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Properties of poly-aminophenylboronate coatings in capillary electrophoresis for the selective separation of diastereoisomers and glycoproteins

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Abstract

The polymerisation of 3-aminophenylboronic acid (APBA) in aqueous environment has been used for the open tubular modification of capillary electrophoresis (CE) capillaries. Being poly-APBA endowed with boronic acid, aromatic rings and secondary amines groups, it posses a variety of functional groups affecting selectivity. Diastereoisomers (e.g. ascorbic and isoascorbic acid) and proteins (e.g. haemoglobins) were successfully separated onto poly-APBA column, by means of a combination of electrophoresis and open tubular electrochromatography. The mechanism of selection was investigated: results indicate an interplay between enhancing or silencing the contribution of the protonable functionalities (amino groups, boronic acid). The properties of APBA polymer coating make it attractive for CE separation and for further application in affinity separations and chip technologies.

Keywords: Coated columns; Diastereomer separation; Aminophenylboronic acid; Glycoproteins; Ascorbic acid; Hemoglobins

1. Introduction

The development of selective materials, innovative stationary phases and molecular selectors and their employment in capillary electrophoresis (CE) and in separative nanotechnologies, has high priority in the scientific community and attracts a lot of interest among the engineers involved in the practical analytic and separation work [1–4]. The selection properties of a material are related to its chemical functionalities and ultimately to the complementarity with the target analyte. The formation of the complex between the analyte and the selective material can be described thermodynamically as a sum of binding energy contributions [5,6]. Thus, a primary research objective is currently the achievement of an adequate understanding of the fundamentals of the recognition mechanism in order to be able to manipulate the recognition as needed. In this respect, the approaches in progress comprise the use of molecular modelling and chemometric for the comprehension of the binding event and for the design of selective polymers [7–9]. Beside the search for guidelines for a more rational approach to impart selection properties, research is devoted to the exploration of new synthetic approaches for the creation of new selective phases [10,11]. Various synthetic ligands are available for the design of selective separation phases. Among these ligands are iminodiacetic acid which has been widely used because of its chelating properties [12,13] and boronic acid, capable of forming reversible covalent complexes with *cis*-diol-containing molecules [14–17]. Examples of separation phases containing boronic acid includes affinity chromatography supports, where aminophenylboronic acid is immobilised onto dextranes [15] or copolymerised with methacrylates [17].

None of the already described materials make use of the direct polymerisation of aminophenylboronic acid monomers. The aqueous polymerisation of aniline monomers and its derivatives by photoinduction or chemical oxidation is however well a known process [18]. The resulting polymers

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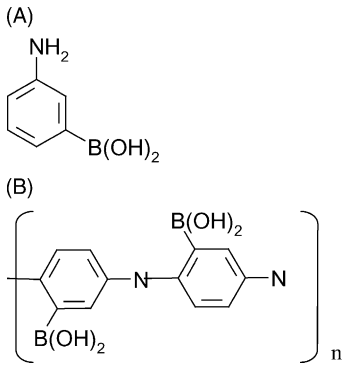

Fig. 1. (A) Monomer of 3-aminophenylboronic acid and (B) polymeric unit.

have a controllable thickness and an excellent ability to coat various different supporting materials (e.g. polystyrene, polypropylene, silica etc.). In the present paper, we describe the polymerisation of aminophenylboronic acid monomers into silica capillaries and the subsequent employment of the poly-APBA columns in CE separations and open tubular CEC separations [19].

The attraction in using this material for separation lies in its chemical structure, which contains an aromatic backbone, boronate groups and secondary amine functionality, as shown in Fig. 1. It was anticipated that the poly-APBA modified columns would have selective properties towards small molecules such as diastereoisomers of ascorbic acid, as well as glycosilated macromolecules such as haemoglobin A1c. An interesting contribution into recognition process was also expected from conjugated aromatic rings and secondary amino groups.

