When this experiment was repeated using ten 0.5 μg loads (a total load of 5 μg or approximately 2.5% of column capacity), complete capture at all flow-rates was observed (data not shown). These results indicate that higher flow-rates may be used to completely capture the target when the load is only a small percent (2.5%) of total column capacity. As the load increases, then increasingly lower flow-rates are required for complete capture. Consequently a substantial increase in assay time occurs when using high concentration samples. For example, a 2 ml sample containing 50 μg of TNFr-IgG needs to be loaded at 0.1 ml min^{-1}, requiring a load time of 20 min, whereas a 2 ml sample containing only 5 μg can be loaded at 1 ml min^{-1}, or in 2 min. If complete capture is not an issue, then loading flow-rates are not as great of a concern. Thus, for example, a more rapid assay (that does not have complete capture) could be developed where quantitation was indirect by reference to a standard curve.

In our experiments complete capture was necessary because without this requirement important structures of low concentration could be lost. There-

fore, we used conditions affording complete capture. Nevertheless, discrimination between the various glycoforms of TNFr-IgG does not appear to occur since we found similar capture efficiencies for both a variably sialylated lot and an asialo TNFr-IgG variant (results not shown).

3.3. Stability of TNFr-IgG in cynomolgus monkey plasma

In analysis of samples from PK studies, it was important to assess the stability of TNFr-IgG in serum or plasma for a time equal to that required for clearance yielding information on drug degradation. Accordingly, TNFr-IgG was added to plasma and samples were incubated for up to 20 days at 37°C. A small decrease (0.9%/day) was observed by the dual column assay method, suggesting that the decrease seen in PK studies (~50% in the first day) is due to clearance and not degradation. The main conclusion, however, is that the drug is not significantly degraded in the autosampler while waiting for automated analysis.

3.4. Assay characteristics

Fig. 2 (profile D) shows a chromatogram for the purification of 5 μg TNFr-IgG spiked into 0.2 ml cynomolgus monkey plasma. Significant events in the purification train (labeled) are: loading, non-specific elution (wash), affinity elution (with simultaneous capture on reversed-phase) and reversed-phase elution. Repeated analyses ($n=7$) yielded a coefficient of variation for the area of the main reversed-phase peak (retention time 24 min) of 2% (data not shown). Recoveries were better than 95% based on external calibration with similarly treated standards. The large peak observed at 4–5.5 min is due to the flow-through of non-binding plasma proteins and other 214 nm absorbing components. This peak is

