# AMINO ACID ANALYSIS IN THE QUALITY CONTROL OF RECOMBINANT HUMAN ALPHA-INTERFERON

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## SUMMARY

The chemical identity of proteins derived from recombinant technology must be verified; they must meet the safety standards specifically established for this type of molecules. Depending upon the molecule under study, amino acid analysis may yield valuable information. The present study evaluates the analytical technique used for recombinant alpha-interferon (alpha-IFN): precision and accuracy of this technique and data processing according to two normalization procedures in the evaluation of the alpha-IFN.

## RESUMEN

La identidad química de las proteínas obtenidas de la tecnología del ADN recombinante deben ser verificadas; deben además cumplir los estándares de seguridad específicos establecidos para este tipo de moléculas. En dependencia de la molécula en estudio, el análisis de amino ácidos puede rendir una información valiosa. En el presente trabajo se reporta la evaluación del análisis de amino ácidos aplicado al estudio del interferón alfa recombinante: se evaluó el procesamiento de datos según dos procedimientos de normalización, la exactitud y la precisión del método

## INTRODUCTION

Quality control procedures play an important role in the biotechnological industry due its singularity. It makes use of living organisms for manufacturing pharmaceuticals (1).

Powerful analytical methods exist to ensure the fidelity and consistency of recombinant protein products. In specific circumstances, depending upon the molecule under study, classical protein chemistry methods such as amino acid analysis may yield valuable information. This method is of maximal value for peptides and small proteins (MW $<20$ kDa) (2).

It includes the complete hydrolysis of the proteins into their amino acid components and their separation by liquid chromatography.

In the conventional method, the free amino acids are separated in a cation-exchange column, using a series of aqueous buffers of increasing pH and ionic strength, at optimal temperatures for each section of the chromatogram. They are detected by post-column reaction with ninhydrin, allowing the reaction to occur in a delay coil at 110°C, and monitoring it by UV absorbance at 440 nm and 570 nm.

More recently, reversed-phase high performance liquid chromatography was introduced using pre-column derivatization with o-phtalaldehyde (3).

Finally the amino acid composition of the sample is estimated by comparison with a standard which contains the natural amino acids at known concentrations.

It is generally agreed that method validation is required to determine whether a given method is a suitable way to provide useful analytical data for a known set of samples. Therefore, analytical methods must be validated for their use in quality control.

In the present paper, we describe the validation of the method used in determining the amino acid composition for the quality control of human recombinant alpha-interferon.

## MATERIALS AND METHODS

### Protein

In this study we used a recombinant alpha-IFN preparation that has previously been evaluated by:

1. identity test using reverse-phase high performance liquid chromatography and gel filtration.

2. purity test by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and silver staining.

3. determination of total protein content by Lowry and Kjeldahl techniques (0.580 mg/mL).

4. determination of alpha - IFN content by ELISA (0. 571 mg/mL).

### Amino acid-free water

Was prepared by reflux over $K_2MnO_4$/NaOH followed by double destilation. This water was used for the preparation of analizer's buffers.

### Amino acid standard

For the calibration of the automatic analyzer, we used a commercial standard of free amino acids (Pharmacia-LKB, Sweden). It contains all the amino acids at a concentration of 2.5 $\mu$mol/mL in 0.1N HCl. A dilution with sodium citrate buffer pH 2.2 was made to obtain a concentration of 0.5 nmol/20 $\mu$L of

**Table 1**

Amino acid composition of alpha-IFN calculated with both methods of normalization. AA: one letter amino acid code. TV: theoretical value from DNA sequence, $\bar{x}$ mean of 15 independent determinations, SD: standard deviation, CV: coefficient of variation (%).