2. Materials and methods

2.1. Reagents

Ammonium persulphate, glutamic acid, 2-morpholinoethanesulfonic acid (MES), *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (TAPS), 2-(cyclohexylamino)ethanesulfonic acid (CHES), tris(hydroxymethyl)aminomethane (Tris), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO), Tween-20, D-isoascorbic acid and L-ascorbic acid, aspartyl-aspartate and sodium hydroxide were from Sigma Chemical Co. (St. Louis, MO, USA). Acetic acid, 3-aminophenylboronic acid monohydrate, monosodium dihydrogenphosphate, disodium monohydrogenphosphate, and acrylamide were from Fluka (Buchs, Switzerland). All other reagents were purchased from commercial sources, at analytical grade and used as delivered. Fused-silica capillaries (50 μm i.d. × 375 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. The derivatisation of the CE column with APBA

Fused silica capillaries, 50 μm I.D. were flushed with 1 M NaOH for 1 h, next 1 h with 1 M HCl, again with 1 M NaOH for 1 h and rinsed with water for 2 h. A suspension in water of 200 mM APBA was prepared by heating and sonicating. Ammonium persulphate was dissolved at a concentration of 50 mM in water and degassed for 10 min prior to use. Poly-APBA coating was obtained by filling the capillaries with a 1:1 (v/v) mixture by volume of the ammonium persulfate and the 3-aminophenylboronic acid solutions, by applying reduced pressure (5 psi; 1 psi = 6894.76 Pa) for 1 min. The polymerisation was carried out for 30 min at 22 °C. Then, the APBA modified capillaries were washed with water for 10 min at low pressure and conditioned with running buffer for 20 min.

2.3. Physico-chemical characterisation of APBA polymer

Poly-APBA was polymerised in free aqueous solvent, by mixing 1:1 the 200 mM APBA monomers suspension with 50 mM ammonium persulphate at room temperature for 40 min; the total volume was 5 ml. Prior to use, the polymer particles were subjected to extensive dialysis against 3 l of water, in a 2500 molecular mass cut-off (MWCO). dialysis tube. The process took 48 h; water was changed regularly three times per day. Dialysed poly-APBA particles were titrated with sodium hydroxide. Titrations procedure was: 1 ml poly-APBA particles was diluted with 2 ml of water, then titrated by addition of 2 μmol of titrant each time, the changes in pH were recorded and plotted in function of the quantity of titrant added. The p*K* values of the polymer were calculated from the curve. The buffering power of the polymer in the various range of pH was calculated with the formula: $\beta = \Delta(\text{titrant added})/\Delta(\text{pH})$.

At the same time, the electroosmotic flow (EOF) in APBA polymer was tested: APBA-modified capillaries were conditioned at pH in the range of 4–9. Runs were performed at 10 kV, acrylamide 3 mM was used as neutral marker. The value of EOF were calculated from the transit time of the marker and plotted as function of pH.

2.4. Measurement of the degree of derivatisation

The degree of derivatisation was measured indirectly for each APBA-modified capillary, by injecting the dipeptide marker glycyl–histidine at a concentration of 1 mg ml^{-1} (injection time: 10 s on Waters instruments and 2 psi s on a Bio-Rad instrument), running the column at pH 6 in 50 mM buffer phosphate and calculating the EOF. The 84% of the derivatised columns showed an EOF value of 9×10^{-5} cm^2 V^{-1} s^{-1}, while in the 16% of the derivatised columns the variation the EOF value was about one order of magnitude greater.

2.5. Peak recovery in APBA columns

A test was devised for monitoring the analyte adsorption (e.g. chemical compounds, proteins) to APBA-modified columns. L-Ascorbic acid was used as test-analyte and the internal standard was methacrylic acid. The modified capillaries were conditioned with 50 mM acetic Tris buffer, pH 5.0. Injections were performed electrokinetically, both with standard procedure, at the inlet of the capillary, and at the outlet of the capillary, replacing the outlet buffer vial with the sample vial and applying the voltage. Applied voltage was 10 kV.

