New multilayer coating using quaternary ammonium chitosan and κ-carrageenan in capillary electrophoresis: Application in fast analysis of betaine and methionine

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ABSTRACT

The aim of this study was to develop a new multilayer coating with crosslinked quaternary ammonium chitosan (hydroxypropyltrimethyl ammonium chloride chitosan; HACC) and κ-carrageenan for use in capillary electrophoresis. A new semi-permanent multilayer coating was formed using the procedure developed and the method does not require the presence of polymers in the background electrolyte (BGE). The new capillary multilayer coating showed a cathodic electroosmotic flow (EOF) of around $30 \times 10^{-9} \, \text{m}^2 \, \text{V}^{-1} \, \text{s}^{-1}$ which is pH-independent in the range of pH 2 to 10. The enhanced EOF at low pH obtained contributed significantly to the development of a fast method of separation. The multilayer coating was then applied in the development of a fast separation method to determine betaine and methionine in pharmaceutical formulations by capillary zone electrophoresis (CZE). The BGE used to determine the betaine and methionine concentrations was composed of 10 mmol L^{-1} tris(hydroxymethyl) aminomethane, 40 mmol L^{-1} phosphoric acid and 10% (v/v) ethanol, at pH 2.1. A fused-silica capillary of 32 cm (50 μm ID × 375 μm OD) was used in the experiments and samples and standards were analyzed employing the short-end injection procedure (8.5 cm effective length). The instrumental analysis time of the optimized method was 1.53 min (approx. 39 runs per hour). The validation of the proposed method for the determination of betaine and methionine showed good linearity ($R^2 > 0.999$), adequate limit of detection (LOD < 8 mg L^{-1}) for the concentration in the samples and inter-day precision values lower than 3.5% (peak area and time migration). The results for the quantification of the amino acids in the samples determined by the CZE-UV method developed were statistically equal to those obtained with the comparative LC–MS/MS method according to the paired *t*-test with a confidence level of 95%.

1. Introduction

Several anionic and cationic polymers have been used as non-covalent modifiers of fused silica capillaries for different applications in capillary electrophoresis (CE) [1]. Polycationic polymers containing quaternary amines such as poly(diallyldimethylammonium and polybrene have been employed in a successive multiple ionic-polymer layer (SMIL) combined with polyanionic polymers such as dextran sulfate [2–7], poly(vinyl sulfonate) [8–11] or poly(sodium 4-styrene-sulfonate) [12,13] for the formation of coatings. SMIL coatings are

particularly attractive due to their simplicity and versatility. The main interaction in the SMIL coating would be the ionic interaction between the polymers layers. Each type of coating generates cathodic or anodic electroosmotic flow (EOF) profiles and stability characteristics. One of the advantages of the use of SMILs with strong polyelectrolytes, such as a sulfated anionic polymer, is the enhanced EOF at pH values below 4.0, in contrast to the uncoated capillary which presents low EOF at the same pH. Other polymers that have been studied as modifiers of capillaries and could also be used in SMILs, forming new coatings with anionic polymers, are chitosan (CTS) and its derivatives. CTS allows covalent crosslinking between the chains of the cationic polymer, as reported in a paper by Huang and co-authors [14]. The crosslinking generates a compact layer, which is rigid and more resistant to changes in the electrolyte conditions, such as pH and type of solution

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and solvent, when compared to a non-crosslinked CTS coating. This crosslinked layer is easily formed and can act as a cationic support for interaction with SMIL coatings and the fixation of anionic polymers. Furthermore, the inclusion of cationic functional groups in the polymer chain improves its interaction with the capillary wall. A derivative of CTS called hydroxypropyltrimethyl ammonium chloride chitosan (HACC) [15] is one such example. The presence of quaternary ammonium groups in the polymer chain contributes to its strong interaction with the internal wall of the fused silica capillaries forming the cationic layer. Anionic polymers, such as carrageenan, also have potential for use in alternative coatings, principally due to the sulfate groups in their structure enabling their interaction with cationic polymers [16]. Inner coated capillaries are commonly employed to minimize undesirable interactions between proteins and the silica surface. However, these coatings can also be tailored to contribute to the development of fast separation methods by CE, within run times of less than a minute [17,18].

Betaine and methionine are amino acids involved in biological activities occurring in plants and biological fluids and they are used in foods and pharmaceutical formulations [19,20]. Many separation techniques have been employed for the determination of these amino acids, mainly gas chromatography [21,22], liquid chromatography [23–26], ion-exchange liquid chromatography [27] and capillary electrophoresis [28–33]. The separation methods developed with these techniques have separation times ranging from 2 to 30 min. Many of these methods were developed for the determination of several amino acids including betaine and methionine, in some cases using derivatization procedures to increase the detectability. In pharmaceutical formulations of hepatoprotective agents these two amino acids have the function of activators of the hepatic system [34,35]. Thus, in these products the amino acids are generally present in high concentrations (> 10 mg mL^{-1}), not requiring low limits of detection. Therefore, CE with UV/Vis detection represents a good option as an analytical technique for the determination of these compounds. With this technique it is possible to develop fast methods with minimal sample treatment using, for example, the short-end injection procedure (SEIP) [36,37]. However, due to specific structural features, some amino acids require special conditions for their separation by CE. As betaine has a pKa value of 1.83 [38], a low pH is required to ensure that it is positive and can be separated from the methionine (pKa1 $=$ 2.17 and pKa2 $=$ 9.34, data from Peakmaster®) and the EOF. An enhanced EOF is desirable to minimize the separation time but at low pH the EOF value is low [39]. In this regard, one way to achieve this particular condition is with the use of coated capillaries.

The aim of this study was to develop a new multilayer coating with crosslinked HACC and κ-carrageenan and apply it in the development of a fast separation method for the analysis of betaine and methionine in pharmaceutical formulations by capillary zone electrophoresis (CZE) using an SEIP. The new multilayer coating was characterized through the measurement of the EOF as a function of pH, the chemical stability in different solutions and the repeatability over several runs. The concentrations of betaine and methionine determined in the samples by CZE-UV were compared with results obtained applying the LC–MS/MS method.

