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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 425–432
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Characterization of recombinant human granulocyte colony stimulating factor (rHuG-CSF) by capillary zone electrophoresis, capillary isoelectric focusing electrophoresis and electrospray ionization mass spectrometry

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Received 6 November 2003; received in revised form 4 February 2004; accepted 6 February 2004

Available online 19 March 2004

Abstract

Recombinant human granulocyte colony-stimulating factor (rHuG-CSF) is a hematopietic cytokine that stimulates and regulates the proliferation and differentiation of neutrophils. Glycosylated and non-glycosylated forms of rHuG-CSF cannot be distinguished by traditional biological assays. In addition, it is very difficult to characterize impurities of the same molecular weight in biologicals. In this study, non-glycosylated rHuG-CSF, two glycosylated rHuG-CSF isoforms and their commercial dosages were successfully separated by capillary zone electrophoresis (CZE) using 50 mM Tricine containing 20 mM NaCl and 2.5 mM 1,4-diaminobutane (DAB) at pH 8.0, which could be employed for the qualitative discrimination assay of rHuG-CSF related products. CZE, capillary isoelectric focusing electrophoresis (CIEF), and mass spectrometry (MS) were used to effectively characterize non-glycosylated rHuG-CSF. It was found that proteins in the samples with different pIs in the CIEF profile could not be detected by CZE, while no difference was observed between these proteins and rHuG-CSF. Further analysis by electrospray ionization mass spectrometry with the resolution of 2000 showed that the components with different pIs in the non-glycosylated rHuG-CSF bulk sample are nearly equal in molecular weight. Therefore, it is necessary to combine several modern analytical techniques for quality control to get well-characterized biologicals.

Keywords: Recombinant human granulocyte colony-stimulating factor; Capillary zone electrophoresis; Capillary isoelectric focusing electrophoresis; Electrospray ionization mass spectrometry; Glycosylated forms

1. Introduction

With the development of techniques in gene cloning, the DNA-derived drugs represent a new therapeutic

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doi:10.1016/j.jpba.2004.02.003

concept for human diseases. Recombinant human granulocyte colony-stimulating factor (rHuG-CSF) is one in the family of hematopoietic growth factors which regulates the proliferation and differentiation of cells of neutrophil lineage [1,2]. Undergoing chemotherapy, rHuG-CSF is now extensively employed to treat neutropenia in cancer patients [3]. There are two kinds of rHuG-CSFs with different expression vectors. One is non-glycosylated protein expressed from engineered *Escherichia coli* cells which contains an extra methionine at its N-terminus. The other is glycosylated protein derived from Chinese hamster ovary (CHO) cells or other mammalian cells which contains an O-linked carbohydrate chain attached to Thr133. Both non-glycosylated and glycosylated forms of rHuG-CSFs are commercially available with the same clinical functions. The only difference between these two forms is that the glycosylated form is more stable in vitro than the non-glycosylated form.

Traditionally, the bioactivity of rHuG-CSF was determined by a growth factor-dependent cell line assay using GNFS-60 [4] and a myeloid bone marrow colony assay [5]. However, both assays give the total bioactivities, which are insufficient to determine the identity and the purity of rHuG-CSF in the dosage forms. For human-use drug, the purity, potency, safety, and efficacy must be well controlled and assessed in order to assure a high quality product. However, unlike traditional synthetic pharmaceuticals, common prescription and over-the-counter drugs, recombinant DNA-derived pharmaceutical proteins are very difficult to be well-characterized by ordinary analytical techniques used for small chemical molecules. Consequently, various advanced analytical techniques have been developed to characterize the DNA-derived proteins, including capillary electrophoresis (CE), mass spectroscopy (MS) such as matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI), as well as their combination. The detection and evaluation of the purity and the heterogeneity of recombinant proteins have been widely reported [6–9]. The advances in analytical instrumentations have been changing the way of quality control in biotechnological companies [10].

