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Evaluation of peptide adsorption-controlled liquid chromatography–tandem mass spectrometric (PAC-LC–MS/MS) method for simple and simultaneous quantitation of amyloid β 1–38, 1–40, 1–42 and 1–43 peptides in dog cerebrospinal fluid

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ABSTRACT

To evaluate the usefulness of the peptide adsorption-controlled liquid chromatography–tandem mass spectrometry (PAC-LC–MS/MS) for reproducible measurement of peptides in biological fluids, simultaneous quantitation of amyloid β 1–38, 1–40, 1–42 and 1–43 peptides (Aβ38, Aβ40, Aβ42 and Aβ43) in dog cerebrospinal fluid (CSF) was tried. Each stable isotope labeled Aβ was used as the internal standard to minimize the influence of CSF matrix on the reproducible Aβ quantitation. To reduce a loss of Aβ during the pretreatment procedures, the dog CSF diluted by water–acetic acid–methanol (2:6:1, v/v/v) was loaded on PAC-LC–MS/MS directly. Quantification of the Aβ in the diluted dog CSF was carried out using multiple reaction monitoring (MRM) mode. The [M+5H^{5+}] and b^{5+} ion fragment of each peptide were chosen as the precursor and product ions for MRM transitions of each peptide. The calibration curves were drawn from Aβ standard calibration solutions using PAC-LC–MS/MS. Analysis of dog CSF samples suggests that the basal concentration of Aβ38, Aβ40, Aβ42 and Aβ43 in dog CSF is approximately 300, 900, 200 and 30 pM, respectively. This is the first time Aβ concentrations in dog CSF have been reported. Additionally, the evaluation of intra- and inter-day reproducibility of analysis of Aβ standard solution, the freeze–thaw stability and the room temperature stability of Aβ standard solution suggest that the PAC-LC–MS/MS method enables reproducible Aβ quantitation.

1. Introduction

Recently, endogenous peptides in biofluids have actively been investigated as biomarkers for discovery of clues to underlying cause of disease, for early and correct diagnostics, and for effective therapy against various diseases [1,2]. Both discovery of functional peptides and analysis of their functions are difficult because many peptides exhibit their functions only at concentrations in the low picomolar range. So far, quantitative analyses of peptide in biofluids have relied almost exclusively on immunological methods such as enzyme-linked immunosorbent assays or radio-immunoassays, because these methods retain sensitivity and selectivity into the low pM peptide concentration range. However, immunological methods have several disadvantages. One is preparation of an antibody, which requires a time-consuming effort to raise a new antibody for each target peptide. Sufficient knowledge of the peptide and the sample composition is often needed to raise an antibody, while there is no guarantee that a specific antibody to the target peptide is available. Another disadvantage is cross-reactivity caused by low specificity of an antibody, which can lead to false positive or negative results. In some cases, immunological assays need time-consuming steps such as overnight incubation, which can lengthen study time considerably.

In comparison with immunological methods, high-performance liquid chromatography–mass spectrometry (LC–MS) methods possess considerably higher selectivity based on the mass-to-charge (m/z) ratios in addition to chromatographic separation, and better reproducibility. Therefore, there is a compelling need for a methodology based on LC–MS for the quantitation of peptides in biofluids [3,4]. Nonetheless, several issues such as the potency to aggregate, bind to proteins that co-exist in the matrix, the adsorption to a solid surface and the carryover from previous injections often cause the difficulty of highly sensitive and reproducible quantification of peptides by LC–MS.

In our previous studies [5,6], we found that the adsorption capacity of a peptide to the column packing changed abruptly and reversibly across a specific amount of organic solvent (the critical point, $f_n = 1$) in the peptide solution. Circular dichroism

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spectral analysis suggested that the abrupt change in the adsorption capacity was caused by the conformational change induced by the organic solvent in the peptide solution. This solvent-induced conformational change was found to be reversible across the critical threshold. Based on this property, we developed a new gradient system, which is named peptide adsorption-controlled liquid chromatography (PAC-LC), for quantitative analysis of peptides in the solution with a higher f_n value than 1 ($f_n > 1$). PAC-LC enables quantitation of peptide in such solutions without any loss of sensitivity due to peptide adsorption to various materials, and found to be superior to a standard LC system in terms of precision, loading volume onto the system, sensitivity and the simultaneous and quantitative analysis of peptides with good precision [7]. Therefore, in this study, we tried simultaneous quantitation of amyloid β1–38, 1–40, 1–42 and 1–43 peptides (Aβ38, Aβ40, Aβ42 and Aβ43) in dog cerebrospinal fluid (CSF) to evaluate the usefulness of PAC-LC–tandem mass spectrometry analysis (MS/MS) for analysis of peptides in biofluids.

