Evaluation of Genetic Stability of Recombinant Human Factor VIII by Peptide Mapping and On-line Mass Spectrometric Analysis

Marc J. Besman[1,2] and Donald Shiba[1]

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Purpose. The genetic stability of a recombinant human factor VIII (rhFVIII) product expressed in Chinese hamster ovary cells (Recombinate™) has been evaluated through comparisons of the protein produced at the beginning, middle and end of a typical production campaign.
Methods. Recombinant human factor VIII was incubated with thrombin, the resulting four polypeptides were isolated by RP-HPLC, subjected to proteolysis with trypsin, and the peptide mixtures were resolved by RP-HPLC. Tryptic peptide mixtures were subjected to on-line mass spectrometric analysis using an electrospray ionization source interfaced to a quadrupole mass analyzer scanning from 1950–200 amu, and the peptide ion data were compared for three lots produced from the beginning, middle and end of a production campaign.
Results. The UV elution profiles for each of the rhFVIIIa polypeptides were highly similar for factor VIII isolated from the beginning, middle and end of production. Total ion data from the peptide maps derived from three lots of rhFVIII were compared by MH^{1+} values as a function of scan range. A total of 918 ions were analyzed for the four polypeptides of rhFVIII produced at the beginning, middle and end of a production campaign. The ions were detected at the same relative retention times, as indicated by the similar scan numbers for the three lots.
Conclusions. These observations support that rhFVIII preparations produced from the beginning, middle and end of a production campaign were highly similar, and demonstrate genetic stability in the manufacturing process of Recombinate™.

KEY WORDS: factor VIII; genetic stability; peptide mapping; mass spectrometry; proteolysis.

INTRODUCTION

Recombinate™, a recombinant form of factor VIII produced in Chinese hamster ovary (CHO) cells, has been demonstrated to be clinically effective in the treatment of hemophilia A, an X-linked genetic disorder (1–4). Factor VIII is a large blood coagulation protein (≈280 kD) which is found as a heterodimer in the circulation complexed to von Willebrand factor (1,5). The protein comprises a number of internally repeated structural domains (A1, A2, A3, C1 and C2) which share ≈30% sequence homology with factor V, and another domain (B) which shares no homology with factor V (1,2); the heavy chain, arising from the N-terminus of the single-chain gene product, constitutes the A1-A2-B domains, while the light chain is described by the A3-C1-C2 domains. Upon activation by thrombin, factor VIII undergoes three highly specific proteolytic cleavages, giving rise to a heterotrimer with greatly increased rate of activation of factor X by factor IXa (1,5,6,7). The restoration of hemostasis in hemophilia A patients is accomplished through the prophylactic administration of factor VIII. A number of factor VIII therapeutic products are commercially available. These vary in purity from low- to high-purity plasma-derived products, to very-high-purity recombinant or plasma-derived products; the latter products' manufacturing processes include immunoaffinity chromatography (8).

Recombinate™ has been licensed for commercial distribution since 1992 (9,10), and was recently designated as a Well-Characterized Biotechnology Product (12) by the Food and Drug Administration. This protein is produced in Chinese hamster ovary cells, harvested from conditioned cell culture medium through a discontinuous batch-refeed process, and isolated using immunoaffinity and ion-exchange chromatographies (10,11,13). The manufacturing process, initiated from a working cell bank and maintained over a maximum number of cell generations, is monitored closely to ensure the consistency of production during the entire duration of the campaign (13).

The evaluation of genetic stability is an important measure of manufacturing process consistency for recombinant protein therapeutics produced in cell culture (14–19). The scope of such considerations includes the production of aberrant protein molecules through alteration of genetic material (i.e. DNA or RNA) or modification of the protein molecule (e.g. post-translational processing). Production of variant or mutant protein may affect biological activity, immunogenicity and/or pharmacokinetics. Conversely, the demonstration of genetic stability in a given production system is generally indicative of manufacturing process consistency, and is desirable in complex, engineered cellular production. Genetic stability may be assessed through an examination of the gene construct, typically through nucleic acid-based sequencing (18). However, a principal advantage of the evaluation of genetic stability at the protein level is that the protein is typically closest to the therapeutic entity administered to the patient. For both approaches, the application of sophisticated and sensitive analytical methods is an essential component to the feasibility of such studies (20–26).