Various methods such as HPLC, CE, and MS have been applied to identify the glycoforms of rHuG-CSF and to analyze the peptide mapping of rHuG-CSF [11–14]. However, most of them use only one or two techniques to detect a high quality sample. Few reports were related to the inconsistent results of the same sample detected by different techniques. Recently, we developed a qualitative method to rapidly and inexpensively characterize glycosylated and non-glycosylated rHuG-CSFs. This method is also effective to control the purity and identity of non-glycosylated rHuG-CSFs in both bulk samples and preparations. To separate two isoforms of glycosylated rHuG-CSF with high resolution, CZE was optimized using different running buffer systems. Capillary isoelectric focusing electrophoresis (CIEF) and electrospray ionization mass spectrometry (ESI–MS) were used to evaluate the purity of non-glycosylated rHuG-CSF. The combination of different techniques, CZE, CIEF, and MS, presented in this study allows the quality control of the rHuG-CSF products with high accuracy.

2. Experiments

2.1. Materials

Non-glycosylated rHuG-CSF (0.5 mg/ml) expressed in *E. coli* was prepared in our laboratory. The preparations of non-glycosylated and glycosylated rHuG-CSFs were commercially available GRANOCYTE® injection (150 μg) and GRAN300® injection (300 μg), respectively. Tricine, NaCl, 1,4-diaminobutane (DAB), ribonuclease A (pI = 9.45), carbonic anhydrase II (pI = 5.90), β-lactoglobulin A (pI = 5.10), and CCK peptide fragment (pI = 2.75) were purchased from Sigma (St. Louis, MO, USA). CIEF gel buffer and Pharmalyte (pH 2.75–10.00) were purchased from Beckman. All buffers were prepared in ultra-pure water made by Mili-Q Water System (Millipore, Bedford, MA, USA).

2.2. Capillary zone electrophoresis (CZE)

The CZE was performed on a Waters Quanta 4000E capillary electrophoretic system with BASELINE 810 software. All determinations were carried out in 50 μm i.d. uncoated fused-silica capillaries (Supelco, Bellefonte, PA, USA) with an overall length of 50 cm and an effective separation length of 42.5 cm. Analytes were monitored using UV detection at 214 nm room temperature. Between each analysis, the capillary was

rinsed with 0.1 M NaOH solution for 2 min, followed by the rinse with water for 2 min and running buffer for 5 min. The running electrolyte was a solution of 50 mM Tricine, 20 mM NaCl, 2.5 mM DAB, at pH 8.0 adjusted by 0.1 M HCl or 0.1 M NaOH. The measurements were performed at an applied voltage of −20 kV. The power supply polarity was reversed with the anodic site at the detector.

2.3. Capillary isoelectric focusing electrophoresis (CIEF)

The CIEF was carried out on a P/ACE 5000 capillary electrophoresic system (Beckman, Palo Alto, CA, USA) with Gold Software 8.1 by using neutrally coated capillaries with a total length of 27 cm (20 cm from the window to inlet) × 75 μm i.d. (Beckman). Ribonuclease A (pI = 9.45), carbonic anhydrase II (pI = 5.90), β-lactoglobulin A (pI = 5.10), and CCK peptide fragment (pI = 2.75) were used as pI determination markers. Anode electrolyte was CIEF gel solution containing 91 mM H_3PO_4 and 2% (v/v) of Pharmalyte (pH 2.75–10). Twenty millimolar NaOH solution was employed as the cathode electrolyte and 10 mM H_3PO_4 was used for rinsing. The samples containing approximately 200 μg of protein were desalted and concentrated to 100 μl using Amicon microcentrifuge tubes with the molecular weight cut-off of 10 kDa. The pI markers and samples were added into CIEF gel solutions containing Pharmalyte before determination. Electrophoretic separation was performed at a constant voltage of 13.5 kV for 2 min and monitored at 280 nm. The capillary was rinsed with pure water for 1 min before filling with sample solution. The focused strips were then rinsed through the detection window by high pressure under 13.5 kV.