The Aβ is a fragment of the amyloid precursor protein (APP) formed by sequential cleavage of APP by β-secretase and γ-secretase [8,9]. Accumulation of Aβ in brain is hypothesized to initiate a pathogenic cascade that eventually leads to Alzheimer's disease (AD) [10]. In particular, Aβ42 has been found to be the initial form of Aβ deposited in diffuse plaques [11]. Therefore, the Aβ in CSF has been extensively evaluated as a potential biomarker for early diagnostic of AD [12] or for the evaluation of pharmacological effects of drug treatment [13,14]. Most of studies used immunological methods for quantitation of Aβ in CSF, and suggested a modest decrease of Aβ42 level in AD patients when compared with controls; approximately 300–500 pg/mL in AD patients and 600–1000 pg/mL in normal controls [15,16]. However, the reported CSF Aβ42 concentration has varied enormously; one study reported a significantly higher CSF Aβ42 concentration of 1770 pg/mL in AD patients and 2440 pg/mL in normal controls [17], whereas other reported a significantly lower CSF Aβ42 concentration of 30 pg/mL in AD patients and 11 pg/mL in normal controls [18]. In addition, there is a report that an elevated concentration of CSF Aβ42 in AD patients when compared with controls [19]. The considerable variation in the CSF Aβ42 measurement would be caused by methodological issues related to sample collection and processing [20–22]. Also, Aβ aggregation, binding of Aβ to carrier proteins and the possibility of post-translational modifications of Aβ could affect the reliability of analysis when using immunoassay-based procedures [23,24]. Although the quantitation of CSF Aβ using LC–MS method have been attempted, there still remain troublesome sample preparation, and various problems related to the sensitivity [3]. Therefore, we also aimed to develop a simple pretreatment method for simultaneous quantitation of Aβ using dog CSF, which includes the same Aβ as human.

2. Materials and methods

2.1. Materials and reagents

Human Aβ38 was purchased from American Peptide Company (Sunnyvale, CA, USA). Human Aβ40, Aβ42 and Aβ43 were purchased from the Peptide Institute (Osaka, Japan). Stable isotope-labeled Aβ38, Aβ40, Aβ42 and Aβ43, including two $^{13}C_9{}^{15}N$-phenylalanines (F*) at the residue position of 4 and 19 as shown below, were obtained from Biosynthesis (Lewisville, TX, USA) for the internal standards (IS).

Labeled Aβ38: DAEF*RHDSGY EVHHQKLVF*F AEDVGSNKGA IIGLMVGG
Labeled Aβ40: DAEF*RHDSGY EVHHQKLVF*F AEDVGSNKGA IIGLMVGGVV
Labeled Aβ42: DAEF*RHDSGY EVHHQKLVF*F AEDVGSNKGA IIGLMVGGVV IA
Labeled Aβ43: DAEF*RHDSGY EVHHQKLVF*F AEDVGSNKGA IIGLMVGGVV IAT

The ultra pure water was prepared by an ultra pure water system (Milli-Q gradient: Nihon Millipore; Tokyo, Japan) from tap water. Methanol and acetonitrile for high-performance liquid chromatography (HPLC) were obtained from Kanto Chemical (Tokyo, Japan). Acetic acid for HPLC was obtained from Nacalai Tesque (Kyoto, Japan). Dimethyl sulfoxide (DMSO) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Individual dog CSF ($n = 5$) was obtained from Oriental Yeast Company (Tokyo, Japan).

2.2. Sample preparation

2.2.1. Preparation of Aβ stock solutions and working solutions

Stock solutions (0.1 mM) of Aβ38, Aβ40, Aβ42, and Aβ43 were separately prepared by dissolving each peptide in DMSO. An Aβ mixture solution with a 1 μM concentration of each Aβ was prepared by collecting 10 μL aliquot of each stock solution into 960 μL of DMSO. These solutions (0.1 mM and 1 μM) were stored in a freezer set at −20 °C until use.

An Aβ mixture working solution with a 10 nM concentration of each Aβ was prepared by diluting a 10 μL aliquot of the 1 μM working solution with 990 μL of water–acetic acid–methanol (3:6:1; v/v/v). Afterwards, Aβ mixture working solutions with a 8, 5, 2, 1.5, 1, 0.5, 0.3, 0.2 and 0.1 nM concentration of each Aβ were prepared using water–acetic acid–methanol (3:6:1; v/v/v). These working solutions were prepared at the point of use.

For the stable isotope labeled Aβ38, Aβ40, Aβ42, and Aβ43, stock solutions (0.2 and 0.5 mM; without purity correction) were separately prepared by dissolving each peptide in DMSO. A stable isotope labeled Aβ mixture solution with a concentration equivalent to 50 nM for Aβ38 and Aβ40, and 200 nM for Aβ42 and Aβ43 was prepared using each stock solution and DMSO. These stable isotope labeled Aβ solutions were stored in a freezer set at −20 °C until use.

2.2.2. Preparation of calibration and QC samples

An assay solution was prepared by adding 4 μL aliquot of the stable isotope labeled Aβ mixture solution into 40 mL of water–acetic acid–methanol (2:6:1, v/v/v). In order to prepare calibration samples with a 1, 2, 5, 10, 20, 50 and 100 pM concentration of each Aβ, 10 μL of the Aβ mixture working solutions (0.1, 0.2, 0.5, 1, 2, 5 and 10 nM) was added to a 900 μL aliquot of the assay solution, followed by the addition of 100 μL aliquot of water. Quality control (QC) samples with a 1, 3, 15 and 80 pM concentration of each Aβ were prepared using the Aβ mixture working solutions (0.1, 0.3, 1.5 and 8 nM) in the same way. Because each Aβ is an endogenous peptide in dog CSF, dog CSF was not used as matrix for calibration and QC samples in this study.