Several considerations affect the development and application of technology to the evaluation of genetic stability of recombinant human factor VIII (rhFVIII):

1. rhFVIII is a protein of 2351 amino acids which is extensively modified post-translationally, and undergoes proteolytic processing during production (1,2,5).
2. The evaluation of genetic stability at the protein level typically encompasses comparative peptide mapping of two or more representative samples of the protein of interest, where proteolysis and chromatographic separation are achieved under highly reproducible conditions.
3. Peptide maps of large proteins are generally very complex, with most peaks comprising multiple peptide components.
4. Automated Edman protein microsequencing of numerous peptides is not practical for routine, comprehensive analysis for all but the simplest of proteins.

[1] Baxter Healthcare, Biotech Group, Hyland Division, 1720 Flower Avenue, Duarte, California 91010.
[2] To whom correspondence should be addressed. (e-mail: besmanm @baxter.com)

NOTATIONS: rhFVIII, recombinant human factor VIII; rhFVIIIa, recombinant human factor VIII which has been activated with human thrombin; RP-HPLC, reversed-phase HPLC; TFA, trifluoroacetic acid; LC/MS, on-line mass spectrometry; TIC, total ion current.

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We have undertaken to evaluate the genetic stability of rhFVIII at the protein level through a combination of peptide mapping with on-line mass spectrometric analysis. The analysis of complex protein biologicals through the structural study of peptides and proteins by mass spectrometry has been reviewed extensively (21,22,27). The analysis of peptide maps by on-line liquid chromatography-mass spectrometry (LCMS) affords the following principal advantages to the evaluation of genetic stability at the protein level.

1. Peptide mapping is a technique widely recognized for its ability to resolve complex mixtures of peptides chromatographically with a high degree of consistency (22–24,26,28).
2. The incorporation of an orthogonal detection method, namely mass spectrometry, allows for the ready identification of multiple ions within a single reversed-phase HPLC (RP-HPLC) peak, providing the means for the interpretation of complex peptide maps (21,22).
3. The ability to apply column eluents directly into the mass spectrometer allows for the seamless transfer of conventional HPLC separation methods already optimized for UV detection.
4. The continuous acquisition of LC/MS data allows for the potential identification of minor constituent ions which may not be detected by UV elution profiles.

We describe below the evaluation of production consistency for protein manufactured from the beginning, middle and end of production, by isolation of four rhFVIIIa polypeptides following incubation with thrombin, proteolysis of each polypeptide with trypsin, and analysis of peptide mixtures by RP-HPLC and on-line LC/MS. Incubation of rhFVIII with thrombin affords four principal polypeptides (5–7), thereby reducing the complexity of peptide mixtures of rhFVIII substantially. Elution profiles and peptide ion data are compared for peptide maps of the B-domain, 73 kD, 50 kD and 43 kD polypeptides prepared from rhFVIII produced at the beginning, middle and end of a typical production campaign. The findings will be discussed from the standpoint of evaluation of genetic stability at the protein level.

MATERIALS AND METHODS

Preparation of Peptide Mixtures

Recombinant human factor VIII was isolated and purified from a Chinese hamster ovary cell line as previously described (9). Samples produced from the beginning, middle and end of a typical production campaign were subjected to anion-exchange chromatography according to the following methods. The Q-Sepharose Fast Flow columns (Pharmacia, 0.8 × 4 cm) were regenerated with 0.1 N NaOH (20 ml) and 0.1 M HCl (20 ml), and equilibrated with buffer-A (20 mM Tris, 5 mM $CaCl_2$, pH 7.4; 40 ml). Samples of rhFVIII (≈ 4–6 mg) were diluted five-fold with buffer-A and loaded onto the columns. The columns were washed with buffer-A (40 ml), and the rhFVIII was eluted with 0.5 M NaCl, 20 mM Tris, 1 mM $CaCl_2$, pH 7.4 (≈ 10 ml). The columns were finally treated with 1 M NaCl, 20 mM Tris, pH 7.4 (10 ml). The regeneration, equilibration, wash and 1 M NaCl steps were performed at 2 ml/min while the sample loading and elution steps occurred at 1 ml/min. The eluant protein concentration was determined by UV absorbance (29) prior to thrombin activation (ϵ_{280} = 0.84 ml mg^{-1} cm^{-1}).

