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Short Communication

Development and validation of a novel sensitive UV-direct capillary electrophoresis method for quantification of alendronate in release studies from biomaterials

A simple, highly sensitive, and robust CE method applied to the determination of alendronate (ALN) was developed from matrices for tissue engineering, characterized by being highly complex systems. The novel method was based on the ALN derivatization with o-phthalaldehyde and 2-mercaptoethanol for direct ultraviolet detection at 254 nm. The BGE consisted of 20 mM sodium borate buffer at pH 10, and the electrophoretic parameters were optimized.The method was validated in terms of specificity, linearity, LOD, LOQ, precision, accuracy, and robustness. The LOD and LOQ obtained were 0.8 and 2.7 μg/mL, respectively. In addition, the method offers higher sensitivity and specificity compared to other CE and HPLC methods using UV-detectors, as well as low cost and simplicity that allowed the rapid and simple quantitation of ALN from bone regeneration matrices.

Keywords:
Alendronate / Biomaterials / Capillary electrophoresis / Scaffolds / UV-detection
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Bisphosphonates (BP) are a class of drugs that prevent the loss of bone mass, used to treat osteoporosis, bone resorption-related diseases such as cancer, and promote fracture repair [1,2]. However, BP are poorly absorbed in the intestines when administered orally [3] and therefore, high doses are needed to be used, leading to gastrointestinal disorders, chronic renal failure, or osteonecrosis of the jaws [4, 5]. To overcome this problem, the BP can be confined in delivery matrices based on composite (polymeric and inorganic) carriers or conjugated with bio-molecular and bio-polymeric systems [6]. Thus, the analyses of the BP release profile from complex matrices/scaffolds as well as the quantitation of its released amount (in the order of part per million –ppm-, part per billion –ppb-) are relevant for the evaluation of the therapeutic effects of these increasing bone-treatment approaches. The analytical determination of bisphosphonates, such as alendronate -ALN- (Fig. 1), is a difficult task since these compounds lack chromophore groups in their chemical structure. In this context, numerous HPLC-based methods have been reported for ALN quantitation, where most of them used ALN derivatization in a precolumn [7,8] or postcolumn system [5–7], ion chromatography [3, 4, 8], inductively coupled plasma [9], HPLC-mass spectrometry methods [5, 10] and ultraviolet(UV)-spectrophotometric methods [1, 2]. Although most of methods previously mentioned reached limits of quantitation (LOQ) in the order of ppb, there are drawbacks associated with the operation costs, high consumption of organic solvents and its consequently cost of discarding, high consumption of sample and reagents, and long analysis time, especially for HPLC methods. In this context, CE-based methodologies might serve as useful alternatives for ALN quantitation, due to their high resolution, short analysis times, low sample and solvent consumption and adequate sensitivity. Few works have been reported about the use of CE for ALN determination, based either on indirect UV-detection [11] or direct UV-detection after the formation of an ALN complex inside the capillary column or after a previous derivatization of ALN with naphthalene-2,3-dicarboxaldehyde (NDA) for fluorescence detection [12, 13]. To date, there have been no reports in the literature regarding either ALN quantification from complex drug delivery matrices such as the ones describes above [6] or about CE-methods based on ALN derivatization with OPA and 2-ME for direct UV-detection. The present work aimed at developing and validating a novel, simple, sensitive, reliable and robust CE-analytical technique to quantify ALN incorporated and released from matrices for bone tissue engineering applications.

Sodium trihydrate alendronate was a gift from Aryl S.A. (Argentina). Sodium decahydrate borate ($Na_2B_4O_7{\cdot}10H_2O$) was purchased from Carlo Erba Reagents (Rodano, Italy). Sodium dihydrogen phosphate (NaH_2PO_4), OPA and 2-MEwere purchased from Sigma Aldrich (St. Louis, MO,

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Abbreviations: 2-ME, 2-mercaptoethanol; **ALN**, alendronate; **BP**, Bisphosphonates; **Nbg**, bioactive glass nanoparticles; **NDA**, naphthalene-2,3-dicarboxaldehyde; **OPA**, o-phthalaldehyde

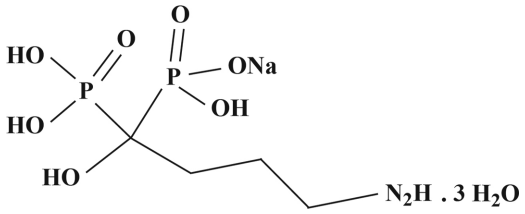

Figure 1. Chemical structure of ALN.

