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In-capillary derivatization approach applied to the analysis of insulin by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

An in-capillary derivatization procedure of insulin for its subsequent capillary electrophoretic analysis (with laser-induced fluorescence detection) was developed. The in-capillary derivatization performed using the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) in a borate buffer pH 8.9, was achieved by successive introduction of plugs of sample and AQC reagent followed by application of a voltage (30 kV). Derivatization reaction results from the differential transport velocities that permit the distinct zones to penetrate each other under the applied field. Reagent/sample molar ratio (R_m) and plug lengths ratio were shown to have an influence on the efficiency of the derivatization reaction. A single peak could be obtained with a high reagent/sample molar ratio ($R_m \geq 68$). The tagged derivative peak intensity and efficiency were improved when reagent solution time injection was at least twice higher than that of insulin sample. The validation of the method showed a good linearity between the corrected area of the derivative peak and insulin concentrations. The relative standard deviations of the migration times and the corrected areas obtained for the tagged derivative were 2.3 and 4.6%, respectively. An efficient derivatization and separation of a mixture of insulin and two glycated forms of insulin was obtained using the technique.

Keywords: Derivatization, electrophoresis; In-capillary derivatization; Electrophoretically mediated microanalysis; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; Insulin; Peptides

1. Introduction

Labeling with a fluorophore is an attractive method to enhance detection sensitivity in CE of amino acids, peptides or proteins due to the low detection limits that can be attained using laser-induced fluorescence (LIF) detection and the wide variety of derivatizing reagents available. Apart from more conventional procedures involving pre- or post-capillary derivatization, new approaches based on in-capillary derivatization have been recently introduced (see [1–3] for reviews). In-capillary derivatization consists of developing the reaction inside the capillary by promoting the mixing of analytes with the reagent. A remarkable feature of this technique is the small reaction chamber volume, which means that the sample consumption and dilution are minimal. In-capillary derivatization can be accomplished by different strategies which may be classified into three groups: zone-passing, at-inlet and throughout-capillary technique. The zone-passing technique is based on derivatization in the middle of the capillary by passing either sample or reagent zone through the other under an electric field applied just after the introduction of the reagent and the sample solutions [4]. This strategy is well appropriated to fast kinetic processes where high reaction yields are achieved in a few

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seconds. In the at-inlet technique, sample and reagent solutions are introduced at the inlet of the capillary either by tandem or sandwich mode, these reactants are mixed by diffusion and allowed to react for a specified time before applying the running voltage. This procedure is especially suitable for low kinetic reactions which take several minutes to develop completely [5]. In the throughout-capillary technique, the sample solution is introduced at the inlet of the capillary previously filled with a running buffer which contains the reagent. When the separation potential is applied, the analyte migrates, mixes with the reagent, allowing the derivatization reaction to occur [6]. A comparison of all derivatization modes discussed above has been reported elsewhere [7,8].

The derivatization of amines, amino acids, peptides or proteins using in-capillary techniques has been mainly reported using *o*-phthaldialdehyde (OPA) [4,6,7,9], naphthalene-2,3-dicarboxaldehyde (NDA) [10], 1,2-naphtoquinone-4-sulfonate (NQS) [8] and 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) [11]. The main problem encountered with covalent labeling involving amino groups of peptides and proteins is the multiple tagged derivatives that are often obtained due to the presence of several functionalities in such molecules. In 1999, Liu et al. [12] reported derivatization conditions with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) able to generate a single and fully tagged peptide or small protein. This labeling agent was earlier introduced by Cohen and Michaud [13] as a strongly labeling reagent coupling primary and secondary amines in a few seconds.

In the present study the potential of AQC to perform in-capillary derivatization of insulin by electrophoretically mediated microanalysis (EMMA) is examined. To our best knowledge, no work has reported yet the use of this reagent for the in-capillary derivatization of amines. As the formation of a single homogeneously tagged product depends on how the derivatization is performed, we have investigated the influence of various factors such as reagent/insulin plug lengths and molar concentration ratio on the yield and on the number of derivatives observed after the in-capillary derivatization reaction. The effect of acetonitrile added to the background electrolyte on the sensitivity was also studied. The in-capillary derivatization method has been validated in terms of linearity and reproducibility. Finally, the method was applied to the CE–LIF analysis of a mixture of insulin and two of its glycated forms which may be found in the plasma of diabetic patients.