| | | METHOD I | | | | | | | | | | | | METODO II | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AA | TV | Z | | | A | | | H | | | R | | | B, Z, A, H, K, R | | |
| | | $\bar{x}$ | SD | CV | $\bar{x}$ | SD | CV | $\bar{x}$ | SD | CV | $\bar{x}$ | SD | CV | $\bar{x}$ | SD | CV |
| B | 12 | 10.66 | 1.059 | 9.93 | 10.89 | 0.969 | 8.89 | 9.58 | 0.693 | 7.23 | 10.21 | 0.974 | 9.53 | 11.23 | 0.838 | 7.46 |
| T | 10 | 8.86 | 0.969 | 10.93 | 9.04 | 0.833 | 9.21 | 7.94 | 0.522 | 6.57 | 8.48 | 0.871 | 10.27 | 9.33 | 0.815 | 8.73 |
| S | 14 | 9.53 | 0.356 | 3.73 | 9.75 | 0.479 | 4.91 | 8.6 | 0.637 | 7.40 | 9.16 | 0.740 | 8.07 | 10.07 | 0.4 | 3.97 |
| Z | 26 | 26.30 | 0.138 | 0.52 | 26.95 | 1.923 | 7.13 | 23.81 | 2.507 | 10.52 | 25.33 | 2.663 | 10.51 | 27.81 | 1.433 | 5.15 |
| G | 5 | 5.12 | 0.413 | 7.83 | 5.21 | 0.163 | 3.12 | 4.6 | 0.351 | 7.63 | 4.90 | 0.366 | 7.46 | 5.39 | 0.342 | 6.34 |
| A | 8 | 7.92 | 0.576 | 7.27 | 8.08 | 0.049 | 0.6 | 7.13 | 0.402 | 5.63 | 7.58 | 0.470 | 6.20 | 8.35 | 0.393 | 4.7 |
| V | 7 | 5.99 | 0.514 | 8.58 | 6.08 | 0.465 | 7.64 | 5.35 | 0.412 | 7.7 | 5.73 | 0.438 | 7.64 | 6.27 | 0.395 | 6.29 |
| M | 6 | 3.64 | 0.418 | 11.48 | 3.71 | 0.399 | 10.75 | 3.26 | 0.256 | 7.85 | 3.48 | 0.360 | 10.34 | 3.83 | 0.364 | 9.5 |
| I | 8 | 6.48 | 0.607 | 9.36 | 6.60 | 0.445 | 6.74 | 5.81 | 0.269 | 4.62 | 6.19 | 0.392 | 6.33 | 6.81 | 0.378 | 5.55 |
| L | 21 | 19.29 | 1.702 | 8.82 | 19.68 | 1.266 | 6.43 | 17.31 | 0.75 | 4.33 | 18.44 | 1.059 | 5.74 | 20.3 | 0.998 | 4.91 |
| Y | 5 | 4.77 | 0.495 | 10.37 | 4.87 | 0.395 | 8.11 | 4.28 | 0.225 | 5.25 | 4.56 | 0.352 | 7.71 | 5.0 | 0.348 | 6.93 |
| F | 10 | 10.02 | 1.012 | 10.09 | 10.22 | 0.781 | 7.64 | 8.98 | 0.458 | 5.10 | 9.56 | 0.545 | 5.70 | 10.54 | 0.626 | 5.93 |
| H | 3 | 3.35 | 0.311 | 9.28 | 3.39 | 0.266 | 7.84 | 3.00 | 0.014 | 0.46 | 3.20 | 0.216 | 6.75 | 3.53 | 0.224 | 6.34 |
| K | 10 | 7.49 | 1.081 | 14.43 | 7.63 | 0.951 | 12.46 | 6.73 | 0.906 | 13.46 | 7.11 | 0.509 | 7.15 | 7.87 | 0.836 | 10.62 |
| R | 10 | 10.48 | 1.007 | 9.60 | 10.68 | 0.694 | 6.49 | 9.41 | 0.646 | 6.86 | 10.0 | 0.00 | 0.00 | 11.03 | 0.598 | 5.42 |
| COMPOSITIONAL CV | | | | 8.81 | | | 7.19 | | | 6.70 | | | 7.77 | | | 6.52 |

each amino acid in the mixture. By peak area integration from the standard, the response factor for each amino acid were determined.