2. Materials and methods

2.1. Chemicals and standards

HACC was synthesized as described by Qin et al. [40] using chitosan (deacetylation degree of 90%) acquired from Purifarma (Sao Paulo, SP, Brazil) and glycidyl trimethyl ammonium chloride (content $\geq$ 90% w/w) purchased from Fluka Chemica (Sao Paulo,

SP, Brazil). Glutaraldehyde (GLU—25% in water) was purchased from Vetec (Duque de Caxias, RJ, Brazil) and κ-carrageenan was acquired from Sigma Aldrich (Sao Paulo, SP, Brazil). Deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA), with a resistivity of 18.2 M Ω cm, was used to prepare all solutions. Solutions of 1.0% HACC (w/v), 12.5% GLU (v/v) and 0.1% κ-carrageenan (w/v), prepared in deionized water and filtered through a 0.45 μm membrane, were used for modification of the capillary surface. Phosphoric acid (content 85%) and tris(hydroxymethyl)aminomethane (Tris) purchased from Labsynth (Diadema, SP, Brazil) and absolute ethanol (content 99.9%) acquired from Vetec (Duque de Caxias, RJ, Brazil) were used to prepare the electrolyte for the separation of betaine and methionine. Sodium dihydrogen phosphate, 2-hydroxy-isobutyric acid (HIBA), acetic acid (99.7%), morpholine ethanesulfonic acid (MES), sodium hydrogen carbonate, and sodium carbonate, all of analytical-grade purity acquired from Sigma Aldrich (Sao Paulo, SP, Brazil), were used to prepare buffer solutions and measure the EOF. The buffer solutions used to measure the EOF and their respective pH values were: phosphate (pH 2.4 and 2.8); Tris/HIBA (pH 3.3–4.2; and pH 7.6–8.9); Tris/acetic acid (pH 4.7 and 5.3); Tris/MES (pH 5.9–7.4); and carbonate (pH 9.5), all with an ionic strength of 20 ($\pm$ 1) mmol L^{-1} and a buffering capacity above 6.0 mmol L^{-1}. Buffer solutions were prepared using the chemicals described above dissolved in deionized water and stored in a refrigerator. Final pH values were measured using a pH meter calibrated with aqueous standards at pH 4, 7 and 10, acquired from Sigma Aldrich (Sao Paulo, SP, Brazil). Betaine hydrochloride (content 99%), L-methionine (content 98%) and L-histidine hydrochloride (internal standard—I.S.; content 98%) were acquired from Sigma Aldrich (Sao Paulo, SP, Brazil). Standard stock solutions of betaine (10,000 mg L^{-1}), methionine (2500 mg L^{-1}) and histidine (2500 mg L^{-1}) were prepared in deionized water and stored in a refrigerator for up to a month. Calibration solutions of betaine and methionine were prepared by diluting the stock solutions with a solution containing histidine (IS—concentration injected 50 mg L^{-1}) and ethanol (concentration injected 30%, v/v).

For the comparative LC–MS/MS method HPLC-grade acetonitrile purchased from J.T. Baker (Mexico) and deionized water, were used to compose the mobile phase. Standard stock solutions of betaine (10 mg L^{-1}) and methionine (10 mg L^{-1}) were prepared in deionized water. Calibration solutions of betaine and methionine were prepared by diluting the stock solutions with deionized water and then with acetonitrile in a proportion of 1:1 before injection into the LC–MS/MS instrument.

2.2. Capillary coating procedure

Initially, the capillary was flushed for 30 min with 1.0 mol L^{-1} NaOH and 15 min with deionized water to enhance the dissociation of the silanol groups. The capillary was then coated using the following steps: flush for 10 min with 0.20% HACC solution and maintain in static contact for 10 min; flush for 5 min with 12.5% GLU solution and maintain in static contact for 15 min; flush again for 10 min with 0.20% HACC solution and maintain in static contact for 10 min to generate the crosslinked cationic layer; and finally flush for 10 min with 0.1% κ-carrageenan and maintain in static contact for 10 min to form the outer anionic layer carrying sulfate groups. Lastly, the new multilayer coating was conditioned for 5 min with BGE (without polymer) before the electrophoretic separations. The EOF measurements to characterize the new coated capillary were performed according to the procedure described by Williams and Vigh [41], using acetone as a neutral marker. A scheme of the crosslinked HACC and κ-carrageenan multilayer coating is shown in Fig. 1.
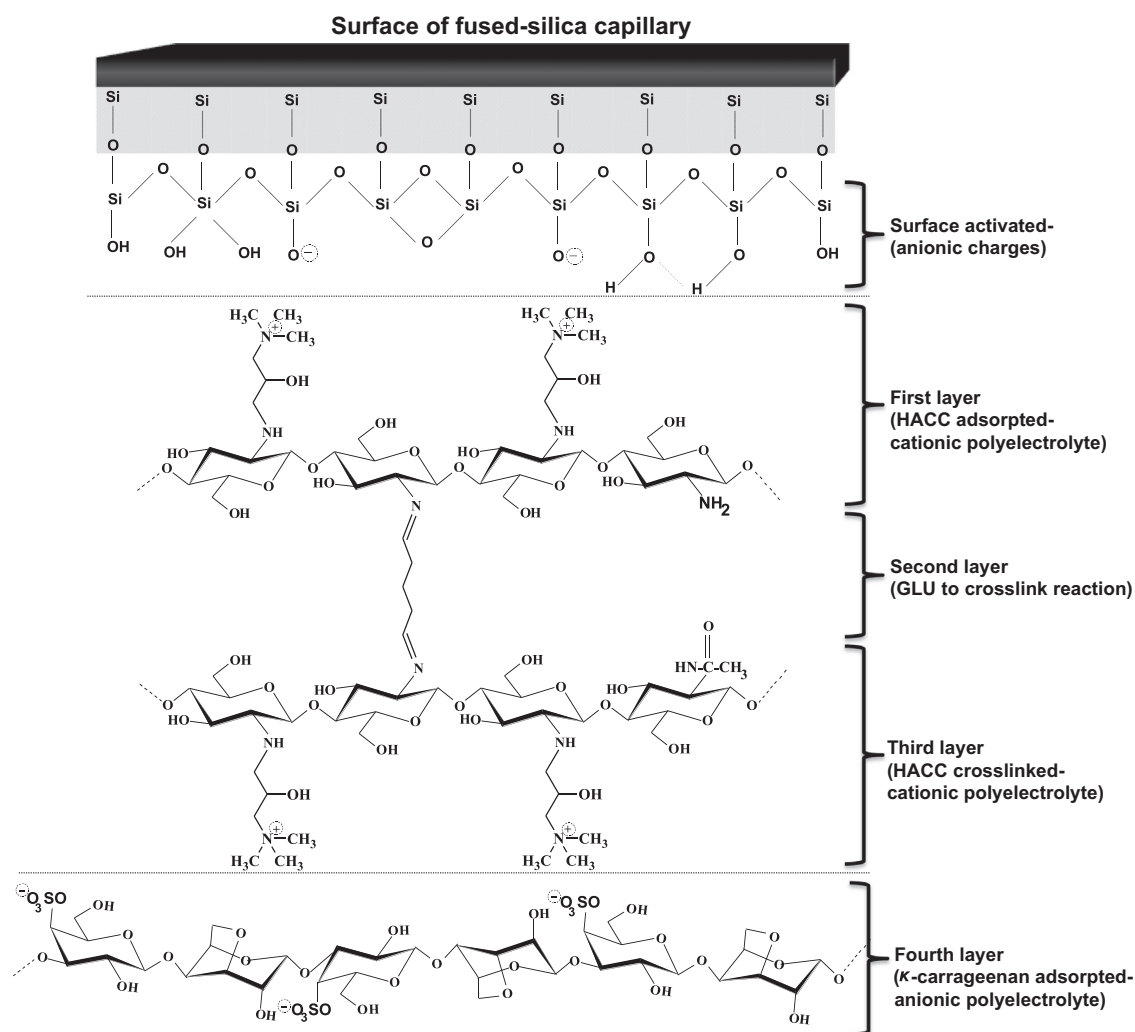
Surface of fused-silica capillary