2. Experimental

2.1. Apparatus

CE experiments were performed using a Beckman P/ACE system MDQ equipped with a UV detector (Beckman Instruments, Fullerton, CA, USA) or coupled to an external ZETALIF-2325 detector (Picometrics, Toulouse, France). The excitation was performed by a Melles-Griot He–Cd laser at a wavelength of 325 nm with a 15 mW excitation power (Carlsbad, CA, USA). Data acquisition and instrument control were carried out using a Beckman P/ACE MDQ system (version 2.2) software.

2.2. Reagents

Buffers and standard solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA) and were filtered through a 0.22-μm pore size membrane filter (Millex, Millipore, France). Sodium hydroxide was obtained from Prolabo (Paris, France). Sodium tetraborate was from Fisher Scientific (Pittsburgh, PA, USA). HPLC-grade acetonitrile was from Fisher Scientific. Insulin (human) was purchased from Sigma (St. Louis, MO, USA). AQC was synthesized by reaction of di-(*N*-succinimidyl)carbonate (Fluka, Buchs, Switzerland) with 6-aminoquinoline (Aldrich, Milwaukee, WI, USA) according to [13].

2.3. Procedures

2.3.1. CZE analysis

Separations were carried out with fused-silica capillaries (Beckman Coulter, Fullerton, CA, USA) of 50 μm i.d. having an effective length of 50 cm and a total length of 60 cm (CE–UV) or 64 cm (CE–LIF). Before each electrophoretic run, the capillary was sequentially flushed with 0.1 M sodium hydroxide, then equilibrated with the separation buffer for 3 min. The working electrolytes (borate buffer, pH 8.9, concentrations ranging from 10 to 80 mM, containing 0–30% (v/v) acetonitrile) were prepared by appropriate dilutions of acetonitrile and 100 mM sodium tetraborate buffer stock solution. The analyses were performed at +30 kV and 40 °C, with photometric detection at 200 nm or LIF detection (excitation wavelength, λ_{ex} = 325 nm).

2.3.2. Derivatization procedures

2.3.2.1. Pre-capillary derivatization. The pre-capillary derivatization was performed using a modified procedure of Liu et al. [12]. One microliter of AQC (10 mM in acetonitrile) was mixed with 3 μL of a 0.13 mM insulin solution prepared in borate buffer 10 mM pH 8.9. The tagging procedure was completed after the solution was vortexed and heated at 54 °C for 10 min. The 1/50 diluted sample was introduced into the capillary by hydrodynamic injection for 10 s at 0.5 psi (1 psi = 6894.76 Pa) and analysed instantaneously.

2.3.2.2. In-capillary derivatization. A solution of insulin with concentrations ranging from 2.0×10^{-7} to 5.2×10^{-5} M in borate buffer 10 mM pH 8.9 was introduced by pressure injection (0.1 psi, 2 or 4 s) into the anodic end of the capillary, previously equilibrated with the running buffer.

A solution of AQC with concentrations ranging from 2.2 $\times$ 10^{-4} to 1.4 $\times$ 10^{-2} M in acetonitrile was subsequently introduced by pressure injection (0.1 psi) for specified periods of time ranging from 1 to 16 s. Then, the voltage was applied between both ends of the capillary.

2.3.3. *In vitro formation and purification of insulin glycated forms P1 and P3*

Glycated forms of insulin (P1, P3) were obtained by incubation of insulin and glucose (Aldrich, Milwaukee, WI, USA) in the presence of sodium cyanoborohydride (Sigma) according to [14]. Monoglycated form P1 corresponds to the glycation of the N-terminal Phe^1 residue of the insulin B-chain. Diglycated insulin form P3 corresponds to the glycation of Phe^1 and Gly^1 of the insulin B- and A-chains, respectively. Isolation and purification of P1 and P3 insulin forms were performed by reversed-phase HPLC according to the procedure described by O'Harte et al. [14].