With standard injections, the analyte travelled throughout the major length of the capillary, being detected after 19.4 cm. In case of outlet injection, the analyte migration was from the outlet to the inlet of the capillary, reaching the detector after only 4.6 cm. Peak areas were measured and compared, after normalisation with the methacrylate internal standard. The difference between the two areas was used as an indicator of the analyte adsorption to the APBA-polymeric film.

2.6. Resolution of diastereoisomers on ABPA columns in CE

Analysis of ascorbic and isoascorbic acid on APBA columns in CE were performed on a Bio-Rad (Hercules, CA, USA) Bio Focus 3000 instrument. Coated capillaries (50 μm i.d.) had an effective length of 19.4 cm and total length of 24 cm. Running buffers were: 50 mM glutamic acid pH 3.22; 100 mM acetic acid pH 4.40; 100 mM acetic acid pH 5.00; 100 mM MES, pH 5.6; 100 mM MES, pH 6.05; 100 mM ACES, pH 6.53; 100 mM ACES, pH 7.20; 100 mM HEPES, pH 7.61; 100 mM TAPS, pH 8.14; 100 mM CHES, pH 8.85; 100 mM CAPSO, pH 9.37; 100 mM CAPSO, pH 10.0.

Applied voltages were 10 and 20 kV. Samples containing 100 ppm ascorbic acid and 100 ppm isoascorbic acid were injected by pressure (2 psi s). The detection wavelength was 265 nm. All runs were repeated three times. Resolution was calculated according to the equation: $R_s = 2(t_2 - t_1)/(w_{t_1} + w_{t_2})$, where w_{t_1} and w_{t_2} are the peak widths at 4σ, measured as time units.

2.7. Separation of glyco-macromolecules on APBA columns in CE

Purification of haemoglobin and glycated haemoglobin was done according to the protocol described by Righetti [20]: haemoglobin was recovered from the erythrocytes of a diabetic patient, focused on an immobilised pH gradient of pH 6.8–7.3. The bands corresponding to the two forms of haemoglobin were cut out of the gel and the proteins recovered by electroelution, following the protocol proposed by Righetti et al. [21].

Analyses of glycated macromolecules were performed onto a Waters Quanta 4000E instrument equipped with UV detection. The detection wavelength was 214 nm. Prior to sample loading, the baseline was recorded at 10 kV, until a flat profile was obtained. Sample injection was *via* hydrostatic pressure (2 psi) for 5 s. The neutral marker acrylamide (concentration: 0.07%, v/v) was added as internal standard, prior to injection, to each sample. Running conditions in 50 mM Tris–acetate at pH 8 with polarity towards the cathode were 10 kV were applied. Running conditions in 50 mM phosphate at pH 6.0 were 5 kV applied voltage. A further set of experiments was conducted adding increasing concentration of the detergent Tween-20 to the buffers (0.05, 0.1, 0.2, 0.5 and 1%).

3. Results and discussion

3.1. The derivatisation of the capillary with APBA polymer

Capillary columns have been modified directly with poly-APBA, polymerised in aqueous environment using chemical initiation. The polymerisation procedure is simple and straightforward: a 1:1 (v/v) ratio of the ammonium persulfate (50 mM) and 3-aminophenylboronic acid (200 mM) solutions was flushed into the capillaries and let to polymerise for 30 min at 22 °C. Study on the polymerisation of monomers of the same family of APBA (e.g. aniline) showed that the polymer in solution forms relatively short chains [22], flocculating in aggregates, while on the supporting surface it deposits in a reasonably thin and ordered film [23,24]. The APBA polymerisation in silica capillary results in polymer grafting to the capillary surface (open tubular modification), while aggregates non-attached to the inner surface of the capillary were eliminated with the washing steps. The thickness of the APBA coating was approximately 50 nm, as indicated from previous experiments [18].

The 70% of the initial bound polymer was stable for the subsequent analysis, while a portion of polymer (ca. 30%) was removed during the application of the electric field. The life of the capillary was approximately 100 runs.

