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Nanoscale liquid chromatography and capillary electrophoresis coupled to electrospray mass spectrometry for the detection of amyloid-β peptide related to Alzheimer's disease

Emmanuel Varesio[a], Serge Rudaz[a], Karl-Heinz Krause[b], Jean-Luc Veuthey[a,*]

[a]*Laboratory of Pharmaceutical Analytical Chemistry, School of Pharmacy, University of Geneva, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland*

[b]*Biology of Ageing Laboratory, Department of Geriatrics, Geneva University Hospital, 1225 Chêne-Bourg, Geneva, Switzerland*

Abstract

Alzeihmer's disease (AD) is a neurodegenerative disorder which is pathologically characterized by the progressive deposit in the brain of a specific form of amyloid, amyloid-β peptides (Aβ). As the latter circulate in the blood, their quantitation in plasma could allow a simple diagnosis of AD. Aβ are present in different variants, one of which contains 40 amino acid residues ($A\beta_{1-40}$). In this work, nanoscale liquid chromatography (nano-LC) and capillary electrophoresis (CE) coupled to mass spectrometry (MS) were compared to determine the most appropriate technique for reaching the usual $A\beta_{1-40}$ concentration in plasma or serum. Both a 50 μm I.D. CE capillary and a 75 μm I.D. nano-LC column were coupled to a single quadrupole mass spectrometer with a sheath-liquid electrospray (ESI) interface or a homemade nanospray interface, respectively. Capillary zone electrophoresis is a powerful separation technique, but its low sensitivity limits its use in the analysis of biological matrices. However, a column-switching set-up with a precolumn of 1 mm×300 μm I.D. packed with a C_{18} PepMap (3 μm) stationary phase and a nanocolumn of 15 cm×75 μm I.D. packed with the same stationary phase was found to be a successful technique which allowed detection of $A\beta_{1-40}$ at the ng ml^{-1} level (a few hundred femtomoles injected) because of its higher sample loading capability.

Keywords: Alzeihmer's disease; Mass spectrometry; Nanoscale liquid chromatography; Amyloid-β peptide

1. Introduction

Alzeihmer's disease (AD) is pathologically characterized by the progressive deposit in the brain of a specific form of amyloid-β peptides (Aβ). Aβ are present in two principal variants, one that contains 40 amino acid residues ($A\beta_{1-40}$) and another hydrophobic extended variant with 42 amino acid residues ($A\beta_{1-42}$) which acts as the initial factor of plaque formation. The more soluble $A\beta_{1-40}$ is the main form which circulates in the plasma and cerebrospinal fluid (CSF) and progressively accumulates in the brain [1,2]. Therefore, it is possible that Aβ is transported into the brain through the blood circulation [3]; the recent identification of circulating Aβ seems to indicate that the quantification of Aβ levels in plasma might allow prediction of the risk of developing AD, might contribute to the diagnosis of AD and could offer an evaluation of new therapies

*Corresponding author. Tel.: +41-22-702-6336; fax: +41-22-702-6808.

E-mail address: jean-luc.veuthey@pharm.unige.ch (J.-L. Veuthey).

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such as drugs and vaccination [4]. $A\beta_{1-40}$ and $A\beta_{1-42}$, together with a series of slightly shorter and longer variants, are generated by cleavage of the amyloid precursor protein (APP). This cleavage is carried out by enzymes such as β-secretase [5] or γ-secretase in its transmembrane domain (Fig. 1) [6,7].

Several analytical techniques have been developed to detect amyloid-β peptides in different matrices. The enzyme-linked immunosorbent assays (ELISA) are the most commonly used techniques and Suzuki et al. developed an Aβ sandwich ELISA for dosing $A\beta_{1-40}$ and $A\beta_{1-42}$ in brain extracts [8]. Another approach using Aβ antibodies is immunoprecipitation (IP), which is either coupled to LC–MS or directly to MS. Takeda et al. achieved a semiquantitative analysis of Aβ in brain extracts by combining IP and MALDI–TOF-MS techniques [9]. Naylor et al. have demonstrated that LC–MS gave better results than MALDI–TOF partly due to salt removal during the LC run [10].