Fig. 1. Scheme of the crosslinked HACC and κ-carrageenan multilayer coating.

2.3. Capillary electrophoresis system and sample preparation

To optimize the separation all experiments were conducted on a CE system (7100 Capillary Electrophoresis System, Agilent Technologies, Palo Alto, U.S.A.) equipped with a diode array detector set at 195 nm, a temperature control device (temperature maintained at 25 °C), and data treatment software (HP ChemStation). The BGE used to determine the betaine and methionine concentration was composed of 10 mmol L^{-1} Tris, 40 mmol L^{-1} phosphoric acid and 10% (v/v) ethanol, at pH 2.1. The standards and samples were introduced at the short-end of the capillary and injected using a hydrodynamic pressure of 50 mbar for 3 s. The separation voltage applied was -28 kV (positive polarity on the injection side). For all experiments, a fused-silica capillary (Microtube, Araraquara, SP, Brazil) of 32 cm (8.5 cm effective length × 50 μm ID × 375 μm OD) was used.

Six samples of hepatoprotective agent in vial form (different brands and lots) were purchased in local pharmacies. Before the analysis in the CE system the samples were prepared by two dilutions: first the samples were diluted to a 1:25 ratio with deionized water in volumetric flasks and then they were appropriately diluted in the injection vial with a solution containing histidine (IS—concentration injected 50 mg L^{-1}) and ethanol (concentration injected 30%, v/v). The samples were prepared in duplicate and injected in duplicate.

2.4. Comparative LC–MS/MS method

The samples were prepared by successive dilutions (30,000 times) with deionized water and before analysis in the LC–MS/MS system they were diluted 1:1 with acetonitrile. The samples were prepared in duplicate and injected in duplicate.

The method using LC–MS/MS analysis was performed on chromatographic equipment consisting of a high-performance liquid chromatography system (1200 Series Agilent Technologies, Germany). Separation was performed on a SeQuant™ ZIC®-HILIC column (150 mm, 2.1 mm ID, 3.5 μm particle size) Merck (Darmstadt, Germany). The runs were performed in isocratic mode using a mobile phase composed of 70% solvent A (H_2O) and 30% solvent B (acetonitrile). The flow rate was set at 100 μL min^{-1}. In all instances, the injection volume was 2 μL. The column temperature was set at 30 °C. The LC system was coupled to a mass spectrometer consisting of a hybrid triplequadrupole/linear ion trap mass spectrometer (Q Trap 3200 Applied Biosystems/MDS Sciex, Concord, Ontario, Canada). Analyst version 1.5.1 was used for the LC–MS/MS system control and data analysis. The mass spectrometer was tuned to the negative and positive modes with the infusion of polypropylene glycol solution (Applied Biosystems, Concord, Ontario, Canada). The experiments were performed using the TurboIonSpray™ source (electrospray-ESI, Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) in positive ion mode.

The capillary needle was maintained at 5500 V. The MS/MS parameters were: curtain gas, 10 psi; temperature, 350 °C; gas 1, 45 psi; gas 2, 45 psi; CAD gas, medium. Methionine and betaine were monitored and quantified using multiple reaction monitoring (MRM). Optimization of the mass spectrometer was performed by the direct infusion of an aqueous solution containing the two analytes investigated herein.

3. Results and discussion

3.1. Crosslinked HACC and κ-carrageenan multilayer coating

3.1.1. Multilayer coating and EOF characterization

The modification of the EOF using κ-carrageenan reported in this paper was aimed at obtaining a semi-permanent, non-covalent coating, i.e., modifying the capillary without the use of the polymer as an additive in the BGE to control the EOF. Therefore, in order to allow maximum fixation of the anionic polymer on the capillary wall, a cationic polymer layer was attached to the silica, composed of HACC crosslinked with GLU, to function as a binding layer between the silica and the κ-carrageenan. The cationic layer was obtained simply by flushing the capillary with solutions of HACC, GLU and once again HACC, similarly to the procedure used to form a layer of crosslinked CTS described in the literature [14]. The modification with κ-carrageenan was performed on top of this cationic layer by flushing the capillary with a solution of the anionic polymer. The cationic layer interacts strongly through electrostatic attraction with the ionized silanols of the silica and also with the sulfate groups of the κ-carrageenan, allowing the fixation of the anionic polymer onto the wall. The presence of amino and quaternary amino groups in the structure of HACC contributes to good κ-carrageenan fixation. The density of the positive charges of the HACC is attributed to amino groups present especially at pH < 5 due to the pKa (around 6.5) [42], this therefore being susceptible to variations in pH. However, the contribution of the charge of quaternary amino groups remains constant regardless of the pH of the medium. Moreover, the presence of hydroxyl groups in the HACC and κ-carrageenan also contributes to the interaction between these polymers via hydrogen bonding.

