Development and Validation of an LC/MS Method of Glatiramer and Its Sequential Amino Acids, at Pharmaceutical Product

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Abstract: Analytical method by liquid chromatography coupled to mass spectrometry (LC/MS) was developed and validated for the quantification of the polypeptide mixture of glatiramer acetate (GA) as an injectable solution, the method was linear, precise, accurate and capable of separating the polypeptide mixture consisting of four amino acids that make up the glatiramer (L-glutamic acid, L-alanine, L-tyrosine and L-lysine), using a Waters Acquity® UPLC BEH300 C18 1.7 µm column, 2.1 × 100 mm, a mobile phase composed of 0.2% formic acid and acetonitrile at a flow rate of 0.2 mL/min, a mass spectrometry detector (WATERS Acquity QDa®). As a result, the fragmented ions related to each molecule were obtained with the following values: 564 m/z for GA, 148 m/z for glutamic acid, 90 m/z for alanine, 182 m/z for tyrosine and 147 m/z for lysine; with retention times of 4.1 min, 11.2 min, 11.3 min, 12.1 min and 10.9 min respectively. The method shows linear and reproducible responses in a range of 128-192 µg/mL GA, with a correlation coefficient (r) of 0.997, recoveries of 99 ± 1.2% and an accuracy of 0.5%.

Key words: Glatiramer, amino acids, polypeptides, chromatography, LC/MS, mass spectrometry.

1. Introduction

The glatiramer acetate (GA) is a chemical compound that belongs to the family of immunomodulators and it is used in the treatment of multiple sclerosis; it is an acetic salt of synthetic polypeptides, containing a mixture of four amino acids: L-glutamic acid, L-alanine, L-tyrosine and L-lysine, with an average molar fraction of 0.129-0.153, 0.392-0.462, 0.086-0.100 and 0.300-0.374, respectively. The average molecular weight of GA varies between 5,000 and 9,000 daltons [1, 2] and it is synthesized by polymerization of the N-carboxyanhydrides of said amino acids. GA is antigenically similar to the basic protein of myelin, so it is speculated that it acts by modifying the immune processes believed to be responsible for the pathogenesis of multiple sclerosis. Chemically, GA is termed as an L-glutamic acid polymer with L-alanine, L-lysine, L-tyrosine, and acetate (salt). Its structural formula is:

\[(\text{Glu, Ala, Lys, Tyr})_x \times \text{CH}_3\text{COOH}\]
\[(\text{C}_5\text{H}_9\text{NO}_4 \cdot \text{C}_3\text{H}_7\text{NO}_2 \cdot \text{C}_6\text{H}_{14}\text{N}_2\text{O}_2 \cdot \text{C}_9\text{H}_{11}\text{NO}_3)_x \times \text{C}_2\text{H}_4\text{O}_2\]

GA is synthesized through amino acid polymerization followed by a subsequent stage of cleavage or partial depolymerization. Due to the stochastic nature of the polymerization and cleavage reactions, the polypeptides obtained by this process vary in sequence, length, and molecular weight (MW), resulting in a characteristic molecular weight distribution (MWD) ranging from approximately 2,500 to 20,000 daltons.
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There are analytical methods used to characterize GA or similar polypeptide mixtures described in the literature, for example by amino acid analysis, by size exclusion chromatography, reverse phase liquid chromatography, capillary electrophoresis, peptide mapping, Edman sequencing; however, the proposed method consists of a chromatographic design coupled to a highly selective mass spectrometry detector, which is capable of discerning from the polypeptide mixture the qualitative-quantitative composition of the GA and its sequential amino acids; designed with the objective of being used as a tool for monitoring critical quality attributes (test and mole fraction) of a formulation, to discriminate batches of GA suitable for pharmaceutical use as an injectable solution [3]. In this work, the analytical method to quantify GA as raw material and finished product was established.

2. Materials and Methods

2.1 Chromatography Conditions

Equipment: Ultra Resolution Liquid Chromatograph (Waters Acquity Class H).

Column: Waters Acquity® UPLC BEH300 C18 1.7 µm, 2.1 × 100 mm.

Flow: 0.2 mL/min.
Injection volume: 10 µL.
Mobile phase: solution A (formic acid 0.2% in water) and solution B (acetonitrile HPLC) in the proportion established in Table 1.
Diluent: HPLC water.
Run time: 8 minutes.
Detector conditions: Waters AcquityQDa® Detector, with the conditions described in Table 2.