Several methods based on liquid chromatography (LC) have been developed for Aβ analysis [11–13], whereas only few publications focus on capillary electrophoresis (CE) separation [14].

In this publication, the potential of both techniques was evaluated for the analysis of $A\beta_{1-40}$ peptide, which was chosen as a model compound of the amyloid-β peptide family.

Therefore, nanoscale liquid chromatography (nano-LC) and CE coupled to mass spectrometry (MS) were compared in order to determine the most appropriate technique to reach the usual $A\beta_{1-40}$ concentration in plasma or serum. Both a 50 μm I.D. CE capillary and a 75 μm I.D. nano-LC column were coupled to a single quadrupole mass spectrometer with a sheath-liquid electrospray (ESI) interface or a homemade nanospray interface, respectively. Generally, ESI is the interface preferred for the on-line coupling of CE and LC with MS, since it generates ions with multiple charges allowing the detection of even large species such as peptides or proteins. Furthermore, m/z data of multiply charged ions can be mathematically deconvoluted to give the original masses of the species [15].

2. Experimental

2.1. Chemicals

Lyophilized synthetic human $A\beta_{1-40}$ peptide

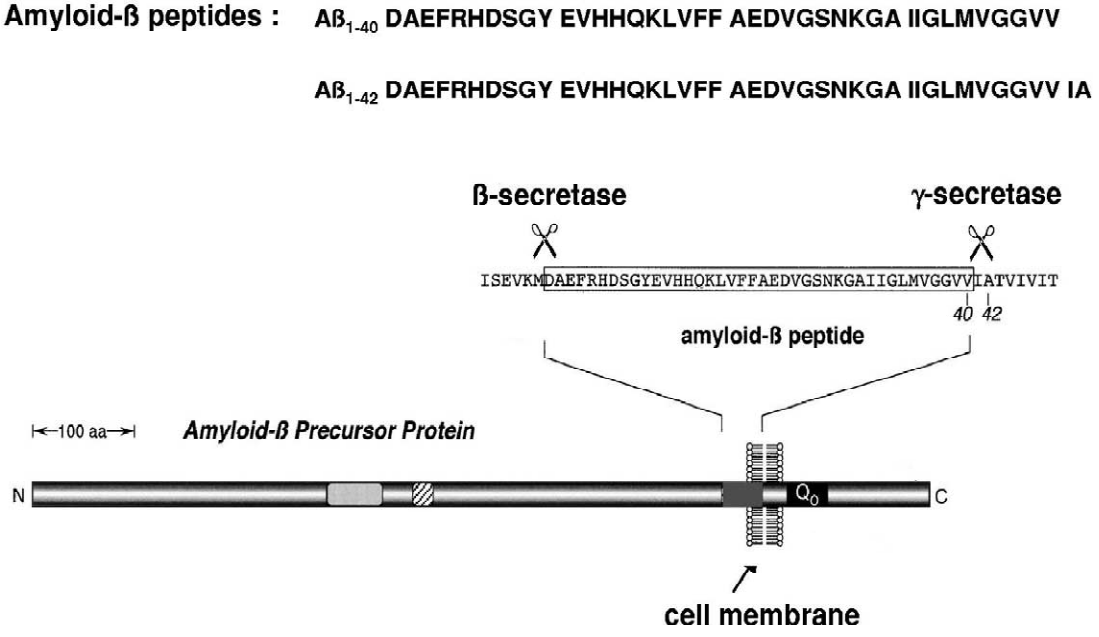

Fig. 1. Commonly proposed mechanism of major Aβ peptides formation.

under the form of trifluoroacetate salt was supplied by Calbiochem-Novabiochem (San Diego, CA, USA).

All reagents were of analytical grade: acetonitrile and isopropanol were provided by SDS (Peypin, France). Formic and acetic acids were purchased from Fluka (Buchs, Switzerland). Trifluoroacetic acid was from Merck (Darmstadt, Germany). Aqueous ammonia (~24%) was from Reactolab (Servion, Switzerland). Deionized water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). Nitrogen for the MS detector was supplied by a nitrogen generator system (Whatman, Maidstone, UK).

