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Amino acids determination using capillary electrophoresis with on-capillary derivatization and laser-induced fluorescence detection

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Abstract

Free amino acids have been derivatized on-capillary with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) and analyzed using a laboratory-made capillary electrophoresis apparatus with laser-induced fluorescence detection. Several parameters that control on-capillary derivatization of amino acids, including pH, mixing time, reaction time, concentration of the derivatization reagents (potassium cyanide and FQ) and solvent of FQ, as well as the temperature of mixing and reaction were optimized. Repeatabilities better than 1.8% for migration time and 7.8% for peak height were obtained. Assay detection limits for the different amino acids ranged from 23 nM for glycine to 50 nM for lysine and glutamic acid. The methods developed were applied to the analysis of several amino acids in pharmaceutical preparations and plasma samples. Results showed a good agreement with those obtained using an amino acid autoanalyzer for the same samples.

Keywords: On-capillary derivatization; Capillary electrophoresis; Laser-induced fluorescence detection; Amino acids; Plasma samples; LIF; Pharmaceutical drugs

1. Introduction

Amino acids are the primary components of proteins and they are essential to life. Amino acid determination in food [1], beverages [2], plants [3], and pharmaceutical drugs [4] has been useful for the characterization of these samples and for quality control. Also, amino acid determination in proteins and peptides is routinely used to estimate their amount and to determine their composition. Besides, amino acids have been determined in body fluids [5,6] because some amino acids are neurotransmitters and other ones have demonstrated to be markers of certain diseases, such as aminoacidopathies. As an example of the latter, propionic acidemia (PA) is the most frequent inborn error of organic acids metabolism in humans and it is caused by a deficiency of the enzyme propionyl-CoA carboxylase [7]. The deficiency of this enzyme results in developmental retardation and it can be detected by an increase in the free glycine (Gly) levels in plasma.

Capillary electrophoresis (CE) is a very powerful separation technique which when combined with laser-induced fluorescence (LIF) detection could provide a fast and sensitivity method for amino acid analysis. However, due to the fact that amino acids (except the aromatic ones) do not present native fluorescence, it is necessary to derivatize them before performing LIF detection. The derivatization reaction can be carried out before introducing the sample in the capillary (pre-capillary), inside the capillary (on-capillary), or between the outlet of the capillary and the detection point (post-capillary). Pre-capillary derivatization can be performed either in the off-line (manual) or in the on-line (automated) mode. Manual pre-capillary derivatization is the most frequently employed mode for derivatization of amino acids in CE because of its simplicity and the wide variety of reagents (fluorophoric and fluorogenic) that can be employed without need of taking into account their slow or fast speed of reaction [8]. However, the method is laborious and requires large sample volumes. In addition, the reaction times are usually long and the derivatization of very diluted samples is problematic [9]. In the on-line mode [10], sample and reagents are mixed just before the capillary inlet using a micro T-piece. This derivatization mode requires special equipment, making its use dif-

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ficult in ordinary practice. Post-capillary derivatization [11] has several advantages over the pre-capillary derivatization mode. The method is automatic, the detrimental effect caused by derivatization on the separation is avoided, and the band broadening due to multiple labeling of proteins [12] can also be prevented. In spite of that, the method has several limitations, such as, loss in separation efficiency due to band broadening caused by post-capillary mixing, incomplete reactions that decrease sensitivity, and the increase in baseline noise caused by the reagent solution added. Band broadening, that seems to be an inherent problem of post-capillary derivatization, would be minimized using sheath-flow cuvettes as reactor and detection cell simultaneously [13,14].

Recently, on-capillary derivatization has been employed because it has several advantages such as low consumption of reagents and sample, short reaction time, needless of extra equipment, and the possibility of automation. Several modes of performing on-capillary derivatization, namely zone-passing, at-inlet, and throughout-capillary, have been described. Zone-passing derivatization is carried out by applying the separation voltage just after the introduction of the labeling reagent solution and the sample solution into the capillary [15]. To perform derivatization in this mode, the compound with lower apparent electrophoretic mobility, either the sample or the reagents, must be introduced first to allow the band of the compound with higher apparent electrophoretic mobility to pass through it. Due to this fact, this strategy is appropriate for fast kinetic processes where high reaction yields are achieved in a few seconds. In at-inlet derivatization strategy, a sample plug and a reagent solution plug are introduced sequentially at the inlet of the capillary, mixed applying a low voltage for a given time (called mixing time), and allowed to react before applying the separation voltage. In this technique, various sample/reagent introduction modes can be used. For example, Taga and Honda compared tandem and sandwich introduction modes in at-inlet derivatization [16]. In the first case, sample and reagent solution are successively introduced in the capillary in the same way described for zone-passing derivatization. In the other case, sample solution is introduced between two plugs of reagent solution, like a sandwich, and it is not necessary to take into account the sample/reagent relative electrophoretic mobility. In both cases, mixing time controls the blend between the sample and the reagent solutions and therefore the derivatization reaction. In throughout-capillary derivatization, the capillary is filled up with a running buffer that contains the derivatization reagent. When the separation potential is applied, the components of the sample simultaneously migrate and mix with the reagent, and the corresponding reaction occurs [17]. A comparison of all on-capillary derivatization modes discussed above has been reported elsewhere [18]. Amino acid derivatization using on-capillary techniques for fluorescence detection has been reported using *o*-phthalaldehyde (OPA) in combination with different thiols [19], OPA/*N*-acetylcysteine (NAC) [17], 5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein (DTAF) [20], and naphthalene-2,3-dicarboxaldehyde (NDA) in presence of sodium cyanide [21].

