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Characterization of a complex glycoprotein whose variable metabolic clearance in humans is dependent on terminal N-acetylglucosamine content

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Abstract

Glycoproteins can be cleared from circulation if they carry oligosaccharide structures that are recognized by specific receptors. High-mannose type and asialo complex oligosaccharides are cleared by the mannose and asialoglycoprotein receptors, respectively. This paper presents the protein and terminal saccharide characterization for nine batches of a glycoprotein developed for pharmaceutical use. Each of these batches was evaluated in human pharmacokinetic (PK) studies, and had similar terminal elimination half-lives, but the initial clearance of this glycoprotein varied between batches. The protein is lenercept, an immunoadhesin comprising the Fc domain of human IgG1 and two tumor necrosis factor (TNF) binding domains derived from the extracellular portion of the TNFR1(p55). Lenercept is manufactured in Chinese hamster ovary (CHO) cells and is extensively *N*-glycosylated but is devoid of high-mannose glycans. The pharmacokinetic variability between these lots *only* correlated with terminal *N*-acetylglucosamine and not with terminal galactose, sialic acid or any polypeptide related parameter. The data emphasize the need for appropriate analytical methods for the characterization of glycoproteins, especially those designed for long half-lives, and show that assessment of the content of all three terminal saccharides is sufficient to ensure consistency of their PK performance properties.

Keywords: Lenercept; TNF receptor; Terminal *N*-acetylglucosamine; Glycoprotein clearance

1. Introduction

Approval to market a pharmaceutical, whether developed using biotechnology or ''traditional'' methods, requires the demonstration of reproducibility of its safety and efficacy as well as demonstration of manufacturing consistency. This typically requires, in addition to adequate clinical trials that show patient benefit, validation of both the manufacturing process and the control system employed to assess the batch-to-batch reproducibility of its operation. Batches of final product are assayed, using validated methods, to ensure that they meet specifications for identity, purity, potency, strength and quality. Many specifications are set on a case-by-case basis and are guided by these considerations and manufacturing process history, to ensure that marketed batches will have the same properties as the batches used for clinical demonstration of safety

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and efficacy. For glycoprotein pharmaceuticals, however, the situation is more complex due to the generally observed product microheterogeneity imparted by the heterogeneity of the attached glycans [1]. The glycans can influence either the intrinsic biological activity or the pharmacokinetic (PK) properties or both [2]. Consequently, additional analytical methods are required for assessment of the key carbohydrate features, which could cause variability from batch-to-batch in either potency or PK properties.

In contrast to the biosynthesis of the polypeptide portion of a glycoprotein, which can be controlled at the DNA level, the biosynthesis of the glycans is controlled by a complex set of enzyme-mediated reactions that can be influenced by genetic, metabolic, and environmental factors [3]. Assessment of the reproducibility of the glycosylation status of a glycoprotein pharmaceutical, especially during the scale-up phase of manufacturing process development, is therefore of paramount importance to ensure maintenance not only of potency, but also of PK properties. This is recognized by the importance of ''equivalence'' assessment if a change is made during the development of the manufacturing process, usually performed by comparative PK studies in animals or humans, even if only slight differences could be detected during the analytical comparisons of the final products obtained from the two processes [4]. When differences are observed in PK parameters, they are frequently attributable to differences in glycosylation [5] although there are examples where small changes in polypeptide structure can also have significant effects.

For the pharmaceutical, which is the subject of this paper, PK changes were observed when the glycoprotein product expression level in the production clone was increased in response to anticipated commercial needs after initial demonstration of clinical efficacy. However, PK variability was also observed from batch-to-batch when the optimized cell culture process was performed reproducibly with tight control of raw materials and manufacturing operations [4]. This variability was established by a series of human PK volunteer trials measuring the PK properties of nine separate batches of drug. These studies allowed the identification of a biochemical parameter, terminal *N*-acetylglucosamine (tGlcNAc), that correlated with the clearance. The data presented here demonstrate that no polypeptide structural feature was responsible for the PK variability and that analysis of terminal saccharide composition was sufficient to attribute the variability to the tGlcNAc content.

The drug is a tumor necrosis factor alpha (TNF) antagonist, with a generic name of lenercept. It is an immunoadhesin comprising the extracellular domain of the human TNF receptor 1 (p55) fused to the CH2 and CH3 domains of human IgG1 [6]. The amino acid sequence is shown in Fig. 1. Expressed in CHO cells, lenercept spontaneously forms a disulfide-linked dimer, creating an Fc domain, which carries two TNF receptor domains [6] and forms a stable complex with TNF in which the activity of the TNF is neutralized [7]. The receptor domain contains three N-linked glycans and one O-linked site while the site in the Fc domain is glycosylated similarly to non-

```
1   LVPHLGDREKRDSVCPQGKYIHPQNNSICCTKCHKGTYLY
          L1       L2          L3       L4
41  NDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEM
                        L5                 L6
81  GQVEISSCTVDRDTVCGCRKNQYRHYWSENLFQCFNCSLC
           L7                        L8       ss
121 LNGTVHLSCQEKQNTVCTCHAGFFLRENECVSCSNCKKSL
                              L9             L10
161 ECTKLCLPQIENVKGTEDSGTTDKTHTCPPCPAPELLGGP
   L11      L12       L13            L14
201 SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
           L15           L16
241 VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE
     L17     L18         L19                L20
281 YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM
    L21 L22  L23   L24 L25          L26
321 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL
       L27           L28                 L29
361 DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
                L30           L31
401 KSLSLSPGK
         L32
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Fig. 1. Amino acid sequence of lenercept. Numbers down the left edge are residue numbers. Expected peptides from digestion with Lys-C, i.e., those ending in **K**, are numbered L1–L32 under the sequence. For clarity, cleavage sites are indicated by **K**, odd-numbered peptides are underlined and glycosylation sites are boxed.

engineered IgG [8]. Initial efficacy of the molecule was demonstrated in a baboon model of septic shock [9]. Lenercept has been clinically evaluated in rheumatoid arthritis and septic shock indications [10,11].

In the work presented herein, we show that the bioactivity of the drug is reproducible from batch-to-batch, that polypeptide properties are either constant from batch-to-batch or have a variability that is unrelated to the PK variability. The status of the glycans was assessed by direct measurement of the three saccharides, which can be terminal in complex-type N-linked glycans, i.e., sialic acid, galactose, or GlcNAc; the first by chemical analysis and the other two by enzymatic methods. In a separate paper [12], we directly show that lenercept molecules carrying the tGlcNAc moieties in the receptor domain were selectively cleared in the initial rapid elimination phase of the PK profile and that this occurs similarly in humans and cynomolgus monkeys. Taken together, these papers provide an important link between certain aspects of glycan structure and a large (and, to our knowledge, unprecedented) dataset from human PK trials. Further, they show the utility of the methods now available to characterize the glycoprotein (both before and after injection) and their potential as tools for monitoring glycosylation, control and consequences.