2.4. Electrospray ionization mass spectrometry (ESI–MS)

The MS analysis was performed on a single quadrupole mass spectrometer SSQ-710 (Finnigan-MAT, San Jose, CA, USA) equipped with a Finnigan atmospheric pressure ionization source operated in the electrospray mode. Constant voltage of +4.5 kV and temperature of 200 °C were applied. The effective mass range was from m/z = 500 to 2000 in normal scan mode at a rate of 3 s per scan. Ion optic settings and sheath gas pressure were optimized on the day of the analysis. Samples were desalted by Amicon microcentrifuge tubes, lyophilized to powder, reconstituted in 0.1% TFA in 50% (v/v) of aqueous methanol, and introduced into the system by a flow injection with the solution of methanol–water–acetic acid (50:49:1, v/v/v) at a rate of 0.2 ml/min.

3. Results and discussion

3.1. Separation by capillary zone electrophoresis

Capillary zone electrophoresis has been introduced as an efficient technique for the separation of DNA-derived proteins [15]. But the protein adsorption is the main problem for this method. The charge on the capillary inner wall is negative, so it will absorb components with a positive charge. Usually, positively charged buffer additives are adsorbed resulting in a positive charge on the capillary inner wall. The pI of rHuG-CSF is about 6. If the pH of the running buffer is above 6, the protein adsorption by the capillary wall can be prevented due to the negative charge in rHuG-CSF. In this study, 50 mM Tricine (pH 8.0) was used as a running buffer. Unlike non-glycosylated rHuG-CSF, glycosylated rHuG-CSF is a heterogeneous product. Moreover, its dosage form contains large amounts of human serum albumin (HSA). Therefore, the preparation of glycosylated rHuG-CSF, GRANOCYTE® injection, is suitable to be used for optimizing the separation conditions. The CZE electropherograms of GRANOCYTE® injection in different buffers are shown in Fig. 1. It was indicated that even though it was difficult to separate the glycosylated rHuG-CSF in the Tricine buffer only, the separation could be greatly improved by the addition of NaCl or a mixture of NaCl and DAB into the running buffer. The peak's drag in Fig. 1(a) showed that the adsorption of rHuG-CSF on the capillary wall was serious even if the separation was carried out in the buffer with the pH above its pI. This may be caused by the locally positive polarity on the surface of rHuG-CSF, although the net charge of the whole protein was negative. The adsorption was significantly reduced by the addition of NaCl into the buffer because high concentration of NaCl greatly increased the ion strength of the buffer. However, two glycosylated

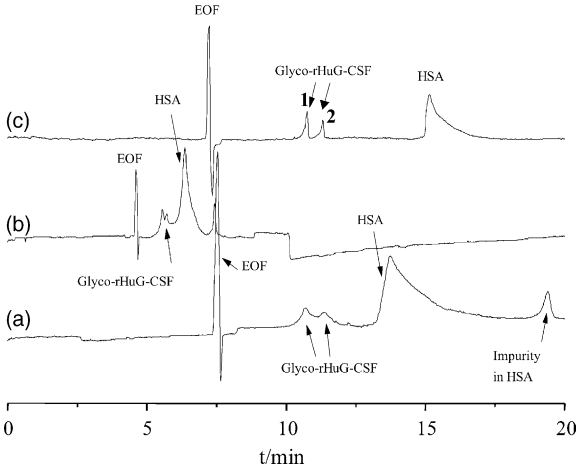

Fig. 1. The separation of GRANOCYTE® injections in different buffers: (a) 50 mM Tricine, pH 8.0; (b) 50 mM Tricine containing 20 mM NaCl, pH 8.0; (c) 50 mM Tricine containing 20 mM NaCl and 2.5 mM DAB, pH 8.0. Separation conditions: uncoated fused-silica capillary 42.5 cm × 50 μm i.d. at 20 kV, 214 nm, and room temperature.