2.2 Method

2.2.1 Preparation of Solutions
In total 0.2% formic acid in water: transfer 2 mL of formic acid to a 1,000 mL flask, add 800 mL of HPLC grade water, sonicate for 10 min, bring to the capacity with water. Filter through a 0.2 µm Teflon filter.
Acetonitrile: 1,000 mL of acetonitrile. Filter through a 0.2 µm Teflon filter.
Reference preparation A: weigh the equivalent of 80 mg of the GA reference substance.
Transfer to a 50 mL vol. flask, add 25 mL of the diluent and sonicate for 10 min, fill with diluent and mix gently.
Reference preparation B: take a 5 mL aliquot of reference preparation A and transfer to a 50 mL flask,

### Table 1: Solution gradient.

| Time (min) | %A  | %B  |
|------------|-----|-----|
| 0.0        | 100 | 0   |
| 1.0        | 100 | 0   |
| 3.0        | 0   | 100 |
| 5.0        | 0   | 100 |
| 6.0        | 100 | 0   |
| 8.0        | 100 | 0   |

### Table 2: Conditions of the mass spectrometry detector.

| Compound       | Polarity | m/z* | Voltage cono (V) | Temperature probe (°C) | Capilar voltage (kV) |
|----------------|----------|------|------------------|------------------------|----------------------|
| GA             | Positive | 564  | 10               | 500                    | 0.8 kV 0.8 kV       |
| L-acidoglutámico | Positive | 148  | 10               | 500                    | 0.8 kV 0.8 kV       |
| L-alanina      | Positive | 90   | 10               | 500                    | 0.8 kV 0.8 kV       |
| L-tirosina     | Positive | 182  | 10               | 500                    | 0.8 kV 0.8 kV       |
| L-lisina       | Positive | 147  | 10               | 500                    | 0.8 kV 0.8 kV       |

* Ratio mass/charge (“m”: molecular mass and “z”: ion charge).
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carry to the capacity with the same diluent. Filter through a 0.2 um nylon filter and inject. The preparation has a concentration of 160 µg/mL GA.

Sample preparation (MP1): weigh 20 mg of the sample into a 25 mL volumetric flask, add 15 mL of diluent, sonicate for 10 min, fill with diluent and mix gently.

2.2.2 Adequability

Evaluate with reference preparation B: according to the procedure, make 6 consecutive injections of 10 µL. With the GA area under the curve of the chromatograms, determine the coefficient of variation (DER), number of theoretical plates (N), symmetry factor and capacity factor. Acceptance criteria: N is greater than or equal to 500 DER. Not more than 3.0%, symmetry factor is not more than 2.0%.

3. Procedure

Make injections of reference preparation B and MP2 solution and determine the percentage of GA titration in the solution using the following formula:

\[
\text{GA Valoration} = \frac{(Rm \times Wo \times (Cs \times D))/(Rs \times Wm \times E )}{100}
\]

where:
- \(Rm\) = sample response;
- \(Rs\) = average response of the GA peak in the reference preparation;
- \(Wm\) = weight of the sample in g;
- \(Wo\) = GA average weight per dosage unit in g;
- \(Cs\) = GA average concentration of the reference preparation B in µg/mL;
- \(D\) = dilution factor;
- \(E\) = asset content according to label in µg;

3.1 Validation Procedure

The validation of the method was carried out both for the raw material and for the finished product. The parameters evaluated were: adequacy of the system, linearity of the system, precision of the system, accuracy, stability of the solution, tolerance, robustness, limit of quantification and detection, repeatability and linearity for the method.

3.1.1 Adequacy of the System

Adequacy of the system was evaluated by analyzing the reference preparation B according to the procedure, making 6 consecutive injections of 10 µL each. With the area under the curve of the chromatograms determines the DER. Acceptance criteria: DER is not greater than 3.0%.

3.1.2 The Linearity of the System

A concentrated preparation of the GA reference substance was performed at a concentration of 1.6 mg/mL and solutions with the following concentration levels 128, 144, 160, 176 and 192 µg/mL were prepared in triplicate following the procedure. The slope, the ordinate to the origin, the correlation coefficient and the coefficient of determination of the concentration concentration vs. analytical response were calculated.

3.1.3 Precision of the System and Accuracy of the System

Six (6) individual samples were prepared at 160 µg/mL and injected into the chromatograph following the procedure. The DER of the analytical response was calculated. It is not more than 3%.

In the system precision and system accuracy, 6 individual simples with a concentration of 160 µg/mL were prepared and injected into the chromatograph following the procedure. The coefficient of variation of the analytical response was calculated. It is not greater than 3%.