2.1.1. Sample preparation

Lyophilized peptide was reconstituted in 5% (v/v) aqueous acetic acid at a concentration of 500 μg ml^{-1}. Acetic acid was used to help peptide solubilization, and aliquots were kept in the freezer at −20 °C. For CE analyses, aliquots were diluted with a 5% (v/v) aqueous acetic acid solution to obtain concentrations of 250 and 50 μg ml^{-1} of $A\beta_{1-40}$ peptide. For nano-LC, concentration of 100 ng ml^{-1} was reached with a 5% (v/v) aqueous acetic acid solution.

2.2. Instrumentation

2.2.1. Capillary electrophoresis

Capillary electrophoresis experiments were performed with an $HP^{3D}CE$ instrument (Agilent Technologies, Waldbronn, Germany). The Chemstation software suite (Agilent Technologies) was used for instrument control, data acquisition and data processing.

For UV experiments, separation was performed in a fused-silica capillary of 56/64.5 cm, 50 μm I.D. (Agilent Technologies) equipped with an extended light-path detection window (i.e. bubble window–bubble factor of 3). Constant voltage of 20 kV was applied throughout all analyses. The capillary was thermostated at 55 °C. UV detection was carried out at 214 and 280 nm. Samples were kept at ambient temperature in the autosampler and injected by hydrodynamic mode (50 mbar for 4 s, ~9 nl injected).

For CE–MS experiments, separation was performed in a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 22/75.5 cm and 50 μm I.D. Constant voltage of 20 kV was applied throughout all analyses. Samples were injected by hydrodynamic mode (50 mbar for 20 s, ~40 nl injected). The on-line coupling of the CE instrument to the mass spectrometer detector was achieved with a commercial orthogonal coaxial triple tube ESI interface (Agilent Technologies). The sheath-liquid, constituted of isopropanol–water (80:20, v/v) in presence of 0.5% (v/v) formic acid, was delivered by a low pressure single-syringe infusion pump model 22 (Harvard Apparatus, South Natick, MA, USA) at a flow rate of 1 μl min^{-1}.

Background electrolye (BGE) was either ammonium acetate buffer (50 m*M*) adjusted to pH 4 or pH 7, or ammonium formate adjusted to pH 3. Electrolyte solutions were filtered through a 0.45-μm pore size syringe filter (Millipore, Milford, MA, USA) and degassed in an ultrasonic bath for 10 min. Before each run, the capillary was preconditioned by flushing water (5 min) and BGE (5 min).

2.2.2. Nanoscale liquid chromatography (nano-LC)

Experiments were performed with an UltiMate Capillary LC system (LC Packings-A Dionex Company, Amsterdam, The Netherlands) mounted with a Z-shaped UV nano flow-cell of 10-mm path length (i.e. cell volume of 3 nl) (LC Packings-A Dionex Company). UV detection was carried out at 214 and 280 nm.

Samples were injected with a FAMOS microautosampler (LC Packings-A Dionex Company) equipped with a 20-μl loop. A column-switching set-up was used with an additional six-port switching valve mounted on the autosampler side panel, as well as a supplementary HP 1050 Series HPLC isocratic loading pump (Hewlett-Packard, Waldbronn, Germany). The UltiChrom software suite (LC Packings-A Dionex Company) was used for instrument control, and also for triggering the HP 1100 Series MSD.

The chromatography was performed on an analytical fused-silica nanocolumn of 15 cm×75 μm I.D. packed with a C_{18} PepMap (3 μm) stationary phase (LC Packings-A Dionex Company). The aqueous mobile phase (A) contained 0.1% (v/v) formic acid solution, and the organic mobile phase (B) contained 0.08% (v/v) formic acid in a water–ace-

tonitrile (5:95, v/v) mixture. A linear gradient started from 5%B to 60%B in 30 min followed by a washing step of 95%B for 4 min. Finally, the column was re-equilibrated with the initial mobile phase. A flow rate of 250 nl ml^{-1} was delivered throughout the entire procedure.