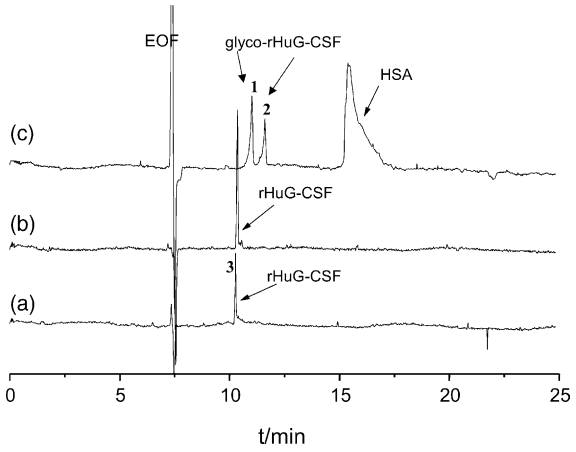

Fig. 2. Capillary zone electropherograms of rHuG-CSF separated under the conditions of Fig. 1. Samples were non-glycosylated rHuG-CSF bulk sample (a), GRAN300® injections (b), and GRANOCYTE® injections (c), respectively. Peak 1: glycosylated rHuG-CSF containing one acetylneuraminic acid. Peak 2: glycosylated rHuG-CSF containing two acetylneuraminic acids. Peak 3: non-glycosylated rHuG-CSF.

species of rHuG-CSFs were not completely resolved (Fig. 1(b)). With the addition of DAB, an additive that can decrease the charges on the capillary surface and amplify the differences in electrophoretic migration, the resolution of separation was significantly improved as shown in Fig. 1(c). Therefore, 50 mM Tricine containing 20 mM NaCl and 2.5 mM DAB, pH 8.0, was used as a routine buffer for the next step of separation.

The separation of a non-glycosylated rHuG-CSF and a glycosylated rHuG-CSF was performed in the buffer described above. As shown in Fig. 2, the glycosylated rHuG-CSF eluted as a double peak compared to the non-glycosylated rHuG-CSF that eluted as a single, sharp peak. This results from the neuraminic acid residue linked with oligosaccharides in the glycosylated rHuG-CSF. The structure of the glycosylated part was identified as NeuAcα2 → 3Galβ1 → 3(±NeuAcα2 → 6)GalNAcol [NeuAc = *N*-acetylneuraminic acid, Gal = galactose, GalNA col = *N*-acetylgalactosamine (reduced)]. So there are two kinds of carbohydrates present in the glycosylated rHuG-CSF. It was found that these two carbohydrates were equal in quantity, differing only in number and the linkage of the sialic acid residues [16]. Acetylneuraminic acids present in the glycosylated rHuG-CSF are negatively charged in the running buffer, which move to the opposite direction from the detector under the separation voltage. Since the velocity of the electro-osmotic flow is higher than the migration velocity of the charged species, the net flow of the negatively charged glycosylated rHuG-CSF is towards the detector. Consequently, glycosylated rHuG-CSF with one acetylneuraminic acid will migrate faster than those with two acetylneuraminic acids. As a result, Peak 1 in Figs. 1 and 2 is assigned to the glycosylated rHuG-CSF containing one acetylneuraminic acid and Peak 2 to that containing two acetylneuraminic acids. To verify peak assignments, all the components in glycosylated rHuG-CSF preparation, except rHuG-CSF, were mixed and analyzed at the same condition, and no peak was observed at the positions. We can also compare the electrophoretic behavior of non-glycosylated rHuG-CSF for the further verification of peak assignments. In the case of non-glycosylated rHuG-CSF (without acetylneuraminic acid) produced in *E. coli* cells, it can be expected that the peak in the CZE separation profile will appear earlier than Peaks 1 and 2. This was validated by analyzing a non-glycosylated rHuG-CSF bulk sample (Peak 3 in Fig. 2). These results indicate that the acetylneuraminic acid in the glycoform greatly affects the mobility of the protein,

and can be used to characterize and to identify proteins with or without carbohydrates.

