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Determination of acidity constants and prediction of electrophoretic separation of amyloid beta peptides

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ABSTRACT

In this paper we describe a strategy to estimate by CE the acidity constants (pK_a) of complex polyprotic peptides from their building peptide fragments. CE has been used for the determination of the pK_as of five short polyprotic peptides that cover all the sequence of amyloid beta (Aβ) peptides 1–40 and 1–42 (Aβ fragments 1–15, 10–20, 20-29, 25–35 and 33–42). First, the electrophoretic mobility (m_e) was measured as a function of pH of the background electrolyte (BGE) in the pH range 2–12 (bare fused silica capillary, I = 25 mM and T = 25 °C). Second, the m_es were fitted to equations modelling the ionisable behaviour of the different fragments as a function of pH to determine their pK_as. The accuracy of the pK_as was demonstrated predicting the electrophoretic behaviour of the studied fragments using the classical semiempirical relationships between m_e and peptide charge-to-mass ratio (m_e vs. $q/M_r^{1/2}$, classical polymer model, q = charge and M_r = relative molecular mass). Separation selectivity in a mixture of the fragments as a function of pH was evaluated, taking into account the influence of the electroosmotic flow (EOF) at each pH value, and a method for the simple and rapid simulation of the electropherograms at the optimum separation pH was described. Finally, the pK_as of the fragments were used to estimate the pK_as of the Aβ peptides 1–40 and 1–42 (^{t}C and D 3.1, E 4.6 and Y 10.8 for acidic amino acids and ^{t}N-D 8.6, H 6.0, K 10.6 and R 12.5 for basic amino acids), which were used to predict their behaviour and simulate their electropherograms with excellent results. However, as expected due to the very small differences on $q/M_r^{1/2}$ values, separation resolution of their mixtures was poor over the whole pH range. The use of poly(vinyl alcohol) (PVA) coated capillaries allowed reducing the EOF and a slight improvement of resolution.

1. Introduction

The acid dissociation constant (K_a, or pK_a in minus logarithm scale) is a fundamental parameter for physicochemical characterization of biologically and pharmacologically relevant compounds [1,2]. Capillary electrophoresis (CE) has been widely used for accurate determination of pK_a of a great variety of polyprotic compounds [1–4]. It is an excellent alternative to potentiometric [2,5], ultraviolet-visible (UV/Vis) spectrophotometric [2,6] and NMR [2,7] determination because it is not limited by sample volume or purity, it can be fully automated and it allows a great versatility in the selection of the separation conditions. The CE determination of pK_a is usually performed measuring the electrophoretic mobility (m_e) of the target compounds as a function of pH within an appropriate pH range in aqueous solutions [4], mixed hydro-organic [8] or non-aqueous media [9] using fused silica capillaries with UV detection [4,8,9]. During the last decade, different interesting alternatives have been proposed to increase the reproducibility and throughput of these typical procedures, such as application of multiplexed [10] and miniaturized instrumentation [11], coated capillaries [12,13], mass spectrometry detection [14,15] or internal standard-based methods [16]. However, most of the applications have been described for small molecules with only a few ionisable groups (less than 4). The determination of pK_as is troublesome for polyprotic compounds with many ionisable groups, especially when expected pK_as are extreme or correspond to the same or similar ionisable groups. This is the case of proteins and polypeptides, which may have dozens of pK_as. An interesting approach to estimate the pK_as of polypeptides may be the study of several fragment peptides with a smaller number of ionisable groups to cover the complete parent sequence. Following a similar strategy, Rickard et al. reported an excellent general set of average pK_a values for amino acids in polypeptides using biosynthetic human insulin (BHI)

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and human growth hormone (hGH) short fragments with a few ionisable groups [17].

The development of rapid, efficient and high-resolution separations in CE requires a previous optimisation. This optimisation can be assisted by modelling the electrophoretic behaviour of the target compounds to avoid excessive experimental work [15,18–21]. Accurate quantitative relationships between the m_e of ionisable compounds and the pH of the background electrolyte (BGE) have proved to be very useful to simultaneously determine their pK_as and the optimum pH for their separation in a complex mixture [15,18]. Moreover, several semiempirical relationships relating the m_e of the analytes to their structure (charge, molecular mass or number of amino acid residues) have been proposed [15,18–21]. In previous works, these classical semiempirical relationships yielded excellent correlations for several peptide hormones [15], neuropeptides [18], apothioneins [19], peptides and glycopeptides from tryptic digests of glycoproteins [20] and quinolones [21] when good estimates of pK_a values were available for charge calculations.