Factor VIII eluted from Q-Sepharose was adjusted with glycerol to 12% [v/v] and incubated with human thrombin (10 U thrombin [Sigma] / mg rhFVIII). Thrombin digestion proceeded at 37°C for 10 minutes, followed by the addition of EDTA (8 mM final). Thrombin-activated rhFVIII (rhFVIIIa) mixtures (≈ 1–2 mg) were separated on a HPLC system (Hewlett-Packard 1090) equipped with a semi-preparative C_4 column (Phenomenex Primesphere, 10 × 250 mm). The HPLC parameters are listed in Table I. The resulting HPLC protein peaks were collected manually and were concentrated by vacuum centrifugation (Savant). Polypeptide concentrations were estimated by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as the standard. Protein purity was evaluated by SDS-PAGE using 4–20% gels (Novex) and Coomassie Blue R-250 staining (30).

Polypeptides of rhFVIIIa were dried by vacuum centrifugation, resuspended in deionized 8 M urea, 0.2 M ammonium bicarbonate, pH ≈ 8 to 238 μg/ml and diluted with water to 61.8 μg/ml. Sequence-grade trypsin (Promega, 0.1 mg/ml in 5 mM acetic acid) was added at a 1:20 (w/w) enzyme:substrate ratio. Tryptic digestions were performed in 2 M urea, 50 mM ammonium bicarbonate, pH ≈ 8 at 60 μg/ml. Digestion was carried out for 18 hours at 37°C, followed by addition of trifluoroacetic acid (TFA) to pH 2. The rhFVIIIa tryptic digestions were evaluated on a C_{18} RP-HPLC column (Vydac, 2.1 × 250 mm), according to parameters listed in Table I.

Mass Spectrometric Analysis of Peptide Mixtures

Peptide mixtures of rhFVIIIa polypeptides (≈ 300 pmol in 100–125 μl) were analyzed by on-line LC/MS by Taylor Technology, Princeton, N. J., according to the following methods. Reversed-phase HPLC was accomplished using a Hewlett-Packard 1090L system fitted with a C_{18} column (Vydac, 1.0 × 250 mm), according to instrument parameters described in Table I. Post-detector column eluent was directed to a Finnigan TSQ700 triple quadrupole mass analyzer fitted with a Finnigan electrospray ionization source, with a spray voltage of 4.5 kV, sheath gas pressure of 60 psi and capillary temperature of 250°C. Scans were performed over a range of 1950–200 amu during a period of 3 seconds. The mass analyzer was tuned and calibrated in the centroid mode, with single quadrupole evaluation of a standard tetradecapeptide; the standard ions (m/z 587.7, 881.0 and 1760.0) were observed within 0.1 amu of known average masses.

Portions of peptide mixtures of the B-domain, 73 kD, 50 kD and 43 kD polypeptides from the beginning, middle and end of a typical production campaign were analyzed by on-line LC/MS consecutively, to minimize the effects of normal instrument variation on the separation and mass spectrometric detection of peptide ions. Total ion chromatograms, UV traces and representative mass spectra for major and minor components were generated for each peptide map. Mass spectra of components identified in the total ion chromatogram (TIC) trace were visualized by summing scans across a given TIC peak, with subtraction of a suitable number of scans on either side of the TIC peak. Charges for ions observed in mass spectra were assigned, MH^{1+} values were calculated, and ion data were entered in a database. The resulting datasets for tryptic peptide