Quantitation of amino acids by CE presents enormous complexity. Only a few papers have dealt with it. Boulat et al. [22] reported that amino acid quantitation in plasma samples is problematic due to the fact that the labeling reaction is influenced by the total amino acid concentration present in the sample. Thus, they reported peak areas decreasing for leucine and phenylalanine when plasma samples were spiked with other amino acids. However, several authors have reported the quantitation of different amino acids in human plasma samples [23–26], in feed samples [27,28], and in pharmaceutical products [28].

However, in spite of these difficulties, the above-mentioned high resolution power of CE combined with the sensitivity of detection provided by LIF, led us to develop methods for amino acids analysis using on-capillary derivatization. In this paper, we explore the effect of several parameters that control on-capillary derivatization of amino acids, namely, separation buffer pH, mixing time, reaction time, concentration of the reagents (potassium cyanide, KCN, and 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)) and solvent of FQ, as well as the mixing and reaction temperature. The applicability of the methods developed to analyze several amino acids in pharmaceutical drugs and in plasma samples is demonstrated.

2. Experimental

2.1. Reagents, standards, and solutions

Borax and KCN were purchased from Sigma (St. Louis, MO, USA), NaOH was from Merck (Darmstadt, Germany), 5-sulfosalicylic acid dihydrate (SSA) was obtained from Panreac (Barcelona, Spain), and 3-(2-furoyl)quinoline-2-carboxaldehyde was from Molecular Probes (Eugene, OR, USA). Acetonitrile (ACN), methanol (MeOH), and 2-propanol (IPOL) were HPLC grade from Scharlau Chemie (Barcelona, Spain). DL-Alanine (Ala), L-glutamic acid (Glu), glycine (Gly), and L-lysine (Lys) were from Sigma. L-Phenylalanine (Phe) and L-serine (Ser) were from Merck. *N*α-Boc Lys was from Fluka (Buchs, Switzerland).

Aqueous solutions of 70 mM borax at different pH values were tested as separation buffer. The 200 mM KCN stock solution was made in 2.5 mM borax and was used either without further dilution or diluted in 2.5 mM borax. Standard amino acid solutions were prepared as 10^{-3} M stock solutions in Milli-Q water (Millipore, Molsheim, France). All stock solutions were stored at 4 °C. Working solutions of mixtures of amino acids were prepared by mixing adequate volumes of each stock solution of amino acid. The final concentration of the amino acid mixtures was adjusted to the desired values with water.

Since FQ in solution, even when stored at −20 °C in darkness, degraded slowly, small aliquots of dried FQ were pre-

pared. To do so, a 50 mM stock solution of FQ was prepared in methanol and 10 μL aliquots of it were placed into 500 μL microcentrifuge tubes. The solvent was removed under vacuum at room temperature using a model RC10-10 centrifugal evaporator (Jouan, Saint-Herblain, France). The dried FQ was stored at −20 °C. It was thawed and solved on the day of the experiment.

Safety precautions: Potassium cyanide is highly poisonous and reacts readily with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer. Neutralization of waste containing KCN should be made by addition of 1% NaOH (about 50 μL/g of cyanide) solution followed by slow addition of bleach (about 70 μL/g of cyanide).

2.2. Samples

Two commercial pharmaceutical drugs containing amino acids were analyzed: Tebetane (capsules from Elfar-Drag Laboratories, Madrid, Spain) and Malandil (doses from Bohm Labs, Madrid, Spain). Tebetane contains three amino acids: Ala, Gly, and Glu. Malandil contains only Lys.

Pools of plasma from healthy and sick donors were generously provided by the Center of Diagnosis of Molecular Diseases (CEDEM, Autonoma University, Madrid, Spain).

2.3. Sample preparation

Samples from pharmaceutical drugs were prepared as follows: 0.1 g of Tebetane or a dose of Malandil was dissolved in 100 and 250 mL of Milli-Q water, respectively. Then, the solution of Tebetane and the solution of Malandil were diluted 100- and 333-fold in water, respectively. Finally, both solutions were filtered through a nylon Tracer syringe filter (0.22 μm pore size) (Teknokroma, Barcelona, Spain).

Pools of plasma were stored upon receipt at −20 °C until analysis. Prior to analysis, the plasma sample was deproteinized by adding 10 μL of sulfosalicylic acid solution (50% in water) to 100 μL of sample. After mixing, the sample was left to stand on ice for 15 min and then centrifuged at 23 000 × *g* for 10 min at room temperature using a model Biofuge Stratos centrifuge (Heraeus, Hanau, Germany). The supernatant was filtered through a 0.22 μm Ultrafree-MC centrifugal filter (Amicon, Bedford, MA, USA) at the same centrifugal force and temperature for 2 min. Finally, the filtrate was diluted 1:50 with Milli-Q water.