2.3.4. *Calculation method*

To estimate the molar ratio (R_m) of the reagent and insulin effectively introduced into the capillary, the following equation was used:

$$R_m = \frac{[AQC]t_{AQC}}{[I]t_I n} \quad (1)$$

where n is the number of potential derivatization sites in the peptide ($n = 3$ for insulin, $n = 2$ for monoglycated insulin form P1, $n = 1$ for diglycated form P3); t_{AQC} and t_I are injection times of AQC and insulin solutions, respectively, at 0.1 psi; [AQC] and [I] are the molar concentrations of AQC and insulin solutions, respectively.

The percentage of derivatized insulin (Y) was calculated by coupling capillary electrophoresis to UV detection at 200 nm and monitoring the corrected peak areas of insulin (A_I) and that of the AQC derivatives (A_{I-AQC}) using conditions permitting a co-migration of all the AQC-insulin derivatized forms (background electrolyte: 10 mM borate buffer pH 8.9) (Eq. (2)):

$$Y = \frac{A_{I-AQC}}{A_I + A_{I-AQC}} \times 100 \quad (2)$$

3. Results and discussion

3.1. Strategy for in-capillary derivatization

In-capillary derivatization can be achieved by successive introductions of a sample solution and the reagent solution followed by application of a voltage, as illustrated in Fig. 1. If the reagent zone moves faster than the sample zone, the former passes over the latter during migration. Reaction occurs while both zones are overlapping each other in the capillary. If the reagent zone moves slower than the sample zone, the introduction order of the sample and the reagent solutions should be reversed.

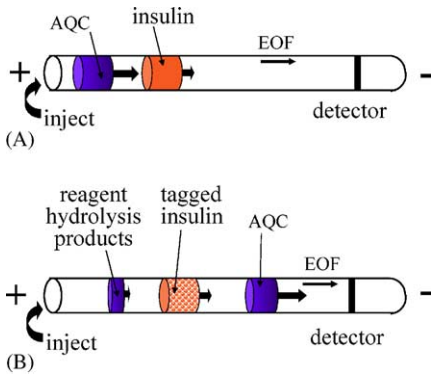

Fig. 1. Schematic representation of the in-capillary microreaction between AQC and insulin before (A) and after reaction (B).

In the model system employed a sample of insulin and AQC reagent solutions were successively introduced at the anodic end of the capillary by hydrodynamic injection. The in-capillary derivatization was performed in borate buffer pH 8.9 according to previous works on pre-capillary derivatization of amino acids [13] and insulin [12]. Moreover, this alkaline background electrolyte allowed a strong cathodic electroosmotic flow (EOF). Insulin exhibiting an isoelectric point of 5.5 was drawn back by its negative electrophoretic mobility. Therefore, the neutral AQC reagent migrated faster than the sample zone toward the cathode, passed the sample zone and reacted with insulin to produce the AQC-derivatized insulin (I-AQC).

Due to the lack of fluorescent properties of insulin, the in-capillary derivatization process was first monitored by CE–UV, as the native and tagged insulin were resolved using 10 mM borate buffer pH 8.9. In these preliminary experiments, the injection time of an insulin sample was kept constant while that of the AQC solution was varied from 0 to 16 s. These conditions correspond to increased value of the molar ratio R_m (AQC to insulin) from 0 to 68, as calculated with Eq. (1). As shown in Fig. 2, the percentage of derivatized insulin (Y), as calculated with Eq. (2), increased as the R_m value raised from 0 to 34. Y attained 100% for R_m equal or superior to 34 indicating that the derivatization of insulin was complete in these conditions.

3.2. Optimization of in-capillary derivatization

Thus, we have further investigated separately and by CE–LIF, the influence of the AQC/insulin molar ratio (R_m) and of the plug lengths of insulin and AQC reagent solutions on the in-capillary derivatization efficiency and particularly on the number of Ins-AQC derivatives formed.