2.2.3. Preparation of dog CSF samples

In order to prepare pooled dog CSF samples with a different dilution factor, 10, 20, 50 and 100 μL of pooled dog CSF were added to a 900 μL aliquot of the assay solution, followed by the addition of 90, 80, 50 and 0 μL aliquot of water, respectively.

In order to evaluate whether the PAC-LC–MS/MS method was able to detect an increase in Aβ concentration in dog CSF spiked with the Aβ working solution of known concentration, the individual dog CSF samples spiked with the Aβ working solution were prepared as follows: 20 μL of individual dog CSF ($N = 5$) was mixed with both a 900 μL aliquot of the assay solution and 80 μL aliquot

of water, followed by addition of 10 μL of the Aβ working solution (0.5 nM). This addition should increase the observed Aβ concentration by 5 pM. The individual dog CSF samples spiked without the Aβ working solution were prepared in the same way.

2.2.4. Preparing of freeze–thaw and room temperature stability test samples

For the freeze–thaw stability test (2, 4, 6, 8 and 10 cycle), 10 μL aliquot of each Aβ stock solution (0.1 mM) was added to 960 μL of DMSO, water–acetonitrile (1:1, v/v) and water–acetic acid–methanol (3:6:1, v/v/v) to yield Aβ mixture working solutions with a 1 μM concentration of each Aβ. Each solution was divided into a 100 μL aliquot and they were stored in a freezer set at −20 °C. Although both water–acetonitrile (1:1, v/v) and water–acetic acid–methanol (3:6:1, v/v/v) solutions were not frozen in a freezer set at −20 °C, the initial freeze cycle lasted for a minimum of 24 h and the subsequent freeze cycles lasted for a minimum of 12 h. The thaw cycle was conducted by thawing at room temperature for a minimum of 1 h. All of the final thaw cycle were conducted at the same time and place.

To prepare freeze–thaw stability test samples with a 0.1 nM concentration of each Aβ, 10 μL aliquot of the stored solutions (1 μM of each Aβ) was diluted with 990 μL of water–acetic acid–methanol (3:6:1, v/v/v), and 10 μL aliquot of the solutions (10 nM of each Aβ) was added to 990 μL of the assay solution. A reference sample was freshly prepared using each Aβ stock solution (0.1 mM) stored in a freezer.

For the room temperature stability test, 10 μL aliquot of each Aβ stock solution (0.1 mM) was added to 960 μL of DMSO, water–acetonitrile (1:1, v/v) and water–acetic acid–methanol (3:6:1, v/v/v) to yield Aβ mixture working solutions with a 1 μM concentration of each Aβ. These solutions were left at room temperature for 26 days.

To prepare room temperature test samples with a 0.1 nM concentration of each Aβ, 10 μL aliquot of the stored solutions (1 μM of each Aβ) was diluted with 990 μL of water–acetic acid–methanol (3:6:1, v/v/v), and 10 μL aliquot of the solutions (10 nM of each Aβ) was added to 990 μL of the assay solution. A reference sample was freshly prepared using each Aβ stock solution (0.1 mM) stored in a freezer.

2.3. LC–MS conditions

PAC-LC system was built using a metal free LC system (GL Science, Tokyo, Japan) equipped with a 1 mL sample loop. Both mobile phase A (water–acetic acid; 100:1, v/v) and mobile phase B (water–acetic acid–methanol; 3:6:1, vv/v) were used to elute Aβ as sharp peaks with low carryover, while mobile phase C (acetic acid–DMSO; 3:7, v/v) was used to wash the analysis line including the column. Analytical column was a Chromolith Performance RP-8e column (4.6 mm i.d. × 100 mm; Merch, Darmstadt, Germany), and the column temperature was set at 50 °C. The total flow rate was set at 0.6 mL/min, and the initial ratio of mobile phase A to B was set at 6:4. A linear gradient elution of 10%/min increase for mobile phase B was started after holding an initial ratio for 4 min in order to recover the adsorption capacity of Aβ to the column packing before Aβ reached the column. After the elution of Aβ from the column, the analysis line was flushed with mobile phase C at a flow rate of 1.6 mL/min for 2 min.

Here, the f_n value, which was developed in our previous study [6], was calculated according to the following equation to evaluate whether the analytical conditions were adequate for Aβ analysis,