2.4. Instrumentation

A laboratory-made CE apparatus was built using a RS/EH50R power supply (Glassman High Voltage, Whitehouse Station, NJ, USA). For LIF detection, the output from a 2060-10S Ar-ion laser (Spectra Physics, Mountain View, CA, USA) (λ_{exc} = 488 nm, 10 mW) was focused on the capillary window using a 41116 quartz lens (Oriel, Stratford, CT, USA) (*f* = 12.7 mm). Fluorescence was collected at right angles to the laser beam using an Oriel 40× microscope and filtered successively through a 550 nm cut-off filter (Schott, Mainz, Germany) and an interference filter centered at 590 nm (Andover, Salem, NH, USA). A 71400 Oriel iris was inserted in the optical train to prevent straight light from reaching the R928 photomultiplier (Hamamatsu, Hamamatsu City, Japan) that was polarized at 600 V. Photocurrent was processed by a 7070 Oriel detection system photometer and a 406 System Gold A/D converter (Beckman, Fullerton, CA, USA). Data were collected on a 486 computer.

Uncoated capillaries (Composite Metal Services, Worcester, UK), 60 cm in length (50 cm to detector) and 50 μm I.D. (375 μm O.D.) were used. If necessary, a laboratory-made special device, made of plastic tubing (5 mm O.D. × 3 mm I.D.), was used to heat 10 cm at the inlet of the capillary where the derivatization reaction took place, using a F3 thermostatic bath (Haake, Karlsruhe, Germany). A small portion of the capillary entrance section (3 cm approximately) was not thermostated. Two silicon septums were used to avoid leaks of water in the places where the capillary passes through the heating device.

2.5. CE procedures

FQ was used as the labeling reagent. This compound generates stable isoindol derivatives when reacting with primary amines in the presence of nucleophilic agents, such as KCN [29]. The derivatization reaction was carried out inside the capillary. To do so, first a plug of a KCN solution was injected into the capillary (46 s) followed by a plug of sample containing the amino acids (8 s), and then by a plug of a FQ solution (46 s). All injections were carried out by gravity (20 cm height). After each injection of sample or FQ, the inlet end of the capillary was washed by immersion in a vial containing Milli-Q water. In the next step, vials containing the separation buffer were placed at both ends of the capillary and a voltage of 3 kV was applied for a given time (mixing time) in the range 0.25–15 min. After mixing, the derivatization reaction was allowed to proceed for a given time (reaction time) in the range 0.25–15 min with the voltage switched-off. Mixing and reaction were studied at temperatures ranging from 24 to 65 °C. Once the reaction time elapsed, separation was performed at 12.5 kV (our home-made equipment can only work safely up to 12.5 kV) at room temperature. Each experiment was made, at least, in duplicate.

New capillaries were first rinsed with 1 M NaOH (100 μL), followed by a rinse with Milli-Q water (100 μL). Between runs, the capillary was rinsed with Milli-Q water (100 μL), followed by a flush with 0.1 M NaOH (20 μL), a rinse with Milli-Q water (100 μL), and finally, washed with the separation buffer (50 μL). Rinses were made manually employing a model 1710 glass syringe (Hamilton, Bonaduz, Switzerland).

3. Results and discussion

Taking into account the respective electrophoretical mobilities, sample and reagents were injected in the above-

mentioned sequence (KCN, sample, and FQ) in order to carry out the derivatization reaction inside the capillary. Under the reaction conditions, the α-amino groups of the amino acids should be electrically neutral to allow its reaction with FQ. Therefore, basic pH buffers (typically in the range 8–11) have to be employed. In these buffers, the electrophoretic mobility (μ_e) of the cyanide ion is opposite to the electroosmotic flow mobility (μ_{EOF}). On the other hand, at these pH values, the amino acids have the carboxylic group negatively charged and the amino group electrically neutral, being then the μ_e of amino acids also opposite to the μ_{EOF}. As the size of the cyanide ion is smaller than that of the amino acids, its μ_e is most probably higher than that of amino acids, even in spite of some amino acids having two negative charges (two carboxylic groups) (μ_e for anions of similar size to cyanide, is around 8×10^{-4} cm^2 V^{-1} s^{-1} [30], while μ_e for amino acids is around 3×10^{-4} cm^2 V^{-1} s^{-1} [31]). It allowed the plug of amino acids to pass through that of KCN. The μ_e of FQ is zero because it is a neutral molecule, so it migrates with the electroosmotic flow. In this way, mixing of the sample, the KCN, and the FQ zones was achieved when positive voltage was applied to the injection end of the capillary.