3.1.4 Linearity of the Method

A concentrated preparation of the GA reference substance was carried out at a concentration of 1.6 mg/mL and solutions with the following concentration levels 128, 144, 160, 176 and 192 µg/mL were prepared in triplicate and independently weighed following the procedure. The ordinate to the origin, the correlation coefficient, the coefficient of determination of the recovery percentage of each sample with respect to the added amount were calculated.
3.1.5 Accuracy and Repeatability of the Method

Six independent samples were prepared at 160 µg/mL in the same way as in the linearity of the method. The amount recovered from the added analyte was calculated as a recovery percentage.

3.1.6 Intermediate Precision

Three samples at 160 µg/mL were worked by two different analysts on 2 different days in the same way as in the linearity of the method. The DER must not be greater than 3%.

3.1.7 Stability of the Analytical Sample

Three solutions containing 160 µg/mL are prepared from a concentrated preparation of the GA reference substance of 1.6 mg/mL, each of which was divided into three parts and kept at room temperature for 0, 24 and 48 hours according to Table 3.

3.1.8 Tolerance

The effect of changing the filter on independent solutions at 160 µg/mL GA was evaluated. The DER in the evaluation of the analyte with respect to the initial condition is 2.0%.

3.1.9 Robustness

For robustness, three independent solutions are prepared at 160 µg/mL GA in different temperature conditions 50, 40 and 25 °C. The absolute difference in the evaluation of the analyte with respect to the initial condition is 2.0%.

3.1.10 Detection limit

To evaluate the detection limit, samples were prepared in triplicate at 1%, 5% and 10% of the GA concentration in the sample equivalent at concentrations of 1.6, 8.0, and 16 µg/mL.

3.1.11 Specificity

The following solutions were prepared: diluent, reference preparation, sample preparation, sample preparation at different pH (10, 2.0) with an oxidizing agent (hydrogen peroxide) and at 60 °C. After following the analytical procedure, the response is due only to the analyte.

### Table 3  Solutions preparation.

| Solution | GA concentration (mg/mL) | t₀ = 0 h | t₁ = 24 h | t₂ = 48 h |
|----------|--------------------------|---------|---------|---------|
| 1        | M₁t₀                     | M₁t₁    | M₁t₂    |
| 2        | 1.6                      | M₂t₀    | M₂t₁    | M₂t₂    |
| 3        | M₃t₀                     | M₃t₁    | M₃t₂    |

### Table 4  Validation results for the method analytic.

| Parameter                  | Criter | Specification | Results |
|----------------------------|--------|---------------|---------|
| System adequability        | Variation coefficient | No more 3.00 % | 0.3%    |
|                           | Symetry factor          | No more 2.00% | 1.03%   |
|                           | Teoric plate             | Upper 500    | 1,531   |
| System precision           | Variation coefficient   | No more than 3.0% | Level 80%, 0.70% |
|                           | Response variation coefficient | No more 3.0% for level | Level 90%, 1.64% |
| System lineality           | Global coefficient variation | No more 3.0% for level | Level 100%, 0.71% |
|                           | Correlation coefficient ($r^2$) | No less to 0.99 | 0.9968  |
|                           | Determination coefficient | No less to 0.98 | 0.9936  |
|                           | Confidence interval IC ($β_1$) | Does not include zero | 32,922-29,892 |
| Method accuracy            | Arithmetic mean of the percentage of recovery | 98.0%-102.0% | 101.1%  |
| Method repetibility        | Variation coefficient   | No more 3.0%  | 0.50%   |
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Table 4 to be continued