$$f_n = \sum_{i=1}^{N} \frac{x_i}{X_i}$$

where X_i and x_i represent the critical threshold (%; v/v) of each organic solvent (i) for the adsorption capacity of each Aβ to the column packing and the contents of each organic solvent in a solution containing Aβ, respectively. In the previous study, the critical contents (%) of acetonitrile for Aβ38, Aβ40, Aβ42 and Aβ43 were found to be 21.6%, 23.9%, 25.5% and 25.6%, respectively. The critical contents (%) of methanol for Aβ38, Aβ40, Aβ42 and Aβ43 were found to be 32.9%, 36.8%, 39.9% and 41.5%, respectively, and acetic acid for Aβ38, Aβ40, Aβ42 and Aβ43 were 33.8%, 37.9%, 41.4% and 41.9%, respectively. The f_B value, which is defined as the f_n value for each Aβ in mobile phase B, calculated using these critical contents showed more than 1 (from 1.67 to 2.08). Whereas the f_{AB} value, which is defined as the f_n value for each Aβ in the eluent produced by mixing mobile phase A and B in a 6:4 ratio, showed less than 1 (from 0.68 to 0.85). In this study, there was no need to consider the solution produced by mixing mobile phase A and the sample solution because the composition of sample solution was the same as the mobile phase B. Based on our hypothesis, each Aβ in both the sample solution and the mobile phase B is expected to lose the adsorption capacity, which suggests no loss of each Aβ due to the adsorption to various materials during handling and loading procedures. In addition, each Aβ in the eluent produced by mixing mobile phase A and the sample solution in a 6:4 ratio is expected to recover its adsorption capacity to the column packing instantly, which suggests rigid retention of each Aβ by the column. This is why the analytical conditions used in this study is decided to be adequate for quantitative analysis of Aβ.

The total eluent flow from the LC system was directed to the 4000 QTRAP tandem mass spectrometry (AB Sciex, Foster City, CA) equipped with the TurboIon Spray ion source (ESI). Quantification of the Aβ was carried out using multiple reaction monitoring (MRM) mode. The MRM transitions of each peptide and IS summarized in Table 1 were monitored with dwell times of 50 ms for each channel. In this study, the $[M+5H^{5+}]$ and b^{5+} ion fragment of each peptide were chosen for MRM transitions in consideration of intensity and specificity. The setting values of the ion spray voltage, ion source temperature, and collision gas were 5500 V, 500 °C, and 11 (instrument unit), respectively. The data were processed using Analyst 1.4.1 software (AB Sciex).

2.4. Calibration

The calibration curve for each Aβ was constructed by the analysis of a 800 μL aliquot of the calibration sample with a 1, 2, 5, 10, 20, 50 and 100 pM of each Aβ using PAC-LC–MS/MS. The peak area ratio for the analyte (Aβ) to IS (stable isotope labeled Aβ) was plotted against the theoretical concentration of the analyte in the calibration sample. The simple linear regression line was obtained using the least-squares method with $1/x^2$ weighting. Acceptance criterion for the calibration curve is that the back-calculated concentration for each calibration sample is within 100 ± 15% of the theoretical concentration (100 ± 20% at the lower limit of quantification; LLOQ). At least a minimum of 6 calibration samples including the LLOQ and the upper limit of quantification (ULOQ) should meet the above criteria [25].

2.5. Data analysis

The intra-batch assay accuracy and precision were determined by analyzing 5 replicates of QC samples (800 μL injection) with 1, 3, 15 and 80 pM of each Aβ. The assay accuracy was defined as the mean determined results expressed as a percentage of the theoretical concentration, and the assay precision was expressed as the coefficient of variation (CV%).

Table 1
Molecular weights, mass spectrometry conditions for MRM transitions for amyloid β peptides and critical contents of each organic solvent for peptide adsorption capacity to the column packing.

Peptide	Molecular weight	Precursor ion $[M+5H]^{5+}$	Product ion		Declustering potential (V)	Collision energy (V)	Critical content (%)[b]		
			m/z	ID			Acetonitrile	Methanol	Acetic acid
Aβ1–38	4131.6	827.3	801.1	b_{36}^{5+}	81	21	21.6	32.9	33.8
Labeled Aβ1–38[a]	4151.6	831.3	805.1	b_{36}^{5+}	81	21	–	–	–
Aβ1–40	4329.8	867.0	843.8	b_{39}^{5+}	81	21	23.9	36.8	37.9
Labeled Aβ1–40[a]	4349.8	871.0	847.8	b_{39}^{5+}	81	21	–	–	–
Aβ1–42	4514.0	903.8	886.2	b_{41}^{5+}	81	21	25.5	39.9	41.4
Labeled Aβ1–42[a]	4534.0	907.8	890.2	b_{41}^{5+}	81	21	–	–	–
Aβ1–43	4615.1	924.0	900.4	b_{42}^{5+}	81	23	25.6	41.5	41.9
Labeled Aβ1–43[a]	4635.1	928.0	904.4	b_{42}^{5+}	81	23	–	–	–

[a] Stable isotoped-labeled Aβ, including two $^{13}C_9{}^{15}N$-phenylalanine at the residue position of 4 and 19.
[b] Critical content (%) of organic solvent for peptide adsorption capacity to the column packing reported in our previous study [6].

The concentration of each Aβ in dog CSF samples (800 μL injection) was determined using the calibration curve constructed from analysis of calibration samples. The obtained concentrations were multiplied by the dilution factor of dog CSF to obtain the actual Aβ concentrations in dog CSF. The matrix effects of any co-eluted materials in dog CSF were also determined by comparing the mean peak area of each labeled Aβ from the calibration samples with the peak area of each labeled Aβ from dog CSF samples.

The stability of each Aβ was evaluated from the analysis of 3 replicates (200 μL injection) of the freeze–thaw stability test samples, room temperature stability test samples and the reference samples with a 0.1 nM concentration of each Aβ. The stability of each Aβ in each solution was defined as the mean peak area ratio (analyte/IS) for the stability test samples expressed as a percentage of those for the reference samples.