2. Materials and methods

2.1. Endoproteinase Lys-C peptide map

Samples from each batch were diluted into a buffer consisting of 6 M guanidine hydrochloride, 360 mM Tris, and 0.2 mM EDTA at pH 8.6 and reduced with 20 mM dithiotheritol (DTT) for 30 min at 37 °C. The reduced samples were *S*-pyridylethylated by the addition of 10 μL of 4-vinylpyridine and incubation for 20 min at ambient temperature in the dark. The *S*-pyridylethylated samples were buffer exchanged into 25 mM Tris, 1 mM EDTA, pH 8 buffer then digested with Endoproteinase Lys-C (1:20 w/w enzyme:substrate) for 4 h at 37 °C. Digestion was terminated by the addition of 150 μL of 10% aqueous TFA. The resulting peptides were separated on a Jupiter C_{18} HPLC column (2.0 × 250 mm, Phenomenex) that was eluted with a linear gradient from 0 to 22.5% solvent B in 45 min, from 22.5 to 26% solvent B in 35 min, and finally from 26 to 35% solvent B in 36 min. Solvent A was 0.1% aqueous trifluoroacetic acid (TFA) and solvent B was 0.1% TFA in acetonitrile. The flow rate was 0.2 mL/min, and the column was maintained at 40 °C. The elution profile was monitored at 214 nm, 254 nm, and 280 nm.

2.2. MALDI-MS analysis of peptides from Endoproteinase Lys-C peptide map

Identification of the collected Lys-C peptides from lot M11-72 was accomplished by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). The mass spectral data was acquired on a PerSeptive Biosystems Voyager Elite mass spectrophotometer operated in the linear mode using delayed extraction (80 ns delay). The accelerating voltage was set at 20 kV with the guide wire voltage set at 10 V. The peptides were ionized using a nominal laser power of 1100. To improve signal quality, the spectra from 128 shots were summed. The instrument was calibrated with a peptide standard solution using a two-point external standard method.

For MALDI-TOF MS analysis of the Lys-C peptides, a 2 mg/ml solution of 2,4,6-trihydroxyacetophenone (THAP) was prepared using a CH_3CN/ammonium citrate (25:75) solution. Normally, 0.5 μL of the collected peptide was applied to the target followed by 0.5 μL of the matrix solution, the target was then vacuum dried [13].

2.3. Sialic acid determination

Sialic acid determinations were performed essentially as described by Anumula [14]. The method is based on acid hydrolysis to release the sialic acid moieties followed by derivatization with *o*-phenylenediamine. The reaction mixture was separated by RP-HPLC and quantification was accomplished by comparison of relative peak area ratios to a suitable standard curve.

2.4. Terminal N-acetylglucosamine determination

The amount of tGlcNAc present in each batch was determined by capillary electrophoresis, with detection by laser-induced fluorescence (CE-LIF), analysis after treatment with β-hexosaminidase and labeling with 1-aminopyrene-3,6,8-trisulfonate (APTS). The β-hexosaminidase digestion of lenercept was performed in a 100 mM sodium citrate-phosphate buffer, pH 5.0, using 20 U β-hexosaminidase (jack bean) per mg of lenercept. After digestion, maltose was added as an internal standard to compensate for variations in CE injection volume. The lenercept and β-hexosaminidase were ethanol precipitated after heating at 95–100 °C for 5 min, the supernatant solution was removed and dried. The labeling reagent, APTS in 15% (v/v) acetic acid, was added directly to this dried pellet, and the labeling reaction initiated by the addition of 1 M sodium cyanoborohydride in tetrahydrofuran. Adding purified water terminated the labeling reaction. Conditions for capillary electrophoresis and fluorescent detection were as follows: capillary length, 20 cm to detector; separation voltage, 17.5 kV, negative-to-positive polarity; temperature, 20 °C; detection, argon-ion laser, 488 nm excitation/520 nm emission. The amount of GlcNAc was determined by comparison with GlcNAc standards subjected to the same incubation and labeling procedures as the lenercept samples. Complete removal of terminal GlcNAc residues was demonstrated by MALDI-TOF MS.

2.5. Terminal galactose determination

The amount of terminal galactose (tGal) present in each batch of lenercept was determined by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) after digestion with β-galactosidase. Lenercept samples were diluted to 1 mg/mL with 100 mM citrate phosphate buffer at pH 4.0 and digested in triplicate overnight at 37 °C with β-galactosidase (bovine testes) using 0.75 units enzyme per mg lenercept.

2.6. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The lenercept samples were first diluted to 1.0 mg/mL then diluted 1:1 with SDS sample buffer. Additionally, the samples for reduced SDS-PAGE analyses were combined with 10 mM DTT. All samples were heated at 90 °C for 5 min. Aliquots (2 μg for silver-stained gel and 20 μg for all other gels) were loaded onto a PAGE-ONE 4–20% gradient gel (OWL Separation Systems), based on the method described by Laemmli [15] along with the molecular weight standards (BioRad). Electrophoresis was performed at 40 mA until the dye front approached the end of the gel, approximately 60 min. After electrophoreses, the gels were either stained or electrophoretically transferred to nitrocellulose membranes for immunoblot analysis. The lenercept bands were visualized with either silver-staining [16] or Coomassie staining. Colloidal Coomassie staining was accomplished using a kit (NOVEX).

2.7. Capillary isoelectric focusing

Charge heterogeneity of the lenercept lots was assessed using capillary isoelectric focusing (c-IEF). Native or enzymatically treated lenercept was separated with a BioRad BioCAP XL capillary, 50 μm × 24 cm. The charged based separation was accomplished using an ampholyte mixture containing 80% cIEF Bio-Lyte Ampholyte 3–10 (2% solution with 0.5% TEMED, 0.2% HPMC) and 20% Bio-Lyte Ampholyte 3–10 (2%, diluted from 40% with H_2O). All lenercept samples (at approximately 1 mg/ml) were mixed with ampholyte at the ratio of 2:1:1 (ampholyte:lenercept:water, v/v). Two p*I* markers, 5.3 and 7.9, were included in the separation mixture to aid in alignment of the electropherograms. Focusing was accomplished at 15 kV (625 V/cm) for 5 min; the focused samples were mobilized at 20 kV (833 V/cm) for 25 min. Detection of the focused protein samples was accomplished at UV 280 nm.

To reduce heterogeneity of the lenercept samples, 1-mg aliquots were digested with 20 μL of carboxypeptidase B (CpB) and/or 400 μL of neuraminidase. After enzymatic digestion, all samples were diluted to 1 mg/mL with water for CE analysis.

2.8. Western immunoblotting of lenercept lots

After SDS-PAGE, the gels destined for immunoblot analysis were electrotransferred to nitrocellulose membranes using a NOVEX electroblot apparatus [17]. The immunoblots were probed with an affinity purified goat anti-human IgG, a mixture of three monoclonal antibodies directed against the TNF-receptor domain, or with a goat anti-Chinese hamster ovary proteins (CHOP) preparation. After this incubation, the membranes were washed to remove excess antibody and were incubated for 90 min with a secondary antibody, rabbit anti-goat (or anti-mouse, for the monoclonals) conjugated with horseradish peroxidase. Immunoreactive bands were visualized by the reaction of the substrate solution ((0.5 mg/mL 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB)), 0.3 μg/mL nickel ammonium sulfate, 0.3 μg/mL cobalt chloride in PBS) with hydrogen peroxide.