Table I. RP-HPLC Parameters

Parameters	C_4 semi-preparative			C_{18} narrow bore			C_{18} narrow bore, LC/MS		
Column dimensions	10 × 250 mm			2.1 × 250 mm			1.0 × 250 mm		
Solvent A	0.1% TFA (v/v) in water						0.1% TFA (v/v) in water		
Solvent B	0.083% TFA (v/v) in 90% (v/v) aq. acetonitrile						0.1% TFA (v/v) in acetonitrile		
Detection	214 nm						214 nm		
Column temperature	40°C			room temperature			room temperature		
Gradient	Time (min)	%B	Flow (ml/min)	Time (min)	%B	Flow (ml/min)	Time (min)	%B	Flow (ml/min)
	0	10	4.7	0	0	0.2	0	1.8	0.05
	5	10	4.7	5	0	0.2	20	1.8	0.05
	7	45	4.7	68	70	0.2	130	63	0.05
	25	70	4.7	70	100	0.4	130.1	100	0.05
	27	100	4.7						

maps of the B-domain, 73 kD, 50 kD and 43 kD polypeptides of rhFVIII produced from the beginning, middle and end of a typical production campaign were sorted and compared for coincident detection of peptide ions in all three production samples.

RESULTS

Preparation of rhFVIIIa Polypeptides

Equivalent anion-exchange chromatography recoveries and purities were observed for the rhFVIII from the beginning, middle and end of production. The Q-Sepharose process was developed to remove Tween 80 from the eluate, and purification parameters, such as flowrates and [Ca^{2+}] were optimized to improve the rhFVIII recovery (data not shown). The Q-Sepharose eluates were normalized to maintain reproducible thrombin activation. After incubation with thrombin, the resulting rhFVIIIa polypeptides were purified by C_4 RP-HPLC to high purity (Fig. 1A), as determined by SDS-PAGE (Fig. 1B). Subsequently, individual rhFVIIIa polypeptides were treated with trypsin to produce peptide mixtures.

Analysis of rhFVIIIa Peptide Maps

In general, the tryptic peptide elution profiles displayed the expected complexity predicted from the number of theoretical peptide bond cleavages. The tryptic peptide maps of the B-domain and 73 kD polypeptides, derived from rhFVIII produced from the beginning, middle and end of a typical production campaign, were highly similar in terms of peak numbers and relative peak intensities (Figs. 2–3). Tryptic peptide maps of the 50 kD and 43 kD polypeptides exhibited equivalent similarity for comparison of rhFVIII from the beginning, middle and end of production (data not shown). The tryptic peptide maps, using UV detection, demonstrated that the rhFVIII produced throughout the production campaign was reproducible, indicative of process consistency.