To determine the κ-carrageenan solution concentration that should be used in the last coating step, the capillary was flushed with different κ-carrageenan solutions ranging from 0.0 to 0.1% (w/v) and the EOF mobility was measured with phosphate buffer, at pH 2.8 and with an ionic strength of 20 mmol L^{-1} (5 min flushing with BGE between runs). The results are shown in Fig. 2a. The EOF mobility was −33.2 (± 2.4) × 10^{-9} m^2 V^{-1} s^{-1} (n=2, anodic, characteristic of crosslinked HACC) with 0.0% κ-carrageenan and decreased gradually with increasing anionic polymer concentration until being reversed with a value of 14.1 (± 2.6) × 10^{-9} m^2 V^{-1} s^{-1} (n=2) at 0.01% of polymer (cathodic, characteristic of κ-carrageenan). At 0.05% of anionic polymer the EOF reached its maximum of around 30.1 (± 1.2) × 10^{-9} m^2 V^{-1} s^{-1} (n=2) and remained practically constant with higher concentrations of polymer. Thus, the concentration of 0.1% κ-carrageenan was used to form the last layer of the multilayer coating.

The variation in the EOF as a function of pH obtained for the new multilayer coating with crosslinked HACC and κ-carrageenan is shown in Fig. 2b. In contrast to the uncoated fused silica capillary, which presented a cathodic EOF with a sigmoidal profile due to the pKa of the silanol groups (pKa∼4–6) [43], the new multilayer coating showed a cathodic EOF but the mobility value observed was practically constant (around 30 × 10^{-9} m^2 V^{-1} s^{-1}) across the pH range studied. Thus, an important difference observed for the new coated capillary was the enhanced cathodic
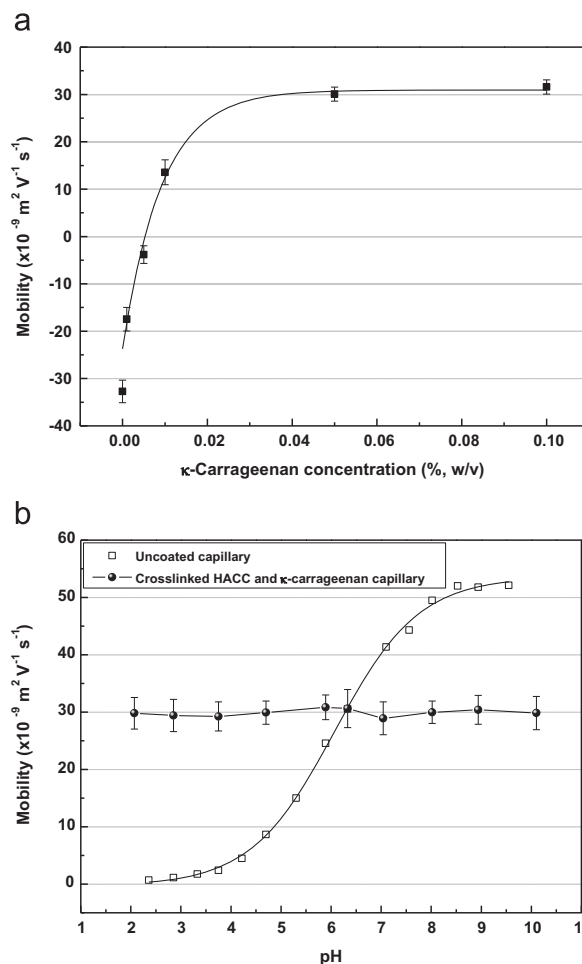
Fig. 2. (a) Variation in the EOF mobility as a function of the concentration of κ-carrageenan used in the last coating step of the procedure applied to construct the multilayer coating (n=2, same capillary). (b) Variation in the EOF mobility as a function of pH for the new capillary coated with crosslinked HACC and κ-carrageenan (n=4, four measurements in two different capillaries coated in the same way) and for an uncoated capillary (n=1). Error bars represent standard deviation of the measurements. Measurement of EOF mobility using the pressure method (± 10 kV/0.75 min) described by Williams and Vigh [41]. Electrolytes with ionic strength 20 (± 1) mmol L^{-1}. Flushing between runs for 3 min with BGE.

EOF at low pH (2.0–4.5). This occurs mainly due to the presence of sulfate groups in the structure of the κ-carrageenan which has a pKa of around 0.5 (pKa of the D-galactose-4-sulfate, determined using Pallas 3.1 TM demo version software calculations), leading to a negative charge density on the wall even at low pH. The reproducibility of the EOF for the new multilayer coating evaluated across the whole pH range (2–10, ten levels) showed an average coefficient of variation (CV) of less than 8% (n=4, intercapillary). The pH-independent cathodic profile of the EOF mobility obtained with the crosslinked HACC and κ-carrageenan system was compared to the other multilayer coatings comprised of polymers describe in the literature. For example, the polybrene and dextran sulfate coating showed a pH-independent EOF of around 37 × 10^{-9} m^2 V^{-1} s^{-1} (pH range of 2–11) and CV of less than 1% (n=3, intercapillary, measured at pH 7) [2]; the polybrene and poly(vinylsulfonate) coating showed a pH-independent EOF of around 49 × 10^{-9} m^2 V^{-1} s^{-1} (pH range of 2–10) and CV better than 2% (n=6, intercapillary, measured at nine pH levels across the entire pH-range) [8]; and the poly(diallyldimethylammonium chloride) and poly(styrene sulfonate) coating showed a weak dependence on pH (range of 4–8) with an EOF of around 42 × 10^{-9} m^2 V^{-1} s^{-1} (at pH 6) and CV of less than 2% (n=11,

intercapillary, measured at pH 4 and 6) [13]. Although the reproducibility of the EOF for new multilayer coating provided a CV of 8%, the result can be considered acceptable, since in the evaluation a wide range and several levels of pH were considered in the calculations. Thus, the EOF characteristics obtained for the new multilayer coating reveal good potential for its application in the development of CE methods.