3.2.1. *AQC/insulin molar ratio (R_m)*

Since three NH_2 groups from the aliphatic ε-NH_2 of Lys^{29} (insulin B-chain) and from the N-terminal Gly^1 and Phe^1 insulin A- and B-chains, respectively, can potentially be tagged in this peptide, the AQC/insulin molar ratio had to

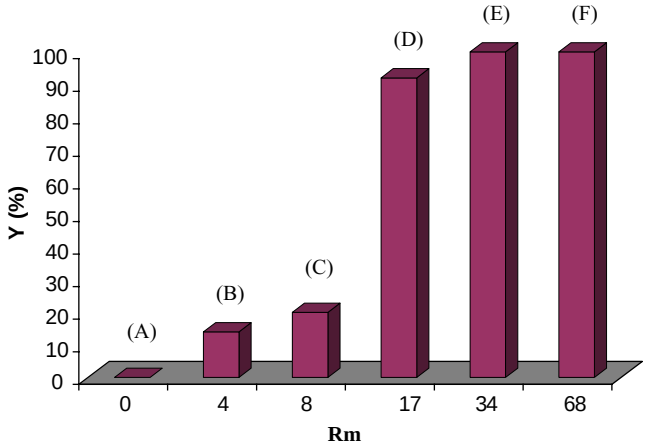

Fig. 2. Percentage of tagged insulin as a function of the AQC/insulin molar ratio (R_m) monitored by CE–UV. Conditions: background electrolyte, 10 mM sodium tetraborate buffer pH 8.9; applied voltage, +30 kV; detection, 200 nm; temperature, 40 °C; injection: 5.2×10^{-5} M insulin, 4 s then 2.6×10^{-3} M AQC, 0 s (A); 1 s (B); 2 s (C); 4 s (D); 8 s (E); 16 s (F).

be optimized to obtain preferentially a single derivative of tagged insulin. The plug lengths of insulin and AQC solutions were kept constant while the concentration of the AQC solutions was varied from 2.2×10^{-4} to 7.0×10^{-3} M (corresponding to R_m values ranging from 4 to 136). Moreover, in order to separate all the fluorescent AQC by-products from the AQC derivatives of insulin, a borate buffer 60 mM pH 8.9 was employed as the running buffer. Fig. 3 shows the series of electropherograms obtained with the in-capillary derivatization procedure compared to that obtained with the pre-capillary derivatization procedure. In this latter case, besides the peak of the AQC reagent ($t = 2.87$ min), only one peak ($t = 4.29$ min) is observed corresponding to the fully tagged insulin derivative as reported by Liu et al. [12]. A continuous increase of the area of the peak observed at 4.29 min with the increase of the R_m was observed. Concurrently, the area of the second peak observed at 3.97 min decreased gradually from R_m 4 to 136. These results indicate that in-capillary derivatization of insulin with low R_m values can lead to multiple tagged derivatives that correspond probably to the mono, di- and tri-tagged derivatives. Under the optimal conditions ($R_m \geq 68$), in-capillary derivatization leads to a migration profile similar to that obtained by pre-capillary derivatization corresponding mainly to the formation of a single I-AQC derivative. However, values of R_m higher than 68 seems to increase the intensity of the peaks attributed to the by-products of AQC (secondary reactions) as demonstrated by the profile obtained with the blank.

3.2.2. Reagent/sample introduction time

In a second part of this work, the influence of the plug lengths of insulin sample and AQC solutions introduced into the capillary were examined under the optimal R_m value (68) determined previously. Here, the introduction time of