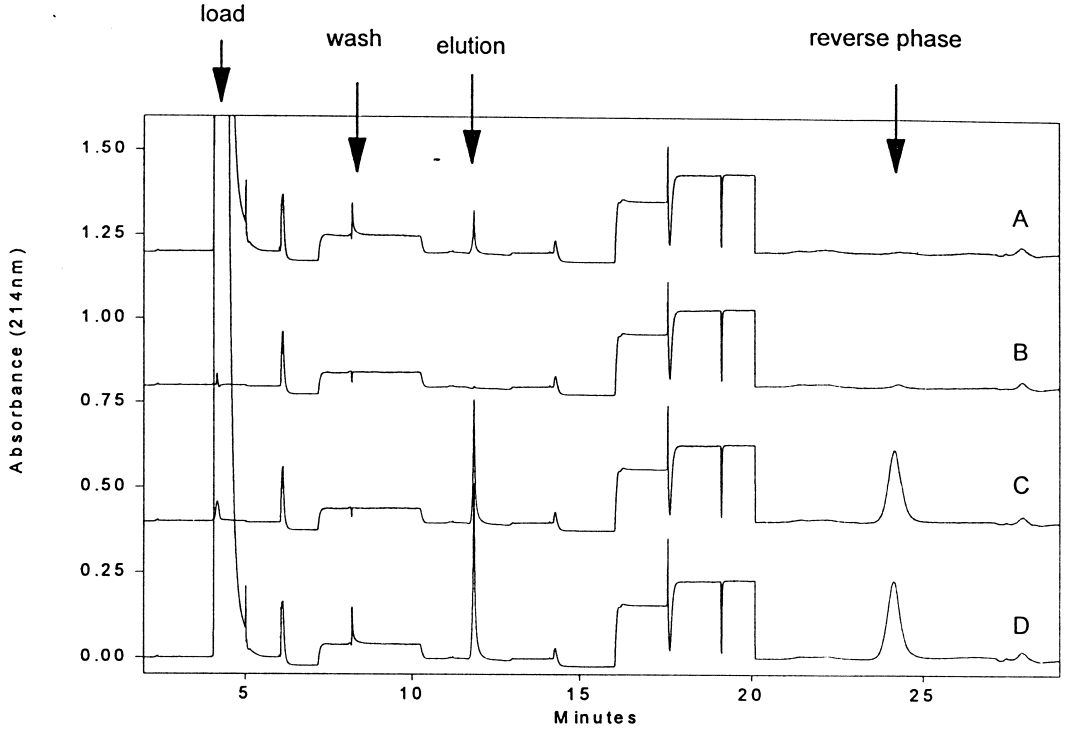

Fig. 2. Chromatograms (offset) showing application of the method. Samples assayed from top to bottom are, (A) cynomolgus monkey plasma, (B) water (blank), (C) TNFr-IgG/loading buffer and (D) TNFr-IgG/cynomolgus monkey plasma. Elution of the affinity column occurs at 12 min. TNFr-IgG elutes from the reversed-phase column at 24 min.

essentially absent in non-plasma samples, i.e., in buffer spiked with 5 μg TNFr-IgG (profile C) and a water blank (profile B).

A standard curve for the recovery of TNFr-IgG spiked into cynomolgus monkey plasma was constructed (not shown) revealing excellent linearity for six loads from 2–15 μg. However, at a load of 20 μg a decline in linearity of peak area versus load was observed. We did not determine whether this effect was due to an overload of either the affinity column or the reversed-phase column, but it is probably the latter due to its small size (column bed volume 16 μl) and hence its small capacity. The regression coefficient for loads up to 15 μg was 0.9998. Recoveries were approximately 95% when compared to detector response for various loads (data not shown). The line of best fit does not pass through the origin because the reversed-phase column has a small amount of carryover from the previous run. This carryover is evident as a small peak (retention time 24 min) observed in a blank run performed immediately after the recovery of a 5 μg load of TNFr-IgG (Fig. 2, profile B). If necessary, and at the expense of assay time, this can be removed by additional washes or by using a different RP column e.g., TSK-phenyl, RP5PW. This alternate column does not carry-over TNFr-IgG to any significant extent (data not shown), but a drawback to its use is its maximum flowrate of 0.5 ml min^{-1}. This means that the non-specific washes and re-equilibration of the support takes longer compared to the POROS support where these washes may be done at 5 ml min^{-1}.

It is worthwhile noting that if sample concentrations are low, then, simply by increasing the sample volume, the total load can be increased until above the 0.2 μg detection limit of this system. The largest sample volume that we loaded was 5.1 ml. Recoveries were similar to those found with smaller sample volumes (data not shown).

The reversed-phase column provides a means to optimize the affinity chromatography steps by monitoring changes in purity and recovery and additionally concentrates, desalts and allows further purification of the captured protein. Recoveries were similar, regardless of starting media (buffer or plasma) (Fig. 2, profiles C and D). Since it was intended to quantify the total drug, a steep gradient was chosen to elute the reversed-phase column so that minimal resolution would occur between TNFr-IgG and possible variants. This allowed the integration of a single peak. However, as a drawback, such an approach allows the possible coelution of contaminating plasma proteins. This possibility was investigated by collecting both the affinity eluate and the eluted reversed-phase peak (from consecutive analysis) and submitting to further analysis by SDS-PAGE. Samples were prepared, run and silver stained using Pharmacia published protocols for PhastGels [18]. Single bands were observed for both the affinity eluate and the eluted reversed-phase peak, indicating the absence of any significant contaminating plasma proteins (Fig. 3). Since no further purification was achieved by the use of the RP column (for this sample) it is possible to recover the protein immediately after elution from the affinity column. However there are many advantages to using a RP column as discussed later. Further evidence of purity was shown by tryptic mapping performed on the main peak recovered from the reversed-phase profile. This was found to be indistinguishable from starting material (data not shown).