2.9. Biological activity of lenercept

The lenercept bioassay is a TNF-neutralization assay [18]. In a typical assay, 96-well tissue culture plates are loaded with lenercept samples and standards ranging in concentration from 0.02 to 2.5 μg/mL. Tumor necrosis factor-α (TNF-α) is then added to each well followed by the KYM1-KD4 cells, which express both p55 and p75 TNF receptors. The plates are incubated for 42–46 h at 36–38 °C in humidified 4–6% CO_2. At the end of the incubation, AlamarBlue™ is added to the wells. The AlamarBlue™ is reduced by viable cells resulting in a fluorescent product, which is monitored at 590 nm. The potency of the sample is quantified by comparing the relative point of inflection (CPE50% or ED50) of its curve with that of a reference standard. Samples are tested on two plates per day on each of two assay days and the results averaged. In studies using lenercept spiked into placebo that were conducted in three laboratories, the assay exhibited accuracy of ±5% of the expected value and overall precision of about 8%.

2.10. Pharmacokinetic studies

Details of the human volunteer trials evaluating lenercept pharmacokinetics are in preparation (Modi, personal communication). Briefly, healthy subjects received a dose of 5 mg as a 5–7 min infusion and serial blood samples were drawn over 28 days for drug concentration determination. Lots M11-70 and 71 were evaluated in 8 subjects each while for all other lots there were 15 or 20 subjects per group. Drug concentrations were determined by an ELIBA (enzyme-linked immunobinding assay) using an anti-TNFR monoclonal antibody to capture lenercept and HRP-labeled TNF to determine the binding activity. The assay has a sensitivity of around 5 ng/mL and a c.v. of 10–20% depending on the dilution required for assay.

3. Results

3.1. Production batches

Batches used for these studies were all produced from the same clone of CHO cells transfected with the plasmid encoding the lenercept molecule [6]. Table 1 summarizes the relevant information on their manufacture and includes the AUC_{1week} values from the pharmacokinetic volunteer trials. Early batches were produced using cell culture conditions (Condition A) in which the level of product expression was ‘‘low’’ but sufficient for clinical evaluation of the drug. Several batches of clinical grade material were produced at the 1000-L cell culture scale and two of these were pooled (to yield Batch M11-1/3) was used for initial clinical evaluation. Positive preliminary clinical data triggered a yield optimization program to meet anticipated commercial requirements. Cell culture conditions and media additions were changed and resulted in an approximate 3-fold yield improvement (Condition B). Several batches of drug were manufactured with this process, also at the 1000-L scale. The sialic acid content of this material (see below) was reduced from around 5 to 3.5 mol/mol of polypeptide. Animal and human PK studies indicated that this material had a reduced AUC_{1week}.

The recovery process for this molecule employed a Protein A affinity column that accomplished most of the purification.

Table 1
Campaign history for lenercept lots

Lot no. M11-	1/3	47	48	70	71	72	73	78	80
Scale	1 kL	1 kL	1 kL	1 kL	1 kL	12 kL	12 kL	12 kL	12 kL
Process	A	B	B*	C	C	C	C	C	C
AUC_{1week}	93	36	40	71	73	64	56	58	72
Sialic acid	4.8	3.2	4.1	4.7	4.8	5.5	4.9	5.2	4.8

Data for AUC_{1week} are μg/h per mL. See text for other details.
* Sialic acid content adjusted during purification.

The function of the subsequent operations was to remove residual CHO cell proteins and DNA and to inactivate possible adventitious agents. One of the chromatographic steps employed an anion-exchange column. Although the initial goal of the recovery process was to avoid glycoform fractionation, this step afforded an opportunity to selectively discard the early-eluting glycoforms (low in sialic acid content) to restore the sialic acid content to levels comparable to that of the initial batch M11-1/3 (4.8 mol sialic acid/mol lenercept). Accordingly, two batches were tested in PK studies: one in which all glycoforms were pooled (Batch M11-47, 3.2 mol sialic acid/mol lenercept) and another (M11-48, 4.1 mol sialic acid/mol lenercept) in which the sialic acid content was adjusted.

The fact that M11-47 and M11-48 showed similar PK (with AUC_{1week} values around 40% of that observed with M11-1/3), despite this significant difference in sialic acid content (3.2 vs. 4.1), required a re-evaluation of the cell culture conditions and a search for the cause of the variability. The third set of cell culture conditions (Condition C) produced a lower (2-fold) yield improvement but restored the sialic acid content similar to batch M11-1/3 and increased the AUC_{1week} in the PK evaluations (Table 1). All subsequent batches were manufactured under these defined conditions and no glycoform fractionation was performed. In some cases the cell culture was performed at the 1000-L scale while most were at the 12,000-L scale.

3.2. Polypeptide structure

Size-exclusion chromatography under native conditions showed that all the batches used in clinical trials were >99% in the expected dimeric form, with traces of higher and lower molecular weight species (data not shown). Although room temperature incubation and shaking of the solution did not induce significant aggregation, long-term high temperature (e.g., 2 years at 40 °C) incubation of the lyophilized material did cause the generation of some aggregated species.

Polypeptide size distributions were also assessed using SDS-PAGE. Staining was accomplished with Coomassie Blue colloidal stain, silver stain and Western blotting with anti human IgG and anti-TNF receptor preparations. No significant differences were observed from batch-to-batch in these patterns. Fig. 2, therefore, presents a composite of the patterns obtained with the various methods. Lanes A and B were 20 μg loads analyzed without and with reduction, respectively. Densitometric analysis of a series of increasing loads (not shown) indicated that approximately 97% of the dye-binding capacity of the non-reduced sample was present in the main band at 120–170 kDa. Traces of a presumptive dimer of the dimeric molecule can be seen above 200 kDa, while a trace of a smaller polypeptide (at 110 kDa) and presumptive monomer bands around 60 kDa can also be seen. In lane B, the sample was reduced, and the main band migrates at around 60 kDa. Smaller fragments can also be seen in the 25–50 kDa range. The silver-stain pattern from a 5 μg load is shown in lane C and reveals the pattern of low molecular weight fragments more clearly, as well as traces of unreduced dimer around 150 kDa. The origin of the fragments was assessed using immunoblotting. In lane D, the blot was probed with goat anti-huIgG while in lane E the blot was probed with a mixture of three monoclonal antibodies directed against TNF-receptor. In both blots, the main band is clearly identified at around 60 kDa. Many of the lower molecular weight bands seen in the silver stain (lane C) are also detected with the anti-IgG. A smeared band is detected at around 45 kDa with the anti-receptor preparation, which is not detected with the anti-IgG antibodies.

