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Quantitative Determination for Nerve Growth Factor Concentration in Commercial Product by SEC－HPLC

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Abstract The commercial product, Mouse Nerve Growth Factor (NGF) for Injection－Nobex®, is consisted of NGF and human albumin. These two components are both proteins and make it difficult to determine the concentration of NGF by conventional methods. In order to establish a new efficiency quality control process for NGF, this work choose Size Exclusion High Performance Liquid Chromatography (SEC-HPLC) to detect stock solutions and products under screened chromatographic conditions: using Shodex KW−802.5 column, at room temperature with the 0.05 M PBS pH=7 buffer as mobile phase, 0.8 mL/min flow rate and detected under 280 nm wavelength. The standard curves with correlation coefficient of 0.999 82 (for stock solutions) and 0.999 97 (for products) were obtained by external standard method. The NGF concentrations tested in HPLC were compared with which in BCA for the same batches, and the RSD values were less than 3%, which corresponded with China Pharmacopoeia (2010). The results show that SEC-HPLC method is sensitive, accurate and reliable for NGF on-line quality control and has the potential for the quantitative determination of other mixed protein preparations.
Keywords Size Exclusion High Performance Liquid Chromatography (SEC-HPLC); NGF; Quantitative Determination

Background

Nerve Growth Factor (NGF), which has the properties of regulating the neuronal differentiation and development, repairing the neuronal damage and regenerating the neurocyte, admittedly, is one of the significant nutritional activity factors for the nervous system [Levi-Montacini R., 1987]. This protein factor consists of 3 subunit compositions (subunit of α, β, and γ), forming a $\alpha_2\beta\gamma_2$ structure with the β subunit as the active group. The β subunit, which has the molecular weight of 26 kD and isoelectric point of 9.3, is a homodimer connected of two 118 amino acids chains by non-covalent bond. The monomer of β subunit is approximately 13.6 kD, and different monomers may have 8 amino acids dissimilarity from the post-transcriptional modification (William et al., 1976).

At present, the nerve growth factor is clinically using for the treatment of nervous system damage as the national first-class new drug (accorded with SFDA, China). It uses human serum albumin and mannitol as excipient by adding to the NGF stock solution in the production process and manufactures three dry powder injection specifications of 18 μg per dose, 20 μg per dose and 30 μg per dose after lyophilization. To the practical production process, the most important quality control aspect lies in monitoring the concentration of NGF in the stock solution and products. Current protein concentration detecting methods, such as Lowry method, Kjeldahl nitrogen determination, BCA method and ELISA method, have various difficult in monitoring NGF, especially that they cannot distinguish the protein excipient of human serum albumin from the target protein of NGF in a quantitative process (in products, the concentration of human serum albumin is much higher than that of NGF). In order to settle these aporias, the method of Size Exclusion High Performance Liquid Chromatography

(SEC-HPLC) was chosen. This method can on-line detect the concentration of NGF in a much higher speed and accuracy, and the most important property is that the detection separation and purification are at the same time. It will largely reduce the cost of qualify control in the practical production process.

1 Results

1.1 Selection of chromatographic conditions

The PBS buffer was chosen as mobile phase for that it is also used as the dissolvent of stock solution. When the PBS at the condition of pH=7.0 and 0.05 mol/L ion concentration, it shows the largest NGF peak area respond and highest column efficiency in the chromatogram (Figure 1 and Figure 2).As a result, the chromatographic conditions are finally selected as follows: Chromatograph: Shimadzu LC−10AT VP Plus HPLC Chromatography; Column: Shodex PROTEIN KW−802.5 No.E703044 Guard

Column: Shodex PROTEIN KW-G No.E712606

Column Temperature: Room Temperature;

Mobile Phase: 0.05 mol/L PBS pH=7 (2.05 g Na_2HPO_4+ 1.29 g NaH_2PO_4•$2H_2O$+4.4 g NaCl)/500 mL