The purity of the recovered material can be attributed to the high specificity of the affinity

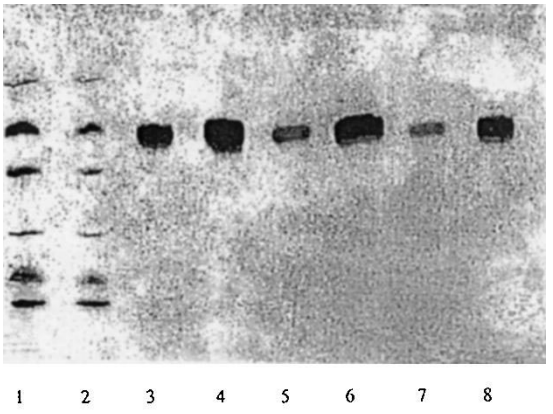

Fig. 3. Scan of SDS-PAGE gel (silver stain) of TNFr-IgG purified from plasma. Samples were taken directly after the affinity step, lanes 4 and 5 (120 and 24 ng, respectively) and after the complete purification by RP-HPLC, lanes 6 and 7 (48 and 10 ng, respectively). Lanes 1 and 2 are molecule weight standards, lanes 3 and 8 are TNFr-IgG (50 ng) starting material.

column and the effectiveness of the non-specific elution washes. Small column volumes coupled with high flow-rates provide the opportunity for extensive wash steps in a short time. Non-specific elution was performed by washing with 100 column volumes of 1*M* NaCl in 80% loading buffer. The peak appearing at 8 min (Fig. 2) is material eluted with this wash. This peak is evident in the purifications of TNFr-IgG spiked into plasma (Fig. 2, profiles A and D) but as may be expected, it is not observed in purifications from buffer or in a water blank (Fig. 2, profiles B and C). The incorporation of 0.05% Tween 20 into this wash solution was very effective at removing non-specifically bound material. However due to foaming and consequent loss of pumping efficiency, its use was discontinued.

Elution of the affinity column is performed using loading buffer adjusted to pH 2.0 with HCl (Fig. 2, 12 min). This agent was chosen since it produces sharp elution peaks and very little refractive index perturbation of the baseline. Peaks of comparable size are observed for similar amounts of sample captured from both loading buffer and plasma suggesting the absence of copurifying plasma proteins (profiles C and D). As expected no affinity elution peak is observed in the water blank (profile B), but, a small peak is observed at this time for a control sample consisting of cynomolgus monkey plasma only (profile A). This observation is not unexpected when such complex biological matrices are used. The composition of this peak was not determined since no peak attributable to TNFr-IgG was observed at the expected elution position in the reversed-phase profile (20–30 min).

3.5. Recovery and quantitation of TNFr-IgG from cynomolgus monkey PK samples

Cynomolgus monkey plasma samples from a PK study were analyzed for TNFr-IgG using the described scheme. Samples from one animal taken at 30 min, 6 h, 1, 6, and 16 days were analyzed and the reversed-phase profiles are shown in Fig. 4 (overlaid). Similar profiles were obtained for all the samples. The main reversed-phase peak (23–25.5 min) was integrated and quantitated as described in the Experimental Section 2.6. The amounts at each time point (for one animal) are shown in Fig. 5. This clearance profile is very similar to that obtained