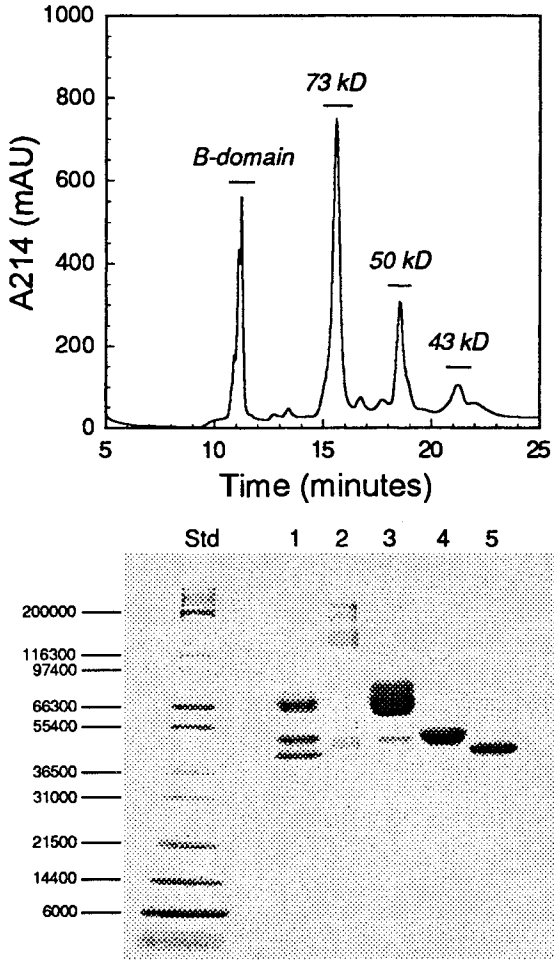

Fig. 1. Isolation of rhFVIIIa polypeptides. Elution profile and fraction collection (indicated by solid horizontal bars) by RP-HPLC (A). Analysis of fractions by SDS-PAGE (B). Lanes contained the following samples: Std—molecular weight standards (Mark 12, Novex); 1—rhFVIIIa; 2—B-domain; 3—73 kD; 4—50 kD; 5—43 kD.

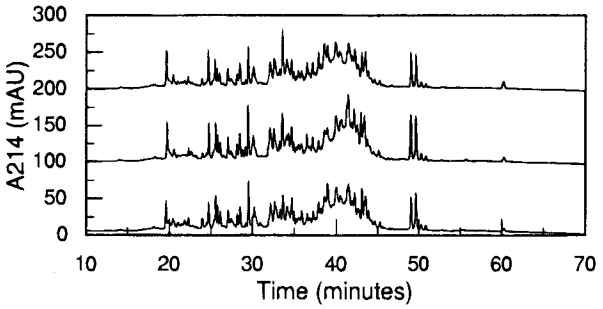

Fig. 2. RP-HPLC elution profile of B-domain tryptic digest of rhFVIII from beginning (bottom trace), middle (middle trace) and end (top trace) of production.

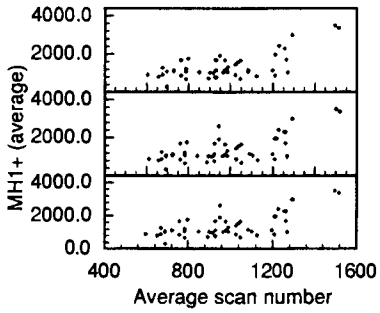

Fig. 4. Peptide ion MH^{1+} profile of B-domain tryptic digest of rhFVIII from beginning (bottom trace), middle (middle trace) and end (top trace) of production.

Analysis of Peptide Mixtures by On-line Mass Spectrometry

Peptide mixtures resolved by RP-HPLC were analyzed by on-line mass spectrometric detection using a triple quadrupole mass analyzer fitted with an electrospray ionization source (21,22). Spectra were acquired continuously, scanning from 1950–200 amu in three seconds. The Base Peak ion traces were complex and unique for each of the four rhFVIIIa polypeptides (data not shown). There was overall similarity between the UV and Base Peak traces, indicating that most UV-absorbing species were ionizable. Base Peak traces were used as guides for the extraction of mass spectra. For those ions of determinate charge, MH^{1+} values were calculated, and ion data were entered in a database programmed to facilitate the interpretation, tracking, manipulation and tabulation of large numbers of peptide ions. The resulting datasets for tryptic peptide maps of the B-domain, 73 kD, 50 kD and 43 kD polypeptides of rhFVIII produced from the beginning, middle and end of production were sorted and compared for coincident detection of peptide ions in all three production samples.

Base Peak elution profiles for the peptide maps of the B-domain, 73 kD, 50 kD and 43 kD polypeptides were highly similar for comparisons of rhFVIII produced from the beginning, middle and end of production. In light of the complexity of the protein as well as the methods used for this analysis, the consistency of both the UV elution profiles and the total ion chromatograms is indicative of a high degree of manufacturing process consistency. These observations indicated an absence of gross genetic instability at the protein level.