Experiments performed to check the degree of derivatisation of the capillary and to check if the polymerisation of APBA gave reproducible derivatisation of the column. The derivatisation was analysed indirectly by measuring the EOF at pH 6.0, using the marker glycyl–histidine. The reproducibility of the derivatisation procedure was estimated from a set of 18 different capillaries coated with APBA and results indicate: the EOF of the 83% of the derivatised columns showed an EOF value of $9{\cdot}10^{-5}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$ (std. 6%), while in the 17% of the derivatised columns the variation the EOF value was one order of magnitude higher. The EOF value reported for pH 6.0 ($\mathrm{EOF} = 9 \times 10^{-5}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$) indicates a reduction of the EOF in APBA coated capillaries of about four times, if compared with the EOF value reported for bare silica capillaries [25].

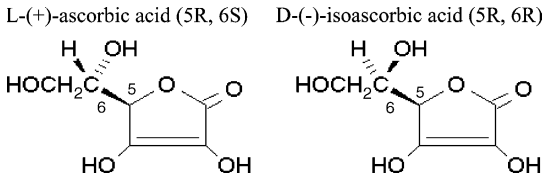

Fig. 2. Comparison between the structure of L- and D-ascorbic acid, named, respectively, ascorbic acid and isoascorbic acid.

3.2. Recognition of diastereoisomers of ascorbic acid on ABPA columns: hypothesis on the mechanisms of selectivity

The recognition properties of poly-APBA were studied analysing migration time of both small molecules and proteins. The diastereoisomers L-(+)-ascorbic acid and D-(−)-isoascorbic acid, shown in Fig. 2, were chosen as suitable analytes for testing the performance of the poly-APBA coating. Normally, the two diastereoisomers are separated in CZE only in the presence of borate buffer at high molarities (200 mM) [28,29]. As recently indicated in a NMR study by Obi et al. [30], such separations results in the formation of different complexes: ascorbic acid forms a 6-member ring complex with borate, while isoascorbic acid forms a 5-member ring complex with borate. A different principle is implicated in the HPLC separations reported where the separation was achieved on aminophases [26], as well as on styrene-type reverse phases [27].

Using an APBA-modified capillary a baseline separation of the diastereoisomers was obtained by running experiments at pH 6.0, as shown in Fig. 3. Applied voltages were both 10 and 20 kV. As default procedure, samples containing 100 ppm ascorbic acid and 100 ppm isoascorbic acid were injected by pressure (2 psi s), analysed at both voltages and detected at 265 nm. The migration order was first isoascorbic and second ascorbic acid, opposite to free zone CE results [28,29], accounting for a mechanism of separation in poly-APBA coated capillaries different from bare capillaries, therefore a mechanism that combines electrophoresis and electrochromatography is supposed. Moreover, the pH of the separation respectively pH 6.0 and 8.5 for poly-APBA and for bare capillaries, thus in the conditions chosen for the separation with poly-APBA columns the EOF is significantly lower.

The highest resolution was obtained by applying 10 kV voltage ($R_s = 1.2$ at 10 kV and $R_s = 0.95$ at 20 kV), contrarily to the capillary electrophoresis dogma assessing that the higher is the field strength the better is the resolution achieved [25]. The same held true in case of polyaniline coatings for the separation of peptides [31]. The possible explanation is a mechanism of separation for the APBA polymer that acts through interactions between the analyte and the polymer coating, as already supposed in case of polyaniline-modified columns.

To elucidate the role of the boronic group in the separation, the migration time of ascorbic and isoascorbic acid was monitored over a pH range from 3.2 to 10.0 and confronted with the protonation state of the poly-APBA coating.

The protonation of APBA polymer versus pH was obtained by measuring the EOF in the capillary (Fig. 4, ◆). Moreover, the titration curve of the polymer as function of the pH was monitored (Fig. 4, ○). Below pH 5.5, poly-APBA is positively charged, thus an inverse EOF is appreciable. Around pH 5.5 poly-APBA is neutral (suggesting a pK for the amino group of about 5.1). In the pH range of 6.5–9.0, the value of the EOF is reasonably steady (ca. 1.6 × 10^{-4} cm^2 V^{-1} s^{-1}). The titration indicates the pKs of boronic acid: $pK_1 = 8.4$ and $pK_2 = 10.5$.

