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High-Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry for the Detection of Amyloid Beta Peptide Related with Alzheimer's Disease

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Recent studies show that quantitative and qualitative differences in amyloid β (Aβ) peptides may be implicated in the development of Alzheimer's disease. New evidence seems to support the existence of a dynamic equilibrium between Aβ peptide in the brain and peripheral blood circulation. The quantitation of Aβ in the blood may allow the development of the potential value of Aβ peptides as a biomarker in the development of Alzheimer's disease. In this communication, quantitation of Aβ peptides using high-performance liquid chromatography coupled with tandem mass spectrometry in a linear ion trap mode is presented. RP-HPLC was performed using a Waters Xterra™ MS C8 column (3.0 mm × 150 mm). $Aβ_{1-40}$ peptide was eluted using a gradient elution program. Eluate from the RP-HPLC column was split to both the UV detector and electrospray ionization MS source. The product ion scan was performed in a linear ion trap mode utilizing the transition of a multiply charged molecular ion of $Aβ_{1-40}$ to a singly charged product ion. The detection limit of 31.25 ng in column load using a 3.0-mm-diameter conventional C8 column was achieved. The $Aβ_{1-40}$ standard calibration curves show excellent linearity from 34 ng to 2500 ng $Aβ_{1-40}$ of column sample load. The product ion scan enhances sensitivity 10 times compared with the best previously achieved by a single-quadrupole instrument in the selective ion monitoring mode. Moreover, the product ion scan of $Aβ_{1-40}$ provides superior selectivity and specificity, which is very important in the quantitation of $Aβ_{1-40}$ in a complex biological matrix.

Key Words: $Aβ_{1–40}$, A-beta peptide, high performance liquid chromatography, mass spectrometer, Alzheimer's disease.

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Alzheimer's disease (AD) is the leading cause of dementia and the most prevalent neurodegenerative disease of aging. A growing body of evidence suggests that amyloid beta (Aβ) peptide plays a primary causal role in the progression of AD.[1] Aβ is found in the cerebrospinal fluid and serum of normal individuals as well as AD patients. Overproduction of Aβ from APP processing errors, Aβ catabolism,[2] clearance,[3,4] and aggregation[5] are also critical in regulating Aβ metabolism. Aβ production is not only restricted to the brain, but is also detected in the media of various culture cells and in biological fluids in both normal individuals and AD patients.[6] New evidence seems to support the existence of a dynamic equilibrium between Aβ in the cerebral spinal fluid (CSF) and various peripheral compartments. A change in Aβ concentration and the composition of its variants in one compartment will perturb the equilibrium of Aβ between the CSF and peripheral compartments. The disturbance in this equilibrium may be a fundamental aspect of AD pathogenesis and progression. Information about Aβ levels and their change in human blood serum may offer a new lead in decoding the pathological mechanisms of AD and may help in developing a treatment to combat this deadly neurological disease of the twenty-first century.

In most studies, Aβ levels in body fluids have been measured by a sandwich ELISA method.[7] Although ELISA is a simple method suitable for routine clinical

TABLE 1

Human Blood Serum Aβ Levels from Different Studies

$A\beta_{1-40}$ Level (ng/mL) in blood			$A\beta_{1-42}$ Level (ng/mL) in blood				
AD	Non-AD dementia	Control	AD	Non-AD dementia	Control	Methods	Refs
0.220 ± 012		0.18 ± 0.86	0.048 ± 0.036		0.53 ± 0.23	Western Blot	19
14.6	13.9		236.4	0.038		SEC-EuIA	20
0.143 ± 0.04	0.12 ± 0.040		0.066 ± 0.024	0.058 ± 0.033		Sandwich ELISA	7
10.9 ± 7.1	7.6 ± 2.9		21.4 ± 4.8	20.6 ± 7.7		SEC-EuIA	9