3.1. Method development

On-capillary derivatization conditions were optimized in order to improve the detection limit of amino acids using LIF monitoring. Previous results in our laboratory and results reported in the literature [32] show that the reaction of amino acids with FQ or 3-(*p*-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), a fluorescent dye chemically similar to FQ, is controlled by the pH of the buffer, the reaction time, the concentration of the reagents, and the reaction temperature. In the present work, the effect of these parameters and others, such as mixing time and derivatization reagent solvent, were explored employing a mixture of five standard amino acids representative of acidic (Glu), basic (Lys), neutral–polar (Ser), and neutral–nonpolar (Phe, Ala) amino acids.

In the case of on-capillary reaction, besides the abovementioned parameters, a critical point is to achieve the mixing and the reaction of the reagents and sample inside the capillary [16]. In this work, a trial-and-error method was used to optimize the mixing and the reaction time in order to obtain the largest peak height for the amino acids studied. Optima mixing times were 0.5 min for Ser, 2 min for Glu, and 3 min for Ala, Lys, and Phe. A decrease in peak height was not observed after the maximum value in any of the cases. Therefore, 3 min was selected as mixing time. Optima reaction times were 1 min for Ser and Lys and 3, 8, and 12 min for Ala, Phe, and Glu, respectively. For every amino acid, a decrease in peak height was observed for reaction times larger than the optimum. A reaction time of 3 min was selected as a trade-off between optimum conditions for Ala, Lys, Ser (the shortest optima mixing times) and Phe and Glu (the longest optima mixing times). The differences founded in optima reaction times could be explained due to different reaction kinetics for each amino acid. In spite of the fact that the maximum of reaction had not been reached for some amino acids, at the reaction time chosen, the repeatability (see below) in peak height was good enough to allow a quantitative analysis of these amino acids.

The pH value is critical for FQ–amino acid adducts formation [32]. The optimum pH for the formation of the FQ derivatives is related to the pK_a of the amino groups, since FQ reacts only with non-ionized amine groups. For pH equal to the pK_a of the amino group, both ionized and non-ionized forms are in equilibrium. When FQ reacts with the non-ionized form, the concentration of this form decreases and, in consequence, the ionization equilibrium is displaced to the formation of the non-ionized form. This behavior allows that the reaction between FQ and the amino acids can take place at pH values around their pK_a values. Also, the nature of the group R of the amino acids can affect the derivatization reaction since it can change the pK_a value of the amino group. Thus, while alkyl and carboxyl groups do not change the pK_a values of the α-amino group (9.6–9.7), aromatic, polar, and other amino groups, slightly reduces their pK_a values (8.6–9.4) [33]. For the amino acid mixture studied, buffers containing 70 mM borax at pH values in the range 8.2–10.0 were tested (see Fig. 1A). Adduct formation, expressed as peak height, increased with pH. At pH values higher than 9.3, several broad peaks appeared in the electropherograms preventing amino acid identification. These peaks were probably due to secondary reactions between FQ and KCN impurities. A pH of 9.3 was selected as optimum even if some amino acids have their amino groups partially protonated.

Reagents concentration has been demonstrated to be of paramount importance in achieving a good yield for the derivatization reaction [32]. Due to the analogies between CBQCA and FQ, we could expect a similar mechanism for the reaction between any of these two reagents and the amino acids. If this hypothesis is true, we will expect a reaction of first order with respect to FQ and KCN concentrations. Therefore, increasing concentrations of FQ and KCN should increase the rate of the labeling reaction. In order to confirm this behavior, different concentrations of KCN and FQ were assayed. As shown in Fig. 1B, the signal of fluorescence for the amino acids increased with the KCN concentration when FQ concentration and FQ solvent were kept constant (6 mM FQ in MeOH). At KCN concentrations higher than 200 mM, several broad peaks appeared in the electropherograms (results not shown). Since in a blank assay, in which amino acids were not injected, these broad peaks were also observed, they did not correspond to amino acids. They were probably due to secondary reactions between FQ and KCN impurities [32]. A concentration of KCN 200 mM was chosen for further experiments. Fig. 1C shows the effect of FQ concentration on the signal obtained for the amino acids studied, when the concentration of KCN was maintained at 200 mM. For Ala, Glu, Phe, and Ser, sensitivity increased with FQ concentration until reaching a plateau at 24 mM, while the signal for