Detecting wavelength: 280 nm

Flow rate: 0.8 mL/min

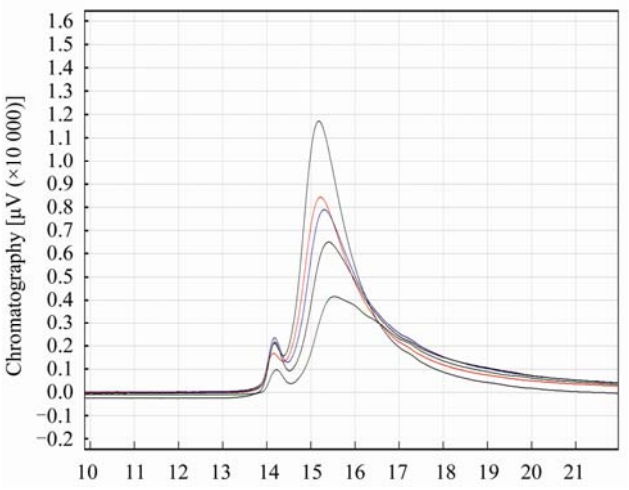

Figure 1 Chromatogram of NGF under different pH condition

Note: The black curve reveals the NGF peak in the pH of 6.96, red curve of pH=6.74; Blue curve of pH=6.53, Brown curve of pH=6.32 and green curve of pH=6.11; All are under the condition of 0.05 mol/L PBS in the speed of 0.8 mL/min

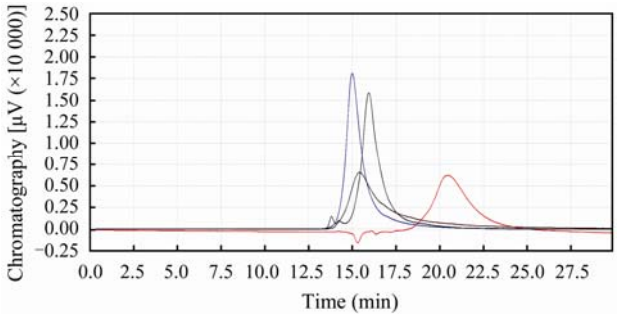

Figure 2 Chromatogram of NGF under different ion concentration condition

Note: The brown curve reveals the NGF peak in the ion concentration of 0.025 mol/L, blue curve of 0.05 mol/L, black curve of 0.10 mol/L and red curve of 0.20 mol/L. All are under the condition of pH=7.0 PBS in the speed of 0.8 mL/min

1.2 Manufacture of the standard curve

1.2.1 Standard curve for stock solution

The standard curve was manufactured using NGF standard by external standard method. After gradient dilution of NGF standard, various samples of various concentrations are detected by SEC-HPLC. We got chromatograms and the data of retention time, peak height, peak area and other parameters in every 40 μL injection. Compiling the data of two parallel experiments in average, the standard curve was got in the linear range of 719 μg/mL to 44.9 μg/mL (has manufactured the standard curve in three different times, and reveals high repeatability that RSD <1.42%) (Figure 3; Figure 4 and Table 1).

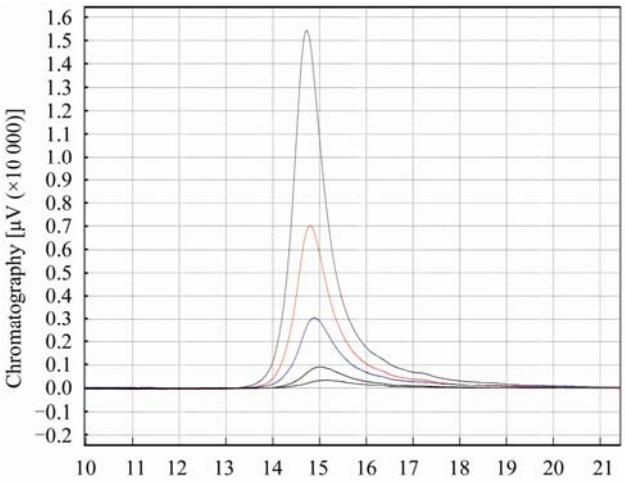

Figure 3 The chromatograms of manufacturing the standard curve for stock solution