In this work, a general equation relating the m_e with pH, pK_as and activity coefficients is used to model the migration behaviour and determine the pK_as of five amyloid beta (Aβ) peptide fragments (1–15, 10–20, 20–29, 25–35 and 33–42) that cover all the sequence of Aβ peptides 1–40 and 1–42. Aβ 1–40 and 1–42 are clinical biomarkers used for Alzheimer's disease diagnosis, which is nowadays one of the most common age-related neurodegenerative disorders [22,23]. Aβ peptides are produced during normal cellular metabolism and are constituents of biological fluids but under pathological conditions they aggregate and form amyloidotic fibrils which are deposited and accumulated in the brain as plaques. As protein aggregation depends on pH [24,25], characterization of the physicochemical parameters of these peptides is important. Once the pK_as of the Aβ peptide fragments were determined by CE, the classical polymer model (m_e vs. $q/M_r^{1/2}$, q = charge and M_r = relative molecular mass) was used to predict the separation of a mixture of the fragments and confirm the accuracy of the pK_as. Finally, we demonstrated that the pK_as of the fragments were good estimates for the ionisable groups of Aβ peptides 1–40 and 1–42 by predicting separations in bare fused silica and poly(vinyl alcohol) (PVA) capillaries.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in the preparation of BGEs and solutions were of analytical reagent grade or better. Acetone, ammonia (25%), boric acid, diethylmalonic acid, hydrochloric acid (25%), methanol, phosphoric acid (85%), sodium acetate, potassium hydroxide, sodium dihydrogen phosphate, sodium formate and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane (Tris) was purchased from J.T. Baker (Deventer, Netherlands). Glutaraldehyde (50%) and PVA (hydrolysis grade 99%, average M_r 90,000) were purchased from Sigma-Aldrich (Steinheim, Germany). Water with conductivity lower than $0.05\,S\,cm^{-1}$ was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). The fragments of Aβ peptides 1–15, 10–20, 20–29, 25–35 and 33–42 and Aβ peptides 1–40 and 1–42 were provided by Bachem (Bubendorf, Switzerland). Their sequences, ionisable groups and M_r are shown in Table 1.

2.2. Electrolyte solutions and samples solutions

The BGEs for the determination of m_e covered the pH range 2–12. They were prepared at the following concentrations (pH was adjusted with 1.0 M HCl or 1.0 M NaOH and the I was calculated to be 25 mM): 15 mM NaH_2PO_4 (pH 2.0), 25 mM NaH_2PO_4 (pH 2.5–3.0), 25 mM sodium formate (pH 3.5–4.0), 25 mM sodium acetate (pH 4.5–5.0), 20 mM diethylmalonic acid (pH 5.5–6.5), 25 mM Tris (pH 7.0–9.0), 30 mM H_3BO_3 (pH 9.5–10.5) and 5 mM H_3PO_4 (pH 11.0–12.0). BGEs were passed through a 0.22 μm nylon filter (Panreac Applichem, Barcelona, Spain).

Individual stock solutions ($1000\,mg\,L^{-1}$) of Aβ 1–15, 10–20, 20–29, 25–35 were prepared in water and Aβ 33–42 in 5% (v/v) DMSO because it was less soluble in water. The working solutions ($200\,mg\,L^{-1}$) contained 3% (v/v) acetone or 5% (v/v) DMSO (Aβ 33–42) as electroosmotic flow (EOF) marker. A mixture of the five peptides ($200\,mg\,L^{-1}$) was also prepared in water. Solvent and storage conditions were especially critical for Aβ 1–40 and 1–42, which were prone to aggregation. Aβ 1–40 and 1–42 were dissolved ($1000\,mg\,L^{-1}$) in 50 mM and 100 mM ammonium hydroxide aqueous solutions, respectively, following the manufacturer's recommendation. A mixture of the two Aβ peptides ($200\,mg\,L^{-1}$) was prepared in 100 mM ammonium hydroxide aqueous solution. All the stock solutions were divided into several aliquots and individually stored at −20 °C. Each aliquot was thawed only once, preserved in the refrigerator between injections and immediately discarded after the analyses.

2.3. Instrumental parameters

All CE-UV experiments were performed in an Agilent HP 3DCE system (Agilent Technologies, Waldbronn, Germany). Unless otherwise indicated, separations were performed at 25 °C in a 57 cm total length (L_T) × 75 μm internal diameter (i.d.) × 365 μm outer diameter (o.d.) bare fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA). All capillary rinses were performed at high pressure (930 mbar). New capillaries were flushed with 1 M NaOH (15 min), water (15 min) and BGE (30 min). The system was finally equilibrated by applying 25 kV of separation voltage for 20 min (normal polarity, cathode in the outlet). Samples were injected at 50 mbar for 3 s. Between runs, capillaries were conditioned by rinsing with 1 M NaOH (1 min), water (1 min) and BGE (1 min). Between workdays or after a change of BGE, the capillary was conditioned by rising with 1 M NaOH (5 min), water (5 min) and BGE (10 min). The system was finally equilibrated by applying 25 kV of separation voltage for 20 min. Capillaries were stored overnight filled with water. For experiments with PVA capillaries, bare fused silica capillaries were coated with PVA following a procedure described elsewhere and cut to a L_T of 57 cm [26,27]. The conditioning, rinsing and separation methods were the same as in bare fused silica capillaries, but without using NaOH to prevent coating damage.

pH measurements were made with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison Instruments, Barcelona, Spain).

2.4. Determination of electrophoretic mobility and acidity constants

The m_e of each Aβ fragment was measured by CE-UV as the difference between the apparent mobility of each peptide, m_{app}, and the mobility of the neutral marker, m_{EOF} [15,18–21]:

$$m_e = m_{app} - m_{EOF} = \frac{L_c L_D}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (1)$$

Where L_C is the capillary length, L_D is the distance from the injection point to the detector and t_{app} and t_{EOF} are the migration time of the peptide and the neutral marker, respectively. Individual solutions of each peptide were injected at each pH and m_e was obtained as the average of five replicates. BGEs indicated in Section 2.2 were run in sequence from low to high pH.