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Capillary zone electrophoresis method for quantification of therapeutic peptide glatiramer acetate

Navid Niaei¹ · Martin Vališ^{2,3} · Jan Petr¹

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Abstract

This study presents the development and validation of a novel capillary zone electrophoresis method for the precise determination of glatiramer acetate and its amino acid constituents. A 120 mmol dm⁻³ phosphoric acid solution adjusted to pH 1.9 with Tris, supplemented with 20 mmol dm⁻³ triethylamine to achieve a final of pH 2.1, resulted in a repeatable analysis of glatiramer acetate. The method demonstrated a limit of detection and quantification of 39.2 μ g cm⁻³ and 130.7 μ g cm⁻³, respectively. This method allows for the rapid control of glatiramer acetate-based pharmaceuticals and distinguishes glatiramer acetate from the amino acids used in its synthesis.

Graphical abstract



Keywords Capillary zone electrophoresis · Glatiramer acetate · Quantitation · Pharmaceutical analysis · Validation

Extended author information available on the last page of the article

Introduction

Multiple sclerosis is a chronic autoimmune-mediated inflammatory neurological disease of the central nervous system, in which the immune response causes damage to myelin, the fatty substance insulating the nerve fibers [1]. Glatiramer acetate (GA) is the first-line drug used for the treatment of multiple sclerosis [2]. It is a non-biological complex drug, a heterogeneous molecule of nano-sized polypeptides. GA has a molecular weight of 5000-9000 Da and contains the amino acids L-lysine, L-alanine, L-glutamic acid, and L-tyrosine in molecular proportions of 39-46%, 13-15%, 30-37%, and 8.6-10%, respectively [3]. Hence, GA is composed of many polypeptides $(> 10^{36}$ possible theoretical sequences). The chemical synthesis of GA involves the activation of reactive groups, polymerization, depolymerization, deprotection, purification, and its formulation with a mannitol solution [4]. In the polymerization step, the growth of linear chains from monomers occurs without cross-linking between the polymer chains. This step is followed by deprotection and cleavage of the polymer. The size of the formed polypeptides and the amino acid sequences rely on factors such as conditions of the reaction, including the duration and temperature of the cleavage process, as well as the comparative reactivity of the activated amino acid monomers. The sequences of polypeptides in GA are considered as extensively reproducible under severely controlled reaction circumstances [4].

Separating and detecting all active GA amino acid sequences is impossible, even with the most technologically advanced multidimensional separation approaches [5]. The relative molar composition of amino acid residues in GA can be determined using liquid chromatography-mass spectrometry [6]. The purification process, employing filtering and size exclusion chromatography (SEC), removes all produced contaminants, minor peptides, and residual amino acids [7]. Molecular mass distribution used to be evaluated by reversed-phase HPLC (RP-HPLC) and polypeptide mapping using capillary electrophoresis [8]. However, RP-HPLC suffers from the poor retention of peptides in reversed phases, leading structurally identical species to typically co-elute in the same fraction [9]. The RP-HPLC method was also employed for GA purity monitoring. Nevertheless, it shares common HPLC drawbacks, including the use of large amounts of expensive solvents and chemicals and, in some cases, long times, thereby increasing production costs. Therefore, a comparable method with lower consumption of chemicals and costs is needed. Such a method can be exceptionally beneficial in routine analytical procedures.

Capillary zone electrophoresis (CZE) serves as a tool that could achieve these targets. CZE separates compounds based on their various electrophoretic mobilities, such as their charge/molecular mass ratio [10]. CZE holds the potential to provide high efficiency and resolution for macromolecules (e.g., peptides and proteins) with good repeatability, minimal reagent consumption, reduced lead times, and costs, along with operation automation [11]. It used to be considered a green analytical technique [12]. Moreover, CZE features a simple separation mechanism based only on the size and charge of the peptide, leading to remarkably accurate separation [13]. CE has proven to be suitable for the analysis of therapeutic peptides [14], therapeutic recombinant proteins [15], immunogenic synthetic peptides [16, 17], therapeutic nucleic acids [18], or vaccines [19, 20].