In Fig. 5 is shown the separation of ascorbic and isoascorbic acid along the pH scale, plotted as resolution (R_s) respect the pH. The curve has a Gaussian profile with maximum at pH 6.4. Above pH 6.4 the resolution of the two diastereoisomers decreases, despite no significant increase in the EOF was measured (Fig. 4, ◆). Moreover, at pH values around the pK of boronic acid, no improvement in the separation

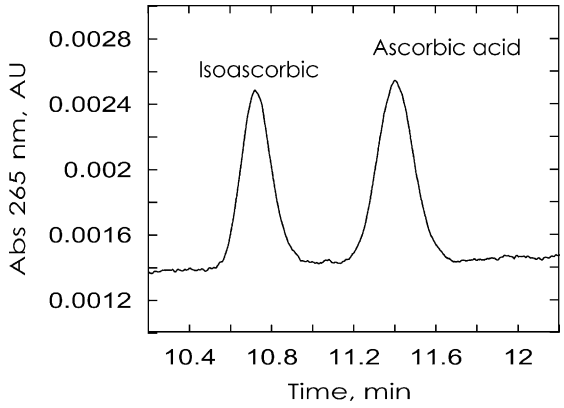

Fig. 3. Separation of ascorbic and isoascorbic acid on APBA modified column. Running conditions were: 100 mM MES Tris buffer, pH 6.0. Experiments were carried out at 10 kV. Sample was injected at a concentration of 100 ppm ascorbic acid and 100 ppm isoascorbic acid.

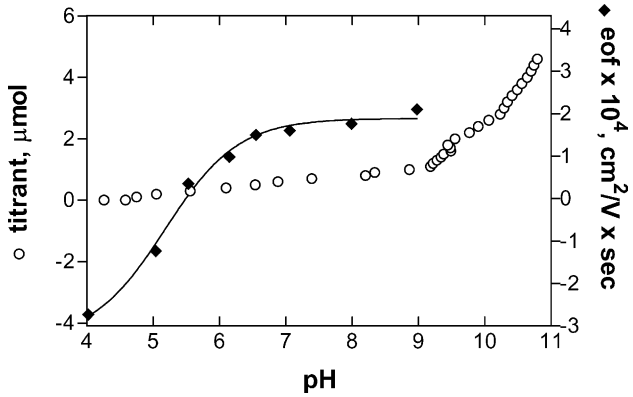

Fig. 4. Plot of the EOF versus the pH in a poly-APBA modified capillary (◆). The plot of the titration of poly-APBA is shown with open circles (○): values of y-axis from 0 to +6 corresponds to the µmol of NaOH added in the titration.

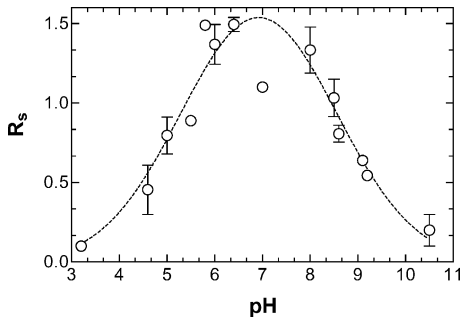

Fig. 5. Influence of the pH of the background electrolyte over the resolution (R_s) of ascorbic and isoascorbic acid. Samples were injected at a concentration of 100 mg l^{-1}. The analysis conditions were: 10 kV and 25 μA. Experimental points were fitted with a Gaussian model.

were observed. Thus, it is hypothesised that the separation of ascorbic and isoascorbic acid on poly-APBA column does not depend on the formation of boronate esters, most likely an analyte–amine interactions might be responsible of the separation, as already described for chromatography [26].