3. Results and discussion

3.1. Calibration, assay accuracy and precision

The results from analysis of 800 μL aliquots of calibration samples using PAC-LC–MS/MS are shown in Table 2. The peak areas were proportional to the concentration, the calibration curve was successfully constructed with the correlation coefficients ranged from 0.9958 to 0.9990, and good accuracy (within ±15%) of the concentration back-calculated from the theoretical concentrations was observed. The LLOQ of the calibration curve achieved 1 pM

Table 2
Calibration values of amyloid β peptides in water–acetic acid–methanol (3:6:1, v/v/v) using PAC-LC–MS/MS.

Peptide	Nominal conc. (pM)	Peak area		Observed conc. (pM)	Accuracy (%)
		Analyte	IS		
Aβ1–38	1.00	2574	11,114	1.07	107.0
	2.00	3911	10,703	1.72	86.0
	5.00	11,692	11,364	4.92	98.4
	10.0	19,943	9526	10.10	101.0
	20.0	45,956	10,547	21.0	105.0
	50.0	107,381	10,468	49.5	99.0
	100	268,108	12,476	104	104.0
	Correlation coefficient = 0.9966				
Aβ1–40	1.00	3653	13,819	1.04	104.0
	2.00	6409	15,591	1.79	89.5
	5.00	15,868	14,767	5.19	103.8
	10.0	32,417	15,194	10.60	106.0
	20.0	55,724	15,806	17.8	89.0
	50.0	148,889	15,193	49.9	99.8
	100	363,580	17,297	107	107.0
	Correlation coefficient = 0.9958				
Aβ1–42	1.00	1812	32,149	1.01	101.0
	2.00	3094	29,496	1.97	98.5
	5.00	8450	32,969	4.97	99.4
	10.0	15,679	31,656	9.71	97.1
	20.0	31,163	28,422	21.6	108.0
	50.0	77,363	31,116	49.2	98.4
	100	161,218	32,626	97.8	97.8
	Correlation coefficient = 0.9990				
Aβ1–43	1.00	834	10,822	0.953	95.3
	2.00	1575	9907	2.18	109.0
	5.00	4060	11,484	5.10	102.0
	10.0	7931	11,756	9.91	99.1
	20.0	15,076	11,226	19.9	99.5
	50.0	35,712	11,461	46.5	93.0
	100	69,884	10,245	102	102.0
	Correlation coefficient = 0.9981				