The aim of our work was to develop a simple CZE method for the precise determination of GA, enabling the rapid control of GA-based pharmaceuticals. In addition, the CZE methodology should separate glatiramer and the four amino acids from which GA is synthesized. This separation is essential for obtaining information about the total concentration of GA and potential amino acid impurities in the final GA product. To the best of our knowledge, there is no publication dealing with such a task.

Results and discussion

Method development

The development of peptide drugs, including GA, has necessitated the separation and analysis of the peptide from its related process impurities and degradation products [21]. In our work, we aimed to establish a novel approach for the precise determination of GA and its amino acids: L-alanine, L-lysine, L-tyrosine, and L-glutamic acid. The isoelectric point (pI) of GA was estimated to be 10 [22], while the pIs of the amino acids are as follows: L-glutamic acid 3.22, L-tyrosine 5.66, L-alanine 6.01, and L-lysine 9.74 [3]. After conducting preliminary tests in the pH range of 2–10, it was found that the use of an acidic buffer is advantageous for the following analysis. This acidic pH ensures the ionization of all the analytes.

First, various phosphate background electrolyte solutions (BGEs) in a pH range between 1.5-3.0 (in 0.1 steps) and concentrations from 20 to 200 mmol dm⁻³ were studied to establish the separation. The results demonstrated that migration times slightly increased with increasing pH probably due to an augmented electroosmotic flow (EOF). High ionic strength buffers resulted in longer migration times due to lower EOF, as well as a reduction in the mobility of ionic analytes. In our experiments, Tris was used as a buffering counter-ion, providing a high buffering capacity for the BGE and improved robustness. Because Tris is a low-conductive BGE component, higher concentrations of the buffer can be employed without causing excessive Joule heating and keeping the current below 'safe levels' (electric currents were below 100 µA at 25 kV) when using high voltage. Increasing the Tris concentration enhanced the separation of GA and the four amino acids. The optimal separation with high repeatability was achieved with a BGE containing 120 mmol dm⁻³ phosphate adjusted to pH 1.9 using Tris. Furthermore, the addition of triethylamine (TEA) was studied to enhance sensitivity. At low pH, TEA was found to be an effective electrolyte medium for increasing sensitivity by blocking the negatively charged silanol groups on the capillary wall (Fig. 1). TEA decreases the adsorption of cationic analytes, including both GA and amino acids, leading to reduced peak widths and increased sensitivity [23]. The pH of the buffer increased with the rising concentration of TEA $(10-40 \text{ mmol dm}^{-3})$. The optimal separation, considering peak shapes and resolution, was achieved with the addition of 20 mmol dm^{-3} TEA at pH 2.1.

Validation

The developed method was validated in terms of linearity, repeatability, and precision. All parameters were assessed by the addition of 1×10^{-4} mol dm⁻³

L-tryptophan as the internal standard (IS) into the mixture of four amino acids and GA. An example of the separation, including the IS, is shown in Fig. 2.

The method precision was assessed for the corrected peak area and migration time, respectively (Table 1). The intraday precision of GA was 0.53% and 1.53% for migration time and peak area, respectively. The RSD values for analyzed amino acids did not exceed 3.36% and 2.01% for migration times and peak areas, respectively. The interday precision related to GA, measured on three different days, was 2.66% and 3.48% for migration time and peak area, respectively.