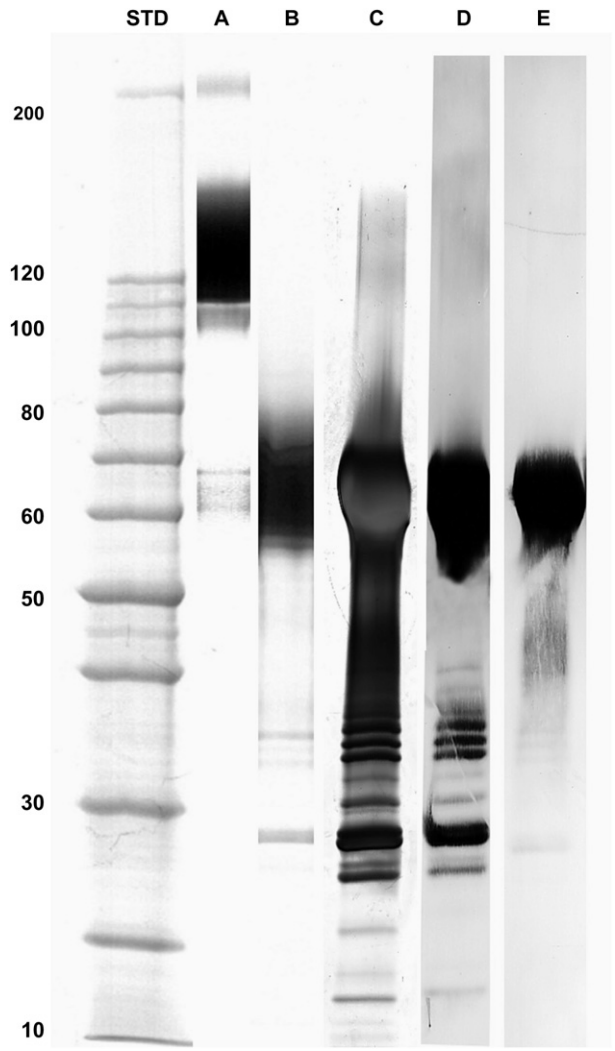

Fig. 2. SDS-PAGE analysis of lenercept. Lane identifications are as follows: STD, molecular weight markers (in kDa); A, Coomassie stain (20 μg, non-reduced); B, Coomassie stain (20 μg, reduced); C, silver stain (5 μg reduced); D, anti-huIgG blot; E, anti-TNFr blot.

3.3. Amino-terminal sequence analysis

Amino-terminal sequence analysis was performed on all batches and demonstrated the presence of two sequences: the major sequence was the anticipated sequence, beginning at residue 1, while a minor sequence, beginning at residue

12, was consistently observed. No other sequences were detected at an estimated sensitivity of 5%. The relative amounts of the two observed sequences was determined by peptide mapping and found to be consistent from batch-to-batch (see below).

3.4. Peptide map

Endoproteinase Lys-C was selected as the enzyme used for creating the peptide map, partly due to the existence of the N-terminal heterogeneity noted above. Fig. 1 shows the sequence and peptides expected from cleavage at lysine residues. Reduction and alkylation of the disulfide bonds was necessary to ensure complete digestion. Variable results were obtained if urea was used in the denaturing buffer for this reaction (data not shown) while 6 M guanidine HCl, combined with good pH control, provided the requisite consistency of the map. After reduction with DTT and alkylation with 4-vinylpyridine, the protein was digested with Lys-C and the resulting peptides were separated by RP-HPLC (see Section 2). A typical map is shown in Fig. 3 with peaks identified according to the numbering shown in Fig. 1. Each of the peaks (or for glycopeptides, the clusters) was collected and analyzed by N-terminal sequencing, amino acid composition, and MALDI-TOF mass spectrometry. The profile was quite reproducible in terms of numbers of peaks, elution times and relative peak areas, with little variation outside the peaks described below. Predicted peptides, low-level incomplete cleavage products, and Lys-C autolysis products accounted for all the observed peaks (data not shown). The glycopeptides eluted as ‘‘clusters’’ and the fine structure of these showed some variability from batch-to-batch.

3.5. N-terminal evaluation

As noted above, Edman sequencing analysis indicated the presence of a sequence beginning at residue 12. Residue 11 is an arginine and is therefore potentially a substrate for endogenous CHO endoproteases, such as trypsin, that cleave after basic residues. The utility of the Lys-C map results from the fact that this enzyme (in contrast to trypsin) does not cleave at the arginine. Consequently, the Lys-C map shows two peaks associated with residues 11–19: the authentic sequence of peptide L2 (residues 11–19) from the molecules with a complete N-terminal peptide and peptide L2a (residues 12–19) from those molecules missing the first 11 residues including the arginine. An expanded view of the peptide map is shown in Fig. 4 in which the elution positions of L2 (at approx. 30.5 min) and L2 clipped (at approx. 29.5 min) are illustrated. Mass spectrometry and sequencing confirmed that these two peaks did not contain co-eluting species and that the sequence from 11 to 19 was not present in any other peak. The two peaks were therefore suitable to use to obtain an estimate of the consistency of the relative amounts of the two sequences: the percent L2 clipped content was determined by peak area (100 × area of L2 clipped divided by the sum of the areas of L2 and L2 clipped). This is therefore the percent of molecules with the minor sequence. Data for the batches are presented in Fig. 5 and show that this event is relatively rare and quite consistent from batch-to-batch, ranging from 5.4% to 8.6%.

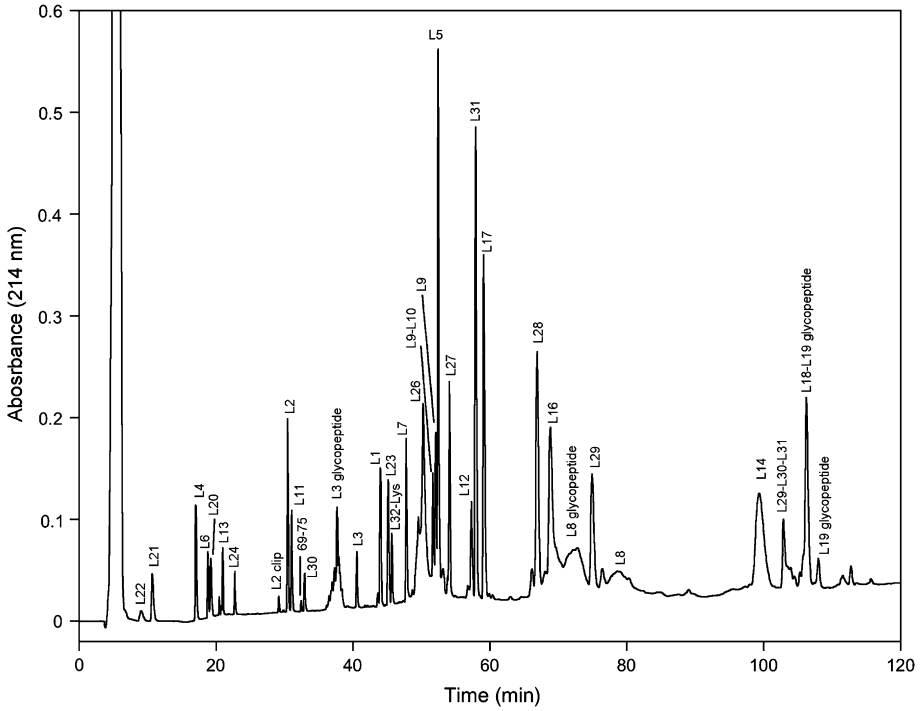

Fig. 3. Peptide map of lenercept. Peaks are identified by peptide number based on expected cleavage pattern for Lys-C, as shown in Fig. 1.