## Protein desalting

Prior to hydrolysis, alpha-IFN was desalted through a PD-10 column (Pharmacia-LKB, Sweden) equilibrated in water. The fraction containing the protein was collected by UV absorbance at 280 nm.

## Acid hydrolysis

Aliquotes (15 $\mu$g, 0.83 nmol) of alpha-IFN solution in water were dried under vacuum in hard-glass tubes with narrow neck. To each tube were added 100 $\mu$L of 6M HCl, containing 0.1% (w/v) phenol and 0.1% (v/v) beta-mercaptoethanol. The bottoms of the tubes were immersed in a dry ice-acetone bath to freeze the acid solution. The tubes were connected to an oil-pump vacuum, evacuated carefully and sealed. The tubes were placed in a fan-assisted oven, and heated at 110°C, for 24h. Following hydrolysis, the acid was removed by rotatory evaporation. The hydrolysates were then dissolved in 200 $\mu$L of sodium citrate buffer pH 2.2. Approximately 0.041 nmol of hydrolyzed protein were analized.

In order to obtain a set of data for statistical processing, 15 independent hydrolyses of the same protein preparation were performed.

## Ion-exchange chromatography

The separation of free amino acids was carried out in an automatic analyzer Alpha Plus 4151 (Pharmacia-LKB, Sweden), equipped with a column (4 x 270 mm) using a cation-exchanger in sodium form and eluted with a set of aqueous buffers following the manufacturer's recommendations. Detection of amino acids in the eluate was made by post-column reaction with o-phtalaldehyde, and the fluorescence emission measured at 425 nm with excitation at 340 nm. Data were acquired with a Shimadzu integrator CR3-A (Japan).

## RESULTS AND DISCUSSION

### Normalization method

Taking into account that the reference for the integration is the standard of free amino acids, we have calculated the error introduced by each amino acid used in both normalization methods. Figure 1 is a bar representation of the individual error (method I) and the average error (method II). Table 2 shows the precision and accuracy of the amino acid standard. These calculations were done after finding the mean for 30 standard sucessions.

Two methods for data normalization were evaluated. Each method included different amino acids for the calculations; method I included only one amino acid (A, Z, H or R), and method II included a group : "B", "Z", "A", "K", "H" and "R". We assumed that all these amino acids are in the protein molecule at the quantities predicted from the DNA sequence, that they are completly free after 24 h hydrolysis, and that they are hydrolytically stable amino acids. The ratio of the sum of the areas to the sum of the theoretical number of these residues was taken in each case, as the area for one residue. This value was used for calculating the number of residues of each amino acid in the sample's analysis.

The experimental values obtained using both methods showed that there is a direct influence of the method used on the calculation of the resulting composition of this protein (table 1). Coefficients of variation (CV) obtained were higher in all cases using method I. Method II showed the lowest CV (6.52%) and good acceptability for almost all amino acids, except "K".

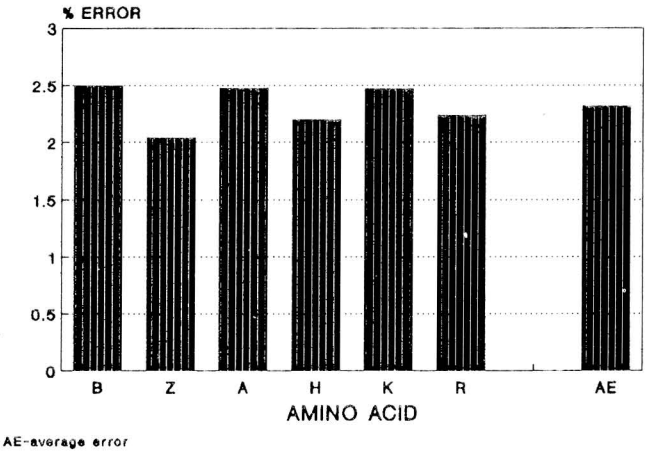


**Fig. 1** Individual and average errors of the amino acid used in both methods of normalization. Values have been taken from the free amino acid standard