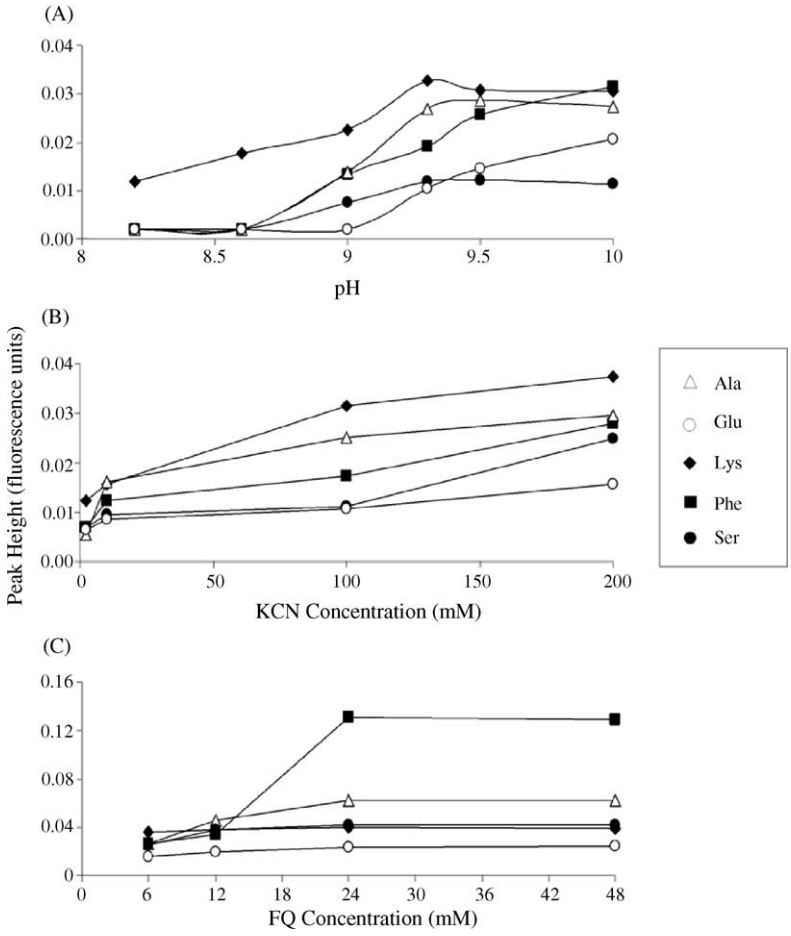

Fig. 1. Effect of pH (A), KCN concentration (B), and FQ concentration (C) on the peak height of FQ–amino acid derivatives. CE conditions for (A): buffer, 70 mM borax at different pH values; capillary, 60 cm length (50 cm to detector) and 50 μm I.D.; separation voltage, 12.5 kV; $T = 24\,^{\circ}C$. CE conditions for (B) and (C): as in (A), except buffer, 70 mM borax (pH 9.3). Reaction conditions for (A) and (B): gravity injection ($h = 20$ cm), 46 s of 100 mM KCN, 8 s sample (each amino acid in concentration ranging from 1×10^{-5} to 8×10^{-5} M), 46 s of 6 mM FQ in MeOH; mixing time, 3 min at 3 kV; reaction time, 3 min at 0 kV. Reaction conditions for (C): as in (A), except KCN concentration, 200 mM.

Lys was kept practically constant along the FQ concentration range studied. Therefore, we selected a FQ concentration of 24 mM for further experiments.

Methanol, the solvent recommended by the supplier, was used in the preceding optimization experiments as FQ solvent, since FQ is scarcely soluble in water. However, we observed that the efficiency of the peaks for the amino acids was slightly lower when methanol instead of water was used as FQ solvent (results not shown). Fig. 2 shows the effect of these two and others FQ solvents on peak height. As expected, low signals for the amino acids studied were observed when FQ was dissolved in water while larger signals were obtained by

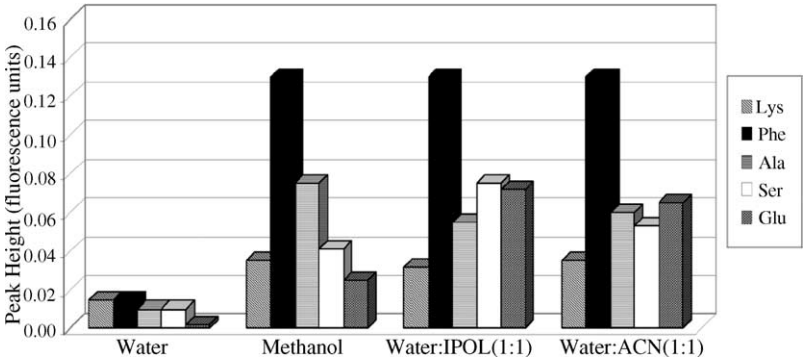

Fig. 2. Effect of FQ solvent on the peak height of FQ–amino acid derivatives. CE and reaction conditions as in Fig. 1C, except FQ concentration, 24 mM. *Abbreviations*: IPOL, isopropanol; ACN, acetonitrile.

using the other solvents. On the other hand, we observed that using either water–isopropanol or water–acetonitrile, good efficiency and improved sensitivity were obtained for all the amino acids studied when compared with water. Besides, when compared to the use of methanol, improvement in sensitivity and efficiency for Ser and Glu were obtained. Results were similar results for water–isopropanol (1:1) and for water–acetonitrile (1:1), and one of them, water–isopropanol (1:1), was chosen as FQ solvent for the rest of the experiments.