Note: Different color peaks reveal the NGF in different gradient dilution concentration

Table 1 Data of the standard curve for stock solution

Peak area	Concentration (μg/mL)				
	719	360	180	89.9	44.9
1st	946 349.5	466 900.1	217 347.9	72 387.9	23 442.9
2nd	1 051 213.0	461 082.5	186 301.4	70 680.6	19 649.2
Average	998 781.3	463 991.3	201 824.7	71 534.3	21 546.1

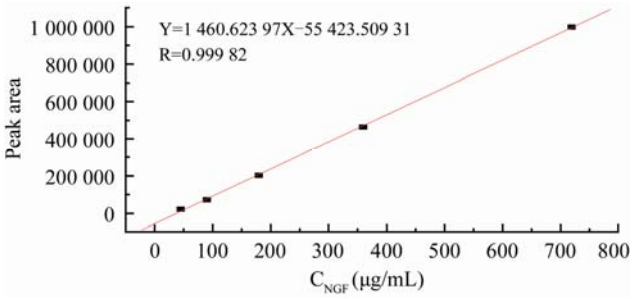

Figure 4 The standard curve for stock solution
Note: The equation reveals the mathematical expression of standard curve and the correlation coefficient of standard curve is 0.999 82

1.2.2 Standard curve for product

Using the external standard method as well, we prepared gradient NGF concentration of products based on the production formula by adding human serum albumin and mannitol into NGF standard (keep the same concentration of human serum albumin 1% V and mannitol 5% V as selling products) to manufacture the standard curve. Detected by SEC-HPLC, we got chromatograms and the data of retention time, peak height, peak area and other parameters in every 90 μL injection. Compiling the data of two parallel experiments in average, the standard curve was got in the linear range of 399.4 μg/mL to 79.9 μg/mL (has manufactured the standard curve in three different times, and reveals high repeatability that RSD <1.42%) (Figure 5; Figure 6; Table 2).

1.3 Detection of NGF stock solution

The NGF stock solution of production batch I010 and H015 were detected by SEC-HPLC based on the chromatographic conditions established above (three parallel experiments in average). The results were calculated through the standard curve and compared with that of BCA method (Table 3). There are tiny differences of results between the two methods, that proving of the reliability of SEC-HPLC quantitative method in detecting NGF stock solution.

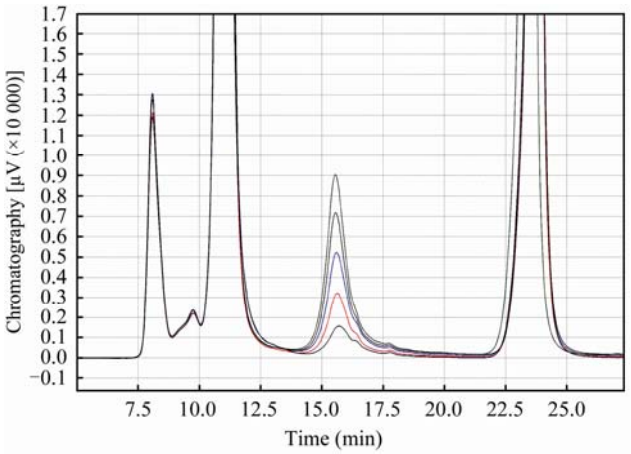

Figure 5 The chromatograms of manufacturing the standard curve for product
Note: The peaks around 15 minutes represent NGF and other peaks for human serum albumin. Different color peaks reveal the NGF in different concentrations