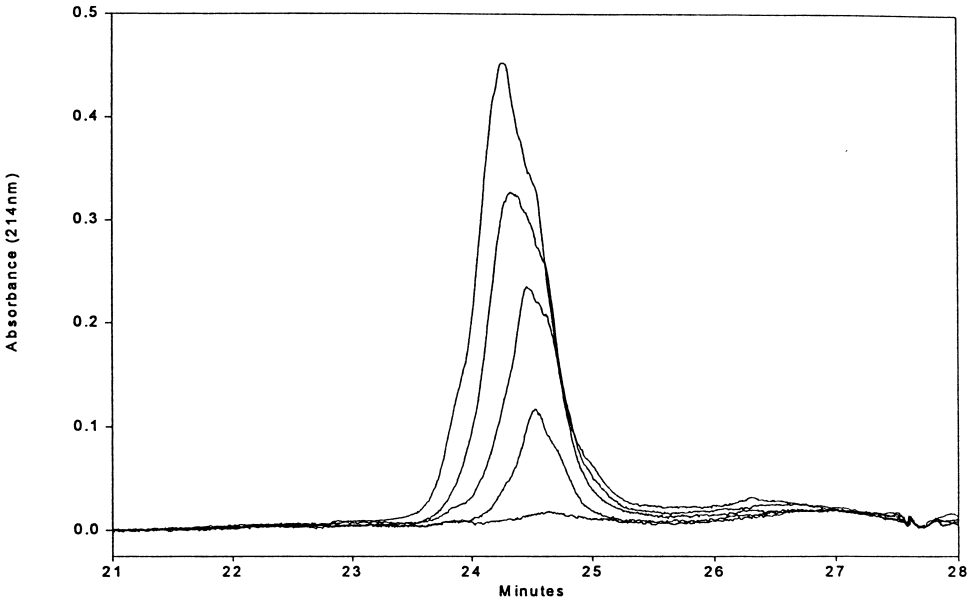

Fig. 4. Overlaid reversed-phase chromatograms obtained from PK samples. Samples were taken at 30 min, 6 h, 1, 6 and 16 days post dosing. TNFr-IgG was eluted using a steep acetonitrile gradient. Details of the separation procedure are given in Section 2.6.

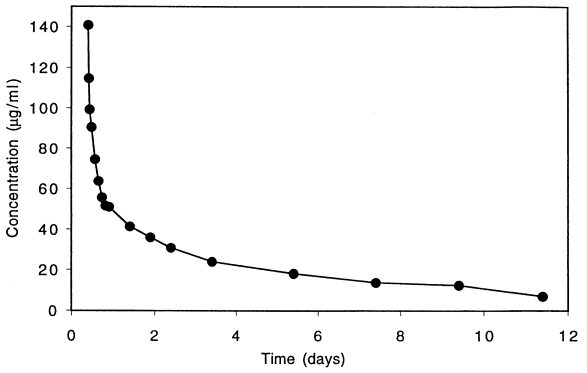

Fig. 5. Typical clearance profile for TNFr-IgG in a cynomolgus monkey. Quantitation data were obtained using the dual column immunoaffinity method described in the Experimental Section.

using an enzyme-linked-immuno-bio assay (ELIBA[1]) for quantitation (not shown). A correlation plot for quantitation using the ELIBA assay versus the dual column assay is shown in Fig. 6. Excellent correlation between the two assays is shown (r^2=0.966) giving rise to believe that complete capture was achieved using the dual column assay. However, the dual column assay consistently produced slighter higher values (slope=1.18). One possible explanation was copurification, but this was completely ruled out by both SDS-PAGE (Fig. 3) and tryptic mapping (not shown) experiments, in which only a single component was detected. The cause of this difference has not been determined and as it has been shown that no copurification occurred, it seems reasonable to postulate that the ELIBA under quantitates the analyte.

The reversed-phase peak containing TNFr-IgG was collected from each analysis and the carbohydrate composition determined, which will be described in another paper.

3.6. Recovery and quantitation of TNFr-IgG from human samples

Because the human PK samples had approximately 100 fold lower concentration of TNFr-IgG (<1.5 µg/ml) compared to the monkey PK samples, the sample size needed to be increased to achieve adequate detection. However, because of practical considerations, sample volume could only be increased tenfold. Consequently, there was a tenfold decrease in the possible load of TNFr-IgG. This fact, coupled with the increased serum load, caused copurifying serum proteins to increase to problematic levels (chromatogram not shown). Therefore the