use, it may not be an ideal method to quantitate Aβ levels in blood, due to specific properties of Aβ and its low concentration in human blood. ELISA measurements cannot distinguish between the full-length Aβ peptides and their truncated structural variants.[8] Aβ measurement by ELISA can be affected by the presence of other proteins, which may cross-react or mask the epitope of antibodies used in the assay. Some studies have revealed the limits of Aβ quantitation by an ELISA method. For example, it has been found that Aβ in serum binds not only to carrier proteins, such as albumin, but also to erythrocytes, which may hinder accurate quantification by immunoassay.[9] The addition of varying concentrations of synthetic peptides to the serum resulted in only 36% recovery of $A\beta_{1-40}$ and 26% recovery of $A\beta_{1-42}$.[9] Because of amphoteric and amphipatic characteristics, Aβ peptides can interact with a large number of serum proteins and other molecules. The interaction of Aβ results in the masking of antigenic epitopes and interferes with immunoassay, making conventional ELISA methods for Aβ inaccurate. Table 1 summarizes the reported Aβ serum levels from different human serum studies. For convenience of comparison, concentrations are reported in ng/mL units. As shown in Table 1, there is a significant variation in mean Aβ blood levels and a large range in values from one study to another. These differences can indeed come from the patient and control samples. However, such a conclusion cannot be reached before we exclude the possibility of inaccuracy in analytical methodology due to the absence of assay standardization in Aβ measurement. In fact, scientists have pointed out the biggest future challenge to research in AD will be the standardization of Aβ measurement methods.[10]

To circumvent limitations of an ELISA method and to permit accurate quantitation of Aβ levels in blood serum, several research groups have explored HPLC coupled with mass spectrometry to qualitatively and quantitatively analyze Aβ in biological fluids.[11–13] Using synthetic Aβ peptide, Thompson et al. demonstrated that high-temperature chromatographic separation on a C4 column coupled with electrospray ionization mass spectrometry is an efficient method for the separation and identification of N-terminal truncated Aβ peptides.[13] Varesio et al. showed nanoscale HPLC and capillary electrophoresis coupled to single-quadrupole electrospray mass spectrometry is an appropriate technique for detecting $A\beta_{1-40}$.[14] Recently, Aβ peptide isoforms in Alzheimer's plaques were successfully quantitated by stable isotope dilution using ^{15}N-labeled Aβ peptide standards spiked in the sample.[15]

The low abundance of Aβ peptides in a complex biological matrix, combined with their hydrophobic nature, make it a challenging task to accurately quantitate Aβ peptides in blood. In this publication, quantitation of Aβ peptides using high-performance liquid chromatography coupled with tandem mass spectrometry in a linear ion trap mode is presented. The product ion scan in a linear ion trap mode utilizing the transition of a multiply charged molecular ion of $A\beta_{1-40}$ to a singly charged product ion enhances sensitivity 10 times compared with the best sensitivity previously achieved by a single-quadrupole instrument in the selective ion monitoring mode. Moreover, the product ion scan of $A\beta_{1-40}$ provides superior selectivity and specificity, which is very important in the quantitation of $A\beta_{1-40}$ in a complex biological matrix. Development of a reliable, robust, and accurate analytical method in this project will help us to draw a clearer picture of Aβ levels and Aβ composition in human blood serum; this will prove valuable in developing reliable AD biomarkers.

MATERIALS AND METHODS

Chemicals

Synthetic human $A\beta_{1-40}$ peptide, ammonium acetate, and HPLC grade solvents (water, acetonitrile, isopropanol) were supplied by Sigma-Aldrich (St. Louis, MO). Glacial acetic acid and Triton X-100 were obtained from Fisher (Fair Lawn, NJ). All chemicals were analytical grade.

Sample Preparation

Siliconized test tubes and HPLC sample vials were used throughout the experiments. $A\beta_{1-40}$ in lyophilized form was reconstituted in 5% (v/v) aqueous acetic acid and 1% Triton X-100. Acetic acid was used to assist peptide solubilization, and Triton X-100 was added to reduce nonspecific binding of $A\beta_{1-40}$ peptide during sample transfer. The reconstituted peptide was kept in the freezer at –20°C until further use. For HPLC-MS-MS analysis, the $A\beta_{1-40}$ aliquot was step-diluted with 5% (v/v) aqueous acetic acid and 1% Triton X-100 to obtain samples with concentrations from 0.39 µg/mL to 25 µg/mL.