3.3. The limit of detection and the peak recovery on APBA-modified columns

In principle, the high UV absorbance of poly-APBA might complicate the detection of molecules which also absorb in the same UV region [32]. In experiments with ascorbic acid, the lower concentration of ascorbic and isoascorbic acid detectable was 1 ppm, which is satisfactory for many practical applications (Fig. 6) [33].

At the same time, the APBA columns were checked for the peak recovery. Ascorbic acid was injected electrokinetically in the capillary both at the inlet, and run towards the detector (transit length: 19.4 cm) as well as from the outlet and let run again to the detector (transit length: 4.6 cm). The peak areas were measured for both running directions, normalised with the internal standard (methacrylic acid) and compared. The peak recovery for the analyte travelling throughout the longer portion of capillary is approximately 87% (±7%) of the control. The loss of analyte due to adsorption was approximately 1.22% cm^{-1}.

3.4. Separation of haemoglobin and glycated haemoglobin on APBA columns

APBA-modified columns were tested for their ability to recognise glycated macromolecules, choosing as example glycated haemoglobin A_{1c}. The A_{1c} protein differs from non-glycated molecule A_0 only by modified one valine residue on the β-chain [34]. At pH 6.0, quite strong adsorption was observed for both, glycated and non-glycated molecules, which was partially compensated by an addition of low concentrations of the neutral detergent Tween-20 in all the following experiments. An appreciable effect of

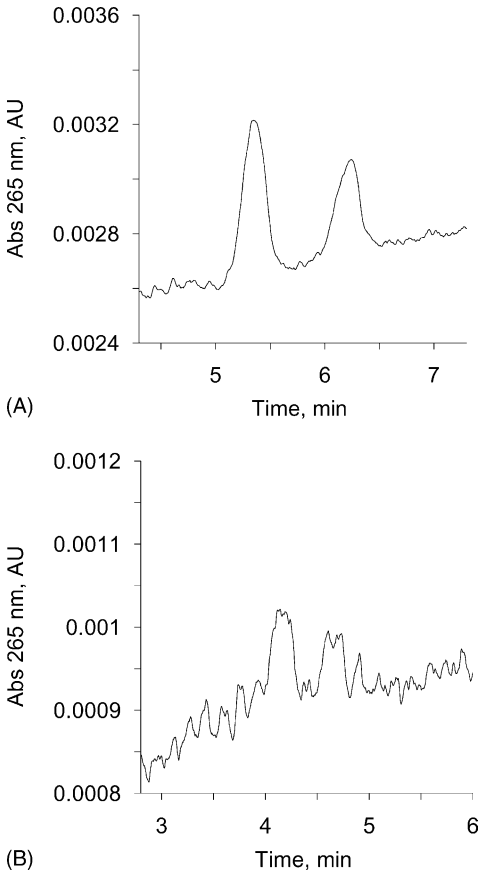

Fig. 6. Assessment of the limit of detection in APBA-coated capillaries. (A) Analysis of 2.5 mg l^{-1} of ascorbic and 2.5 mg l^{-1} isoascorbic acid. (B) Analysis of 1 mg l^{-1} of ascorbic acid and 1 mg l^{-1} of isoascorbic acid. Running conditions: 200 mM Tris–acetate, pH 8.0, 20 kV.

reduction of the adsorption was observed upon addition of Tween-20 in proportions of 0.05–0.5% (v/v). In these conditions, haemoglobin A_0 and A_{1c} were effectively separated onto APBA-coated capillary, as shown in Fig. 7. An addition of 1% (and greater concentrations) of Tween-20 resulted again in a loss of resolution.

Experiments with different A_0/A_{1c} ratios run at pH 6.0 allowed in first place the identification of A_{1c} as the latter component migrating. While a detailed explanation of the analyte–APBA polymer interactions contributing to the separation of A_{1c} and A_0 haemoglobin is quite hard to perform, because of the complex nature of these analytes, it is possible to speculate to the phenomenon. Haemoglobin A_{1c} and A_0 differs in isoelectric points (p*I* values: 6.9 and 7.0, respectively), thus at pH 6.0 it might be the case that this small difference gain significance, especially if the selection mechanism implies charge-interactions combined with hydrophobic interactions between the proteins and the poly-APBA.