The method's linearity was determined using six concentration levels (60, 190, 310, 500, 650 μ g cm⁻³ GA) across three independent sample preparations and replicates (*n* = 18). The corrected peak area vs. GA

Fig. 1 The effect of triethylamine concentration on the separation of a mixture of GA and related amino acids. BGE: 120 mmol dm⁻³ phosphoric acid adjusted with Tris to pH 1.9 with addition of TEA; applied voltage + 25 kV; UV detection 200 nm; sample: 5 mmol dm⁻³ L-alanine (Ala) and L-lysine (Lys), 0.5 mmol dm⁻³ L-tyrosine (Tyr) and L-glutamic acid (Glu), and 100 µg cm⁻³ GA

Fig. 2 The separation of a mixture of GA and related amino acids with L-tryptophan as the internal standard. Conditions: capillary: 48.5 cm \times 50 µm id., BGE: 120 mmol dm⁻³ phosphoric acid adjusted with Tris to pH 1.9 with addition of 20 mmol dm⁻³ TEA (final pH 2.1); sample: 5 mmol dm⁻³ L-alanine and L-lysine, 0.5 mmol dm⁻³ L-tyrosine, L-glutamic acid, 0.1 mmol dm⁻³ L-tryptophan, and 100 µg cm⁻³ GA



Table 1 Precision data for GA and related aminoacids

Compound	Intraday precision $(n=3)$		Interday precision $(n=9)$				
	RSD% (t)	RSD% (area)	RSD% (t)	RSD% (area)			
GA	0.53	1.53	2.66	3.48			
L-Lysine	0.45	2.01	1.87	2.98			
L-Alanine	1.41	1.06	3.09	1.82			
L-Tyrosine	2.75	0.74	6.25	1.58			
L-Glutamic acid	3.36	1.07	6.86	2.40			
L-Tryptophan	2.04	-	4.72	-			

Determined for 5 mmol dm $^{-3}$ L-alanine and L-lysine, 0.5 mmol dm $^{-3}$ L-tyrosine, L-glutamic acid, 0.1 mmol dm $^{-3}$ L-tryptophan, and 200 $\mu g~cm^{-3}~GA$

concentration plot was linear, with a correlation coefficient value of 0.9989. The limit of detection (LOD) and quantification (LOQ) were 39.2 and 130.7 μ g cm⁻³, respectively. The standard deviation of the signal (SD) used to calculate LOD and LOQ values was 0.229. The method's linearity in the range of 131–650 μ g cm⁻³ GA is deemed acceptable for the product quality control.

Subsequently, the precision of GA determination was evaluated at three distinct concentration levels, 100, 300, and 600 μ g cm⁻³ GA, with three runs for each of the three independent replicate samples. The results for corrected migration time and peak area are presented in Table 2. The RSD values, which are required to be below 10% for pharmaceutical analysis, meet the stipulated criteria.

Finally, the real sample, copaxone injection solution, was analyzed. The concentration found was 39.2 ± 2.7 mg cm⁻³, corresponding closely to the declared value of 40.0 mg cm⁻³. In addition, the recovery was assessed by spiking a known amount of GA (300 µg cm⁻³) into the injection solution, resulting in a recovery rate of 97.9%, well within the predefined requirement of 90–110%. Based on our data, the CZE method is deemed feasible for the quantification of GA in pharmaceutical formulations (Fig. 3).

Conclusion

In our work, we developed a simple CZE method for the precise determination of GA, enabling the control of GA-based

Table 2 Precision data for GA determination

Concentration/ µg cm ⁻³	Intraday precision $(n=3)$		Interday precision $(n=9)$	
	RSD% (t)	RSD% (areas)	RSD% (t)	RSD% (area)
100	0.13	0.56	3.69	2.29
300	0.19	0.58	1.53	1.66
600	0.62	0.39	2.41	6.89



Fig. 3 Application of the CZE method for the quantification of GA in the copaxone injection solution. Tryptophan was used as the internal standard (IS). Conditions: capillary: $34.0 \text{ cm} \times 50 \text{ }\mu\text{m}$ id., BGE: 120 mmol dm⁻³ phosphoric acid adjusted with Tris to pH 1.9 with addition of 20 mmol dm.⁻³ TEA (final pH 2.1)

pharmaceuticals. The CZE methodology not only separated GA but also the four amino acids from which GA is synthesized. Hence, our setup allows us to gather information about the total concentration of GA and potential amino acid impurities in the final GA product. Moreover, the use of greener CZE methodology can be the key advantage over the traditional HPLC, especially for the sustainable future.

