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Short communication

RP-HPLC determination of recombinant human interferon omega in the *Pichia pastoris* fermentation broth

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Abstract

A rapid and valid reversed-phase high performance liquid chromatography (RP-HPLC) method for determination of recombinant human interferon omega (rhIFNω) in the yeast *Pichia pastoris* fermentation broth was developed. The method is based on the hydrophobicity of rhIFNω followed by RP-HPLC separation with UV detection. The chromatography analysis was performed on EC 250/4 NUCLEOSIL 300-5 C18 (250 mm × 4 mm i.d., 300 Å, with a particle size of 5 μm) column. The compositions of the mobile phase A and B were 999:1 (v/v) water/TFA and 999:1 (v/v) acetonitrile/TFA at a flow rate of 1.0 ml min^{-1}. Detection was done by spectrophotometry at 280 nm and the column temperature was 30 ± 1 °C. Calibration curve was linear ($r = 0.9986$, $n = 7$) in the range of 0.074–0.555 mg ml^{-1} for rhIFNω and the regression equation was $y = 2.02 \times 10^6 x - 1.27 \times 10^5$. Limit of detection for rhIFNω was 0.053 mg ml^{-1}. The values of R.S.D. (%) of intra-day and inter-day precision were <5.65 and <5.68 ($n = 6$), respectively. The R.S.D. (%) values and the average recovery rate of recovery experiment were <1.23 ($n = 3$) and 97.97%.

Keywords: Recombinant human interferon omega (rhIFNω); *Pichia pastoris*; Fermentation broth; RP-HPLC; Quantitative analysis

1. Introduction

IFNω, belong to the type I IFN, was found by three independent groups in 1985 [1–3]. The mature human IFNω protein shows about 60% sequence identity with various IFNα species but is longer by six amino acids at the C-terminal end. It is composed of a single polypeptide chain of 174 amino acid residues and has a molecular weight of 22.17 kDa [4]. It is a glycoprotein glycosylated in the alone potential N-linked site of Asn^{80}-Met^{81}-Thr^{82} in its mature protein. Recently, IFNω has been found to have a wide range of biological activities, and is hopeful to be used as a new potential antivirus and antitumor agent [5–9]. The yeast *Pichia pastoris* expression system has been utilized extensively for the generation of heterologous recombinant proteins [10]. The high-level expression of recombinant human interferon omega (rhIFNω) in yeast *P. pastoris* has been obtained [11].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA) are usually used for determination of recombinant proteins in fermentation process [4,12]. But the repeatability and the accuracy of both methods are influenced by many factors and the longer period of determination is needed. Therefore, the fermentation process cannot be adjusted and controlled timely according to the detected result, and consequently, the quality control would be interfered in commercial production.

The application of hydrophobicity of protein to separate rhIFNω is reported in this paper. The developed method was successfully used in the quantitative analysis of rhIFNω in yeast *P. pastoris* fermentation broth.

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2. Experimental

2.1. Chemicals and reagents

The standard sample (98.50% purity) and the fermentation broth of rhIFNω were the gifts from Southwest Pharmaceuticals Co. Ltd. (Chongqing, China). Acetonitrile was of HPLC grade and was purchased from Dima Technology Inc. (Richmond Hill, USA). Trifluoroacetic acid (TFA) was purchased from shanghai Chemical Agent Co. (Shanghai, China). Water used for sample preparation and HPLC separation was ARIUM 611UF grade, with resistivity at least 18.2 MΩ cm and TOC maximum 4 ppb (Sartorius AG, Germany).

2.2. Apparatus and chromatographic conditions

Experiments were carried out in a Waters 2695 Liquid Chromatograph with autosampler and Waters 2996 Diode array detector (Waters, Milford, USA). The system is twice a year operationally qualified using its software built-in procedures.

EC 250/4 NUCLEOSIL 300-5 C18, 250 mm length, 4 mm internal diameter, 300 Å aperture and 5 μm particle size was used as the analytical column (Macherey-Nagel GmbH & Co., Germany). The compositions of the mobile phase A and B were 999:1 (v/v) water/TFA and 999:1 (v/v) acetonitrile/TFA at a flow rate of 1.0 ml min^{-1}. The elution strategy was the combination of the linear gradient and isocratic elution with the changes of the mobile phase A and B (0–9 min, B: 0–70%; 9–13 min, B: 70%; 13–15 min, B: 70–100%; 15–17 min, B: 100%; 17–24 min, B: 100–0%). When the mobile phase A and B were respectively 30% and 70%, the elution needed to be become an isocratic elution for 4 min. In all HPLC runs, the mobile phase was filtered through 0.45 μm nylon membrane and degassed before use. The detection wavelength was set at 280 nm. The column was operated at ambient temperature (30 ± 1 °C). Injection volume was 20 μl.

2.3. Pretreatment of fermentation broth

Fermentation broth of 10 ml was centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 μm nylon filter membrane and was stored at −20 °C.