USA). All chemicals were of analytical grade and used without further purification. Ultrapure water was obtained from EASY pure RF equipment (Barnstead, Dudubuque, IA, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations, Westboro, MA, USA) before use.

All separations were performed with a P/ACE™ MDQ Capillary Electrophoresis System, equipped with diode array detector (190-600 nm) and data was processed by Karat V.8 software (Beckman, Framingham, MA, USA). An uncoated fused-silica capillary of 50 cm length (30 cm to detector) and 75 μm i.d. (MicroSolv Technology, Eatontown, NJ, USA) was used. ALN quantitation was performed using a BGE consisting of 20 mM sodium borate at pH 10.0. All samples were introduced into the capillary by pressure at 0.5 psi for 5 s. The instrument was operated in normal polarity mode with constant voltage of 15 kV and the detection was performed at 254 nm. Cartridge temperature was maintained at 35°C during runs.

A stock standard solution containing 1 mg/mL of ALN was prepared in 10 mM sodium phosphate buffer at pH 7.4 (diluent). The ALN calibration curve ranged from 5.0 to 50.0 μg/mL.The derivatization reagent was prepared from OPA and 2-ME. Briefly, 10 mg of OPA were dissolved in a 0.05 M sodium hydroxide (NaOH), and 50 μL of 2-ME were added. Then, 0.05 M NaOHwas added to reach a final volume of 10 mL achieving a final concentration of 0.1% w/v OPA and 0.5% v/v 2-ME.

Nanocomposite scaffolds made of alginate and bioactive glass nanoparticles (Nbg), containing ALN were prepared as mentioned in a previous work [6]. Phosphate buffer (10 mM, pH 7.4) was used as medium for the ALN release study in order to mimic physiological conditions [14,15]. This buffer was also employed for sample preparation/derivatization. Five samples of 13 mm diameter were withdrawn from the release mediumat regular time intervals. Sample pH was adjusted to 13 (same as the derivatization reagent) to keep the derivatization conditions.The basic medium is necessary for the stabilization of the amine group of ALN to allow reaction with aldehyde groups of OPA [15]. After this, derivatization was carried out by mixing the sample withdrawn from the release study with the derivatization reagent in relation 92:8 (v/v). Prior to the CE analysis, the mixture was heated and kept at 60°C during 15 min in order to allow a faster formation of the derivatized compound. The use of 2-ME has shown the best results when used as a nucleophile for this reaction. In addition, different 2-ME/OPA ratios (concentrations of OPA from 0.1 to 0.5% w/v and 2-ME from 0.5 to 1.0 % v/v) were tested, and suitable results were obtained by using 0.1% w/v OPA and 0.5% v/v 2-ME in 0.05 M NaOH solution. The 2-ME/OPA ratio used was 5 μL of 2-ME/mg of OPA, which was previously reported as suitable for ALN-derivatization [15]. Organic solvents, as methanol, were also assessed for reagent preparation and no relevant changes were observed. Different wavelengths (i.e. 200, 214, 254 and 333 nm) were tested for the detection of OPA-derivatized ALN, and 254 nm was selected due to the higher sensitivity obtained. The CE cartridge temperature was kept at 35°C during runs, which offers best conditions for the stability of the ALN-derivatized compound, as it was reported recently [16]. The BGE consisted of 20 mM sodium borate buffer at pH 10, however different concentration ranges (10–50 mM) and pH values (7–11) were tested previously to achieve suitable conditions in terms of resolution, peak shape and adequate current. Therefore, 20 mM was set as the optimal concentration. The pH value is important in the stability of the derivatized compound during the run. Hence, pH 10 was selected as the best condition. Finally, parameters as the applied voltage and the hydrodynamic sample introduction were optimized to obtain suitable resolution and analysis time of ALN. The applied voltage varied from 12 to 15 kV, and the application of pressure during runs (from 0.1 to 0.2 psi) was also evaluated. Runs were finally performed under normal polarity at 15 kV, where the current reached values close to 90 μA, and the application of 0.2 psi during runs showed an increase in the signal/noise ratio, which ultimately results in sensitivity improvement. The hydrodynamic injection method was tested using different pressures (from 0.1 to 1.0 psi) and times of injection (from 1 to 10 s), being 0.5 psi at 5 s the best condition to obtain adequate peak symmetry, peak area and resolution of ALN signal.