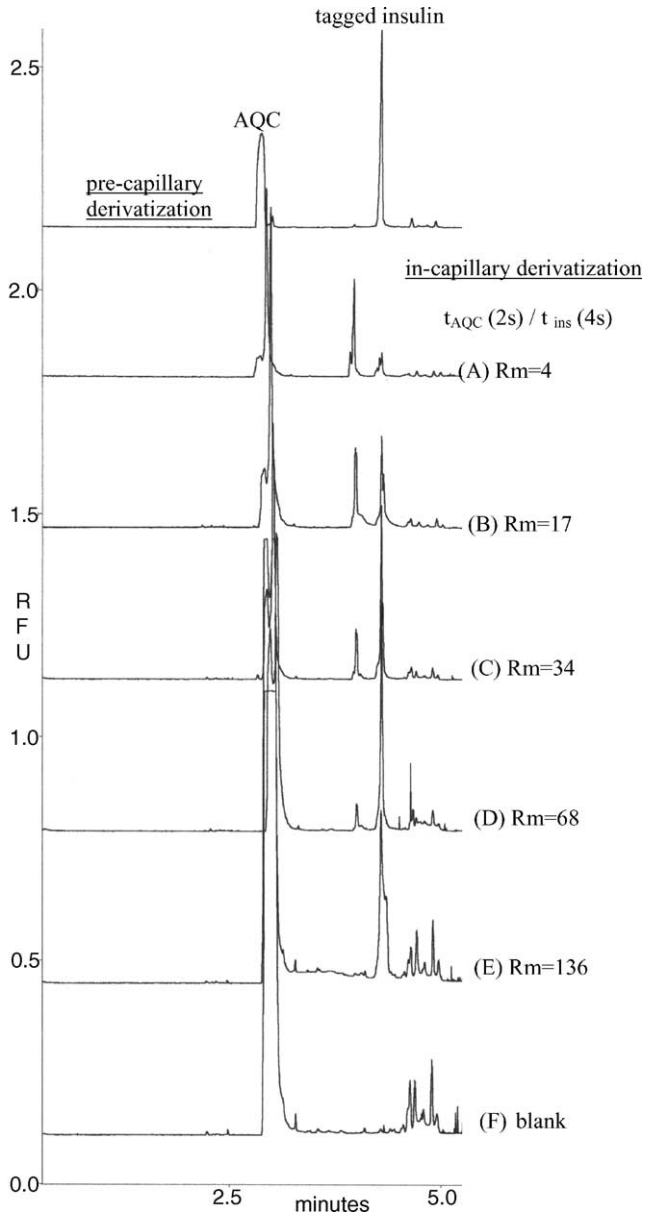

Fig. 3. Influence of the AQC/Ins molar ratio (R_m) on the number of I-AQC peaks obtained by CE–LIF. Conditions as in Fig. 2 except: borate buffer concentration 60 mM pH 8.9, LIF detection ($\lambda_{ex} = 325$ nm); injection: 3.4×10^{-5} M insulin, 2 s then AQC, 4 s, 2.2×10^{-4} M (A); 8.8×10^{-4} M (B); 1.75×10^{-3} M (C); 3.5×10^{-3} M (D); 7.0×10^{-3} M (E); blank: injection of borate 10 mM pH 8.9, 2 s then AQC 7.0×10^{-3} M, 4 s (F).

the insulin sample was remained constant (2 s) while that of AQC solution was varied from 1 to 8 s. As AQC was dissolved in acetonitrile, the time was limited to 8 s to avoid current leakages. In order to keep the AQC/Ins molar ratio constant the concentration of the AQC solutions was varied from 1.75×10^{-3} to 1.4×10^{-2} M. As shown in Fig. 4, the shape of the I-AQC peak observed at 4.29 min depends greatly on the plug length of the reagent solution: a broad and small peak is observed for AQC injection times less than 4 s indicating probably the presence of multiple tagged derivatives. As general trend, an increase of the area