For the column-switching set-up, the loading solvent consisted of a 0.1% (v/v) trifluoroacetic acid (TFA) aqueous solution delivered at a flow rate of 50 μl min^{-1}. A nano-precolumn of 1 mm×300 μm I.D. (LC Packings-A Dionex Company) packed with the same stationary phase (C_{18}-PepMap) as the analytical column was used for sample preconcentration and clean-up.

Coupling nano-LC to MS was achieved by a liquid junction-based nanospray interface. This interface consisted of a PEEK micro-tee assembly (UpChurch Scientific, Oak Harbor, WA, USA) equipped with a platinum wire acting as an electrode, and a fused-silica PicoTip (5 cm×20 μm I.D.) as nanospray needle (New Objective, Cambridge, MA, USA). The whole set-up was mounted on a homemade three-axis translational stage.

2.2.3. Mass spectrometry

Measurements were carried out in the positive electrospray ionization mode and were performed on a single quadrupole HP Series 1100 MSD (Agilent Technologies, Palo Alto, CA, USA). For CE–MS experiments, the electrospray voltage was set at 4500 V. The nebulizing pressure (i.e. nitrogen) was set at 10 p.s.i. Nitrogen was also used as drying gas at a flow rate of 7 l min^{-1} and a temperature of 100 °C. MS acquisition was performed in the selected ion monitoring (SIM) mode. Selected masses were the multiply charged ions of $A\beta_{1-40}$ peptide corresponding to $[M+5H]^{5+}=866.7$ amu and $[M+6H]^{6+}=722.2$ amu with a dwell time of 559 ms for each ion. For nano-LC–MS coupling, a homemade nanospray interface was used and the electrospray voltage was set at 2000 V. Nitrogen was used as drying gas at a flow rate of 7 l min^{-1} and a temperature of 200 °C. MS acquisition was performed in the selected ion monitoring (SIM) mode. Selected masses were multiply charged ions of $A\beta_{1-40}$ corresponding to $[M+2H]^{2+}=2165.0$ amu, $[M+3H]^{3+}=1444.0$ amu, $[M+4H]^{4+}=1083.0$ amu, $[M+5H]^{5+}=866.6$ amu and $[M+6H]^{6+}=722.2$ amu with a dwell time of 173 ms for each ion.

3. Results and discussion

3.1. Capillary electrophoresis

Preliminary analyses by CE were performed with UV detection. According to its theoretical p*I* value of 4.9, $A\beta_{1-40}$ migrated under its anionic form with ammonium acetate buffer at pH 7 as BGE, or under its cationic form when the pH value of the BGE was ~4. Fig. 2 shows electropherograms obtained for a 250 μg ml^{-1} peptide standard solution analyzed at both pH with a fused-silica capillary equipped with an extended path length detection window (i.e. bubble cell). One disadvantage of CE–UV is its