Instrumentation

High-Performance Liquid Chromatography

A Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD), consisting of a SCL-10AVP controller, SIL-10AD VP autoinjector, SPD-10A VP UV-VIS detector, and LC-10AT VP pump, was used. RP-HPLC was performed using a Waters Xterra MS C8 column (3.0 mm × 150 mm) with a particle size of 3.5µm (Waters Corporation, Milford, MA). A 10-µL aliquot of synthetic $A\beta_{1-40}$ was injected onto the HPLC column. The mobile phase consisted of solvent A (5% acetonitrile, 5% isopropanol, 10 mM ammonium acetate, and 90% water, pH = 7) and solvent B (45% acetonitrile, 45% isopropanol, 10 mM ammonium acetate, and 10% water, pH = 7). $A\beta_{1-40}$ peptide was eluted using a gradient elution program of 20% to 85% B in 15 min, a 5-min hold at 85% B, followed by a return to 20% B for a 10-min equilibration. The flow rate was 0.4 mL/min. Eluate from the RP-HPLC column was split to both the UV detector and electrospray ionization MS source. An eluate stream of approximately 50 µL/min was directly introduced into the TurboIonSpray source.

Mass Spectrometry

Mass spectrometry experiments were performed on either a hybrid quadrupole/linear ion trap 4000 Q TRAP MS/MS system or Q TRAP MS/MS system (Applied Biosystems, Foster City, CA). All analyses were performed in the highly sensitive linear ion trap (LIT) mode. Analyst software was used for data analysis. Enhanced multiple scans were performed with a hybrid quadrupole/linear ion trap 4000 Q TRAP system to acquire multiply charged molecular ions of $A\beta_{1-40}$ peptides. Enhanced resolution scans using the 4000 Q TRAP system were also used to examine and to confirm charge states of the multiply charged molecular ion. The enhanced product ion scan was initially performed with a 4000 Q TRAP system to generate the product ion fragmentation pattern of the $A\beta_{1-40}$ molecular ion. The transition from a multiply charged molecular ion to the most abundant singly charged product ion was used to detect and quantitate $A\beta_{1-40}$ peptide using the Q TRAP system in the positive electrospray ionization mode, equipped with a TurboIonSpray Source. The following conditions were used for LC-MS/MS quantitation of $A\beta_{1-40}$ peptides using the Q TRAP system: declustering potential, 40.0; entrance potential, 10; collision energy, 60; collision energy spread, 0; curtain gas, 30.0; collision gas, high; ionSpray voltage, 2500; temperature, 350°C; ion source gas 1, 50 L/min; ion source gas 2, 50 L/min; interface heater, on; fixed LIT fill time, 100 msec; Q1 resolution, low; scan rate, 250 amu/sec; Q3 entry barrier, 8 V; settle time, 0 msec; and pause between mass ranges, 5.00 msec.

RESULTS AND DISCUSSION

The electrospray mass spectrum permits unambiguous identification of $A\beta_{1-40}$. When a 10 µg/mL $A\beta_{1-40}$ standard solution is infused into the mass spectrometer via an automatic syringe pump, the enhanced multiple scan in the linear ion trap mode reveals two multiply charged ions of $A\beta_{1-40}$ centered at 1083.2 amu and 1444.3 amu (Figure 1). Algorithmic transformation of these charged states (1083.2 = $[M+4H]^{4+}$ and 1444.3 = $[M+3H]^{3+}$) yields a singly charged peak centered at mass 4330, which is consistent with the theoretical molecular weight of $A\beta_{1-40}$ peptide. The mass of $A\beta_{1-40}$ peptide was previously confirmed by MALDI-TOF experiments conducted on a MALDITM-LR spectrometer from Waters Corporation (Beverly, MA) as 4330.16 amu. To confirm the charge state of the multiply charged molecular ion of $A\beta_{1-40}$ peptide, the high resolving power of the enhanced resolution scan mode