Although there is no standard available to verify Peak 2, we believe it is scientifically rigorous to assign Peak 2 to the glycosylated rHuG-CSF containing two acetylneuraminic acids. Usually, the glycosylation of a given protein will not change when a production line is fixed. rHuG-CSF produced by CHO cell line is identified to contain two O-linked carbohydrate moieties differing only in number (one and two) of sialic acid residues. Since GRANOCYTE® injection is a commercialized biologic for human therapeutic use, it is reasonable to believe that the main component, glycosylated rHuG-CSF, is highly purified and is well characterized. Therefore, we believe that the glycosylated rHuG-CSF in GRANOCYTE® injections used in this study contained two O-linked carbohydrate moieties, and there was no possibility that more than two acetylneuramic acids were attached to rHuG-CSF.

It is a great interest to characterize a rHuG-CSF in its preparation. As there are large amounts of other components in a dosage form, for example, human serum albumin, that keeps the bioactivity of the rHuG-CSF, it is very hard to develop an effective method for the quality control. We used the CZE conditions described above to analyze glycosylated rHuG-CSF preparation, GRANOCYTE® injection, and non-glycosylated rHuG-CSF preparation, GRAN300® injection. The results in Fig. 2 show that GRAN300® injection gave the same single peak (Fig. 2(a)) as its bulk sample (Fig. 2(b)), because the additives in the non-glycosylated rHuG-CSF have no absorption at 214 nm. However, there is a large peak in the CE profile of GRANOCYTE® injection, which is caused by the carrier protein HSA. The big difference of the migration time between HSA, non-glycosylated rHuG-CSF, and two glycosylated isoforms of rHuG-CSFs would not affect the characterization of rHuG-CSF species in the preparations.

Furthermore, as shown in Figs. 1 and 2, Peak 2 is lower than Peak 1, suggesting that the contents of two rHuG-CSF glycoforms might be different in their injections. However, it was reported that these two carbohydrates should be in equal amounts [16]. It seems possible that some of the glycosylated rHuG-CSF molecules with double acetylneuraminic acid residues in GRANOCYTE® injection lost one acetylneuraminic acid during the storage, the sample preparation or other processes. But no peak in the position of Peak 3 was observed in Fig. 2(c), supporting that the glycosylated rHuG-CSF with only one acetylneuraminic acid might be stable. So it was suggested to employ the ratio of two rHuG-CSF glycoforms and the peak intensity at the position of Peak 3 as one of the indexes for quality control between batches and between different storage conditions.

In addition to developing a capillary electrophoretic separation of glycosylated and non-glycosylated rHuG-CSF, quantifying both types of rHuG-CSF in either bulk products or preparations could be achieved by using highly purified rHuG-CSF as a reference.

Based on the CZE detection, all three samples were very pure. However, this did not mean the identity and purity of these samples were perfect, as CZE separation is based only on one of the chemical properties of each protein. Therefore, CIEF was employed for further characterization of the non-glycosylated rHuG-CSF.

3.2. Separation by capillary isoelectric focusing electrophoresis (CIEF)

CIEF is an important technique for analyzing the charged variants of recombinant proteins based on pI differences. Compared with the traditional technique, CIEF characterized by isoelectric focusing in slab gel with visualization in discrete bands is much easier and more accurate [17]. In this study, CIEF was used to determine the identity and purity of the non-glycosylated rHuG-CSF by its apparent pI. Four components with different pIs, ribonuclease A (pI = 9.45), carbonic anhydrase II (pI = 5.90), β-lactoglobulin A (pI = 5.10), and CCK peptide fragment (pI = 2.75), were employed as standard markers. The CIEF profile of the pI markers showed that the retention time of each protein is proportional to its pI, which indicated that no electro-osmotic flow existed in the capillary. Therefore, capillary could be used for further test.