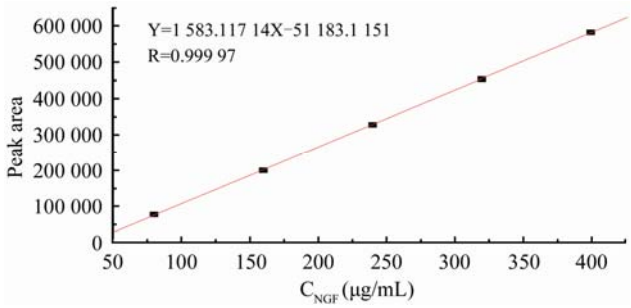

Figure 6 The standard curve for product
Note: The equation reveals the mathematical expression of standard curve and the correlation coefficient of standard curve is 0.999 97

Table 2 Data of the standard curve for product

Peak area	Concentration (μg/mL)				
	79.9	159.8	239.7	319.6	399.4
1st	89 749.5	197 126.6	324 580.6	431 244.6	656 574.1
2nd	64 289.0	202 969.3	331 340.0	476 448.5	508 261.5
Average	77 019.3	200 048.0	327 960.3	453 846.6	582 417.8

Table 3 Compare of the SEC-HPLC results and BCA results

Batch	BCA Method (μg/mL)	SEC-HPLC Method (μg/mL)	RSD (%)
I010	519	539	2.67
H015	462	472	1.51

1.4 Detection of NGF product

A dose of Mouse Nerve Growth Factor (NGF) for Injection - Nobex® (18 μg per dose) was dissolved in 0.2 mL PBS buffer forming the NGF product sample that the calculated theoretical concentration is 90 μg/mL. The sample was also detected by SEC-HPLC based on the chromatographic conditions established above (three parallel experiments in average) and got the result of 91.2 μg/mL. The two results are nearly the same with the RSD of 0.93%, that proving of the reliability of SEC-HPLC quantitative method in detecting NGF product.

2 Discussion

The method of using HPLC to separate and determine protein has been proposed from the 1970s. And in recent years, its quantitative measurement is getting more and more attention by researchers. However, there is almost RP-HPLC method seen in the literatures. Practically, in the production process of NGF, the quality control method without degenerating the protein and bring in impurities is needed. We focus on the SEC-HPLC because of its moderate condition and the potential of on-line detecting.

As a routine monitoring method which requires simple, speedy and accuracy, SEC-HPLC also provide the same environment as the stock solution, which will prevent the difficulty of changing dissolvant and break in the continuous production process. Compared to BCA method that must formulate standard curve for every detecting, HPLC method can achieve the advantage of on-line monitoring, which means you don't need to break in the production process but detecting the concentration while the solution flow is processing. Meanwhile, in the product quality control, once of the HPLC detection can separate human serum albumin and NGF as well as directly calculated the concentration from the peak area of NGF, showing the convenient and speedy of this method. This work has set up the technical standards of SEC-HPLC quantitative determination for NGF with the actual test results corresponding with China Pharmacopoeia. It's believed that the SEC-HPLC quantitative determination method will become the dominant quality control process in the future. It will assuredly save cost and raise the accuracy in the production process of NGF.

3 Materials and Methods

3.1 Materials

This work uses the NGF stock solution and products (Mouse Nerve Growth Factor for Injection - Nobex®, Xiamen Bioway Biotech Co. Ltd., China) as subjects. The NGF stock solution is from the production batch of I010, H015. And the injection specification is 18 μg NGF per dose with human serum albumin (20% Human Serum Albumin, Octapharma Pharmazeutika Produktionsges m.b.H) 1%V/mL and mannitol 5%V/mL, after lyophilized into dry powder formulations.

3.2 Standard

NGF standard was purified from NGF stock solution by Sephacryl S-100 column in PBS buffer. The purity of standard is 99.1% after corroborated by HPLC (Figure 7) and MS (Figure 8), and the result is corresponded with China Pharmacopoeia (2010).And the concentration of NGF standard is 719 μg/mL.