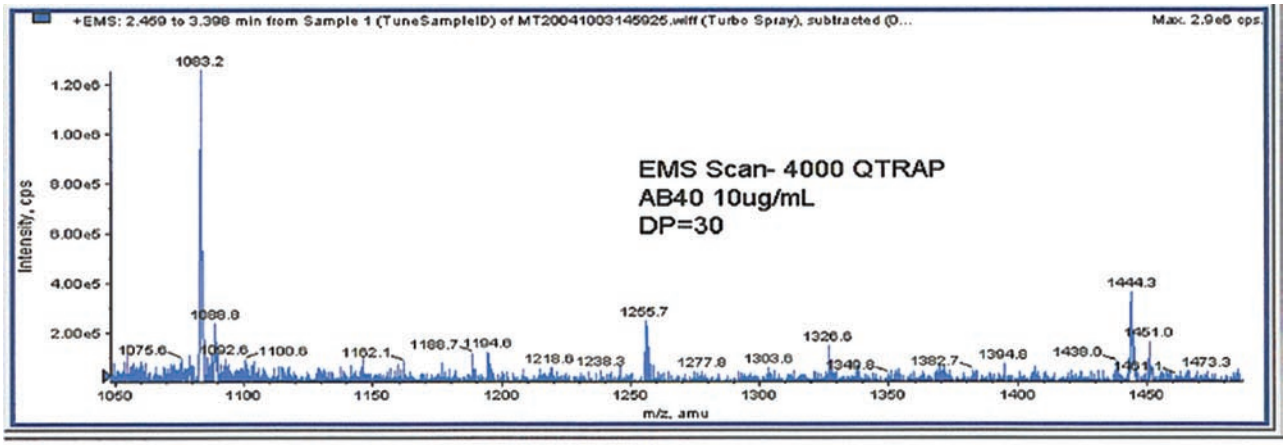

FIGURE 1

Enhanced multiple scan of $A\beta_{1-40}$ in the linear ion trap mode shows multiply charged ions of $A\beta_{1-40}$ corresponding to $[M+4H]^{4+}$ = 1083.2 amu and $[M+3H]^{3+}$ = 1444.4 amu. Enhanced multiple scans were performed on a hybrid quadrupole/linear ion trap 4000 Q TRAP system, and the sample was introduced into the electrospray ionization source using an automatic syringe pump at a rate of 10 µL/min.

was used to verify the multiply charged (+4) molecular ion centered at 1083.2 amu (Figure 2). Enhanced resolution scans of the ion at 1444.3 amu confirm three charges as calculated (data not shown).

In the subsequent method development for quantitation, the predominant multiply charged molecular ion $[M+4H]^{4+}$ = 1083.2 amu was chosen as the precursor ion in the first quadrupole. Nitrogen was