| Method lineality          | Correlation coefficient ($r$) | Determination coefficient de ($r^2$) | Variation regression coefficient ($\text{CV}_y/x$) | Aritmetic medium recovery % | Percent recovery variation coefficient |
|---------------------------|-------------------------------|-------------------------------------|-----------------------------------------------|-----------------------------|---------------------------------------|
|                           | No more 0.99                  | No less to 0.98                     | No more than 3.0%                             | 98.0%-102.0%                | No more than 3.0%                     |
|                           | 0.99882                       | 0.98764                             | 0.73%                                         | 101.0%                      | 0.73%                                 |
| Intermediate precision    | Arithmetric mean of analyte titration | 98.0%-102.0%                      | 101.2%                                       | A1D1                        | 101.6%                                |
|                           |                               |                                    |                                               | A1D2                        | 1017%                                 |
|                           |                               |                                    |                                               | A2D1                        | 1015%                                 |
|                           |                               |                                    |                                               | A2D2                        | 0.55%                                 |
|                           |                               |                                    |                                               | A1D1                        | 0.28%                                 |
| Intermediate precision    | Variation coefficient of analyte titration | No more to 23.0%                 |                                               | A2D1                        | 0.15%                                 |
|                           |                               |                                    |                                               | A2D2                        | 0.21%                                 |
|                           | General variation coefficient | No more to 3.0%                    |                                               | 0 h                         | Inicial                               |
|                           |                               |                                    |                                               | 24 h                        | 0.3%                                  |
|                           |                               |                                    |                                               | 48 h                        | 1.0%                                  |
| Solution stability        | Absolute difference from the initial condition | No more to 2.0%                  |                                               | Nylon                       | Reference                             |
|                           |                               |                                    |                                               | PTFE                        | 0.8%                                  |
|                           |                               |                                    |                                               | PVDF                        | 0.48%                                 |
| Sturdiness column         | Absolute difference in valuation | No more to 2.0%                   |                                               | 45 °C                       | Initial                               |
| temperature               |                               |                                    |                                               | 50 °C                       | 0.30%                                 |
|                           |                               |                                    |                                               | 40 °C                       | 0.70%                                 |
| Cuantification limit      | Coefficient of variation of the response | No more than 8.0% for level       |                                               | Level 1%, 1.86%             | Level 1%, 1.86%                       |
|                           |                               |                                    |                                               | Level 5%, 1.87%             | Level 5%, 1.87%                       |
|                           |                               |                                    |                                               | Level 10%, 0.85%            | Level 10%, 0.85%                      |
|                           |                               |                                    |                                               | 0.9964                      | 0.9929                                |
| Detection limit           | The signal/noise ratio should not be less than 3 | 1.86%                             |                                               |                              |                                       |
|                           |                               |                                    |                                               |                              |                                       |

4. Results

The application of the method showed linear and reproducible responses in a range of 128-192 µg/mL GA, with a correlation ($r$) of 0.997, recoveries of 99 ± 1.2% and an accuracy of 0.5%.

A WATERS Acquity® UPLC BEH300 C18 1.7 µm, 2.1 × 100 mm column was used, with a mobile phase composed of 0.2% formic acid and acetonitrile at a flow rate of 0.2 mL/min per gradient and using a detection system of mass spectrometry (WATERS Acquity QDa®) at 564 m/z for GA, 148 m/z for glutamic acid, 90 m/z for alanine, 182 m/z for tyrosine and 147 m/z for lysine.

GA retention time was 4.1 min and the case of amino acids, they were 11.2 min, 11.3 min, 12.1 min and 10.9 min for glutamic acid, alanine, tyrosine and lysine, respectively.

The chromatography results and the response vs. concentration correlation obtained are illustrated in Figs. 1 and 2.

4.1 Method Linearity

The linearity was for this method 120,000 µg/mL to 200,000 µg/mL, and the parameters for the graphic were: $r^2 = 0.9976$, $m = 0.9958$ (Fig. 2).
After performing all the tests on each parameter and obtaining the acceptance criteria, the results were concentrated and show in Table 4.

5. Discussion

The present investigation focused on the design and validation of an analytical method by LC/MS to characterize a polypeptide mixture based on GA in an injectable pharmaceutical dosage form (solution), more particularly, on a method capable of characterizing mixtures of polypeptides that comprise the four sequential amino acids that make up the GA (glutamic acid, alanine, tyrosine and lysine). A wide variety of analytical methods for the characterization of glatiramer acetate or mixtures of similar polypeptides are currently described in the literature, some based on analytical techniques for amino acid determination such as: size exclusion chromatography, reverse phase liquid chromatography, electrophoresis capillary, peptide mapping, or even Edman sequencing; these techniques depend on the physical, chemical or physicochemical affinity of the molecule of interest to an indicator, support or determined specific separation process, projecting results with high variability, low accuracy and certainty. Due to the aforementioned, a chromatographic method was developed coupled to a mass spectrometry detector that has a very high level of selectivity due to the tandem action of both analytical techniques (high resolution liquid chromatography and mass
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spectrometry). High resolution liquid chromatography favored the separation of GA components and, on the other hand, mass spectrometry further favored the specificity of the method when performing a molecular sieve in relation to the mass/charge of the molecules that make up GA and subsequently it was fragmented to obtain ions that were registered by a mass detector that only responded to the mass/charge ratio, complying with all the parameters of its validation as shown by the results obtained and presented in Table 4.

6. Conclusions

The developed method has a greater advantage over the currently available methods. This method is selective, fast and economical because the analysis quantifies the different amino acids of the polypeptide mixture and of GA in a single analytical run. It was also validated and can be used for raw materials and finished products, since it was validated for both cases.

References

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