Figure 2A shows electropherograms of standard solutions with different concentrations of ALN and the blank (10 mM sodium phosphate buffer at pH 7.4, treated under the same conditions than the derivatized standard solutions of ALN), migration time for ALN was 4.5 min.

CE system validation was accomplished following the International Conference on Harmonization (ICH) guidelines [17] in terms of specificity, linearity, LOD and LOQ, precision, accuracy and robustness. Table 1 shows the results of the validation study. The specificity of the proposed CE method was carried out by a forced degradation study. The study was performed using a solution containing 1 mg/mL of ALN which was exposed to acid hydrolysis (0.1 N HCl), basic hydrolysis (0.1 N NaOH), oxidation (1% v/v H_2O_2) and photolytic degradation (natural light at 25°C and UV-light at 254 nm at 25°C, respectively). In all cases, each reaction was carried out over 24 h. Before injection, samples were diluted to achieve 25 μg/mL ALN. No significant changes were observed by acid and basic hydrolysis as well as photolytic degradation when comparing to the 25 μg/mL ALN standard solution. This outcome suggests that ALN is stable under the mentioned conditions. When ALN was exposed to oxidation conditions, the ALN peak was not detected in the electropherogram, which indicates its complete degradation.

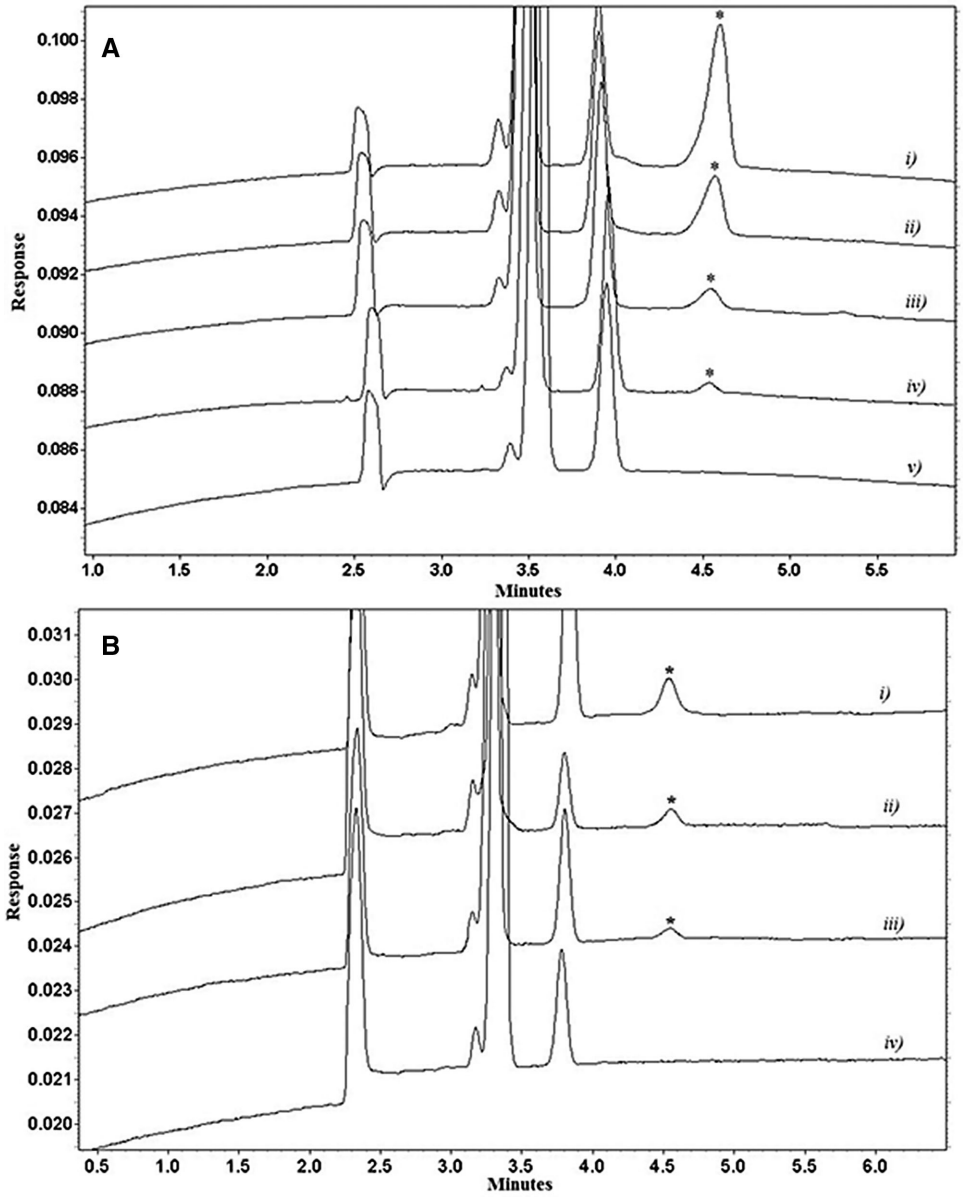

Figure 2. (A) Electropherograms of ALN standard solutions: (i), ii), iii) and iv) at 50, 25, 10, and 5 μg/mL, respectively, and *v)* blank (10 mM sodium phosphate buffer at pH 7.4, treated under the same conditions than the derivatized standard solutions of ALN). (B) Electropherograms of (i) 10 μg/mL ALN standard solution; (ii) sample of the release study at day 14; (iii) sample of the release study at day 30; (iv) blank (10 mM sodium phosphate buffer at pH 7.4, treated under the same conditions than the derivatized standard solutions of ALN). *ALN peak. BGE: 20 mM sodium borate pH 10; electrophoretic conditions: see in text

However, the oxidation of an amine results in an amine oxide which cannot react with OPA. Linearity was evaluated using five concentration levels of ALN standard solutions prepared from 1 mg/mL ALN solution by using the phosphate buffer (10 mM, pH 7.4) as diluent, covering the concentration range from 5.0 μg/mL to 50.0 μg/mL. The LOD (S/N = 3) and LOQ (S/N = 10) values achieved were 0.8 μg/mL and 2.7 μg/mL respectively. Precision was evaluated for intra-and inter day assay by using a 25 μg/mL ALN standard solution and it was expressed as RSD values for migration times and peak areas. The RSD values obtained in the study of precision were lower than 2. The accuracy of the method was evaluated by recovery studies. Three different concentrations samples (low, medium and high concentrations) from the release study were injected and then spiked with enough concentration of ALN to obtain a 30% higher response than the sample measured previously. The recovery values presented were good and they were obtained with high precision (Table 1).