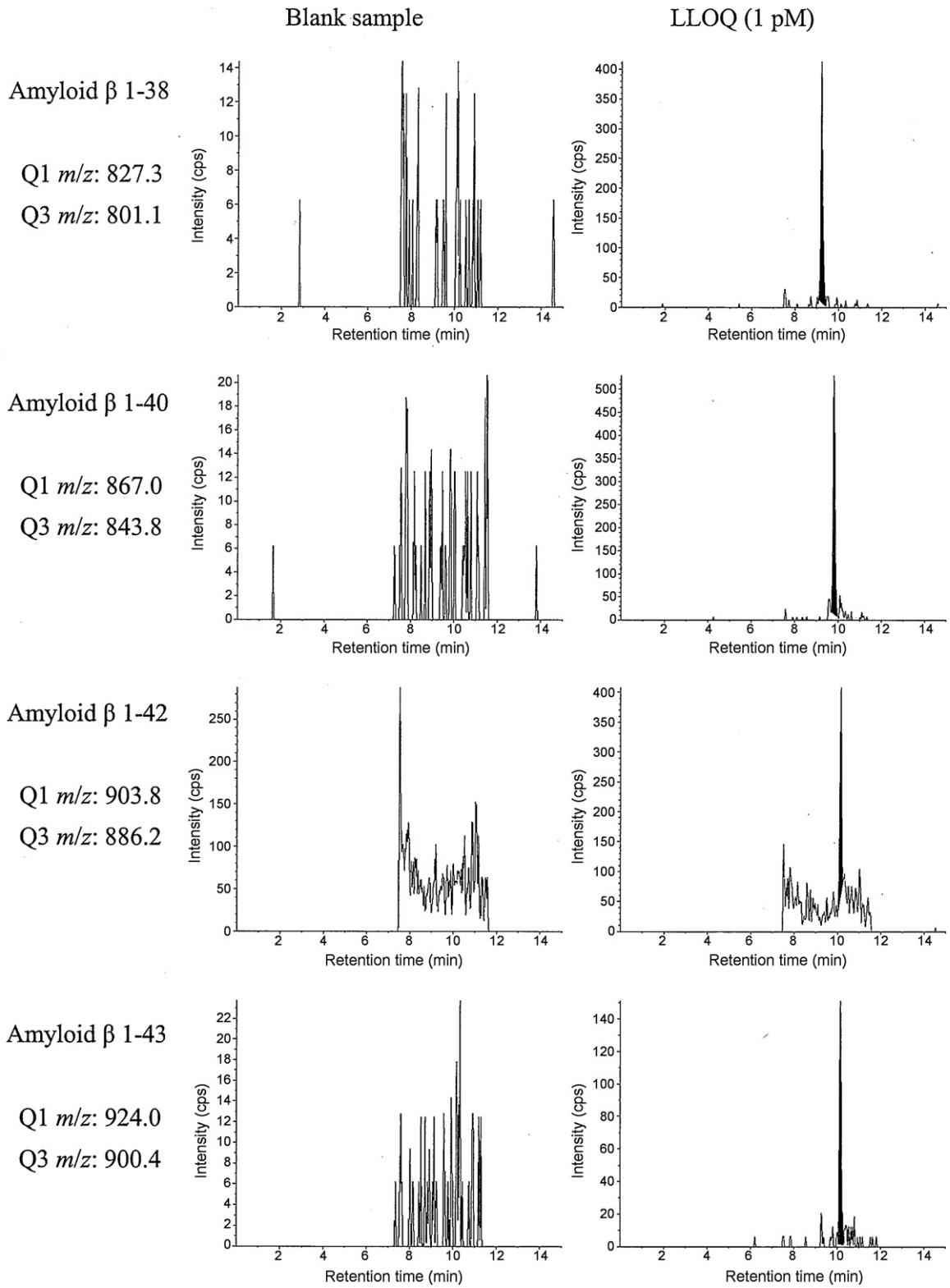

Fig. 1. Representative MRM chromatograms of Aβ38, Aβ40, Aβ42 and Aβ43 in water–acetic acid–methanol (3:6:1, v/v/v) at blank sample (left) and the lower limit of quantification (LLOQ) of the calibration curves (1 pM, right).

for each Aβ. The representative MRM chromatograms of the LLOQ and blank samples are shown in Fig. 1. At all QC samples (1, 3, 15 and 80 pM for each Aβ, $n = 5$), the intra-day accuracy was within ±15% and the precision was within 15% (Table 3), which met the requirements of bioanalytical guidance for low molecular compounds [25]. Although data are not shown, the inter-day evaluation on three different days was found to produce similar accuracy and precision as well as the intra-day evaluation. The results of this study indicate that PAC-LC–MS/MS enables simultaneous quantitation of Aβ38, Aβ40, Aβ42 and Aβ43 in water–acetic acid–methanol (3:6:1, v/v/v) with good reproducibility.

Table 3
Intra-batch assay accuracy and precision for the determination of amyloid β peptides in water–acetic acid–methanol (3:6:1, v/v/v) using PAC-LC–MS/MS.

Ppeptide	QC sample	Nominal concentration (pM)	Mean observed concentration (pM)	Accuracy (%)	CV (%)
Aβ1–38	LLOQ	1.00	1.03	103.0	13.6
	LQC	3.00	3.12	104.0	7.1
	MQC	15.0	14.7	98.0	8.2
	HQC	80.0	78.3	97.9	3.1
Aβ1–40	LLOQ	1.00	0.946	94.6	14.2
	LQC	3.00	3.03	101.0	10.9
	MQC	15.0	15.2	101.3	7.2
	HQC	80.0	78.1	97.6	8.3
Aβ1–42	LLOQ	1.00	1.10	110.0	13.6
	LQC	3.00	3.15	105.0	4.1
	MQC	15.0	14.9	99.3	8.1
	HQC	80.0	77.6	97.0	4.4
Aβ1–43	LLOQ	1.00	1.03	103.0	8.7
	LQC	3.00	3.32	110.7	5.4
	MQC	15.0	13.9	92.7	7.9
	HQC	80.0	76.7	95.9	6.9

LLOQ: lower limit of quantitation sample.
LQC: low quality control sample.
MQC: middle quality control sample.
HQC: high quality control sample.

3.2. Quantitation of Aβ in dog CSF

The Aβ concentrations in pooled dog CSF, which has not been published yet, were calculated using both the observed concentrations and the dilution factor (Table 4). The Aβ concentrations in pooled dog CSF are approximately constant regardless of dilution factor. The results of this study indicate that the basal concentration of Aβ38, Aβ 40, Aβ42 and Aβ43 in dog CSF is approximately 300, 900, 200 and 30 pM, respectively. The representative MRM chromatograms of Aβ38, Aβ40, Aβ42 and Aβ43 and IS in dog CSF at dilution factor of 20 were shown in Fig. 2.

The obtained Aβ42 and 43 concentrations in pooled dog CSF were almost constant, whereas the IS peak area for Aβ42 and Aβ43 decreased with the decrease of dilution factor. These results suggest that the use of stable isotope labeled Aβ as IS is highly effective in order to minimize the influence of matrix effects caused by any co-eluted materials in dog CSF for the reproducible Aβ quantitation.

Next, in order to evaluate whether the method shows quantitative response, the individual dog CSF samples spiked with and without the Aβ working solution were analyzed. The observed Aβ concentrations are shown in Table 5. The mean concentrations for the individual dog CSF samples with the Aβ working solution increase approximately 5 pM compared to basal Aβ, which corresponds to the added Aβ concentration to dog CSF. The results of this study suggest that PAC-LC–MS/MS enables simultaneous quantitation of Aβ38, Aβ40, Aβ42 and Aβ43 in dog CSF using the simple sample preparation.

3.3. Freeze–thaw and room temperature stability of Aβ in standard solution

The effects of 2, 4, 6, 8 and 10 freeze–thaw cycles on the stability of each Aβ (1 μM) in DMSO, water–acetonitrile (1:1, v/v) and water–acetic acid–methanol (3:6:1, v/v/v) were evaluated. Although both water–acetonitrile (1:1, v/v) and water–acetic acid–methanol (3:6:1, v/v/v) solutions were not frozen in a freezer set at −20 °C, each Aβ was found to be stable during at least ten freeze–thaw cycles regardless of solution composition (Table 6). In addition, each Aβ in water–acetonitrile (1:1, v/v) was found to be

Table 4
Concentrations of amyloid β peptides in dog CSF from analysis by PAC-LC–MS/MS.