2.4. Preparation and storage of standard rhIFNω solution

The standard rhIFNω was dissolved and diluted by ultrapure water. The solution of rhIFNω which concentration was 3.7 mg ml^{-1} was packed into 0.2 ml tubes and stored at −20 °C.

2.5. Identification of rhIFNω

To confirm the chromatographic peak of rhIFNω form the complex chromatogram of the yeast *P. pastoris* fermentation broth, the spectrum and the chromatographic identifications of rhIFNω must be combined.

The chromatographic effluent of rhIFNω was investigated by UV absorption spectrum. The chromatography with the diode array detector could carry out automatically the all-wavelength scan of UV absorption spectrum. Firstly, the chromatographic analysis of the standard rhIFNω solution and the pretreatment of fermentation broth were performed. Then, the chromatographic effluent of the main peak of the standard rhIFNω solution with the retention time of 10.118 min was automatically scanned and the characteristic spectrum of rhIFNω was obtained.

The chromatographic identification of rhIFNω in the yeast *P. pastoris* fermentation broth was investigated by chromatographic analysis. Twenty microliters of standard rhIFNω solution was added to 180 μl fermentation broth and mixed fully. The chromatographic analysis of the fermentation broth added standard rhIFNω solution was performed.

2.6. Validation of the method

The linearity of rhIFNω in standard solutions was investigated at seven concentration levels of 0.0740, 0.0925, 0.1850, 0.2775, 0.3700, 0.4625 and 0.5550 mg ml^{-1}. Working samples were prepared in duplicate from the standard rhIFNω solutions of 3.7 mg ml^{-1}. Aliquots of each solution were diluted in the mobile phase A to obtain solutions of the required concentration.

The intra-day and inter-day precision was evaluated by the data for six times determinations of the sample of the

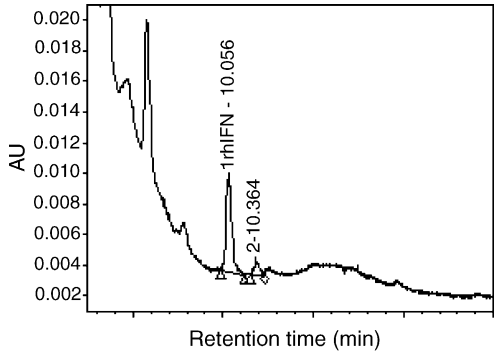

Fig. 1. Typical RP-HPLC chromatogram showing the separation of rhIFNω in the yeast *P. pastoris* fermentation broth. Column: EC 250/4 NUCLEOSIL 300-5 C18 (250 mm × 4 mm i.d., 300 Å, with a particle size of 5 μm). Mobile phase A: 999:1 (v/v) water/TFA. Mobile phase B: 999:1 (v/v) acetonitrile/TFA. Flow rate: 1.0 ml min^{-1}. Injection volume: 20 μl. Elution gradient: the mobile phase B (0–9 min, 0–70%; 9–13 min, 70%; 13–15 min, 70–100%; 15–17 min, 100%; 17–24 min, 100–0%). Wavelength used for UV detection: 280 nm. Column temperature: 30 °C. The peaks 1 and 2 present the peaks of the chromatographic effluent of rhIFNω and the impurity close to rhIFNω in the yeast *P. pastoris* fermentation broth.

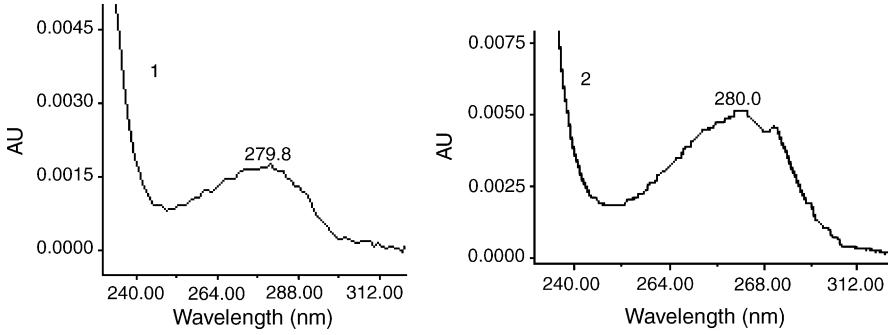

Fig. 2. UV absorption spectrum of the chromatographic effluents of rhIFNω. The curves 1 and 2 present respectively the standard rhIFNω and the rhIFNω in the yeast *P. pastoris* fermentation broth.

same batch number in 1 day and one time per day in 6 days.

For determination of accuracy, the recoveries of the added rhIFNω were determined by adding a known amount of standard rhIFNω to the fermentation broth at the three concentrations of 0.370, 0.463 and 0.555 mg ml^{-1}. Each level was repeated three times ($n = 3$) and the amounts of rhIFNω were found by the assay method.

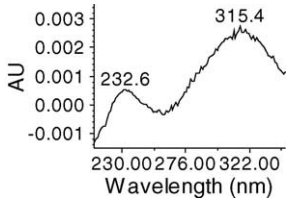

Fig. 3. UV absorption spectrum of the chromatographic effluents of the peak 2 shown in Fig. 1.