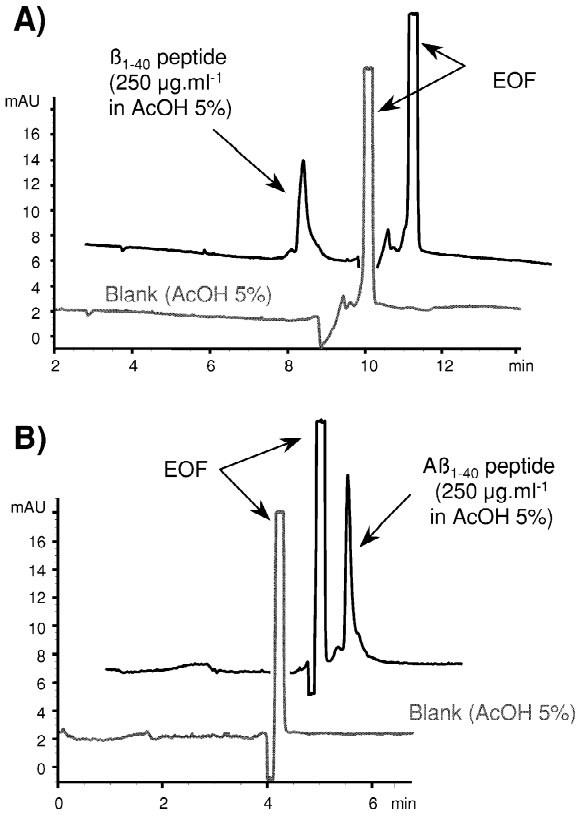

Fig. 2. CE–UV separation at two different pH: (A) 50 m*M* ammonium acetate (pH 4.0); (B) 50 m*M* ammonium acetate (pH 7.0). See text for experimental details.

relatively low sensitivity due to the small quantity injected and to the short optical path length afforded by the small internal diameter (I.D.) of the capillary. Therefore, a high peptide concentration was necessary to perform UV detection at a wavelength of 214 nm. Nevertheless, CE possesses the great advantage of tuning the ionic form of the peptide by adjusting the electrolyte's pH. Thus, the use of a BGE at acidic pH permitted analysis of the amyloid peptide under its cationic form without adsorption on the capillary wall.

To overcome the sensitivity limitation, two strategies were considered: firstly, increasing the amount of analyte introduced in the CE capillary, and secondly, enhancing detection sensitivity. The first approach can be performed either by chromatographic techniques, such as on-capillary membrane preconcentration or by electrophoretic methods such as field amplified sample stacking [16,17]. After several unsuccessful experiments with the latter technique (data not shown), the second approach was investigated by coupling CE with MS to attain the expected biological concentration of $A\beta_{1-40}$ peptide (i.e the ng ml^{-1} range).

Before performing any separation with the CE–MS interface, a standard solution of ~200 μg ml^{-1} of $A\beta_{1-40}$ was prepared and infused in the MS at a flow rate of 1 μl min^{-1} using a low-pressure syringe pump. The MS signal gave two main multiply charged ions at 722.2 amu ($[M+6H]^{6+}$) and 866.7 amu ($[M+5H]^{5+}$), and a minor ion observed at 1083.1 amu ($[M+4H]^{4+}$).

For successful CE–MS coupling, different interfaces have been developed. However, because of its instrumental simplicity and ability to enhance the ionization process by chemical reaction, the coaxial sheath-liquid electrospray interface is by far the most popular and suitable configuration for CE–ESI-MS [18]. The sheath-liquid is used for closing the electrical circuit and for helping peptide protonation. In this study, the sheath-liquid was a solution of 0.5% (v/v) formic acid in water–isopropanol (20:80, v/v) delivered at a flow rate of 1 μl min^{-1}. Several unsuccessful attempts were made under negative electrospray ionization with an alkaline sheath-liquid (data not shown).

In order to analyze the peptide under its cationic form, two different acidic buffers were compared. The formate buffer (pH 3.0) gave a slightly higher efficiency than the acetate buffer (pH 4.0), probably due to a lower pH value decreasing the silanophilic interactions of the peptide with the capillary walls. Better sensitivity was achieved with CE–MS than with CE–UV. A concentration of 50 μg ml^{-1} (~40 nl injected) was detected by CE–MS (Fig. 3), while only 250 μg ml^{-1} was detected by CE–UV. However, this sensitivity is not sufficient for the analysis of biological fluids since the expected concentrations are between 25 and 650 ng ml^{-1} [4,19].

3.2. Nanoscale liquid chromatography

Before performing chromatography, a 100 μg ml^{-1} standard $A\beta_{1-40}$ solution was infused in the MS through the liquid junction-based nanospray interface. Since the nanospray needle was positioned relatively close to the MS ion sampling orifice, a lower electrospray voltage (i.e. 2000 V) was applied than in the CE–MS experiment (i.e. 4000 V). Therefore, and as shown in Fig. 4, five multiply charged ions were monitored for $A\beta_{1-40}$ peptide. These ions were further used to tune the MS quadrupole in the SIM mode. By deconvolution, the molecular weight (MW) of $A\beta_{1-40}$ peptide was calculated with a mass of 4328.5 Da (specified MW at 4328.9 Da from the supplier). This mass was previously confirmed by MALDI–TOF experiments conducted on a Voyager DE-STR from PE Biosystems (Foster City, CA, USA), where a MW of 4328.2 Da (i.e. a molecular ion $[M+H]^{+}$ of 4329.2 Da) was determined (data not shown).