Comparison of On-line LC/MS Data for Peptide Mixtures from the Beginning, Middle and End of Production

For each rhFVIIIa polypeptide, only those peptide ions with determinate charge were evaluated further by comparing MH^{1+} and retention time for each stage in production (i.e. beginning, middle and end). Thus, 918 ions were analyzed for tryptic peptide maps of the B-domain, 73 kD, 50 kD and 43 kD polypeptides of rhFVIIIa produced from the beginning, middle and end of a typical production campaign. These groups of ions were sorted by scan number, since the comparative identification of a given peptide ion in the three peptide maps should be matched by MH^{1+} value and retention time. The ion data for the B-domain and 73 kD polypeptide are depicted in Figures 4 and 5, respectively.

For each of the four polypeptides of rhFVIIIa, no unique MH^{1+} ions were observed in the tryptic peptide LC/MS data, when comparing protein produced from the beginning, middle and end of a typical production campaign. Moreover, the ions were observed at similar relative retention times, as indicated by the similar scan numbers for the three lots (Figs. 4–5). Therefore, the rhFVIII produced from the beginning, middle and end of a typical production campaign was considered to be highly similar, indicative of genetic stability in the manufacturing process.

DISCUSSION

Preparation of rhFVIIIa Polypeptides

Fractions of rhFVIII pooled after Q-Sepharose chromatography were incubated with human thrombin. Under physiologi-

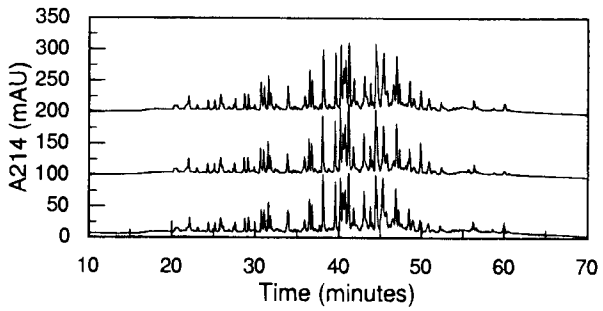

Fig. 3. RP-HPLC elution profile of 73 kD tryptic digest of rhFVIII from beginning (bottom trace), middle (middle trace) and end (top trace) of production.

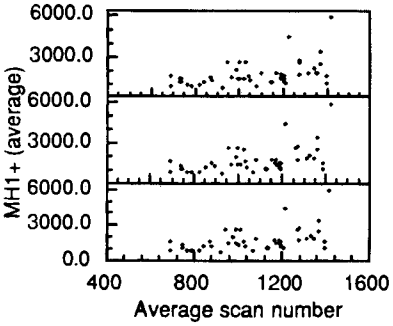

Fig. 5. Peptide ion MH^{1+} profile of 73 kD tryptic digest of rhFVIII from beginning (bottom trace), middle (middle trace) and end (top trace) of production.

cal conditions, thrombin activates factor VIII to the active heterotrimer through three specific proteolytic cleavages (5). The generation of four rhFVIIIa polypeptides prior to complete proteolysis was necessary for the evaluation of genetic stability because of the size and complexity of the intact protein. The principal advantage of fragmentation of rhFVIII with thrombin is that it effects quantitative cleavage at three sites (5,7). While the rhFVIIIa polypeptides each exhibit a degree a heterogeneity (Fig. 1B), they arise through proteolysis at three sites with high specificity. A measure of the specificity of thrombin is the integrity of the polypeptides' N-termini: under the conditions described above, each of the rhFVIIIa polypeptides isolated by RP-HPLC yielded essentially a single N-terminal sequence (data not shown).

We sought to isolate each rhFVIIIa polypeptide to a high degree of homogeneity, since cross-contamination would potentially lead to artifactual results and complicate the evaluation of genetic stability. Ion-exchange chromatography was an ineffective method due to polypeptide interactions (data not shown). Upon evaluation of a number of reversed-phase immobilized phases, we found the isolation of rhFVIIIa polypeptides was accomplished optimally with a PrimeSphere C4 column. The electrophoretic bands observed for each rhFVIIIa polypeptide were consistent with previous reports (6,7). We also observed that RP-HPLC isolation of rhFVIIIa polypeptides without prior anion-exchange chromatography was ineffective in the complete removal of buffer components which interefered with mass spectrometry.