The same samples as those in Fig. 2 were analyzed by CIEF assay. Because salt in the samples may affect pI determination, it is necessary to desalt the samples before CIEF determination. To obtain the accurate pI of an unknown sample, ribonuclease A (pI = 9.45) and CCK peptide fragment (pI = 2.75) were added into the sample as the running markers. Fig. 3 shows the CIEF electropherogram of a non-glycosylated

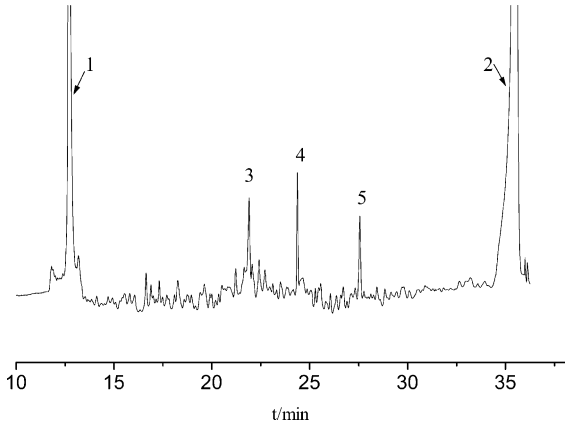

Fig. 3. Capillary isoelectric focusing electropherogram of the non-glycosylated rHuG-CSF bulk sample. The experiment was carried out on P/ACE 5000 CE system. Separation conditions: neutral coated capillary 20 cm × 75 μm i.d. Peak identification: Peak 1: ribonuclease A (pI = 9.45). Peak 2: CCK peptide fragment (pI = 2.75). Peaks 3, 4, and 5: non-glycosylated rHuG-CSF bulk sample.

rHuG-CSF bulk sample. It was found that the sample contains three ingredients with pIs of 6.75, 6.02, and 5.09, respectively. To verify the possibility that the degradation of the rHuG-CSF occurred during the CIEF analysis, GRAN300 injection was analyzed at the same CIEF conditions. As shown in Fig. 4, only

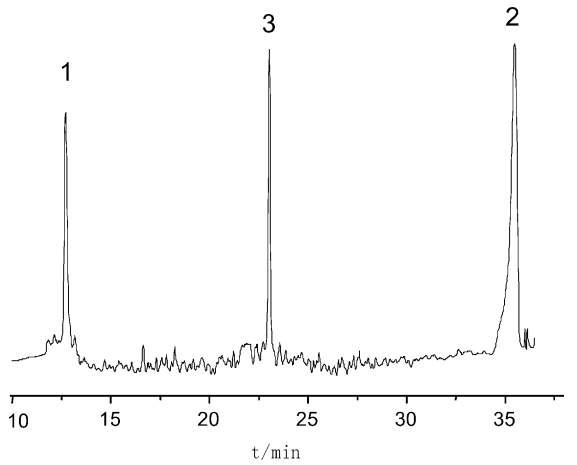

Fig. 4. Capillary isoelectric focusing electropherogram of GRAN300 under the same conditions of Fig. 3 except that the concentrations of marker ribonuclease A and CCK peptide fragment were decreased by half. Peaks 1: ribonuclease A (pI = 9.45). Peak 2: CCK peptide fragment (pI = 2.75). Peak 3: GRAN300® injections (non-glycosylated rHuG-CSF preparation).

one ingredient with the apparent pI of 6.10 was observed, and interestingly, the protein in GRAN300 had a different pI compared to the three molecules in the bulk product as shown in Fig. 3. Different pIs in CIEF imply the changes of protein structure, including the changes of amino acid sequence, post modification, and spatial structure. To know the details of the three peaks in Fig. 3, a further analysis by electrospray ionization mass spectrometry is required. Evidently, it is problematic for the *E. coli*-derived rHuG-CSF bulk sample described above to be a human-use drug. However, the rHuG-CSF bulk sample, indeed, showed bioactivity in in vitro assay. Therefore, if we only use bioassay and CZE for quality control, it is impossible to discriminate the difference between these two samples. Consequently, analytical methods are complementary to each other, and it is better to use different approaches to control the quality of the same sample.