Nano-LC is a well established technique for the analysis of peptides and proteins in various matrices (i.e. biological fluids [20], cell lysates [21,22], protein digests [23]). Such a miniaturized LC system is generally used so as to have a compatible mobile phase flow rate with ESI-MS (i.e. no post-column splitter is necessary), and to maintain the benefit of high sensitivity due to the reduced inner diameter (I.D.) of the chromatographic columns. This theoretical gain in sensitivity (F) can be calculated by using the following equation: $F=(ID_{\text{conventional}}/ID_{\text{reduced}})^2$ [24]. However, in order to achieve this sensitivity improvement, the same amount of sample should be injected onto the conventional and the miniaturized column. This can be achieved either by using the

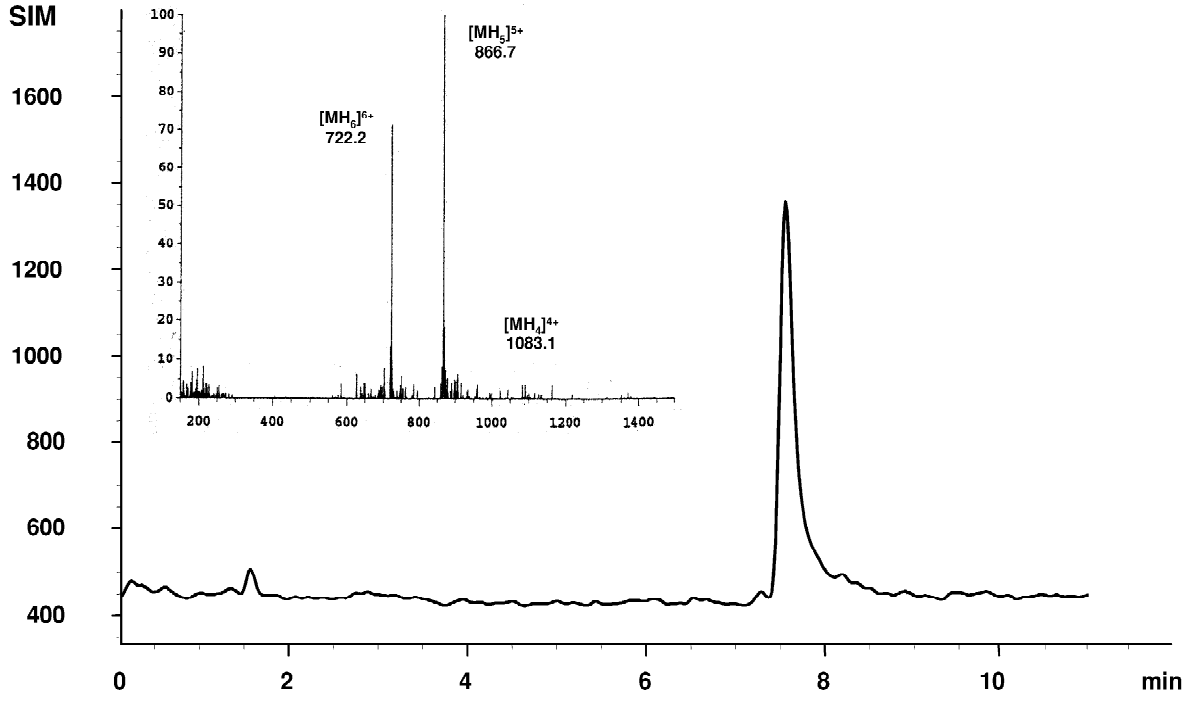

Fig. 3. Electropherogram obtained in CE–MS for a 50 μg ml^{-1} $A\beta_{1-40}$ solution. Insert: MS spectrum of $A\beta_{1-40}$ when infusing a standard solution with a syringe pump using the regular ESI interface. See text for experimental details.