It should be stressed that the measurement of glycated haemoglobin has a paramount importance in clinical diagnostic for monitoring of diabetes. To analyse the sensitivity of APBA columns for A_{1c}, samples of A_0 were spiked with

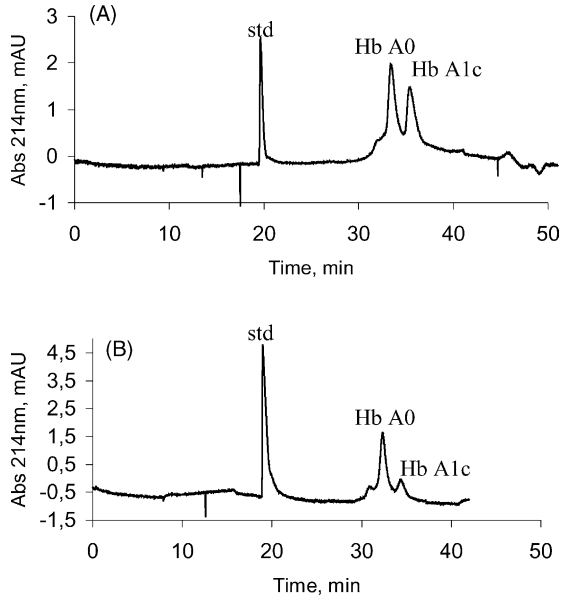

Fig. 7. Separation of haemoglobin A_0 and glycated haemoglobin A_{1c} onto poly-APBA column (37 cm × 50 μm i.d.). Running conditions: buffer pH 6.0, Tween 0.5%; cathodic direction; 10 kV, 52 μA. Internal standard: glycyl–histidine. (A) Separation obtained for a sample containing a 60:40 ratio of haemoglobin. (B) Separation of A_0 and A_{1c} haemoglobin of a sample containing a ratio 88:12 of A_0:A_{1c}.

increasing concentrations of A_{1c} (1, 3, 5, 7, 12, 15, 20 and 23%), run on the APBA column and the peak areas were measured. Fig. 7B shows the run of A_0:A_{1c} (88:12) as observed in these experiments, which shows two well-defined peaks. In clinical analysis, A_{1c} should be detected in the range of concentrations 1–12%, thus improvements should be made in order to use the APBA columns for the screening of diabetes. One way to improve the separation and the sensitivity of measurements will be to use derivatised narrow and ultra-narrow capillaries with open-tubular mode or to perform brush-like derivatisation.

4. Conclusions

Poly-APBA have been used for the open tubular modification of capillaries and tested for the separation of small analytes to macromolecules. The poly-APBA coating provides a straightforward and cost-effective modification procedure. Although poly-APBA confers a strong hydrophobic character to the capillary, which has the disadvantage of favouring adsorption phenomena, resulting in asymmetrical peak shape and tailing, poly-APBA coated columns were successfully used for separating diastereoisomers of ascorbic acid in a pH range between 6.0 and 7.0. The separation find its reasons in a mechanism of interaction of the analytes with the modified capillary column, thus separation could be ascribed to both electrophoresis and electrochromatography. Moreover, poly-APBA was tested for the discrimination of haemoglobin and glycated haemoglobin, proving a fairly high selectivity towards the sugar-modified protein. Poly-APBA mechanism of separation is an interplay between hydrophobic backbone and dissociable chemical groups (i.e. amino group and hydroxyls), providing interesting separation abilities. The characteristics of the poly-APBA coating might be of interests in the development of bi-dimensional CE, where poly-APBA could be used for the coating of a portion of the capillary column. Apparently this coating might be very helpful in the downscaling separation, and in developing microfluidic devices.

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