Peptide	Dilution factor	Observed concentration (pM)	CSF concentration (pM)	Mean CSF concentration (pM)	IS area of CSF sample	Mean IS area of calibration samples	Matrix effect (%)
Aβ1–38	100	2.86	286	290	12,184	12,210	99.8
	50	6.08	304		11,464		93.9
	20	15.4	308		8936		73.2
	10	26.3	263		9475		77.6
Aβ1–40	100	9.48	948	909	15,809	16,334	96.8
	50	18.5	925		17,191		105.2
	20	46.4	928		14,878		91.1
	10	83.3	833		16,620		101.8
Aβ1–42	100	1.97	197	198	32,939	30,599	107.6
	50	4.09	205		25,692		84.0
	20	9.90	198		18,324		59.9
	10	19.1	191		12,658		41.4
Aβ1–43	100	<LLOQ	NC	34.1	12,235	11,071	110.5
	50	<LLOQ	NC		8836		79.8
	20	1.67	33.4		4911		44.4
	10	3.47	34.7		3175		28.7

NC: not calculated.

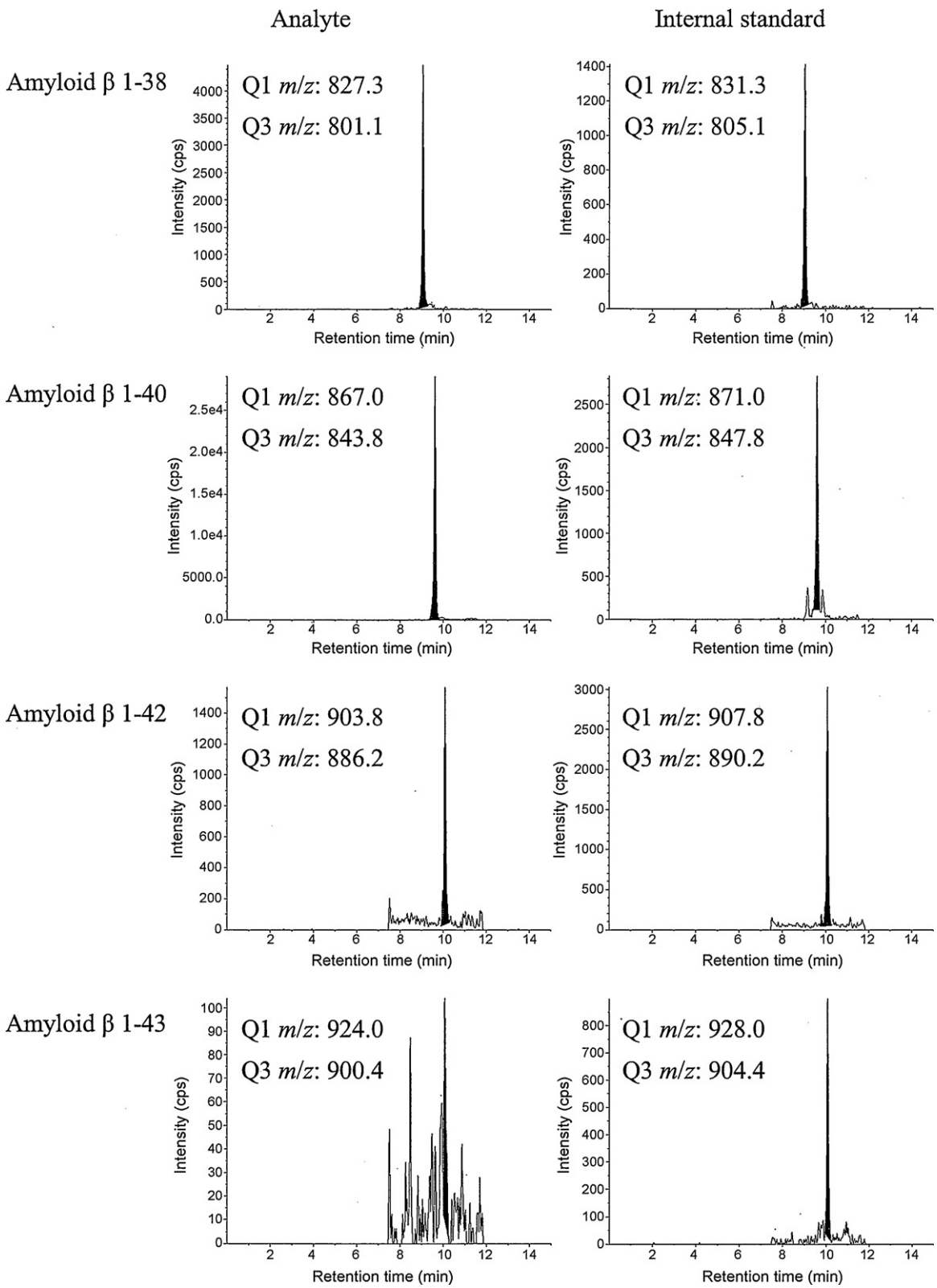

Fig. 2. Representative MRM chromatograms of Aβ38, Aβ40, Aβ42 and Aβ43 (left) and internal standards (right) in dog CSF (dilution factor 20).

stable at room temperature at least for 26 days, while DMSO and water–acetic acid–methanol (3:6:1, v/v/v) caused gradual decrease of each Aβ. The results of this study suggest that Aβ solutions should be stored at −20 °C and water–acetonitrile (1:1, v/v) is a preferable solution to prepare Aβ working solutions among the solutions tested.