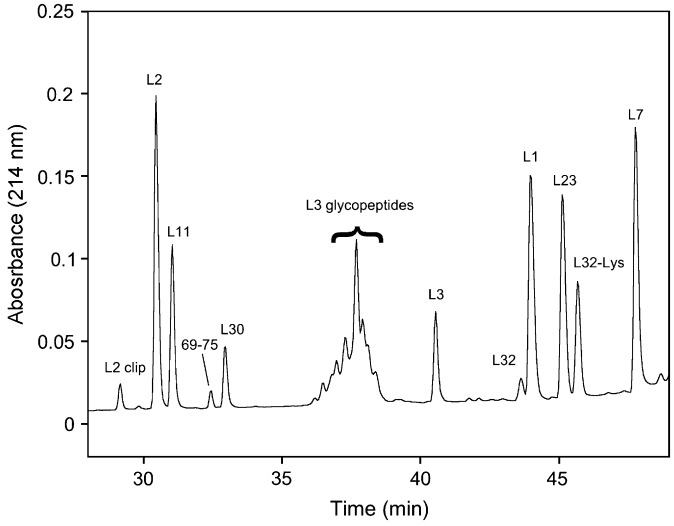

Fig. 4. Expanded view of peptide map showing N-terminal clip (L2 and L2 clipped), C-terminal (L32 and L32-Lys) and the L3 glycopeptide cluster (see text).

3.6. C-terminal evaluation

The status of the carboxyl-terminus of lenercept was evaluated by peptide mapping in a fashion similar to that described above for the amino terminus. Two peptides were identified in the Lys-C map as the anticipated C-terminal peptide (L32, residues 402–409, SLSLSPGK) and the peptide missing the C-terminal lysine (L32-Lys, residues 402–408, SLSLSPG). These are also shown in Fig. 4. These peaks do not contain co-eluting peptides and are sufficiently well resolved to allow for quantification of their relative proportions. The extent of processing was calculated using the ratio of the peak area of L32-Lys to the total peak area of both peaks. Results obtained for the batches of lenercept are summarized in Fig. 5. These data demonstrate that the lysine is removed from the majority of molecules (range from 50 to 89%).

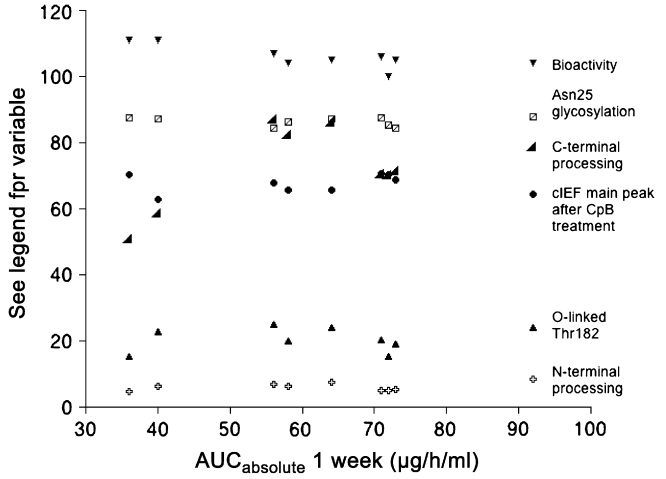

Fig. 5. Plot of polypeptide parameters against AUC_{1week} for lenercept lots. Open crosses, N-terminal clip (%); triangles, occupancy of Thr184 (%); half boxes, C-terminal processing (%); circles, main peak by cIEF (%); open squares, occupancy of Asn25 (%); inverted triangles, specific activity in bioassay (% of reference material).

3.7. Hydroxylysine content

This was determined from the peptide map in a manner analogous to that used for the N- and C-terminal quantification. Two peaks in the peptide map were identified by mass spectrometry as peptide L12 (residues 165–174) with hydroxylysine or lysine at residue 174, respectively. The content of hydroxylysine was estimated from the areas of the two peaks. Since this peptide contains only one lysine, this ratio provides a direct estimate of hydroxylysine content in moles/mole polypeptide, ranging from 0.12 to 0.26 moles hydroxylysine/mole polypeptide. The values are low and quite consistent from batch-to-batch. This unusual amino acid has been observed before [19] but its significance is not known.

3.8. Deamidation and isomerization

The extent of deamidation of lenercept was evaluated mainly by peptide mapping. The identification of peaks in the Lys-C map provided an indication that there is one site with detectable deamidation present when final batches were examined (residue 346 in peptide L28) and both the aspartate and isoaspartate forms were detected. Comparison of the batches showed that the non-deamidated form represented 91–93% of this peptide by integration of the appropriate peaks.

The relatively high susceptibility of this residue to deamidation suggested that this might also be occurring during the sample handling required for the proteolytic digestion step of the method. Therefore, a sample was prepared as normal and incubated under the digestion conditions for various lengths of time before addition of the Lys-C. All samples were then incubated with the enzyme for the standard time and the degradations peaks were analyzed. This experiment showed that the longer the pre-incubation, the greater the level of degradation observed. Extrapolation of the time course to "zero" incubation time indicated that an "apparent" level of ~8% corresponded to a true value of ~3–4% before analysis. The data indicate that deamidation is not a significant phenomenon for this protein.

3.9. Isoelectric focusing

Overall charge heterogeneity of lenercept was assessed by c-IEF. Several profiles are shown in Fig. 6. This figure also illustrates that galactosidase and hexosaminidase treatments, while expected to affect the pharmacokinetics (see below), have little effect on the pattern. This result is expected because the released monosaccharides (galactose and *N*-acetylglucosamine) are uncharged. Since the c-IEF pattern is complex and clearance is not controlled by sialic acid, this method does not provide any useful information for untreated batches. The underlying charge heterogeneity of the lenercept lots was, therefore, monitored by c-IEF after neuraminidase and

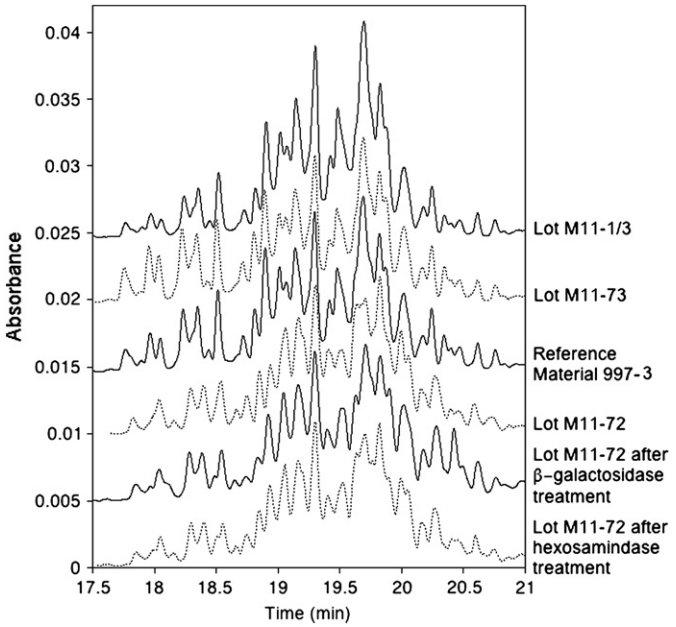

Fig. 6. Capillary IEF profiles of lenercept lots. From top to bottom: lot M11-1/3; lot M11-73; reference material 997-3; lot M11-72; lot M11-72 after galactosidase treatment; lot M11-72 after hexosaminidase treatment.