3. Results and discussion

3.1. Studies of chromatographic conditions

To achieve the separation of rhIFNω in the yeast *P. pastoris* fermentation broth, the chromatographic conditions, such as chromatographic columns, mobile phases, flow rates, elution modes, wavelengths of detection and column temperatures, were studied during optimization. The optimal separation conditions of the chromatography were achieved. The composition of mobile phases and the combination of the gradient and isocratic elution were found to be important to improve the separation efficiency. The typical RP-HPLC chromatogram of rhIFNω in the fermentation broth was shown in Fig. 1.

3.2. Identification of rhIFNω

3.2.1. Spectrum identification

The curve 1 in Fig. 2 shows the characteristic spectrum of rhIFNω with a maximum absorption at 280 nm. The separated peak of rhIFNω in the yeast *P. pastoris* fermentation broth (see peak 1 in Fig. 1) with the retention time of 10.056 min also had the consistence of the characteristic spectrum with that of the standard rhIFNω solution. However, such characteristic spectrum was not showed in the impurity peak (peak 2 in Fig. 1), which spectrum plot with a maximum absorption at 315.4 nm shown in Fig. 3. Since the impurity peak does not contain proteins, we concentrate our attention to the peak 1 in Fig. 1 as the peak for the rhIFNω.

3.2.2. Chromatographic identification

By comparison the chromatograms of the original fermentation broth and that of the original fermentation broth added standard rhIFNω solution, the area of the peak with the retention time of 10.056 min was significantly increased. The result indicated that the chromatographic peak was the peak of rhIFNω in the yeast *P. pastoris* fermentation broth.

Therefore, the separated peak of rhIFNω in the complex fermentation broth could be identified by the characteristic spectrum and the retention time consisted with that of the standard rhIFNω.

Table 1
The evaluation of intra-day and inter-day precision ($n = 6$)

No.	Intra-day precision			Inter-day precision		
	Retention time (min)	Concentration (mg ml^{-1})	R.S.D. (%)	Retention time (min)	Concentration (mg ml^{-1})	R.S.D. (%)
1	10.054	0.278	0.23	10.007	0.263	5.68
2	10.041	0.287	3.38	9.843	0.293	5.13
3	9.986	0.288	3.98	9.957	0.263	5.54
4	9.984	0.284	2.51	9.808	0.289	3.65
5	10.171	0.265	4.46	10.041	0.287	2.78
6	10.007	0.262	5.65	10.054	0.278	0.35

Table 2
Accuracy evaluation in rhIFNω of the fermentation broth ($n = 3$)

No.	Concentration found in added broth (A) (mg ml^{-1})	Concentration in original broth (B) (mg ml^{-1})	$A - B$ (mg ml^{-1})	Theoretical concentration after the addition (C) (mg ml^{-1})	$(A - B)/C$ (recovery, %)	R.S.D. (%)
1	0.431	0.068	0.363	0.370	98.11	0.15
2	0.524	0.066	0.458	0.463	99.03	1.09
3	0.601	0.064	0.537	0.555	96.76	1.23

Table 3
Analysis results for rhIFNω in the fermentation broth

Sample	Concentration of rhIFNω (mg ml^{-1})	R.S.D. (%)
1	0.135	1.48
2	0.267	0.75
3	0.324	0.53

3.3. Validation of the method

The linearity of the method was studied in a concentration range of 0.074–0.555 mg ml^{-1}. The correlation of the mean peaks area response versus concentration of rhIFNω showed excellent linearity over the range studied. The linear equation was $y = 2.02 \times 10^6 x - 1.27 \times 10^5$ ($r = 0.9986$) where x is the concentration of rhIFNω, and y is the mean peak area response. Limit of detection (LOD) (S/N 2:1) for rhIFNω was 0.053 mg ml^{-1}.

Table 1 gives the intra-day and inter-day precision values of measured concentrations of rhIFNω, as calculated of linearity plot. In two situations, the R.S.D. (%) values are <5.65 and <5.68 ($n = 6$), respectively, demonstrating that the method was precise.

The respective HPLC area responses from the accuracy determination study are shown in Table 2. Good recoveries are obtained for each concentration, confirming that the method is accurate. The R.S.D. (%) values and the mean recovery rate are <1.23 ($n = 3$) and 97.97%.

3.4. Analysis of real samples

Concentrations of rhIFNω in the fermentation broths of three different batches were analyzed by RP-HPLC. The results are listed in Table 3.

4. Conclusion

A rapid, convenient, accurate and stable RP-HPLC method for quantitative determination of rhIFNω in the yeast *P. pastoris* fermentation broth was developed. It is valid for linearity and precision in the studied concentration range. Thus the proposed method could overcome the shortages of conventional SDS-PAGE and ELISA methods, such as bad repeatability and accuracy and long determination time, and be employed for the assay of rhIFNω on quality control of commercial production absolutely.

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