3.1.2. Chemical stability and repeatability

The chemical stability of the new multilayer coating was evaluated using different solutions and solvents, comparing the EOF mobility before and after flushing the coated capillary with the test solution. The BGE used in these experiments was a mixture of Tris and HIBA, pH 3.3 (ionic strength 20 mmol L^{-1}). The EOF mobility showed good stability after flushing the capillary with the acidic solutions (hydrochloric acid and acetic acid, both at 0.1 mol L^{-1}) and with organic solvents (ethanol and acetonitrile). The variation in the EOF mobility in these cases was less than 5.3% ($n=3$). With a 5 mol L^{-1} urea solution the variation in the EOF was more expressive, but the value was still acceptable (12%, $n=3$). Employing a 0.1 mol L^{-1} NaOH solution led to the greatest percentage variation in the EOF (195%, $n=3$) and reversal of the EOF from cathodic to anodic. An anodic EOF is characteristic of the crosslinked HACC. Thus, the anionic layer of κ-carrageenan was removed by the NaOH solution. However, after flushing with an acid solution (15 volumes, solution of pH 3.0) the fraction of amino groups of the crosslinked HACC layer was protonated increasing the positive charges available to interact once again with the anionic polymer. On repeating the last step of the capillary coating procedure with κ-carrageenan solution, the coating is easily recovered. The values for the EOF mobility after flushing the coated capillary three times with 0.1 mol L^{-1} NaOH and regenerating only the layer of κ-carrageenan were 29.9 × 10^{-9} m^2 V^{-1} s^{-1}, 27.9 × 10^{-9} m^2 V^{-1} s^{-1} and 28.7 × 10^{-9} m^2 V^{-1} s^{-1}, respectively. The CV for these EOF mobility values was less than 3%, showing a good capacity for the regeneration of the new multilayer coating. The chemical stability of the crosslinked HACC layer for all test solutions described above showed a variation in the EOF of less than 5%. This chemical stability of the crosslinked HACC layer is an important feature since in cases where the last layer is removed it enables its fast reconstruction with κ-carrageenan. This is possible mainly due to the crosslinking reaction which leads to the formation of a resistant cationic layer of HACC, a phenomenon similar to that described in the literature when capillary coating of chitosan crosslinked with GLU is compared with a non-crosslinked coating [14]. When compared with an SMIL coating comprised of polybrene and dextran sulfate [2], the new multilayer coating proposed herein, despite having a lower chemical stability under alkaline conditions, showed greater stability in 0.1 mol L^{-1} HCl. This result suggests that a major advantage of the new coated capillary, that is, the enhanced mobility of the EOF at low pH, can be exploited with good chemical stability of the coating, contributing to good performance in methods where these pH conditions are employed. A point worth noting in relation to the κ-carrageenan polymer is its involvement in the formation of rigid gels in the presence of potassium ions [44]. Hence, these ions should not be used in the BGE composition in order to avoid changes in the properties of the multilayer coating.

Another important point to be considered is the repeatability associated with the new multilayer coating over several runs. This study was performed in BGEs at pH 2.36 (phosphoric acid/sodium dihydrogen phosphate) and at pH 8.94 (Tris/HIBA), both with an ionic strength of 20 ($\pm$ 1) mmol L^{-1}. The experimental conditions used for 150 runs at each pH were: injection of acetone as neutral marker, flushing the capillary between runs for 1 min (equivalent to 5 volumes of flushing) and an applied voltage of 25 kV for

2 min. The results showed that at pH 2.36 the EOF remained stable at around 30 × 10^{-9} m^2 V^{-1} s^{-1} for 130 runs, and then showed a slight decrease to 27 × 10^{-9} m^2 V^{-1} s^{-1}, maintaining this value until the end of the runs. The CV of the EOF measurements was less than 5% for 150 runs at pH 2.36. The EOF at pH 8.94 remained practically constant at around 32 × 10^{-9} m^2 V^{-1} s^{-1} and the CV was less than 1.5% for 150 runs, demonstrating the good stability of the EOF in the coated capillary.

3.2. Application of the new multilayer coating in betaine and methionine separation

3.2.1. Selection of BGE components and internal standard

The development of a fast separation method with direct detection by CZE is dependent on the right choice of the BGE components, which should be based on the mobility curves of the analytes and their structural characteristics since these will influence the detectability. The effective mobility curves for betaine and methionine shown in Fig. 3 verify that the difference between the mobility of the two analytes is small and both present positive mobility only at very low pH (pH < 3.0). At pH greater than 3.0 the resulting charge of the betaine is very close to zero (mobility almost zero), hindering the separation by CE. According to the curve for the difference in the mobility of the analytes, the most appropriate pH range for the separation is from 2.0 to 2.5, that is, where the maximum difference in mobility occurs. However, at pH around 2.5 the mobility of the analytes is very low ($< 10 × 10^{-9}$ m^2 V^{-1} s^{-1}) and this causes the migration of the analyte peaks to values very close to the EOF peak. This can result in resolution problems in the separation, especially in the case of betaine which has the lowest mobility. At pH around 2.5 other molecules that absorb UV radiation and are present in pharmaceutical formulations, such as the preservatives methylparaben and propylparaben, migrate as neutral species exactly at the time of the EOF, generating a peak with a large area and a low efficiency. Therefore, to prevent possible resolution problems, pH 2.1 was chosen for the BGE, at which high mobility values are observed for the species with an acceptable difference between their mobility values. At this very low pH the phosphoric acid can provide the counter-ion dihydrogen phosphate for the BGE. This counter-ion was selected for the application of the method. Phosphoric acid has a pKa value of 2.16 and therefore provides buffering capacity for the BGE. The selection of the co-ion
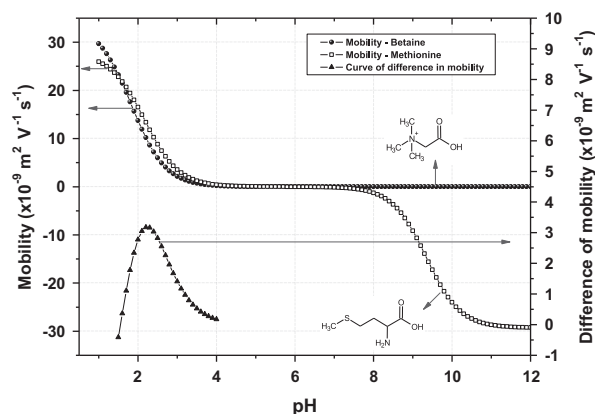