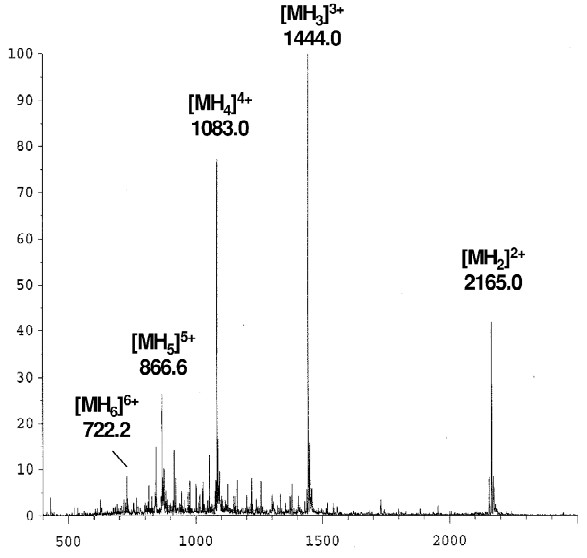

Fig. 4. MS spectrum of $A\beta_{1-40}$ when infusing a standard solution with a syringe pump using the nanospray interface. See text for experimental details.

sample focusing effect during the injection (e.g. by using an injection solvent with a lower eluent strength than the mobile phase), or by using a column-switching set-up. In this study, only the column-switching approach was evaluated.

Indeed, this technique combines both a sample pre-concentration and a clean-up step, as well as a fast injection mode. With a 300 μm I.D. pre-column, a loading flow rate of 50 μl min^{-1} was used to completely flush the 20-μl loop in less than 30 s (vs. 100 min for direct injection with a regular nano-LC set-up).

Fig. 5 shows the column-switching set-up used with the single quadrupole MSD detector. As mentioned previously, the injection volume might be somewhat larger in column-switching experiments than with a regular nano-LC set-up. In this study, both 10 μl (partial loop) and 20 μl (full loop) of $A\beta_{1-40}$ peptide solution (100 ng ml^{-1}) were injected. A preconcentration time of 2 min was chosen to flush the injection loop as well as the connecting PEEK tubings. The loading solvent constituted of

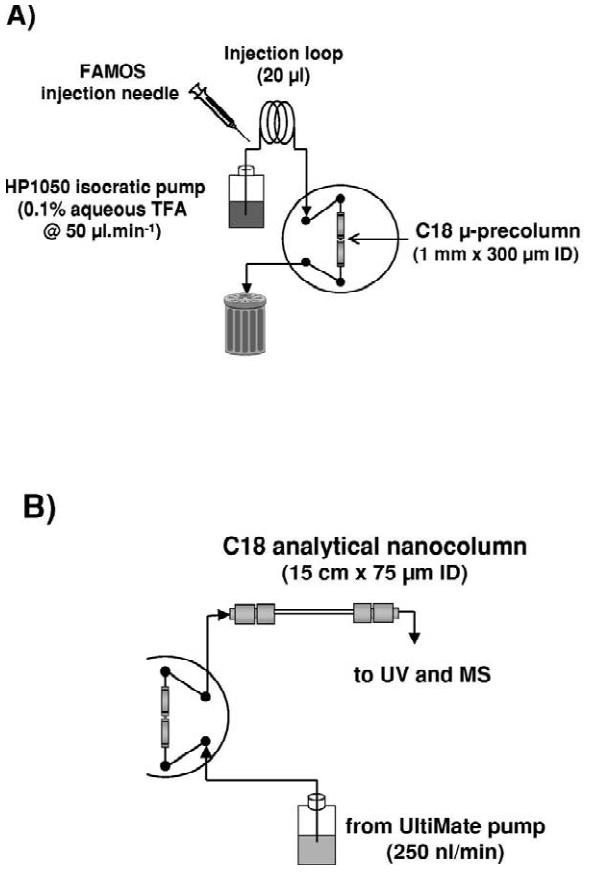

Fig. 5. Column-switching set-up for sample preconcentration and clean-up (A) followed by amyloid peptide analysis (B). See text for experimental details.