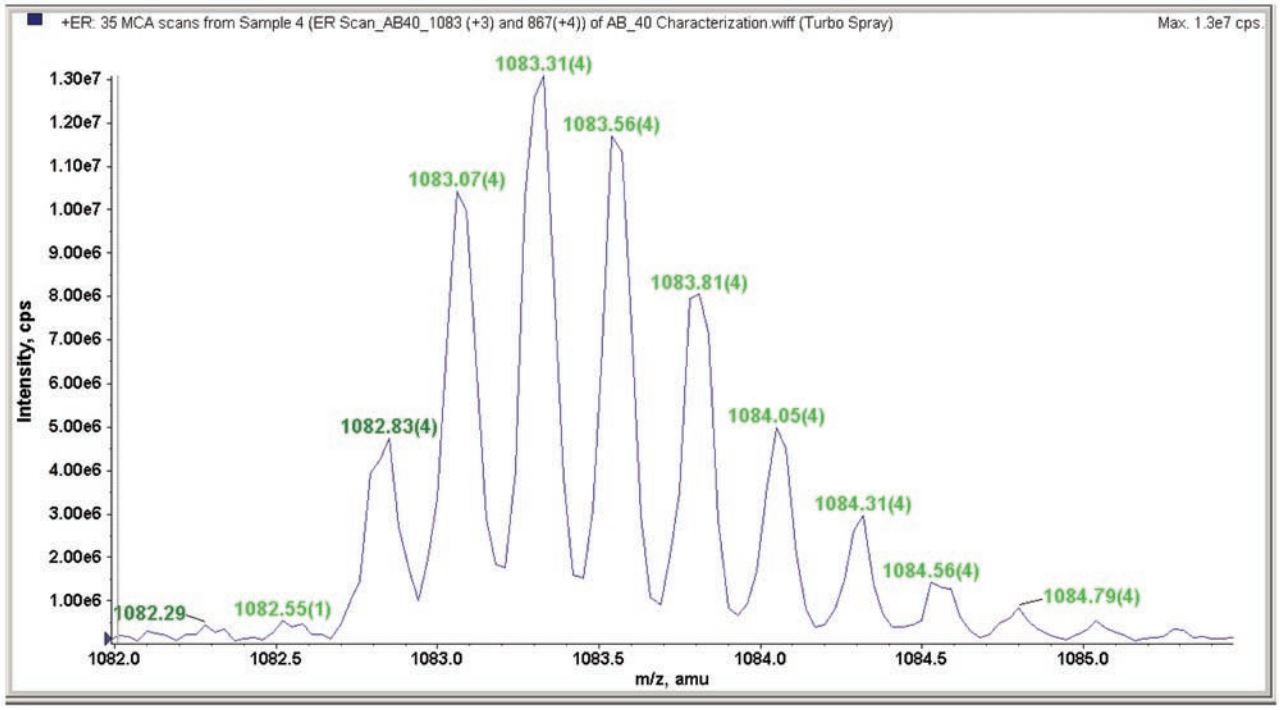

FIGURE 2

Enhanced resolution scan of $A\beta_{1-40}$ reveals (+4) charge state of molecular ion $[M+4H]^{4+}$ = 1083.2 amu. Enhanced resolution scans were performed on a hybrid quadrupole/linear ion trap 4000 Q TRAP system, and the sample was introduced into the electrospray ionization source using an automatic syringe pump at a rate of 10 µL/min.

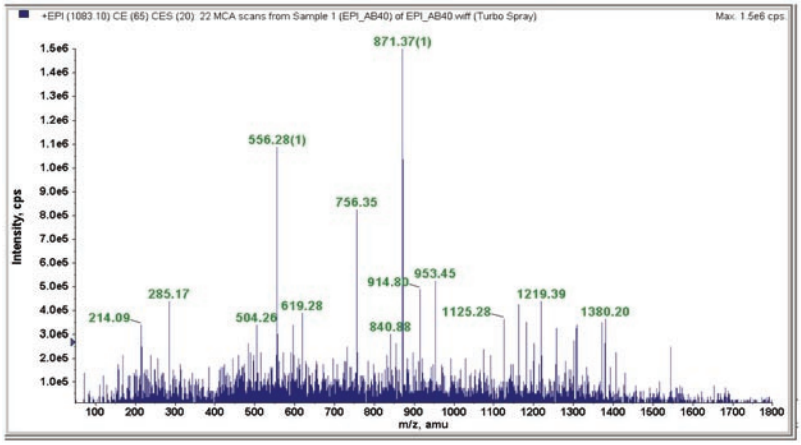

FIGURE 3

Enhanced product ion scan of multiply charged ions of $A\beta_{1-40}$ at $[M+4H]^{4+}$ = 1083.2 amu shows a singly charged product ion with *m/z* 871.4 amu. Enhanced product ion scans were performed on a hybrid quadrupole/linear ion trap 4000 Q TRAP system, and the sample was introduced into the electrospray ionization source using an automatic syringe pump at a rate of 10 µL/min.

applied in the second quadrupole as the collision gas to fragment the precursor ion. The product ions were then trapped in the third quadrupole operated in the linear ion trap mode, enabling a highly sensitive full scan MS/MS analysis of $A\beta_{1-40}$. Figure 3 shows the enhanced product ion scan of multiply charged ions of $A\beta_{1-40}$ at $[M+4H]^{4+}$ = 1083.2 amu. The predominant product ion appears to be a singly charged species at 871.4 amu. The peptide sequence as deciphered by Pro ID software (Applied Biosystems, Foster City, CA) corresponds to Asp-His-Arg-Phe-Glu-Ala-Asp (Aβ1–7) (Figure 4). The enhanced product ion scan of the molecular ion $[M+3H]^{3+}$ = 1444.3 amu generates a similar but not identical fragmentation pattern, with a predominant product ion at 871.4 amu (data not shown).

In the RP-HPLC chromatograms, $A\beta_{1-40}$ shows a retention time of 8.06 min, as monitored by the MS/MS transition from the multiply charged molecular ion of 1083.4 amu to the singly charged ion of 871.4 amu (Figure 5). Reversed-phase HPLC is a well-established technique for the analysis of peptides and proteins in various biological matrices.[16,17] However, $A\beta_{1-40}$ is a very hydrophobic peptide, with poor recovery in conventional reversed-phase HPLC with an acidic mobile phase. Some studies have found that the retention behavior and recovery of Aβ peptide can be significantly improved when the column is eluted under basic conditions. For example, a Zorbax Extend-C18 column significantly improves the Aβ peptide sample recovery when a mobile phase of acetonitrile/water with 20 mM NH4OH is used. Most silica-based reversed-phase HPLC columns can be run only under acidic conditions, due to dissolution of the porous silica network under basic conditions. Only a few polymer reversed-phase HPLC columns and modified silica columns can be used under an extended pH range from 1 to 13. A Waters Xterra C8 column, modified for use in acidic pH as well as basic pH, was chosen for its proven reliability and stability. HPLC elution with a mixture of isopropanol/acetonitrile/water/10 mM ammonium acetate (pH = 7) provided excellent separation of $A\beta_{1-40}$ peptide, as shown in Figure 5. The mobile phase used was also compatible with electrospray ionization in the positive-ion mode.