**Table 2**
Precision and accuracy of the amino acid standard SD: standard deviation,CV: coefficient of variation, R: recovery (%), AA: one letter amino acid code.

| AA | SD | CV | R |
|---|---|---|---|
| B | 0.025 | 5.01 | 99.8 |
| T | 0.031 | 6.26 | 99.0 |
| S | 0.025 | 5.01 | 99.8 |
| Z | 0.020 | 4.05 | 98.6 |
| G | 0.027 | 5.45 | 99.0 |
| A | 0.024 | 4.80 | 99.8 |
| V | 0.027 | 5.45 | 99.0 |
| M | 0.019 | 3.78 | 100.4 |
| I | 0.025 | 5.03 | 99.4 |
| L | 0.026 | 5.25 | 99.0 |
| Y | 0.026 | 5.30 | 98.0 |
| F | 0.028 | 5.60 | 100.0 |
| H | 0.022 | 4.40 | 100.0 |
| K | 0.024 | 4.91 | 97.6 |
| R | 0.022 | 4.44 | 99.0 |

### Precision and accuracy of the analytical method

Precision of an analytical method is a measure of its repeatability. It refers to the agreement between successive independent analytical measurements on the same sample. Precision is usually expressed as the standard deviation (SD) or coefficient of variation (CV). Assessing the precision of an analytical method taking into account the CV , is useful because this parameter is independent of the dimension of the value obtained and from its absolute value. In general, a value for CV lower than 10% can be accepted as a good one (4).

Accuracy is generally recognized as the difference between the mean of a set of results obtained with one method and "the true value" of the sample. It is usually expressed as the percent of recovery (%R) (Table 3.). The accuracy assessment is under the influence of systematic errors, and, in the case of amino acid analysis, it is most seriously influenced by the effects of the chemical reaction and structural characteristics of the protein itself, in particular, it is related to the stability of different amino acids and variations in peptide bond strength. The accuracy of

**Tabla 3**
Accuracy of the analytical technique calculated for both methods of normalization.

| | METHOD I | | | | METHOD II |
|---|---|---|---|---|---|
| | Z | A | H | R | B, Z, A, H, K, R |
| R(%) | 90.26 | 91.84 | 81.07 | 86.34 | 95.03 |

protein composition data is also dependent on the amount of care taken in sample hydrolysis prior to analysis.

An analysis showing low recovery for some amino acids can be explained and accepted as correct on the basis of their chemical behavior during the acid hydrolysis. "S" and "T" are partially dehydrated under acid hydrolysis, other amino acids are somewhat labile to oxygen, and the amino acids with bulky side chains participate in a cluster forming chemically resistant bonds.

On the other hand, values beyond those true values for "B", "Z", "A", "H", "K", "G" and "R", which are stable under hydrolysis and are not particularly hydrophobic give a warning about manipulation or contamination errors.

## CONCLUSIONS

We obtained for our technique in the case of alpha-IFN the following parameters: with the method I a CV oscillating between 6.7 and 8.81% and a recovery in a range of 81.07-91.84% for different amino acids used for the calculations. The method II showed a CV of 6.52% and a recovery of 95.03%. It has been shown that amino acid analysis by ion exchange chromatography with o-phtalaldehyde detection provides the biotechnologist with assurances of reproducibility in amino acid identification and quantifications for the quality control of alfa-IFN.

Similar studies have been reported in the literature (5,6), having found differences in accuracy between 87.3% and 93.5%, and precision errors in the range of 2.5%-5.3%. These results support the observation that errors in amino acid analysis do not reflect the intrinsic accuracy of the method, they rather reflect the experience of the various analysts and the levels of contamination for different laboratories.

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