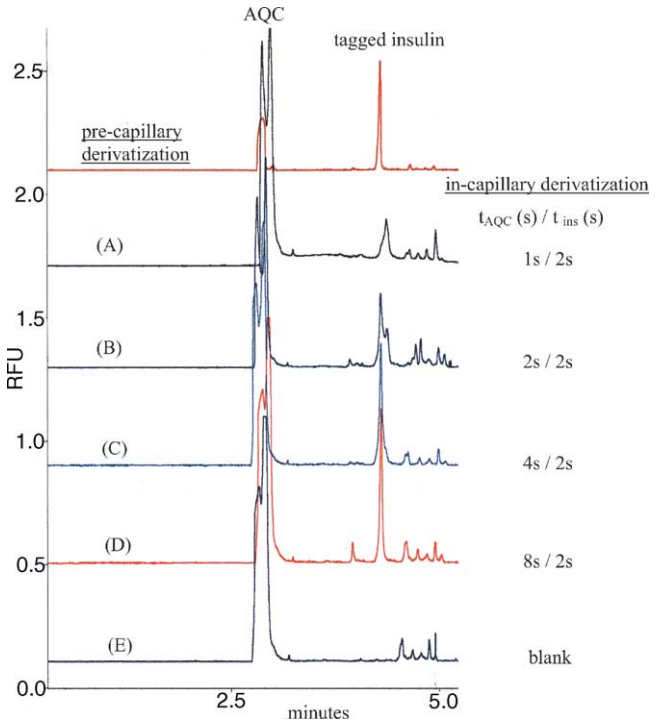

Fig. 4. Influence of the plug lengths of AQC and insulin solutions on the yield of derivatized insulin and on the number of I-AQC peaks. Conditions as in Fig. 3 except: injection: 3.4×10^{-5} M insulin, 2 s then AQC 1.4×10^{-2} M, 1 s (A); 7.0×10^{-3} M, 2 s (B); 3.5×10^{-3} M, 4 s (C); 1.75×10^{-3} M, 8 s (D); blank: injection of borate 10 mM pH 8.9, 2 s then 1.4×10^{-2} M AQC, 1 s (E).

and efficiency of the peak with the increase of the AQC solution time injection was observed. Optimal conditions could be attained when the injection time of the reagent solution is at least twice higher than that of the sample. These results are in good agreement with previous works on in-capillary derivatization [4] which demonstrated that longer is the contact time between the reagent zone and the sample zone, the higher is the in-capillary derivatization efficiency.

3.2.3. Effect of organic modifier

The effect of the addition of acetonitrile (ACN) to the running electrolyte on the fluorescent intensity of the derivatized insulin was studied. The percentage of solvent was varied in the range from 0 to 30% ACN. This percentage should be limited to 30% in order to prevent the precipitation of sodium tetraborate. A continuous increase of the fluorescent intensity of the derivatized insulin peak with the increase of the ACN percentage was observed: the value of the corrected area of the I-AQC peak was two fold higher with 30% ACN.

Finally, the optimal in-capillary derivatization procedure corresponded to an introduction time of 2 and 4 s for insulin and AQC (3.5×10^{-3} M) solutions, respectively, with a working electrolyte composed of borate buffer 60 mM pH 8.9 with 30% acetonitrile.

3.3. Analytical performances of in-capillary derivatization procedure: calibration curve, reproducibility and sensitivity of the method

The linearity of the in-capillary derivatization CE–LIF method was investigated using an insulin solution with concentrations ranging from 3.4×10^{-5} to 1.7×10^{-6} M and using the optimized conditions. Linear regression analysis, plotting the corrected area of the I-AQC derivative peak (y) versus the molar concentration (x) gave the following equation: $y = 1.000 \times 10^{10}x - 13\,610$ with a satisfactory correlation coefficient ($R = 0.998$). The reproducibility of the in-capillary microreaction was studied through six derivatization reactions of a 5.2×10^{-5} M insulin solution. The relative standard deviations (R.S.D.s) of the migration times and the peak corrected areas obtained for the I-AQC derivative were 2.3 and 4.6%, respectively.

The sensitivity of the in-capillary derivatization CE–LIF method was studied by calculating the limit of detection corresponding to a signal-to-noise ratio of 3, estimated by plotting the I-AQC derivative peak height versus the concentration of insulin sample injected. Using the same background electrolyte (borate buffer 10 mM pH 8.9) and the