carboxypeptidase treatment. These enzymatic treatments remove the known charge heterogeneity arising from sialic acid and C-terminal lysine variability, respectively, both of which were independently assessed. The c-IEF pattern after these treatments is greatly simplified (Fig. 7), and the acidic peaks are attributed to heterodimers of N-terminally clipped and deamidated polypeptide chains. Evaluating the proportion of molecules in the main peak assessed the charge heterogeneity of each batch and the batch-to-batch consistency is demonstrated in Fig. 5. These enzyme treatments had no effect on the biological activity of the molecule (data not shown).

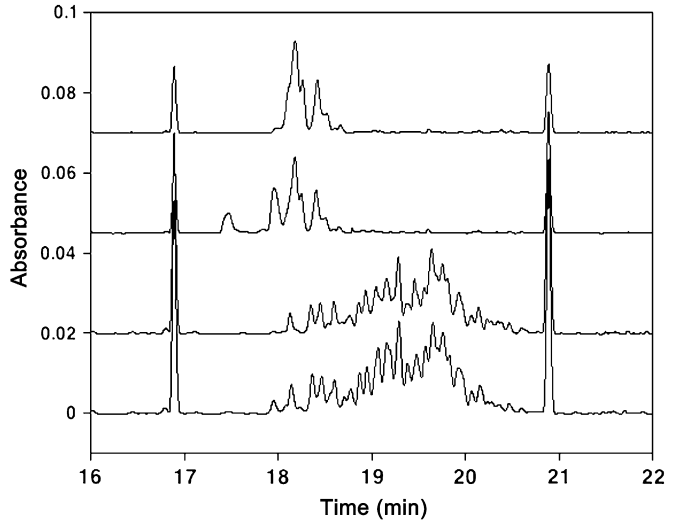

Fig. 7. Capillary IEF profiles of lenercept: A, untreated reference material; B, after carboxypeptidase treatment; C, after neuraminidase treatment; D, after treatment with both enzymes.

3.10. Oxidation

Methionine residues in proteins can be oxidized, and surface residues, which are more susceptible to this reaction, can be identified by incubation with *t*-butylhydroperoxide [20]. This method was used to identify potential sites of oxidation. Peptide L16 was found to be the most susceptible to oxidation while L7, L26 and L31 were affected to a lesser extent. In the control sample, a trace peak was observed to elute where L16ox appeared in the oxidized sample. This peak in the control sample could not been identified. However, if it were L16ox, it would represent only approximately 3% of the L16 peptide. The other sites possibly susceptible would therefore be expected to be present at much lower levels. These data indicate that oxidation, like deamidation, is not a significant event for this molecule.

3.11. Occupancy of N-linked glycosylation sites

Variable carbohydrate status could be introduced if the extent to which potential glycosylation sites were occupied (i.e., the ‘‘occupancy’’) were itself variable. This was assessed using peptide mapping in which unoccupied peptides were resolved from occupied forms. Batches were analyzed before and after removal of the glycans by peptide-*N*-glycosidase F (PNGase F) and this permitted the identification of the glycopeptides. In the case of Asn^{25}, it was observed that in the intact materials (i.e., prior to the PNGase F treatment), this site is only partially occupied (see Fig. 4). This partial occupancy was remarkably consistent from batch-to-batch, with 87% of the polypeptide chains being glycosylated. The glycopeptide cluster and the unoccupied peptide are indicated in Fig. 4, while the deglycosylated form and the unoccupied form of the peptide were also resolved in the map after deglycosylation (data not shown). These two peptides could be resolved because the deglycosylated form has an aspartic acid after the glycan is removed, while the unoccupied form is an asparagine.

The other two-glycosylation sites in the receptor domain are on one peptide (L8 at Asn^{116} and Asn^{122}) with no intervening amino acids, which would allow their easy separation for individual analysis. Two peptide clusters were identified by sequencing and mass spectrometry with the appropriate sequence, with the major cluster (~95% of the total area) having two glycans and the minor one having only one, although it was not possible to determine which asparagine was occupied in this latter peptide. These data indicate that these two sites are almost completely occupied in all batches. A similar approach allowed the determination that the glycosylation site in the Fc domain was completely occupied and no peptide was found in the analysis of the peptide map for the unoccupied form of this peptide.

3.12. O-linked glycosylation

Early work during the development of lenercept included monosaccharide composition analysis, which showed that

N-acetylgalactosamine was present at low levels. This indicates the presence of O-linked glycans on some molecules. The presence of O-linked glycans was also observed on the larger fragment of the soluble TNF receptor isolated from urine [21]. Fig. 8 shows the region of the L13 peptide that was identified by mass spectrometry as the site of the O-linked glycans. Two peaks running slightly ahead of the unmodified L13 peptide were identified as bearing the O-linked core structure of GalNAc-Gal with one or two sialic acids. The site of glycosylation was inferred from sequence analysis in which the signal from Thr^{182} was significantly depressed compared to the signal from the unmodified peptide. Fig. 8 shows that the majority of L13 is in the unmodified form. Quantification of the proportion of L13 occupied by O-inked glycans was by integration of the various forms of the peptide. Fig. 5 shows that the occupancy of the site ranges between 13 and 24%.

The effect of this O-linked glycosylation was investigated by lectin chromatography. Jacalin lectin has been shown to bind to O-linked glycans [22] and a sample of lenercept was passed over the column. Most of the material flowed through the column and some material only eluted when the eluent contained galactose. Lys-C peptide mapping indicated that the flow through material was devoid of the peaks associated with the O-linked glycans (see above) while the bound material was found to have approximately 47% of the sites occupied with these glycans. Mass spectrometry analysis showed that the distribution of the N-linked glycans in the two fractions was indistinguishable (data not shown). These data suggest that the Jacalin lectin does indeed have a high specificity for O-linked glycans and that the fractions were separated solely into two populations of lenercept: O-glycosylated and non-O-glycosylated. Since lenercept is a dimer, and the lectin will bind a molecule with O-glycans on either of the two polypeptide chains, the fact that approximately 50% of the sites are occupied in the bound material suggests that dimers in which both sites are O-glycosylated are rare. Samples of the flow through and bound materials from the Jacalin lectin column displayed essentially the same activity, indicating that there is little effect of the presence or absence of the O-linked structures on the ability of lenercept to bind and neutralize TNF.