3.3. Analysis by electrospray ionization mass spectrometry

ESI–MS is an effective technique to determine the molecular weight of proteins precisely. Modifications and changes of amino acid species in a protein can be deduced from the accurate molecular weight. In order to characterize the identity in the CIEF profile, mass spectrometry was employed. The non-glycosylated rHuG-CSF is composed of 175 amino acids with the theoretical molecular weight of 18798.9 Da. As shown in Fig. 5, the observed molecular weight of the rHuG-CSF bulk sample was 18801 Da, which was very close to the theoretical value. Although no other peaks with different masses are observed in Fig. 5, we still cannot say the bulk sample was pure as the resolution of the MS used in this study was not high enough to show the difference of the molecular weights of the three components in the bulk sample. In the case of non-glycosylated rHuG-CSF, the masses of multiple charged ions are less than 2000 Da. For example, the mass of a rHuG-CSF ion charged with 15 protons is 1256.5 Da. Because the resolution of 2000 (FWHM, $m/\Delta m$, m: the mass of an ion) for SSQ 710 allows mass selection of two ions differing by less than 0.63 Da (Δm) when the ion mass (m) is 1256.5 Da, it is possible for SSQ 710 to resolve two proteins with the mass difference of 9.4 Da (0.63 × 15). Accordingly, it is unequivocal to identify the increase or