Fig. 3. Effective mobility curves for methionine and betaine and curve for the difference in the mobility of the two molecules. Curves constructed in Excel® using the following data: betaine pKa = 1.83 [38] and μ_e = 34.1 × 10^{-9} m^2 V^{-1} s^{-1} (electrophoretic mobility determined by pressure using 10 kV/0.75 min through the Williams and Vigh method [41], BGE 22 mmol L^{-1} phosphoric acid, ionic strength 10 mmol L^{-1}, pH 2.0); methionine pKa$_1$ = 2.17 and μ_e = 27.7 × 10^{-9} m^2 V^{-1} s^{-1}; pKa$_2$ = 9.34 and μ_e = −29.3 × 10^{-9} m^2 V^{-1} s^{-1} (data from Peakmaster® software).

component of the BGE for the separation and direct detection of betaine and methionine was carried out seeking a molecule that does not absorb at the same wavelength as the analytes, to avoid interference in the detection. Thus, the co-ion chosen was Tris, because this has no carbonyl groups in its structure. These groups are responsible for allowing the direct detection of the analytes, especially betaine at 195 nm. The internal standard selected for use in the method, without compromising the analyte separation time, was histidine.

After the selection of the components of the BGE an experimental run was performed on the capillary coated with the new multilayer coating, the BGE being comprised of 10 mmol L^{-1} Tris and 40 mmol L^{-1} phosphoric acid. The BGE had a conductivity of less than 0.4 S m^{-1}, ionic strength of around 18 mmol L^{-1} and buffering capacity of approximately 40 mmol L^{-1}. When these separation conditions were tested, on the injection of a sample the co-migration of the analytes was observed. This occurs due to the small difference in the effective mobility values for the analytes. Additionally, the enhanced mobility of the EOF (30×10^{-9} m^2 V^{-1} s^{-1}) in the coated capillary did not allow an effective separation of the analytes. One approach to resolving this problem is to adjust the EOF mobility by reducing its intensity, which was carried out through the addition of ethanol to the BGE. Ethanol was also added to the sample to adjust the conductivity of the plug, leading to an effective separation of the analytes.

3.2.2. Influence of the organic solvent on the separation system

The influence of ethanol in the sample and BGE was investigated to optimize the resolution and separation time of the analytes using concentrations of this organic solvent of 10, 20 and 30% (v/v). Based on the results obtained it was possible to verify an improvement in the resolution of the analytes with an increase in the ethanol content of the sample and BGE, and this effect was more pronounced when ethanol was added to the BGE, because it caused a decrease in the EOF mobility of the coated capillary. However, increasing the ethanol concentration in the BGE causes an increase in the separation time, affecting the instrumental throughput of the method. This decrease in the EOF mobility is strongly related to the simultaneous change in two physico-chemical properties of the BGE: the viscosity and dielectric constant [45]. With increasing ethanol concentration in the BGE the viscosity increases, while the dielectric constant decreases. According to the Smoluchowski Equation the mobility is directly proportional to the dielectric constant and zeta potential, and inversely proportional to the viscosity [45]. Thus, the use of ethanol in the BGE causes a decrease in the EOF mobility allowing this electrophoretic parameter to be adjusted as desired. This behavior is a result of the influence of the organic solvent in the dielectric properties of the electric double layer and the charges on the capillary surface [45,46]. Besides the effect on the EOF mobility, the organic solvent influences the effective mobility of the betaine and methionine, this being dependent on the actual mobility at that time (which is affected by the viscosity, among other factors), the pKa, and the pH. All of these three parameters are dependent on the organic solvent [47,48]. A similar influence of the organic solvent on the effective mobility of the BGE components is observed and other parameters of the BGE, such as pH and conductivity, also are affected. Therefore, the use of an organic solvent in the CZE system modifies the separation conditions, contributing to an improvement in the selectivity of the analyte separation. Thus, seeking an adequate separation resolution and a time ratio which provides fast analysis, the conditions chosen were 30% ethanol in the sample and 10% ethanol in the BGE. The EOF mobility measured under this condition using ethanol in the BGE was 22×10^{-9} m^2 V^{-1} s^{-1} with a good
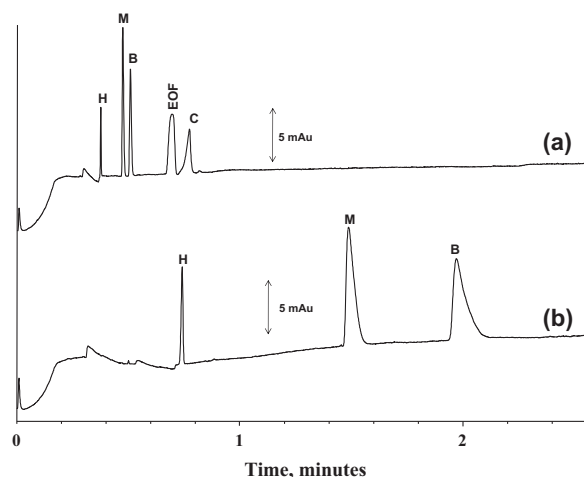
Fig. 4. Electropherogram of a hepatoprotective sample obtained employing the optimized method in a capillary with multilayer coating comprised of crosslinked HACC and κ-carrageenan (a) and in an uncoated capillary (b). Experimental conditions: capillary 32 cm × 50 µm ID, BGE 10 mmol L^{-1} Tris, 40 mmol L^{-1} phosphoric acid and 10% (v/v) ethanol, at pH 2.1, voltage 28 kV, injection 50 mbar/3 s (short-end injection, L_{det}=8.5 cm). Injected sample diluted 50 times using 30% (v/v) of ethanol, with informed concentration of 10 mg mL^{-1} methionine and 50 mg mL^{-1} betaine. Data acquired at 195 nm. Legend: H-histidine (IS); M-methionine; B-betaine; EOF-electroosmotic flow; C-citrate.

repeatability (CV < 1.1% for 35 measurements of EOF mobility), representing a reduction of 27% in the EOF mobility compared with the BGE without ethanol.