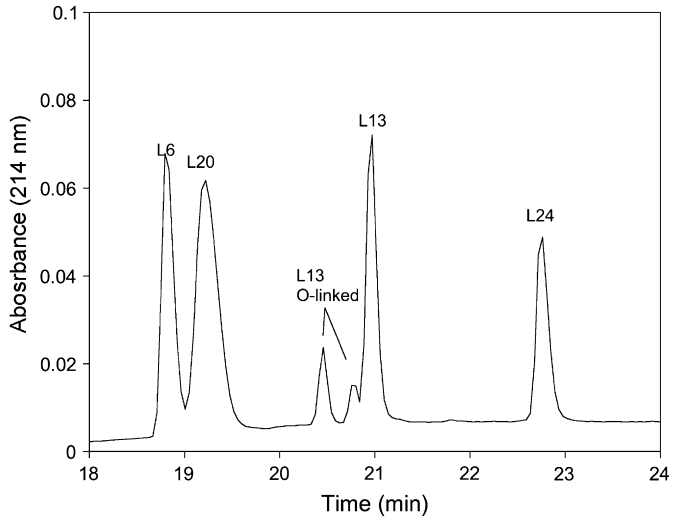

Fig. 8. Expanded view of peptide map showing forms of peptide L13.

3.13. Bioassay

The mechanism of action of lenercept is based on its ability to bind TNF through its normal receptor recognition site and thereby prevent it from binding to that site on receptors in the patient, thus blocking its mediating role in the inflammatory process. Although assays that monitored the binding event were successfully used to assess the activity of lenercept (not shown), regulatory authorities prefer a biological read-out, wherever possible, for testing of such pharmaceuticals. Therefore, and the cell-killing inhibition assay described in Section 2 was developed. The biological activity of all the batches described herein was, within experimental error (i.e., ±15%), the same as that of the in-house reference.

3.14. Terminal sugars

Sialic acid content was determined by the method described by Anumula [14] in which the sialic acid is simultaneously released by acid hydrolysis and derivatized with OPD. This method has an interassay coefficient of variation (c.v.) of ~5% for the derived value of moles sialic acid per mole of protein, which includes the variation from the protein concentration determination. Values for the batches are presented in Fig. 9. It can be seen that cell culture condition B resulted in lower sialic acid content (3.5 mol/mol for Batch M11-47) than condition A or C (range of 4.8–5.6) and that the anion-exchange chromatographic modification boosted the value for M11–48 from an initial value of 3.5 to 4.4.

Terminal galactose content was determined by incubation of the batches with β-galactosidase and measurement of the released galactose by HPAEC-PAD. Results are presented in

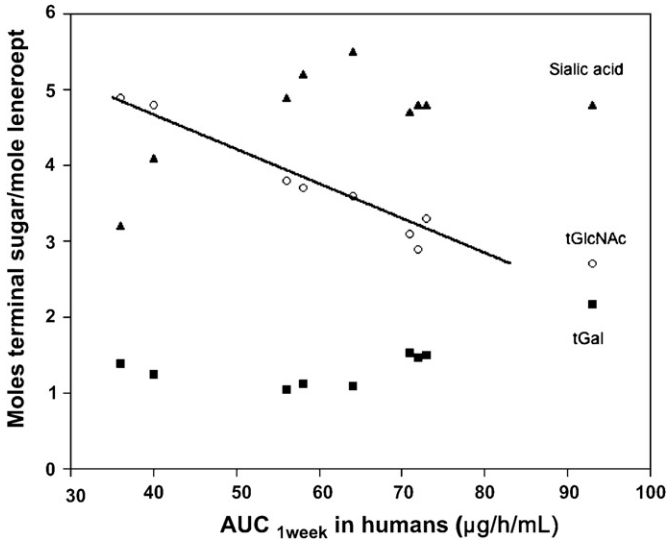

Fig. 9. Correlation of AUC_{1week} with terminal sugar content for lenercept lots. Circles are terminal galactose content; triangles are sialic acid content; squares are terminal GlcNAc content. tGlcNAc = 6.3 − 0.042 × AUC ($R = 0.96$).

Fig. 9. Conditions were optimized to ensure maximal galactose release. Assessment of the extent of release was performed by: (1) PNGase F treatment of the sample (with or without prior galactosidase treatment) followed by mass spectrometric analysis of the N-linked glycans [15]; and (2) release of the N-linked glycans from the protein followed by galactosidase treatment of the free glycans. These comparisons indicated that the extent of galactose release was the same whether the glycans were free or still linked to the protein. Small amounts (<10%) of glycans with masses consistent with the presence of tGal remained in both cases after extensive digestion and are interpreted as indicative of the presence of small amounts of lactosamine repeat structures in which the galactose residue is internal.

Terminal *N*-acetylglucosamine (tGlcNAc) was similarly determined enzymatically by incubation of batches with hexosaminidase. Released GlcNAc was measured, after fluorescent labeling, by capillary electrophoresis (see Section 2) and results are presented in Fig. 9. Completeness of release (>95%) was demonstrated in a similar fashion as for the galactosidase treatment by MALDI-TOF MS on both protein-bound and free glycans. This assay was validated and demonstrated a variability (% CV) of 5–8% (data not shown).

The data from the polypeptide analyses and terminal sugar analyses are shown in Figs. 5 and 9, respectively, with the *X*-axis in each case being the AUC_{1week}. It can be seen that the only parameter which correlates with this is the tGlcNAc content. Other parameters either vary little from batch to batch or vary independently from the AUC_{1week} for the batch. The sums of the terminal saccharide contents (not shown) indicate good consistency of the number of measured antennae per molecule from batch to batch, especially considering that the three numbers are derived from separate methods, each with its own experimental error.

4. Discussion

The data presented in this paper were generated partly to provide documentation for regulatory approval to market lenercept and partly to determine the cause of the variability in the pharmacokinetics from batch to batch that was observed in human volunteer trials. Since the formulation composition (other than the drug) was the same for all batches, the cause was considered to arise either from some aspect of the polypeptide portion or from the carbohydrate portion. Detailed examination of a wide range of characteristics has revealed that one specific carbohydrate structure was responsible.

The size and complexity of a molecule like lenercept inevitably results in some chemical changes from the ''perfect'' molecule during its manufacture, either due to the cell line and culture conditions producing it or to the recovery process operations. Peptide mapping was therefore extensively used in these studies. Although Edman degradation confirmed the presence of two N-terminal sequences (starting at residues 1 and 12), the peptide map was used to provide a more reliable quantification for this parameter. All the batches from the production clone consistently had approximately 7% of this secondary sequence (12–409, and this did not change during the cell culture or recovery processes (not shown). A second cell line, from an independent transfection, also showed consistency, with values of 45 ± 4% ($n = 3$) of this secondary sequence. The consistency of the data suggest that this second sequence could arise from a secondary signal peptidase cleavage whose efficiency was in some way related to either the site of integration of the gene encoding the protein sequence or to the signal peptidase itself in a distinct cell line. This cleavage site was also observed in studies of the soluble TNF receptor obtained from human serum [23].