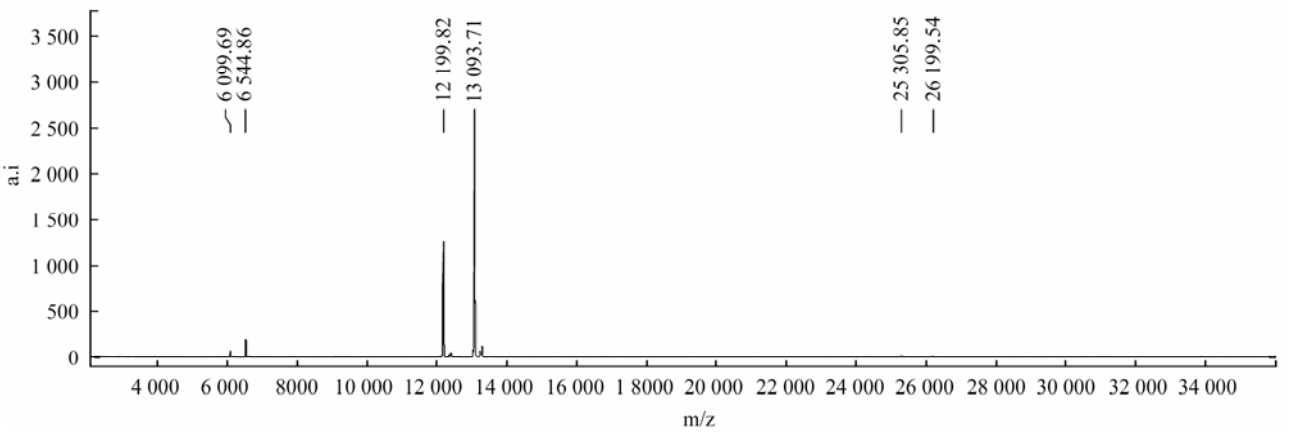

Figure 7 HPLC chromatogram of NGF standard

Note: The chromatogram reveals the exclusive NGF peak and suggests that the purity of standard is 99.1% from statistics

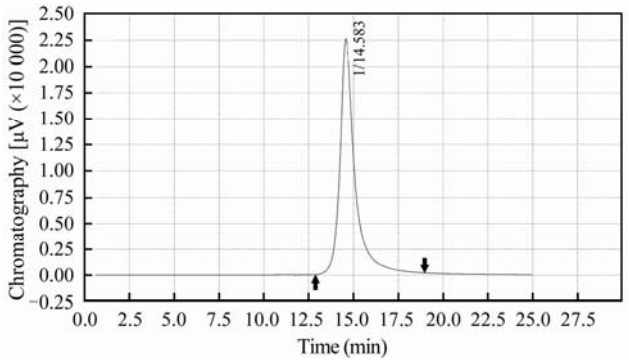

Figure 8 Mass spectrogram of NGF standard

Note: It reveals that mainly exists the NGF; The three peak groups represent the NGF double-charged peak, single-charged peak and dimer peak. Meanwhile, the doublet of every peak group reveals the 8 amino acids dissimilarity from the post-transcriptional modification

3.3 Method

In order to prevent the protein denaturation and bringing in impurities, we chose SEC-HPLC other than the traditional RP-HPLC method which will introduce in impurities such as acetonitrile and methanol. This work used Shimadzu Shodex KW-802.5 column that exclusion range of 6~15 kD.

Author Contributions

Da Huang and Hongwei Ren designed research; Da Huang and Jianrong Zhang performed research; Da Huang analyzed data; Da Huang wrote the paper; and Yiming Xu, Liming Lu, Lingyan Ma, Yongchao Fu, Minxiang Li and Lingyuan Xiong provided support data and advisory. The authors declare no conflict of interest.

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