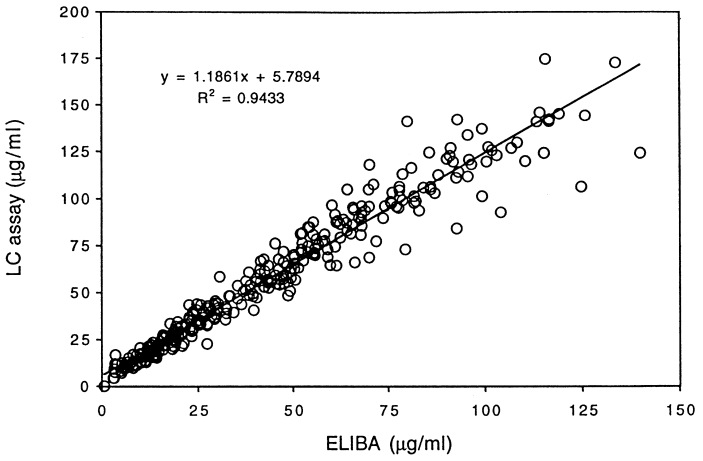

Fig. 6. Correlation plot for quantitation of PK samples using ELIBA and dual column immunoaffinity method.

[1]An ELIBA assay is similar to an ELISA sandwich assay but involves binding that reflects the biological functioning of the molecule e.g. receptor-ligand pair.

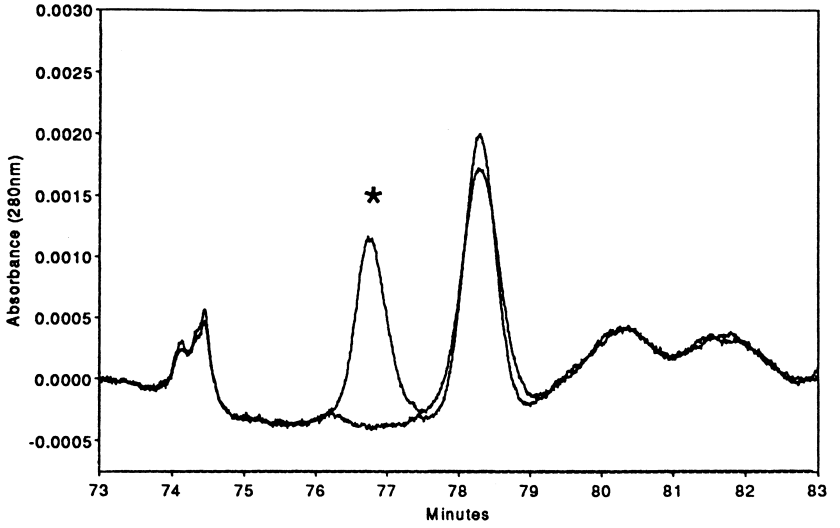

Fig. 7. Typical chromatogram (RP section) for the recovery of TNFr-IgG from human serum. The TNFr-IgG peak is marked with a '*'. Overlaid is the chromatogram of human serum alone.

method was modified in order to purify TNFr-IgG from human serum at these lower concentrations (see 2.7).

As a result of these changes (i.e., modified non-specific washes, increased resolution on the reversed-phase column), TNFr-IgG was baseline resolved from other co-purifying human serum proteins. The chromatogram in Fig. 7 shows the recovery of TNFr-IgG (1 μg) spiked into human serum (1 ml). For comparative purposes the analysis of human serum alone is shown plotted on the same axes. The TNFr-IgG peak is marked with a '*'. All other peaks are components from serum and are common to both chromatograms.