So far, it is reported that methodological issues related to sample collection and processing cause a considerable variation in CSF Aβ analysis, especially freezing CSF causes a highly significant loss of Aβ [20]. The reported study showed that sodium dodecyl sulfate (SDS)-heat denaturation prior to freezing was effective in preventing loss by freezing. However, SDS is an undesirable material for

Table 5
Effects of addition of amyloid β peptides into dog CSF samples on the observed concentration by PAC-LC–MS/MS.

Peptide	Dog #	Observed concentration (pM)		Difference of concentration (pM)	Mean difference of concentration
		CSF	Spiked CSF		
Aβ1–38	No. 1	8.90	13.40	4.50	
	No. 2	7.05	12.10	5.05	
	No. 3	7.76	10.90	3.14	4.59
	No. 4	7.58	12.80	5.22	
	No. 5	7.45	12.50	5.05	
Aβ1–40	No. 1	29.2	33.4	4.20	
	No. 2	22.8	27.8	5.00	
	No. 3	23.3	27.7	4.40	4.42
	No. 4	24.1	27.2	3.10	
	No. 5	23.6	29.0	5.40	
Aβ1–42	No. 1	6.94	12.00	5.06	
	No. 2	4.74	10.60	5.86	
	No. 3	6.10	10.10	4.00	5.07
	No. 4	6.59	11.70	5.11	
	No. 5	5.87	11.20	5.33	
Aβ1–43	No. 1	1.57	6.17	4.60	
	No. 2	<LLOQ	6.07	NC	
	No. 3	1.17	6.07	4.90	4.86
	No. 4	1.25	6.03	4.78	
	No. 5	1.23	6.38	5.15	

Table 6
Freeze–thaw and room temperature stability of amyloid β pepitdes (1 μM) in DMSO, water–acetic acid–methanol (3:6:1, v/v/v) and water–acetonitrile (1:1, v/v).

Solution	Freeze–thaw cycle	Storage time (day)	Storage temperature (°C)	Stability (%)			
				Aβ38	Aβ40	Aβ42	Aβ43
DMSO	0	0	−20	100.0	100.0	100.0	100.0
	2	4	−20	94.7	97.7	99.2	97.3
	4	6	−20	104.3	106.1	101.2	110.1
	6	11	−20	106.8	94.3	102.4	101.6
	8	18	−20	111.3	100.0	103.6	114.4
	10	26	−20	105.4	99.7	104.0	98.1
	–	26	Ambient	86.3	75.5	79.6	75.9
Water–acetic acid–methanol (3:6:1, v/v/v)	0	0	−20	100.0	100.0	100.0	100.0
	2	4	−20	105.1	105.2	100.4	104.3
	4	6	−20	102.5	100.9	96.4	103.9
	6	11	−20	96.8	94.5	89.6	93.8
	8	18	−20	101.5	91.5	88.4	103.1
	10	26	−20	94.0	93.0	90.8	94.9
	–	26	Ambient	55.1	59.4	56.8	61.1
Water–acetonitrile (1:1, v/v)	0	0	−20	100.0	100.0	100.0	100.0
	2	4	−20	114.5	105.2	100.8	102.3
	4	6	−20	111.6	111.2	106.0	106.2
	6	11	−20	113.1	103.0	100.0	107.8
	8	18	−20	100.7	104.5	95.6	105.4
	10	26	−20	107.5	99.0	97.2	107.4
	–	26	Ambient	108.2	112.1	104.8	112.8

LC–MS-based analysis. The results suggest that the dilution of CSF with water–acetonitrile (1:1, v/v) before freezing has potential to stabilize Aβ in CSF over a long term. Then, the long-term stability of Aβ in CSF diluted with various solutions will be evaluated in the future study.

4. Conclusion

The results of the present study indicate that PAC-LC–MS/MS method enables simultaneous quantitation of Aβ38, Aβ40, Aβ42 and Aβ43 in dog CSF with good reproducibility using the calibration curve consisted of Aβ standard solutions and a simple sample preparation of only dilution. This is the first time Aβ concentrations in dog CSF have been reported. The use of stable isotope-labeled Aβ38, Aβ40, Aβ42 and Aβ43 as the IS enhances the reliability of the method, and the simple sample preparation of only dilution enables to change target matrix from dog CSF to human CSF easily. Consequently, PAC-LC–MS/MS method will contribute to researches in the development of an accurate and early diagnosis method of AD, to find out the mechanism of pathogenesis of AD, and to discover a fundamental treatment of AD in the future. In addition, PAC-LC–MS/MS method will have easily applicability to the quantitation of other peptides in biological fluids.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.03.032.

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