The calibration curve utilizing the transition from $[M+4H]^{4+}$ = 1083.2 amu to 871.4 amu in the enhanced product ionization mode is shown in Figure 6. The calibration curve was obtained using the total ion count (TIC) in the enhanced product ionization mode. The

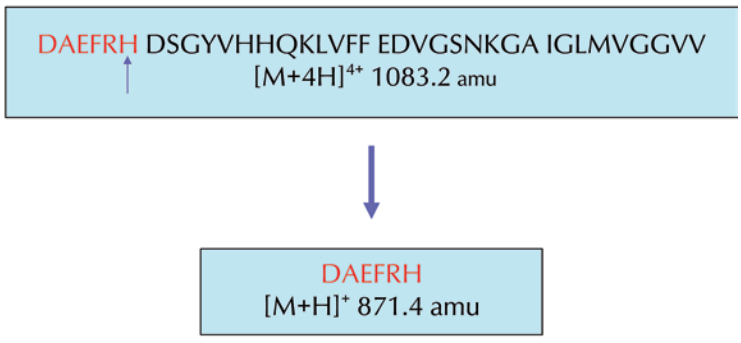

FIGURE 4

The fragmentation of multiply charged $A\beta_{1-40}$ with $[M+4H]^{4+}$ at *m/z* 1083.2 amu forms the singly charged peptide at 871.4 amu. The peptide sequence was derived from Pro ID software (Applied Biosystems, Foster City, CA).