The temperature of the mixing and reaction plays a major role in the kinetics and yield of the reaction and therefore on the sensitivity of the technique [34,35]. In order to explore the effect of this parameter on the signal/noise ratio, the set-up described in Section 2 was used to thermostat the inlet end of the capillary, where reaction took place. The effect of the temperature in the range 24–65 °C was studied. In general, peak height increased with temperature, but several broad peaks (not due to amino acids) appeared at temperatures higher than 45 °C, probably due to the formation of by-products of the reaction between FQ and KCN impurities. For Phe and Ala, peak height increased 15% and 45%, respectively, at 45 °C with respect to 24 °C. For Ser and Glu, fluorescence was more than two-fold higher at 45 °C than at 24 °C. One exception was Lys, for which peak height decreased 42% at 45 °C with respect to 24 °C, probably due to the formation of three derivates at higher temperatures (see below). Due to the above-mentioned reasons, we selected 45 °C as the optimum temperature, like a compromise between the largest peak height for amino acids and the absence of peaks due to impurities. The set-up used to thermostat the inlet end of the capillary is clearly advantageous over the way of performing heating carried out by other authors, that consists on the immersion of the capillary tip on a vial of buffer previously heated [34].

For mixing and reaction carried out at 45 °C, the mixing time and the reaction time were reconsidered due to the fact that the viscosity of the solutions used decreases with temperature and therefore electroosmotic flow driving the mixing should probably increase. Optimum mixing time at 45 °C was 6 min for all the amino acids tested, except for Ser, that presented a maximum peak height at 3 min and decreased slightly at higher mixing times. The optimum mixing time being larger at 45 °C (6 min) than at 24 °C (3 min) could be due to the fact that the reaction velocity increases with temperature and amino acids need several minutes (at 3 kV) to reach the capillary heated zone. Each of the reaction times tested, between 0.25 and 15 min, produced the same peak height. In consequence, the lower reaction time (0.25 min) was selected. The effect of the concentration of KCN in the range 25–200 mM at 45 °C was tested again. In all the cases, a maximum was obtained at 50 mM KCN (results not shown). Finally, the effect of the FQ concentration (6–48 mM) in water–isopropanol (1:1) on the sensitivity, when reaction temperature is 45 °C, was studied. For all the amino acids assayed, sensitivity increased with FQ concentration until reaching a plateau at 24 mM (results not shown). Therefore, this FQ concentration was selected for further experiments.

In summary, Fig. 3 shows the electrophoretic separation of the standard amino acid mixture under the optimum conditions at both reaction temperatures, 24 and 45 °C. For every amino acid other than Lys, higher peaks were obtained at 45 °C than at 24 °C. Three peaks were obtained for Lys derivatization at 45 °C and only one at 24 °C. Lys contains two primary amino groups and therefore three different reaction products can be formed. At 24 °C, labeling reaction velocity is slower than at 45 °C and probably, at 24 °C, FQ only reacts with the most favorable Lys amino group. To prove this hypothesis, a Lys derivative with the α-amino group blocked with a *tert*-butoxycarbonyl group (*N*α-Boc Lys) was derivatized inside the capillary at 24 and 45 °C. Only one peak was obtained for this compound at these two temperatures (results not shown). For these reasons, those samples where only Lys determination was of interest, were analyzed using the method described at 24 °C. The rest of amino acids were analyzed using the method described at 45 °C that provided, in general, the best sensitivity and resolution.

3.2. Figures of merit

The methods developed were tested for the quantitative analysis of several samples. First, the capabilities of the method were studied (see Table 1). The repeatabilities (run-to-run precision) for migration time and peak height were calculated as the relative standard deviation (RSD) of six con-

Table 1
Figures of merit of the developed methods

Amino acid	Repeatability[a] (RSD, %)		Calibration equation[b]	Correlation coefficient	LOD[c] (nM)
	Time	Peak height			
Lys[d]	1.8	5.8	$h = -0.09 + 15313C$	0.9990	50
Ala	0.3	7.6	$h = 0.006 + 2956C$	0.9966	37
Gly	1.5	7.6	$h = 0.0025 + 2665C$	0.9998	23
Glu	1.0	7.8	$h = 0.0005 + 1139C$	0.9998	50

[a] $n = 6$.
[b] h: peak height; C: analyte concentration (M).
[c] S/N = 3.
[d] The method developed at 24 °C was used.