3.2.3. Separation of the betaine and methionine in the new coated capillary and in an uncoated capillary

The separation of betaine and methionine in a hepatoprotective sample using the optimized method was carried out in a modified capillary coated with crosslinked HACC and κ-carrageenan and the results were compared to those obtained for an uncoated capillary (Fig. 4). It was observed that although the resolution of the separation in the coated capillary (Rs=1.37) is lower than that in the uncoated capillary (Rs=3.7), the separation efficiency for betaine and methionine in the coated capillary (see data in Table 1) is close to or better than that in the uncoated capillary (methionine 50.7×10^3 plates per meter, peak width at the baseline 0.09 min; betaine 29.9×10^3 plates per meter, peak width at the baseline 0.15 min). Moreover, the analyte separation time for the coated capillary (betaine $t_{migr} \sim 0.51$ min) was almost a quarter of that for the uncoated capillary (betaine $t_{migr} \sim 2.10$ min), highlighting fast separation as an advantage of the method developed. Another comparison carried out by injecting the same sample into an uncoated capillary without the addition of ethanol to the sample and BGE showed that, although there was an improvement in the analyte resolution (Rs=3.8), the separation efficiency decreases considerably (methionine 23.9×10^3 plates per meter, peak width at the baseline 0.14 min; betaine 14.6×10^3 plates per meter, peak width at the baseline 0.28 min) and a significant increase in the separation time is observed (betaine $t_{migr} \sim 2.5$ min —electropherogram not shown). The low EOF mobility at pH around 2.1 in the uncoated capillary (EOF peak not detected until 10 min) increased the analyte separation time and this causes an increase in the analyte residence time inside the capillary, leading to a reduction in the separation efficiency.

Another advantage of the new coated capillary is the possibility of developing a method of separation without the capillary flushing step between runs [49]. This strategy can be applied in the analysis of samples with a known matrix which is not too

complex and contains few components, as in the case of drugs. A hepatoprotective sample was injected 31 times into the new coated capillary without flushing between runs and the 31 electropherograms obtained showed only the peaks of the betaine, methionine, EOF and citrate, which is present in the sample matrix. No other peaks which could interfere in the separation of the proposed method without flushing were observed, confirming its applicability. In an uncoated capillary it would not be possible to employ this strategy, since the EOF peak migrates with a lower mobility and could interfere with the peaks of the analytes, migrating onto them during the separations, i.e., EOF peaks of the initial injections can migrate between the analyte peaks of the subsequent injections, interfering in the separations. The instrumental analysis time (IAT) for the optimized method using the new coated capillary without flushing between runs was 1.53 min (approx. 39 runs per hour). The proposed method without flushing allowed an increase of 13 runs per hour in relation to the method employing flushing between runs (IAT 2.3 min using 0.33 min of flushing—corresponding to 26 runs per hour).

3.3. Method validation

The evaluation of the proposed method optimized for the determination of betaine and methionine in pharmaceutical formulations was performed in accordance with the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)

Table 1
Figures of merit of the optimized method for the determination of betaine and methionine in pharmaceutical formulations by CZE using a capillary multilayer coated with crosslinked HACC and κ-carrageenan. For other conditions see experimental section.

Figure	Analyte	
	Betaine	Methionine
Regression equation-calibration range (mg L^{-1})[a]	100–700	50–500
Regression equation-slope (L mg^{-1})[a]	0.0042	0.0213
Slope standard deviation[a]	0.0002	0.0006
Regression equation-intercept[a]	0.0498	0.1054
Intercept standard deviation[a]	0.0097	0.0161
Regression equation-coefficient of determination (R^2)[a]	0.9997	0.9999
F significance[b]	4.0×10^4	1.4×10^5
Limit of detection, LOD (mg L^{-1})[c]	7.62	2.49
Limit of quantification, LOQ (mg L^{-1})[c]	23.1	7.56
Intra-day precision, CV (%)—peak area[d]	1.2 (2.3)	1.1 (1.3)
Intra-day precision, CV (%)—migration time[d]	1.1 (1.2)	1.1 (1.1)
Inter-day precision, CV (%)—peak area[d]	1.6 (2.1)	1.5 (1.9)
Inter-day precision, CV (%)—migration time[d]	1.7 (3.3)	1.6 (3.0)
Number of plates (N m^{-1})[e]	49.9×10^3	47.4×10^3
Peak width (min)—at the base	0.035	0.032
Peak tailing factor [e]	1.11	1.12
Resolution (betaine:methionine)[e]	1.37	

[a] Curve with six levels of betaine and seven levels of methionine, prepared in two authentic replicates. External calibration curve with internal standard correction.
[b] Tabulated F: $F_{1/10} = 4.96$ for betaine and $F_{1/12} = 4.75$ for methionine.
[c] Limits of detection and quantification calculated according to the equations: $LOD = (3.3 \times s)/S$; $LOQ = (10 \times s)/S$, where s is the intercept standard deviation and S is the slope of the external analytical curve equation.
[d] Coefficient of variation (CV) values for instrumental precision measured in the same solution: intra-day precision with 8 preparations at the same concentration and inter-day precision with 4 preparations on one day and 4 the next day. Intra-day precision $n = 8$ and inter-day precision $n = 8$. CV values in brackets refer to a pharmaceutical sample diluted 50 times.
[e] $N = 16 (t_i/w_b)^2$, where t_i is the migration time of analyte given in min and w_b is the peak width at the baseline, in the same units t_i. N results divided by 0.085 m (L_{det}). $Rs = 2 (t_n - t_{n-1})/(w_n + w_{n-1})$, where t is the peak migration time and w is the width of the base. Concentration of betaine and methionine around 700 and 350 mg L^{-1}, respectively. Tailing factor of the peaks for $n = 3$.

Table 2
Verification of the selectivity of the method for determination of betaine and methionine in pharmaceutical formulations using standard addition method. Determination coefficients > 0.99.

Analyte	Concentration (mg L^{-1})[a]		Recovery (%)[b]
	Added	Found[b]	
Betaine	100	98	98
	200	198	99
	300	302	101
	400	431	108
	500	498	99
Methionine	50	49	98
	100	99	99
	150	151	101
	200	203	102
	250	248	98

[a] Recovery using hepatoprotective sample diluted 50 times. Regression equations: betaine $y = 0.0046$ (± 0.0004)$x + 0.9519$ (± 0.0741) and methionine $y = 0.0201$ (± 0.0011)$+ 3.9369$ (± 0.2438). Concentrations of betaine and methionine in the samples were 10.3×10^3 mg L^{-1} and 9.8×10^3 mg L^{-1}, respectively.
[b] Average results for two measurements of one replicate ($n = 2$).