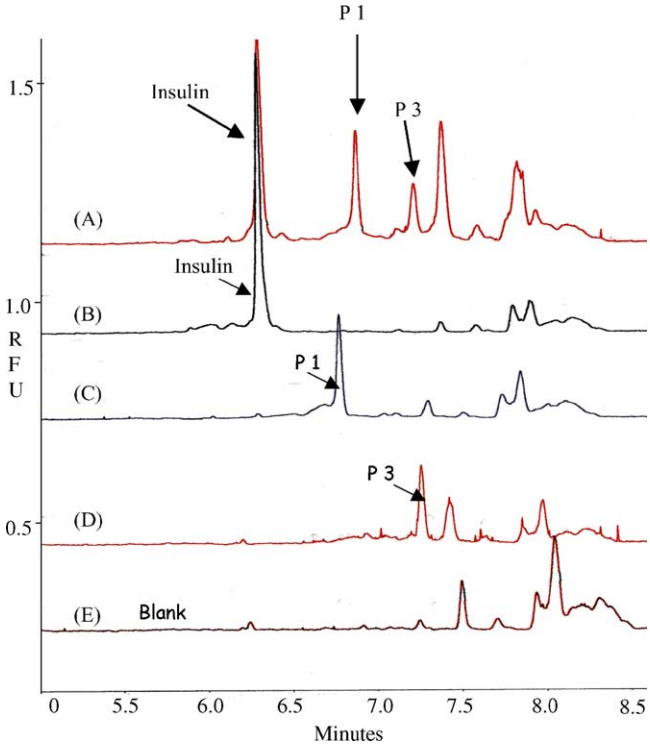

Fig. 5. In-capillary derivatization and separation of a mixture of insulin and two of its glycated forms: P1, P3. Conditions as in Fig. 3, except: background electrolyte, 80 mM sodium borate buffer pH 8.9–ACN (90:10, v/v); injection: mixture of insulin, mono- and diglycated forms 3.4×10^{-5} M each, 2 s then AQC 7.0×10^{-3} M, 4 s (A); 3.4×10^{-5} M insulin, 2 s then AQC 1.1×10^{-3} M, 4 s (B); 3.4×10^{-5} M P1, 2 s then AQC 1.1×10^{-3} M, 4 s (C); 3.4×10^{-5} M P3, 2 s then AQC 1.1×10^{-3} M, 4 s (D); blank: injection of 10 mM borate buffer pH 8.9, 2 s then AQC 7.0×10^{-3} M, 4 s (E).

same introduction time of insulin solution (2 s), in-capillary derivatization of insulin with AQC followed by CE–LIF analysis results in a 25-fold increase in sensitivity compared to spectrophotometric UV detection. The limit of detection (LOD) of insulin in-capillary derivatization with LIF detection was 5.0×10^{-7} M; this value is comparable to that obtain for pre-capillary derivatization (2.0×10^{-7} M).

3.4. Application of the in-capillary derivatization procedure

Finally, the in-capillary derivatization procedure was applied to the CE–LIF analysis of a mixture of insulin and two of its glycated forms: P1 and P3 which correspond to a mono- and diglycated insulin forms, respectively. In order to improve the selectivity of the separation, the borate buffer concentration was increased to 80 mM. Peak identities were determined by in-capillary derivatization of insulin, P1 and P3, separately. As shown in Fig. 5, the method leads to an efficient in-capillary derivatization of the three compounds and a good selectivity is observed. Although the concentrations of insulin, P1- and P3-forms in the mixture are identical (3.4×10^{-5} M), the peak areas of the corresponding AQC derivatives decrease with the number of glucose residues present in the insulin. This result is consistent with the number of amino groups potentially tagged in the native compound (3, 2 and 1, respectively) and add support to the hypothesis of a fully tagged insulin obtained under our conditions.

4. Conclusions

This work demonstrates that in-capillary procedure for derivatization of amine group by AQC is greatly dependant on the molar ratio of the sample and AQC, and on the lengths of the plugs introduced into the capillary. Conditions allowing a single tagged product could be found when this approach was applied to the derivatization of insulin. In addition, the in-capillary derivatization method has been validated in terms of linearity of the response and reproducibility. Compared to pre-capillary derivatization, in-capillary derivatization was superior in terms of simplicity and ease of automation and it can be performed on very small amount of peptide or protein. Although the sensitivity attained is not as high as expected with a LIF detection, the ability to perform a rapid and selective on line derivatization is demonstrated. In addition, the method described allows a rapid and simultaneous CE–LIF analysis of insulin and its glycated forms.

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