0.1% (v/v) aqueous TFA solution was delivered at a flow rate of 50 µl min^{-1}. After 2 min, the valve was switched and the analyte was backflushed in the analytical column and the mobile phase gradient was started. The chromatographic conditions were slightly adapted from Thompson et al. [13] for the nano-LC system requirements. Fig. 6 shows the resulting chromatogram obtained in these experimental conditions. The chromatogram represents the trace of the four most intense multiply charged ions (SIM mode). It can be noted that no peak was detected by UV in these conditions. The limit of detection was estimated at ~25 ng ml^{-1}, which fulfills the analytical requirements in terms of the monitoring range.

Works are in progress to find a suitable sample preparation procedure to analyze the other amyloid peptides present in biological fluids such as serum or plasma (e.g. $A\beta_{1-39}$, $A\beta_{1-40}$, $A\beta_{1-42}$). Indeed, $A\beta_{1-40}$ peptide was found to be fairly soluble in aqueous solutions, but $A\beta_{1-42}$ peptide is known to present poor solubility in aqueous solvents; therefore, the analytical conditions have to be adapted [25].

4. Concluding remarks

Evaluation of both CE–MS and nano-LC–MS was conducted for the analysis of $A\beta_{1-40}$. It was reported that both techniques are complementary. Indeed, CE possesses the great advantage of tuning the sepa-

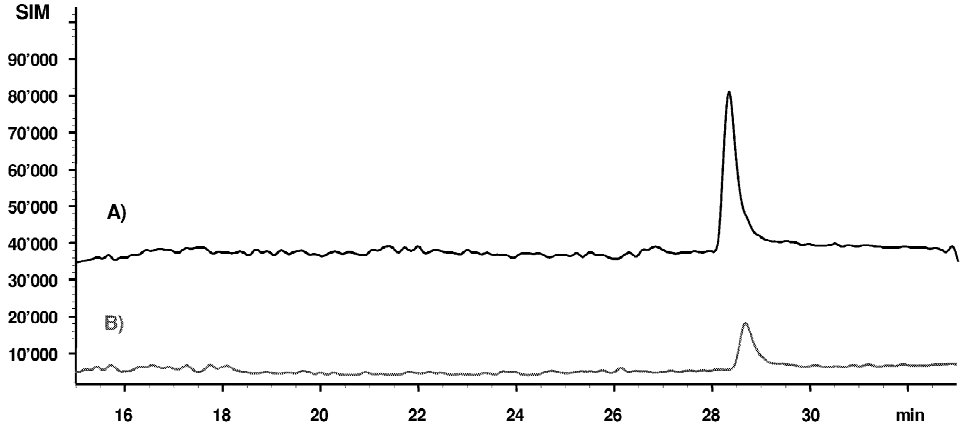

Fig. 6. Chromatograms obtained in nano-LC for a 100 ng ml^{-1} $A\beta_{1-40}$ peptide solution: (A) 20 µl injected (i.e. 460 fmol), (B) 10 µl injected (i.e 230 fmol).

ration by adjusting the pH of the buffer electrolyte solution which allows good flexibility during method development. However, the lack of sensitivity remains a major drawback for analysis in the biological range concentration and CE–MS would be better applied in quality control during peptide synthesis.

This study demonstrated that a column-switching set-up, enabling large injection volumes, is necessary to reach the required sensitivity when using nano-LC–MS. Moreover, a reduction of the analytical column inner diameter could further improve these limits of detection. However, when working with a nano-LC column, the LC–MS interface should be modified according to the low flow rate delivered (e.g. 250 nl min^{-1}). Therefore, nano-LC–MS is certainly a more appropriate technique than CE–MS, in terms of sensitivity, to monitor Aβ peptides in biological fluids.

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