The carboxyl-terminal amino acid of lenercept is a lysine, and this residue was removed in a substantial and variable proportion (~50–90%) of the chains, probably due to variable levels of carboxypeptidase activity [24] during cell culture and initial recovery. This phenomenon has frequently been observed for antibodies [25] but so far has no identified consequence. This parameter varied from batch-to-batch, but did not correlate with the PK variability.

Hydroxylysine was identified as being present at residue 174 (normally a lysine) in about 17% of the polypeptide chains. Although this was quite consistent from batch-to-batch and uncorrelated with the PK variability, its presence is unusual. Further, the mechanism by which it was incompletely but consistently incorporated, and only in this one site on the protein, remains to be determined.

Analysis of the polypeptide chains, either in native form by size-exclusion chromatography or by gel electrophoresis in denatured form, revealed little variability from batch-to-batch. The dimeric form of the molecule was quite stable and aggregation was detectable at only trace levels. The presence of small levels of proteolytic ''clips'' was detected by denaturing electrophoresis in a consistent banding pattern and, in such forms, the resulting fragments remained associated in the native state, presumably through disulfide bonds or non-covalent interactions. The observed fragments were all identified as parts of the Fc domain or the receptor domain by immunoblotting.

Deamidation was not observed to any great extent and was consistent from batch-to-batch. The importance of understanding the extent of deamidation generated by the analytical method used to measure the phenomenon was highlighted by the time-course studies where extrapolation to zero time was required for an accurate estimate (see also a review of these issues for peptide mapping [26]). Oxidation is another common degradation pathway for proteins [27] but lenercept did not appear to have any particularly sensitive sites, and the batches displayed little evidence for it.

The polypeptide characterization described above was performed to meet the requirements for submission for approval to market lenercept, but is presented here to establish that no polypeptide parameter is related to the variable pharmacokinetic performance of the batches. A common analytical method used for the evaluation of proteins, and often recommended by Health Authorities for inclusion in submissions [28], is isoelectric focusing (IEF). In addition to enabling the monitoring of polypeptide charge heterogeneity arising

from, e.g., deamidation, IEF can provide important information on sialic acid content for glycoproteins. In two particular cases, for example, a consistent isoelectric focusing pattern is used to assure batch-to-batch consistency of erythropoietin [29] and recombinant follicle stimulating hormone [30]. In the case of lenercept, the IEF pattern is extremely complex, largely due to the multiplicity of sialic acid residues contained in the dimeric form. Yim [31] was one of the first to employ c-IEF to examine the glycoform distribution of a complex glycoprotein (tissue-type plasminogen activator), and he observed a series of approximately evenly spaced peaks separated on the basis of their sialic acid content. The intensity distribution across these peaks appeared to be close to the Gaussian that would be expected if two assumptions are made: each sialic acid affects the isoelectric point similarly and the attachment of sialic acids is random. For lenercept, the distribution is clearly not truly Gaussian and the spacing between the peaks is not even, yet the pattern is remarkably consistent from batch-to-batch, even after allowing for some intensity fluctuations. These observations suggest that (1) a sialic acid present on one part of the molecule may not have the same effect on p*I* as one attached somewhere else and (2) a non-random distribution of sialic acids (from site to site) may be occurring. Interpretation of the pattern is further complicated by the influence of C-terminal lysine variation, deamidation, and N-terminal heterogeneity. These complexities and the observation that the pharmacokinetic variation was not correlated with sialic acid content eliminated the utility of c-IEF for batch evaluation. However, removal of the identified sources of IEF variability, sialic acid content and C-terminal lysine heterogeneity, by enzymatic removal of both charged groups, did result in a c-IEF evaluation that was useful. Although the cause(s) of the residual heterogeneity in the c-IEF profile have not been definitively established (probably deamidation and N-terminal heterogeneity) the resulting c-IEF pattern was remarkably consistent from batch to batch, thereby confirming that no polypeptide charge-related parameter was related to the pharmacokinetic variation. As expected, however, the enzymatic removal of the (uncharged) terminal galactose or GlcNAc had no effect on the c-IEF profile, again demonstrating that the c-IEF profile was insensitive to the key parameter controlling the pharmacokinetic variability.

The specificity of the clearance receptors for particular glycans is mediated by the terminal saccharides in the glycan structures. Therefore, measurement of the terminal saccharide content in a glycoprotein would be expected to be useful in understanding, or even predicting, their pharmacokinetic performance. The data in Fig. 9 clearly show that the only parameter that is correlated with the pharmacokinetic performance is the terminal GlcNAc. The receptor responsible for clearing the protein bearing tGlcNAc is likely to be the mannose receptor [32]. A crystal structure of mannose binding to a C-type lectin [33] shows that the structural features of the mannose involved in the interaction are the same as those present on a tGlcNAc residue.

Although the most frequently encountered source of variability in the pharmacokinetics of glycoproteins is related to sialic acid [34], it is the absence of sialic acid that exposes the underlying galactose residues that can consequently be recognized by the asialoglycoprotein receptor [35]. Furthermore, the binding affinity is highly dependent on the number and orientations of the galactose residues [36]. A glycan with a single galactose has extremely low affinity and glycans with two terminal galactose residues are only weakly bound. Thus, asialotransferrin circulates in serum with a half-life comparable to its sialylated form, as a consequence of its carrying predominantly biantennary glycans [36]. In most cases where sialic acid controls the pharmacokinetic parameters, it is not the amount of sialic acid that is the parameter of interest but rather the extent to which it is absent. While the overall content of terminal galactose is important, the content and distribution of glycans with two or more terminal galactose residues, if they can be measured, would be the most useful information to help understand the PK variability.

The preceding paragraph was implicitly based on the assumption that if the sialic acid is absent, the underlying galactose is present and therefore exposed for potential interaction with the asialoglycoprotein receptor. (The observed correlations with sialic acid content also assume a reasonably consistent antennarity.) In many cases it is true that galactosylation efficiency is sufficiently high for this to be a reasonable assumption, and this has led to the general focus on sialic acid content, rather than terminal galactose content, when considering the PK of glycoproteins. For lenercept, however, the comparisons of high and low sialic acid content batches showed that this did not hold and that it was neither sialic acid content nor terminal galactose, but rather terminal GlcNAc which was responsible. This is most easily understood when it is appreciated that the batch-to-batch variation in the biosynthetic pathway was not in sialylation but rather in galactosylation. The variation in sialic acid content occurred because of the variation in the amount of galactose, to which the sialic acid is attached as the final biosynthetic step of complex glycans. It is concluded that the specific clone selected and used for production of all the batches described herein did not galactosylate as efficiently as ‘‘normal’’ CHO cells.

The data presented in this paper demonstrate that glycosylation adds another layer of complexity to the development of protein pharmaceuticals. The common microheterogeneity of the glycans and potential for variability from batch-to-batch result in the need for appropriate analytical methods to assess the key structural features that may affect potency or PK properties. Although there are many methods available for glycan characterization, they are not always suitable for use in the quality control system, which is responsible for ensuring that each batch will have the correct properties. It is first necessary to determine the key features of a (glyco)protein which affect performance and then to develop suitable tests that are able to routinely assess those features. For lenercept the studies presented here established that tGlcNAc is the key feature that influences PK and a suitable assay for its measurement was developed and validated.

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