Analysis of rhFVIIIa Peptide Maps

The experimental design for the evaluation of genetic stability of recombinant human factor VIII was influenced in several ways by the size and complexity of the protein. Factor VIII is extensively modified post-translationally, and comprises a heterodimer of 2331 amino acids in the processed form produced by Chinese hamster ovary cells (5,11,13). A partial reduction of complexity was achieved through separate proteolyses of the four rhFVIIIa polypeptide substrates. However, particular attention was paid to the consistency of isolation of the rhFVIIIa polypeptides, so as to minimize the introduction of potential artifacts. In this study, proteolytic methodologies were developed to allow for efficient production of peptide mixtures of rhFVIIIa polypeptides without prior chemical modification, again so as to minimize sample manipulation. Peptide maps were produced from rhFVIIIa polypeptides in the presence of 2 M urea, which effected substrate denaturation and allowed for efficient proteolysis. In order to minimize the introduction of potential artifacts attributable to sample handling or instrumentation, proteolytic digestions of rhFVIIIa polypeptides isolated from rhFVIII from the beginning, middle and end of production were carried out concurrently, as were the RP-HPLC and on-line LCMS analyses.

The RP-HPLC elution profiles for tryptic peptide maps of the four rhFVIIIa polypeptides were unique and exhibited complexities generally consistent with expectations (Figs. 2–3). In addition, for each of the four rhFVIIIa polypeptides, peptide maps were highly similar for rhFVIII from the beginning, middle and end of production, indicative of the absence of gross genetic instability in the production of this therapeutic protein.

A number of investigators have reported the use of peptide mapping in the evaluation of production consistency of recombinant DNA-derived biologicals (19,25,26,28), and variants generated by changes in cell culture conditions, chemical degradation or stress conditions have been detected with this technique (19,26,31,32). Researchers have attempted to identify and control numerous experimental parameters which can influence the consistency of peptide map elution profiles (23,26). However, factor VIII differs in several important respects from the proteins described in these published reports, both in size (i.e. 2331 amino acids) and complexity (i.e. proteolytic processing, numerous post-translational modifications). Moreover, on-line LCMS analysis of peptide maps of rhFVIIIa polypeptides has indicated that a large number of RP-HPLC peaks contain multiple peptides (Figs. 4–5). Therefore, while the consistent UV elution profiles for peptide maps of rhFVIII from various stages in production provided a general indication of production consistency, on-line mass spectrometry allowed for a more rigorous evaluation of genetic stability.

Analysis of Peptide Mixtures by On-line Mass Spectrometry

Continuous monitoring of the RP-HPLC column eluents by both ultraviolet and mass spectrometric detection was accomplished by on-line LCMS. The Base Peak ion and ultraviolet detector traces exhibited overall similarity, as would be expected for a mixture of UV-absorbing, ionizable components. Whereas the UV intensity of a given peak reflected the combination of concentration and molar extinction coefficient for its constituent peptides, its ion detector signal was a function of several factors: abundance, mass:charge ratio of an observed ion, mass analyzer scan range and ionization potential.

Interpretation of Mass Spectrometric Data

The degree of charging of a peptide ion by positive electrospray ionization has been discussed in the literature (22), and is generally believed to be correlated with its content of basic amino acid residues, though instrument acquisition parameters may influence the relative abundance of the different charge forms. The scan range of the mass analyzer places limitations on the ability both to detect all peptide ions and interpret their charge states. Moreover, we have observed that the relative abundances of different charge states of a given peptide may change substantially, even when similar peptide mixtures were analyzed consecutively on a single instrument (data not shown). Therefore, for the purposes of this study where consistency was paramount, only those ions for which charge could be assigned unambiguously were considered for comparative analysis.