[50] using the validation parameters: linearity, limits of detection and quantification, precision and selectivity. The results are shown in Table 1.

With the aim of obtaining quantification models through the use of ordinary least square regression, external calibration curves with internal standard addition employing six levels of betaine (100–700 mg L^{-1}) and seven levels of methionine (50–500 mg L^{-1}) standards, both in authentic replicates ($n = 2$) were randomly constructed. Some statistical assumptions, including homoscedasticity (Cochran's test), lack of fit through a priori test hypothesis (ANOVA) as shown in Eq. (1) [51], and normality test (Shapiro–Wilk) of residuals were verified (95% confidence interval).

$$F_{calc} = \frac{S_{y,x}^2}{S_y^2} = \frac{\sum_{i=1}^{p} m_i (\overline{y}_i - \hat{y}_i)^2 / (p - 2)}{\sum_{i=1}^{p} \sum_{j=h}^{m_i} (y_{ij} - \overline{y}_i)^2 / (m - p)} \quad (1)$$

The regression coefficients are shown in Table 2. The values calculated in the Cochran's test (0.742 for betaine and 0.617 for methionine) are both lower than the tabulated values (0.781 for betaine and 0.727 for methionine), indicating homoscedastic behavior. The p-values obtained in the ANOVA (0.08 and 0.22) and normality of residuals (0.431 and 0.371 for betaine and methionine, respectively) are considered satisfactory within the confidence interval considered, since they are higher than $\alpha = 0.05$. Additionally, the regression significance values (F significance, $F_{sig} = 4.0 \times 10^4$ and 1.4×10^5, for betaine and methionine curves, respectively) are statistically acceptable, since they are higher than at least ten times the tabulated F ($F_{1/10} = 4.96$ and $F_{1/12} = 4.75$, for betaine and methionine, respectively). Thus, the linearity can be considered to be acceptable within the concentration ranges mentioned above and the optimized method can be used to quantify betaine and methionine in the pharmaceutical samples.

The limits of detection and quantification obtained are considered to be suitable for the concentrations of the two amino acids in the samples of the pharmaceutical formulations. The results for the inter-day precisions showed low CV values (< 3.5%) for the migration time and peak area using standard and sample solutions. The separation efficiency, peak symmetry and resolution observed for the analytes are considered suitable for the separations.

The matrix effect of the proposed method was evaluated using the method of standard addition due to difficulty in obtaining a matrix free of the active ingredient. The results obtained are

Table 3
Results for the determination of betaine and methionine in pharmaceutical formulations using the optimized CZE-UV method and the comparative method of LC–MS/MS. For experimental conditions see experimental section.

Sample	Betaine (mg mL^{-1})		Methionine (mg mL^{-1})	
	CZE-UV[b]	LC–MS/MS[c]	CZE-UV[b]	LC–MS/MS[c]
A1[a]	–	–	9.1 ± 0.1	8.9 ± 0.6
A2[a]	–	–	9.4 ± 0.1	9.5 ± 0.5
B1[a]	9.9 ± 0.3	9.4 ± 0.2	9.2 ± 0.2	9.2 ± 0.8
B2[a]	9.8 ± 0.2	9.5 ± 0.2	9.2 ± 0.1	8.9 ± 0.8
B3[a]	9.7 ± 0.5	9.5 ± 0.3	9.1 ± 0.3	8.8 ± 0.3
B4[a]	9.4 ± 1.0	9.4 ± 0.3	9.0 ± 0.7	8.9 ± 0.4

[a] A–B represents different brands, and 1–4 different lots.
[b] Data obtained at 195 nm, and results expressed as mean with confidence level of 95% for $n=4$.
[c] Data obtained at 118.086/59.0 for betaine and 150.115/104.1 for methionine. Regression equation of betaine: slope 1.51×10^6 L mg^{-1}, R^2 0.999, LOD 0.01 mg L^{-1}, LOQ 0.03 mg L^{-1}. Regression equation of methionine: slope 2.56×10^6 L mg^{-1}, R^2 0.999, LOD 0.02 mg L^{-1}, LOQ 0.07 mg L^{-1}. Parameters using eight levels of concentration.

reported in Table 2. The slopes of the standard addition curves (betaine 0.0046 ± 0.0004 and methionine 0.0201 ± 0.0011) and external standard calibration curves (betaine 0.0042 ± 0.0002 and methionine 0.0213 ± 0.0006) were compared. The similarity of these slopes, i.e., ratio between the slopes of the calibration curves around the unit, indicates that the method is not influenced by matrix effects [52]. Moreover, the recovery results showed good agreement (97–108%, recovery) with the reference values. Thus, the method described herein showed appropriate selectivity for betaine and methionine in pharmaceutical formulations.

3.4. Sample analysis

The results for the quantification of the betaine and methionine in the hepatoprotective samples using the optimized CZE-UV method and the comparative LC–MS/MS method are shown in Table 3. Statistical analysis using the paired t-test for the determination of betaine and methionine in the samples applying the two methods showed that the results are statistically equal with a confidence level of 95%, because $t_{calc} < t_{critical}$ (betaine $t_{calc}=2.23$, $t_{critical}=2.45$, NDF=6; methionine $t_{calc}=1.09$, $t_{critical}=2.23$, NDF=10).

4. Conclusions

A novel capillary multilayer coating using crosslinked HACC and κ-carrageenan prepared by simple flushing with solutions of the modifiers was developed. The application of the multilayer coating in the development of a method for the determination of betaine and methionine in pharmaceutical formulations using CZE showed good performance. The new coated capillary was crucial to obtaining a fast separation of the analytes using the sequential injection mode. The pH-independent cathodic EOF mobility obtained with the new modified capillary verified its potential for application in other CE methods, especially given the simplicity and stability of the modification. Moreover, the multilayer coating permits the use of an organic solvent in the BGE to promote the adjustment of the EOF without the destruction of the polymeric layer. Other molecules with structural characteristics similar to those of betaine could also be determined by CZE using fast methods such as that developed in this study exploiting the properties of these new coatings.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.01.047.

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