The robustness of the CE method was studied by making variations in different parameters such as time of injection ± 1 s, cartridge temperature ± 2°C, run voltage ± 1 kV, and electrolyte pH value ± 2%. Variations were evaluated for a 25 μg/mL ALN standard solution in six replicates and analysed by the Student test [18] for migration time, theoretical plates and ALN concentration. No significant changes have been found ($p > 0.05$), except for time of injection and voltage.

Derivatized samples were analysed to quantify the amount of ALN released from the scaffolds used. Figure 2B shows electropherograms of a10 μg/mL ALN standard solution, two samples from the release study and the blank.

Table 1. Method validation for the CZE-UV assay for the determination of ALN

Parameter		Value	
Linearity range (μg/mL)		5.0–50.0	y = 1333.4x − 3536
r^2		0.994	
LOD (μg/mL)		0.8	
LOQ (μg/mL)		2.7	
Precision			
Intraday ($n = 4$)	RSD of migration time	0.6	
	RSD of peak area	0.9	
Interday ($n = 16$)	RSD of migration time	0.5	
	RSD of peak area	0.8	
Accuracy[a)]		104.8 (1.8) 105.4 (0.8) 101.6 (0.5)	

a) Percentage recovery i.e. values obtained from three samples with different concentrations from the release study. RSD values in parenthesis.

The main advantage of the CE-analytical technique developed here is the possibility of using a simple method for ALN quantitation. The preparation of the derivatization reagent as well as the simplicity and low cost that characterizes the CE-based methods in terms of low sample, solvent and reagents consumption in comparison to HPLC-based methods, make this analytical technique a suitable alternative for ALN determination. In addition, the possibility of using a conventional detection mode as UV absorption also contributes to the low cost and simplicity of the proposed CE-method. The strength of the work lies in its simplicity rather than sensitivity. However, the LOD (0.8 μg/mL) and LOQ (2.7 μg/mL) fully comply with the requirements of this research. Moreover, this methodology is suitable in terms of linearity, precision, accuracy and robustness.

It is worth noticing that this method also allowed rapid and simple release studies of ALN from composite biomaterials used for tissue engineering for the first time and is potentially applicable to the quantitation of small amounts of ALN released from these complex regeneration matrices, which could be applied in quality control and stability studies.

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The authors have declared no conflict of interest.

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