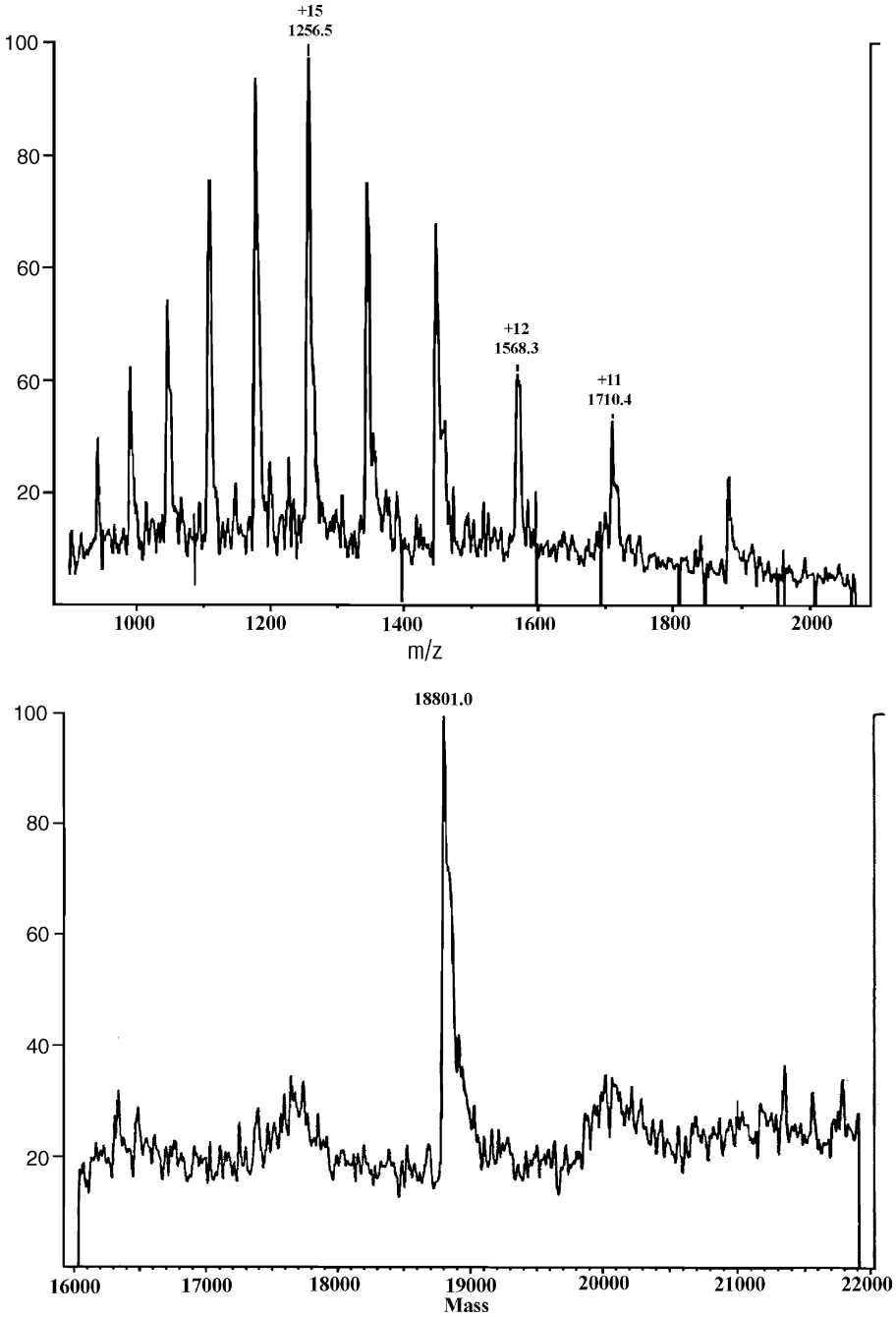

Fig. 5. Mass spectrometry of rHuG-CSF. The top is the electrospray ionization (ESI) mass spectrum and the bottom is its deconvoluted mass spectrum.

decrease of one amino acid in rHuG-CSF. However, a substitution of one amino acid by another one in rHuG-CSF cannot be identified with current resolution if the mass difference of these two amino acids is smaller than 9.4 Da. Therefore, the three components in Fig. 3 are not the results of the insertion or deletion of an amino acid in the rHuG-CSF sequence. As the change of molecular weight by post-protein modification is normally larger than 10 Da, the three peaks in Fig. 3 are not the results of protein modification either. But we still did not get a satisfactory explanation for the three peaks in Fig. 3 by CIEF and MS with the resolution of 2000. For elucidating the details of the three peaks in Fig. 3, on-line direct coupling of CIEF with electrospray ionization–mass spectrometry of high resolution is essential, which has already been used to detect model proteins, human hemoglobin variants, and phosphorylated proteins [18–20].

4. Conclusions

Capillary zone electrophoresis has been successfully used to investigate the purity of the biomolecules in the pharmaceutical industry. Because of the high efficiency of CZE separation, small amount of active protein in dosage forms can be detected and quantified even if a large amount of additives, such as HSA, were present. It has also been shown in this study that CZE is capable of separating glycoforms based on the number of acetylneuraminic acids contained in the carbohydrate structure, and with this information the analysis is much useful to discriminate whether a degradation of glycosylated rHuG-CSF molecules occurred or not. The analysis is very fast with the time of less than 20 min, which is suitable for a routine quality control.

In contrast to CZE, CIEF has the special ability of characterizing the protein heterogeneity. In this study, three ingredients with different pI values were distinguished by CIEF for the “pure” sample by CZE, indicating that CIEF is a necessary purity control tool for biological proteins.

As ESI–MS is a tool for highly accurate mass determination, it can be used to characterize the modification and the identity of a DNA-derived protein. However, it is impossible to identify the impurities caused by the identical molecular weight. Therefore, each analytical tool is complementary to the other, and it is preferable to employ several analytical methods based on different principles for the quality control.

Usually it has been considered difficult to characterize protein molecules analytically, so its production process is locked in place and strictly controlled, which may prevent the progress in drug industries. The present study showed that it is possible to combine several analytical tools to help us understand the potential effects of the changes in a manufacturing process on the end product, by which the evaluation of a remodeled production line may be expedited.

Acknowledgements

We thank National Natural Science Foundation Committee for financial support (No. 692350220).

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