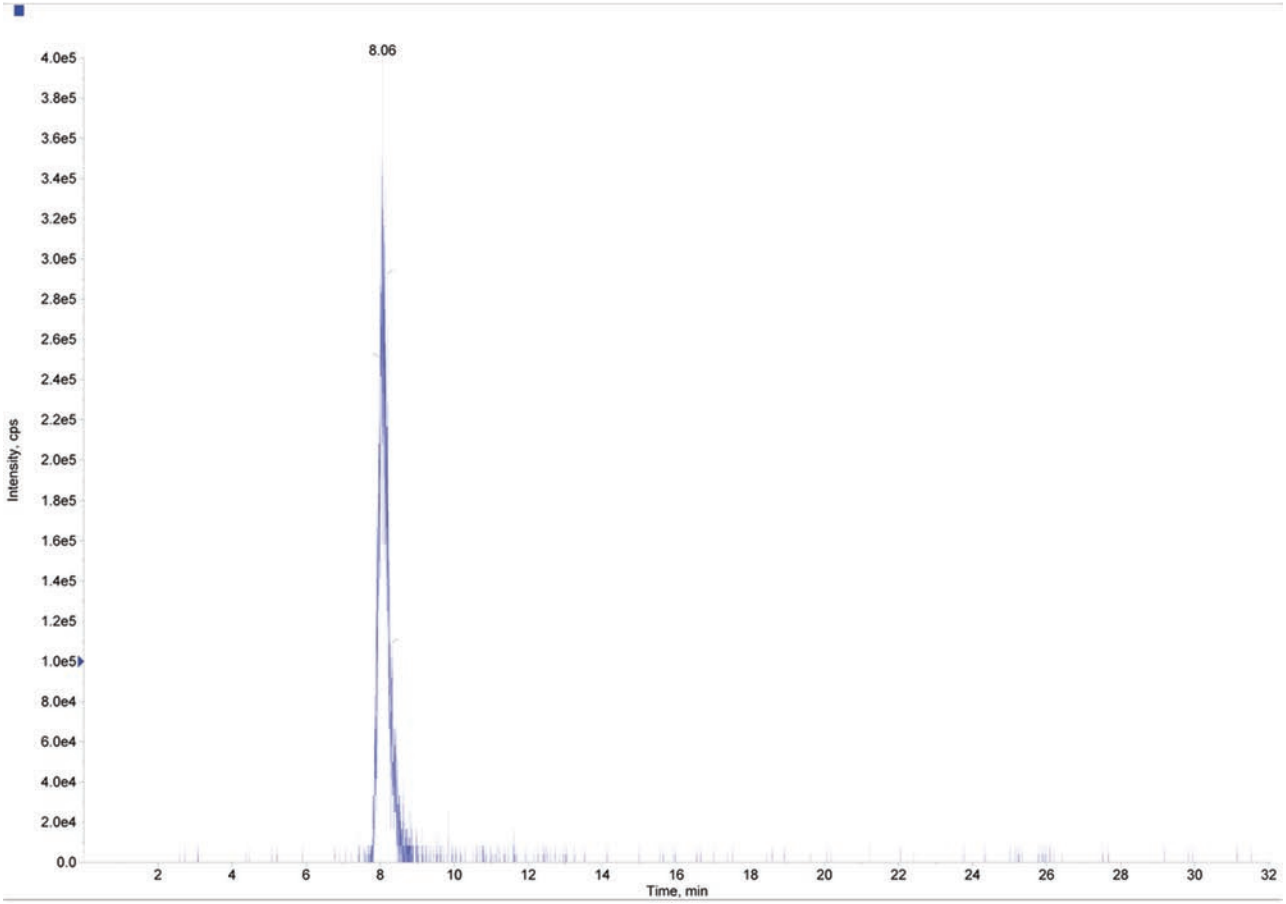

FIGURE 5

The RP- HPLC chromatogram of $A\beta_{1-40}$ monitored by the transition from (+3) multiply charged ion of *m/z* 1083.2 amu to singly charged ion of *m/z* 871.4 amu. Enhanced product ion scans were performed on a 2000 Q TRAP system, and the sample was introduced into the TurboIonSpray ionization source through post-column splitting from the RP-HPLC system. The peak represents TIC from a column load of 1000 ng $A\beta_{1-40}$ peptide. The RP-HPLC-MS/MS experiments are detailed in the text.

$A\beta_{1-40}$ standard calibration curves show excellent linearity from 34 ng to 2500 ng $A\beta_{1-40}$ of column sample load. The dynamic range of quantitation was close to three orders of magnitude for $A\beta_{1-40}$. The LC/MS/

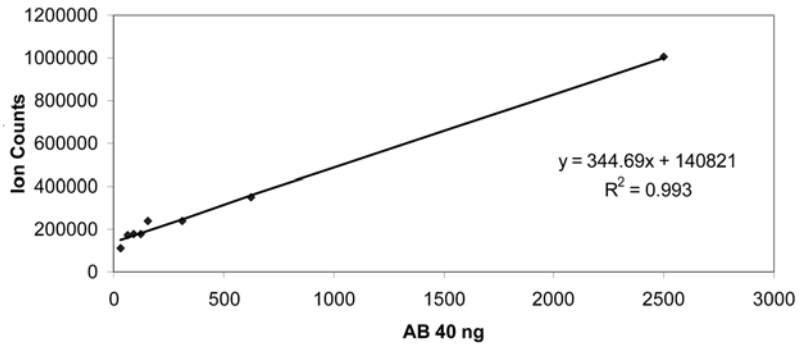

FIGURE 6

Calibration curve of $A\beta_{1-40}$ from 34 ng to 2500 ng $A\beta_{1-40}$ column sample load. The correlation coefficient was 0.993. The calibration curve was obtained using TIC obtained from an enhanced product ionization scan utilizing the transition from $[M+4H]^{4+}$ = 1083.2 to *m/z* 871.4 amu.

MS method using a conventional RP-HPLC column coupled with the Q TRAP system in the Enhanced Product Ion scanning mode was capable of detecting $A\beta_{1-40}$ at low nanogram levels with good specificity and linearity of response. The transition of multiply charged molecular ions of proteins or peptides to a singly charged product ion provides a useful tool to qualitatively and quantitatively analyze peptides. Multiple reaction monitoring in the triple quadrupole mode is commonly used in quantitation using LC-MS/MS analysis.[18] The advantage of multiple reaction monitoring using a single transition and several summed transitions is that it allows detection and quantitation of several target compounds simultaneously. However, in the present studies, enhanced product ion scans using a linear ion trap mode was chosen as the quantitation tool due to its 5- to 10-fold higher sensitivity than multiple reaction monitoring.