3.7. Analysis of process samples

In addition to the quantitation and recovery of TNFr-IgG from monkey and human PK samples, TNFr-IgG concentrations were determined over the time course of an experimental fermentation run. Samples which were taken at time zero and then daily for 12 days were analyzed together. These are shown graphed against time in Fig. 8. The TNFr-IgG concentrations determined by a protein A based LC assay as well as cell viability are also plotted. The protein A assay has a detection limit of 10 μg/ml and concentrations do not approach this until day 5, hence values are not available for the first 5 days using this assay. Excellent agreement between the two assays occurs for days 5–9. After day 9 the values diverge. The concentrations on days 10, 11, and 12 determined using the dual column assay were 141, 105, and 93 μg/ml respectively, whereas the concentrations on these same days determined using the protein A assay were 148, 152, and 156 μg/ml respectively. This disparity at later time points was observed for two other fermentation time courses (data not shown). The cause(s) of this difference has not been identified but it correlates with cell viability. Cell viability decreases rapidly after day 9 and protein concentrations (by the dual column assay) begin to decrease after day 10. It is quite possible that, after cell death followed by cell lysis, released proteases begin degradation of TNFr-IgG. The differences in assay values could be explained by proteolysis coupled with the differences in specificity of the two affinity columns. Protein A binds to the Fc region whereas the dual column assay uses a monoclonal antibody that recognizes the receptor domain. The difference between these two assay results indicates the importance of a thorough understanding of the assay's specificity in highly variable sample compositions, as will be the case in animal samples.

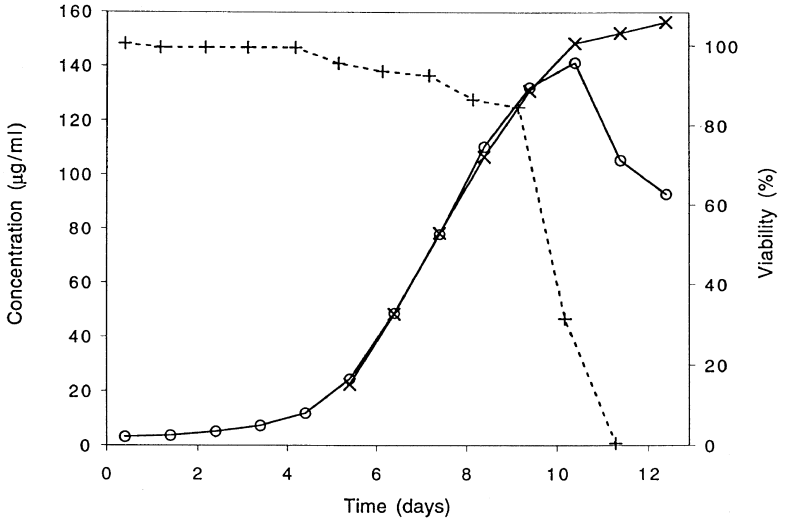

Fig. 8. Graph of TNFr-IgG concentration (left side, *Y* axis) determined by both dual column immunoaffinity assay (◦ – – ◦) and Protein A assay (× – – ×) versus fermentation time. Also plotted is cell viability (+ – +) (right side, *Y* axis) versus time.

4. Conclusion

An automated, dual column, immunoaffinity assay to recover and quantitate TNFr-IgG has been developed. Sample preparation is minimal including only centrifugation and dilution. Methods for antibody immobilization and subsequent testing of the affinity column for acid stability and capacity have been developed. The flow-rate required for complete capture depends on the amount of sample, relative to the column capacity. For complete capture with the used antibody and with a load at 2.5% of column capacity, a loading flow-rate of 0.5 ml/min or less is required, whereas for complete capture of a load near total column capacity, a loading flow-rate of 0.1 ml/min or less should be chosen. Detection is performed by absorbance measurement at 214 nm and quantitation by external calibration with similarly treated standards. A dynamic range of 0.5–15 μg sample load is obtained. Samples were quantitated and recovered from monkey and human PK samples (plasma and serum respectively) as well as from cell culture fluid and buffers. Quantitation is reproducible, with a coefficient of variation of about 2%. The procedure was used to produce PK profiles of TNFr-IgG in cynomolgus monkeys and humans. In addition it has been used for process monitoring. The observed PK clearance profiles were similar to those obtained when quantitation was performed by ELIBA. Sufficient material was recovered from clinical samples for partial characterization and was shown to be pure by both SDS-PAGE and tryptic mapping. The new method provides a means for the complete recovery of TNFr-IgG from PK samples and coupled with newly developed characterization methods can identify the structures involved in the clearance of TNFr-IgG.

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