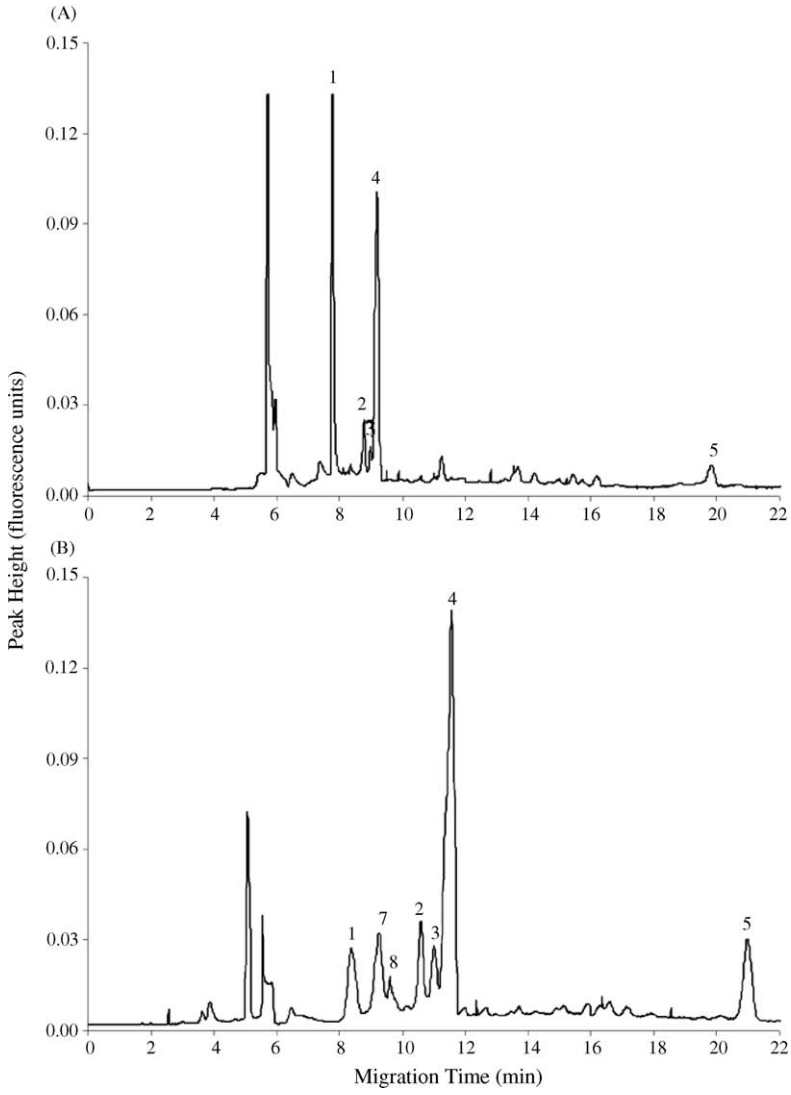

Fig. 3. Separation of a standard mixture of amino acids derivatized with FQ under the optimized conditions at: (A) 24 °C; (B) 45 °C. Peak identification: (1, 7, and 8) Lys; (2) Phe; (3) Ser; (4) Ala; (5) Glu. CE conditions as in Fig. 1B. Reaction conditions for (A): 46 s of 200 mM KCN, 8 s sample (each amino acid in concentration 2×10^{-5} M), 46 s of 24 mM FQ in water–isopropanol (1:1); mixing time, 3 min at 3 kV; reaction time, 3 min at 0 kV. Reaction conditions for (B): 46 s of 50 mM KCN, 8 s sample, 46 s of 24 mM FQ in water–isopropanol (1:1); mixing time, 6 min at 3 kV; reaction time, 0.25 min at 0 kV.

secutive injections of a solution containing a mixture of standard amino acids (6×10^{-6} M of each amino acid in the final solution). The repeatability was better than 2% for migration time and 8% for peak height. The linearity of the method was determined by using six standard solutions containing each amino acid in concentration ranging from 1×10^{-7} to 4×10^{-5} M. Good linearities were obtained in this range, with correlation coefficients, in general, higher than 0.999. The detection limit (LOD) (S/N = 3) shown in Table 1 is the assay detection limit. It is important to note that this assay detection limit [36] refers to the minimum amount of unlabeled amino acid that should be present in the sample to carry out the derivatization. Assay detection limits were in the range 23–50 nM. These values are about three orders of magnitude worse than those provided by the use of sheath-flow cuvettes [37]. However, developed methods are sensitive enough for the determination of amino acids in several instances, as it is shown below.

3.3. Applications

The methods developed were used to analyze the amino acids contained in some pharmaceutical formulations. As an example, Fig. 4 shows an electropherogram from a sample of Tebetane. Samples of Tebetane and Malandil were analyzed in duplicate with a RSD of the results lower than 4%. In Table 2, the comparison between the results obtained for the analysis of the two pharmaceutical products using the developed methods and the results provided by the Center of Diagnosis of Molecular Diseases (CEDEM), using an amino acid autoanalyzer with post-column derivatization [38], is shown. Determination error (ε) was calculated as the relative

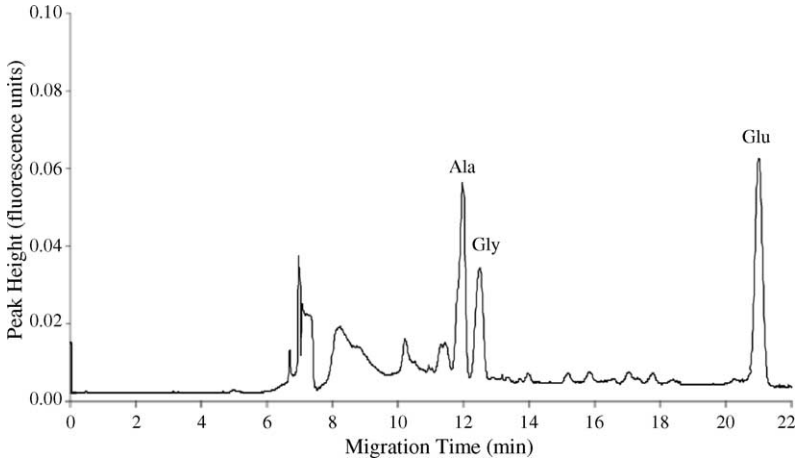

Fig. 4. Electropherogram of a Tebetane sample. CE conditions as in Fig. 1B. Reaction conditions as in Fig. 3B.