The combination of reversed-phased HPLC with mass spectrometry had been previously reported using a 75-nm-diameter nano-HPLC column coupled with a single-quadrupole mass spectrometer in the selective ion monitoring mode using multiply charged ions of $A\beta_{1-40}$ corresponding to $[M+2H]^{2+}$ = 2165.0 amu, $[M+3H]^{3+}$ = 1444 amu, $[M+4H]^{4+}$ = 1083.0 amu, $[M+5H]^{5+}$ = 866.7 amu, and $[M+6H]^{6+}$ = 722.2 amu with a dwell time of 173 ms for each ion.[14] A detection limit of 0.25 ng in column load was achieved using this approach, which fulfills the analytical requirements in terms of the expected range of $A\beta_{1-40}$ in biological fluids.[14] The detection limit in the present communication is 31.25 ng in column load using a 3.0-mm-diameter conventional C8 column. This level of sensitivity is far from sufficient for the direct analysis of Aβ peptide in biological fluids. However, the detection limit could be potentially enhanced up to 1600-fold to reach 0.02-ng levels, if the current method is adapted to a 75-nm-diameter nanoflow HPLC column. This estimation is based on the theoretical gain in sensitivity (F) calculated by using the equation

$$F = \left(\frac{D_{conventional}}{D_{reduced}} \right)^2$$

Subsequent experiments using online microcapillary RP-HPLC coupled with MS/MS demonstrated a significant sensitivity enhancement over conventional RP-HPLC. Using an UltiMate™ Capillary/Nano LC system (LC Packings, Dionex Corporation, Sunnyvale, CA) equipped with a Waters XterraTM C8 capillary RP-HPLC column (0.3 mm × 150 mm), a 50-fold sensitivity enhancement was achieved for $A\beta_{1-40}$ peptide quantitation, which is slightly less than the theoretical 100-fold improvement predicted in going from a 3.0-mm-diameter conventional column to a 0.3-mm capillary column. The less than ideal improvement in sensitivity reflects those factors that will compromise the performance of capillary and nanoflow HPLC, such as dead volumes, gradient delay, etc.

The product ion scan mode was chosen instead of the selective ion monitoring mode in order to achieve superior specificity in $A\beta_{1-40}$ quantitation. In the product ion scan mode, the multiply charged molecular ion of $A\beta_{1-40}$ $[M+4H]^{4+}$ =1083.0 amu was selected in the first quadruple of the mass spectrometer. The molecular ion selected was subsequently collided with gas in the second quadruple to generate fragments of the molecular ion, called product ions. The product ion of $A\beta_{1-40}$, identified as the singly charged species at 871.4 amu, was collected and quantified. Quantitation of $A\beta_{1-40}$ peptides from patient serum samples presents a high level of challenge due to the complexity of the biological matrix. Selective ion monitoring may not achieve the specificity required for quantitation of $A\beta_{1-40}$ in patient serum, because a molecular ion with a similar *m/z* ratio could be generated from biomolecules coexisting with $A\beta_{1-40}$ peptide in blood samples. A product ion scan, on the other hand, monitors the product ion (*m/z*) 871.4 amu, which is specifically derived from the multiply charged molecular ion of $A\beta_{1-40}$ $[M+4H]^{4+}$ = 1083.0 amu, and therefore provides superior specificity for $A\beta_{1-40}$ identification and quantification compared with selective ion monitoring in the selective ion scan mode. This is significant when taking into consideration the complexity of a biological matrix. Work is in progress to transfer the established HPLC-MS/MS method to a nanoflow HPLC-MS/MS system, to directly determine the $A\beta_{1-40}$ concentrations in blood serum samples from both patients with Alzheimer's disease and normal controls. The analytical conditions presented here will also be adapted to analyze $A\beta_{1-42}$, a more hydrophobic and, consequently, a more challenging amyloid peptide for quantitation in biological fluids.

CONCLUSION

The results from this study will establish an accurate and reliable analytical method to qualitatively and quantitatively analyze Aβ peptides. The method presented in this communication can be adapted to nanoflow HPLC-MS/MS to further enhance the sensitivity up to 1000-fold for direct quantitation of $A\beta_{1-40}$ in blood samples. The direct quantitation of $A\beta_{1-40}$ in biological fluids using HPLC-MS/MS may provide a new avenue to identify possible biomarkers for the development of Alzheimer's disease.

The research results and methodology in this project will establish the groundwork to address these important but challenging issues in the future. The experimental results from this project and the analytical method it furnishes will provide a solid foundation to study the link between $A\beta_{1-40}$ peptide deposition in human serum to Alzheimer's disease, to understand the pathological mechanism of Alzheimer's disease, and ultimately to develop diagnosis and treatment strategies for Alzheimer's patients.

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