Experimental

Phosphoric acid ($\geq 85\%$), sodium hydroxide ($\geq 98\%$), hydrochloric acid (\geq 37%), tris(hydroxymethyl)methylamine (Tris, \geq 99.9), and amino acids such as L-alanine, L-lysine, L-tyrosine, L-glutamic acid, and L-tryptophan were purchased from Sigma Aldrich (St. Louis, MO, USA). TEA was purchased from Fluka (Buchs, Switzerland). All chemicals were of analytical-grade purity. GA standards were obtained from Sigma Aldrich (COP-1; St. Louis, MO, USA). Copaxone injection solutions in prefilled syringes at a concentration of 40 mg cm⁻³ (Teva; LOT 11967) were generously provided by Prof. Martin Vališ (University Hospital Hradec Králové). Standard stock solutions and BGEs were prepared using ultrapure water with a resistivity of 18.2 MQ cm (MilliQ, Millipore, France). BGEs were prepared by dissolving the appropriate quantity of phosphoric acid in ultrapure water. To adjust the pH of the BGEs, a titration of phosphoric acid solution with Tris (1 M) to pH 1.9 was performed, followed by adding TEA to achieve pH 2.1. All BGE solutions were filtered using 0.45-µm pore-size membrane filters. Stock solutions were stored at +4 °C.

Instrumentation

All CE experiments were conducted using the Agilent 7100 capillary electrophoresis system with a diode-array detector

(Waldbronn, Germany). Data processing was carried out using the ChemStation software provided with the CE instrument. The analysis utilized 50 μ m ID bare-fused silica capillaries (Molex; Lisle, IL, USA) with a total length of 48.5 cm and an effective length of 40 cm.

CZE method for GA concentration determination

All fused silica capillaries were preconditioned before any use with 0.1 mol dm⁻³ NaOH (20 min), water (10 min), 0.1 mol dm⁻³ HCl (5 min), and water (3 min) at 935 mbar, respectively. Capillary preconditioning was performed before each run with cycles of water, 0.1 mol dm⁻³ HCl, water, 0.1 mol dm⁻³ NaOH, water, and BGE at 935 mbar for 2 min each. Hydrodynamic injection was performed at 100 mbar for 10 s. Separation occurred with an applied voltage of 25.0 kV (normal polarity, cathode in the outlet) at 25 °C. UV-absorbance detection was set at 200 nm (4 nm bandwidth). All samples were diluted with milli-Q water, protected from light, and stored at 4 °C.

CZE method validation

The repeatability of the method was assessed by adding of 1×10^{-4} mol·dm⁻³ of L-tryptophan as the IS to a mixture of analyzed amino acids and GA. Concentrations ranged from 5×10^{-4} to 5×10^{-3} mol dm⁻³ for amino acids, and 100 µg cm⁻³ for GA, with three replicates at each concentration level. Interday precision was determined based on the repeatability obtained on three different days.

The method's linearity was evaluated using six concentration levels: 60, 190, 310, 500, and 650 μ g cm⁻³ GA. Three independent sample preparations and replicates (*n*=18) were performed. The corrected peak area, calculated as the ratio of GA peak area to the IS peak area, was used to assess linearity. The LOD and LOQ were calculated from these equations: LOD=3 SD/s and LOQ=10 SD/s, where SD is the standard deviation of the signal intensity and s is the slope of the calibration curve.

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Data availability The data are available from the corresponding author upon reasonable request.

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Authors and Affiliations

Navid Niaei¹ · Martin Vališ^{2,3} · Jan Petr¹

- ⊠ Jan Petr jan.petr@upol.cz
- Department of Analytical Chemistry, Faculty of Science, Palacký University Olomouc, 17. listopadu 12, 77146 Olomouc, Czech Republic
- ² Department of Neurology, University Hospital Hradec Kralove, Hradec Králové, Czech Republic
- ³ Faculty of Medicine in Hradec Králové, Charles University in Prague, Hradec Králové, Czech Republic