Table 2
Determination of amino acids in pharmaceutical formulations

Pharmaceutical formulation	Amino acid	Developed method (mg of amino acid)[a]		Reference method (mg of amino acid)[a]	ε (%)[b]
		Value	RSD (%)		
Tebetane	Ala	100.5	2.7	88.4	13.7
	Gly	45.5	2.0	41.7	9.1
	Glu	258.5	3.7	265.0	2.4
Malandil	Lys	41.1	1.9	36.4	12.9

[a] Amounts are expressed as mg of amino acid per capsule or dose.
[b] ε: determination error.

error between the value obtained by the developed method and the value given by the CEDEM (reference value). A good agreement between the results provided by both methods was observed.

The method developed at 45 °C was also used for the analysis of Gly in plasma samples. Fig. 5 shows a comparison between the electropherograms of plasma samples from a healthy donor (A) and from a patient suffering from propionic acidemia (B). It can be noted the marked increased in the level of Gly in the plasma sample corresponding to the sick individual. For the quantitative analysis of Gly in these plasma samples, three aliquots of each sample were prepared and each one was analyzed in triplicate. The Gly concentration obtained using the developed method was 271 μM (RSD 4.3%) from the plasma of the healthy donor and 1206 μM (RSD 5.7%) from the plasma of the patient suffering from propionic acidemia. In order to check the accuracy of the developed method, these values were compared to those given by the

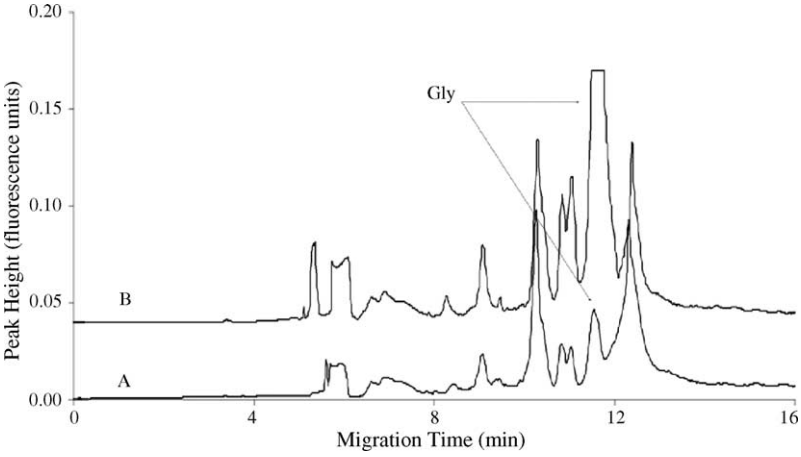

Fig. 5. Separation of FQ–amino acids in a plasma sample from: (A) a healthy person; (B) a patient suffering from propionic acidemia. CE conditions as in Fig. 1B. Reaction conditions as in Fig. 3B.

CEDEM, 268 and 946 μM, respectively. Normal values for Gly in healthy patients are in the range 226.36 ± 79.89 μM. Although the developed method showed some disagreement with the traditionally used method for very high concentrations of Gly, the method presented an excellent accuracy for analysis of Gly at low concentrations in plasma (determination error of 1.1%). This result indicates the feasibility of the developed method to show clearly if the Gly content in plasma is in the normal range or above it.

The main advantage of the developed methods over the employment of an amino acid autoanalyzer and, in general, over HPLC methods, that are the methods usually employed for amino acid analysis, is the use of small sample volumes. For instance, in the case of the plasma samples analysis, 50 μL is enough to carry out sample preparation and several runs. In addition, the methods developed in this work have reasonable repeatability in terms of peak height and migration time. Limits of detection are in the nM range, which is lower than that of the amino acid autoanalyzers and of the most employed CE methods with UV detection.

4. Concluding remarks

Two on-capillary derivatization methods, at 24 and 45 °C, for amino acid analysis by CE have been developed. These methods allow a sensitive determination of amino acids using LIF detection. Several parameters that control on-capillary derivatization have been optimized. Higher sensitivity is obtained for derivatization performed by the method developed at 45 °C than for that at 24 °C for all the amino acids tested, except Lys. For Lys analysis, the method developed at 24 °C has been employed because, besides the higher sensitivity, only one peak for this amino acid was obtained. In both methods, reaction plus separation take less than 30 min. The applicability of the methods developed for the analysis of several amino acids in pharmaceutical drugs and in plasma samples has been demonstrated. The concentration values provided by the methods developed agree quite well with those obtained with a standard method. The developed methods could be useful in quality control of pharmaceutical compounds. Also, the preliminary results obtained, seem to indicate the applicability of these methods for aminoacidopathies diagnosis. To this purpose, the feasibility of the developed methods for the analysis of more amino acids than that used in this work is being studied in our laboratory. In our opinion, most probably the developed methods should also be useful to determine amino acids in other matrixes, such as foods and beverages.

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