Comparison of On-line LCMS Data for Peptide Mixtures from the Beginning, Middle and End of Production

The MH^{1+} ion data acquired for peptide maps of rhFVIIIa polypeptides were compared as a function of scan number. The plots were highly unique for each rhFVIIIa polypeptide, and provided two dimensions of identity for each peptide: namely, its protonated mass and its retention time, for which both properties are highly dependent on the peptide's composition and sequence (Figs. 4–5). Other investigators have characterized

complex peptide mixtures using two-dimensional plots of m/z vs. scan number (24,33). These contour plots appear very complex because there is no correction for baseline threshold, leading to spurious background signals, and because multiple charge states were observed for many peptides. In contrast, the plots shown in Figures 4 and 5 appear simpler because only interpreted data is displayed, and only MH^{1+} is plotted.

For comparisons of the four rhFVIIIa polypeptides derived from three stages in production, the two-dimensional plots of MH^{1+} vs. scan number were highly consistent, both in protonated mass and retention time (Figs. 4–5). This observed conformity applied equally to the simplest (data not shown) and most complex plots (Fig. 4), the latter being of particular note because of the heterogeneity and extent of post-translational modification intrinsic to the B-domain.

Considerations in the Detection of Protein Variation by Peptide Mapping and LCIMS

A number of previous reports have described the application of peptide mapping with UV detection for the evaluation of product consistency (18,19,31,34). One study described the construction of a single arginine-glutamic acid mutant substitution in tissue plasminogen activator; the mutant and wild-type proteins were admixed in several stoichiometries, digested with trypsin, and analyzed by RP-HPLC (19). The UV elution profiles indicated the detection of the mutant peptide at ≈5% abundance (19), though this amino acid substitution, chosen for its predicted effect on tryptic digestion (i.e. loss of a trypsin digestion site), cannot be viewed as equally representative of all amino acid substitutions. In addition, other unrelated peak differences in these chromatograms were not addressed, and our extensive evaluation of digestion conditions of factor VIII has also indicated that proteolytic artifacts can give rise to minor differences in elution profiles (data not shown), suggesting such observations are somewhat typical of the experimental method. Consistent with our observations, the mass spectrometric analysis of a monoclonal antibody has been reported previously (35), and indicated that variants present in ≈10% abundance could be detected. Notwithstanding limitations in the ability to address this question unambiguously, we believe that an anomalous ion signal could be detected at similar abundance by on-line LCMS, and that the recurrent detection of ions for material produced at the beginning, middle and end of production are consistent with the absence of such variation.

CONCLUSIONS

Peptide mixtures of the B-domain, 73 kD, 50 kD and 43 kD polypeptides, isolated from rhFVIII produced from the beginning, middle and end of a typical production campaign, were generated under highly reproducible conditions. These peptide mixtures were analyzed by RP-HPLC, with both ultraviolet detection and continuous on-line mass spectrometry with electrospray ionization. The rhFVIII produced from the beginning, middle and end of a production campaign shared the same rhFVIIIa tryptic peptide map elution profiles, evidence that the peptide map preparation and rhFVIII were highly reproducible.

For each of the rhFVIIIa polypeptides, the peptide map total ion chromatograms for rhFVIII produced from the beginning, middle and end of a typical production campaign were compared, and found to be highly similar. For peptide mixtures of rhFVIII produced from the beginning, middle and end of a typical production campaign, mass spectra for the LCMS data of these three samples were analyzed and interpreted; ion charges were assigned, and MH^{1+} ion data were entered in a database. Lists of ions were sorted according to scan number, and then compared for the coincident observation of the ions in all three samples. For comparisons of protein derived from three stages in production, the ions detected were observed consistently for the B-domain, 73 kD, 50 kD and 43 kD polypeptides of rhFVIII. Moreover, the ions were observed at the same relative retention times, as indicated by the similar scan numbers for the three lots (Figs. 4–5). Therefore, the rhFVIII produced from the beginning, middle and end of a typical production campaign can be considered to be highly similar, and the data support the demonstration of genetic stability in the manufacturing process of recombinant anti-hemophilic factor, the active drug substance in Recombinate™.

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