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# Quantification of *N*-acetyl-seryl-aspartyl-lysyl-proline in hemodialysis patients administered angiotensin-converting enzyme inhibitors by stable isotope dilution liquid chromatography–tandem mass spectrometry

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ABSTRACT

We developed a sensitive, selective and accurate method based on liquid chromatography with tandem mass spectrometry (LC–MS/MS) to determine N-terminal thymosin-β peptides of Ac-SDKP and Ac-ADKP in human plasma samples. Quantification of Ac-SDKP and Ac-ADKP was performed using solid phase extraction (SPE) based on $C_{18}$, reversed phase LC separation, and stable isotope dilution electrospray ionization-MS/MS in multiple reaction-monitoring (MRM) mode. The Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were synthesized for the internal standards. These MRM monitoring ions were $m/z$ 488 → 129 (quantitative ion)/226 for Ac-SDKP, $m/z$ 496 → 137 for Ac-SDKP-$^{13}C_6$, $^{15}N_2$, $m/z$ 472 → 129 (quantitative ion)/226 for Ac-ADKP, and $m/z$ 479 → 129 for Ac-ADKP-$d_7$, respectively. Lower limit of quantitation (LLOQ) of Ac-SDKP and Ac-ADKP was 0.1 ng/mL in human plasma. Recovery values were ranged from 94.7% to 106.3% for inter- (RSD: 0.6–3.5%) and intra- (RSD: 0.4–4.9%) day assays. Plasma Ac-SDKP levels were significantly higher in hemodialyzed subjects treated with angiotensin-converting enzyme inhibitors of enalapril (27.3 ± 24.6 ng/mL, $n$ = 10) and trandolapril (12.3 ± 16.9 ng/mL, $n$ = 18) than healthy (0.4 ± 0.2 ng/mL, $n$ = 7) and hemodialyzed subjects (0.6 ± 0.2 ng/mL, $n$ = 34). This analytical method would be useful to measure N-terminal thymosin-β peptides in human plasma for the clinical study.



## 1. Introduction

*N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetrapeptide normally present in human organs and fluids such as blood, plasma and urine samples. Ac-SDKP has a regulatory effect on the proliferation of hematopoietic stem cells [1,2], and was generated from thymosin $\beta_4$ by enzymatic N-terminal cleavage in vitro and in vivo [3]. Cavasin et al. reported that prolyl oligopeptidase (POP) is involved in release of Ac-SDKP from thymosin $\beta_4$ in vitro, and POP inhibitor indicates lower endogenous levels of Ac-SDKP in vivo [4]. On the other hand, Ac-SDKP was hydrolyzed almost exclusively by angiotensin-converting enzyme (ACE). Thus, after treatment with ACE inhibitors, Ac-SDKP levels was increased to 5-fold in plasma [5]. This systematic Ac-SDKP level was useful to facilitate the detection of defects in compliance with ACE inhibitor treatment in both patients and physicians [6]. Recent reports were discussed that a part of the anti-inflammatory Ac-SDKP is due to its direct effect on bone marrow stem cells and macrophage, inhibiting their differentiation, activation, and cytokine release [7], and that a part of the prevention of aortic fibrosis effect could also be due to reduced expression of the profibrotic cytokine TGF-$\beta_1$ and inhibition of Smad2 phosphorylation [8]. Moreover, Ac-SDKP would be biomarker to the malignant phenotype of cancer [9].

Among diagnosed interventions for hemodialysis-induced hypotension, the use of renin–angiotensin system such as ACE inhibitors is a promising approach by translating into better blood pressure control as well as incremental nephroprotective and cardioprotective effects [10]. However, the few useful and reliable assays to screen endogenous Ac-SDKP in human plasma such as hemodialysis whom was administered ACE inhibitors have been reported.

The old papers described that the enzyme immunoassay (EIA) was developed for the investigation of distribution of N-terminal thymosin β tetrapeptides [11,12]. Junot et al. reported that specific EIA was developed for sensitive and selective monitoring Ac-SDKP

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in mouse plasma and tissues [13]. However, EIA methodology may give erroneous values which were due to non-specific binding to the antibody leading to an accurate estimation of real concentration in human fluids such as blood, plasma and urine samples. On the other hand, an assay to screen endogenous Ac-SDKP in human plasma has been reported by using liquid chromatography with electrospray mass spectrometry (LC-ESI-MS) [14]. Unfortunately, the recovery in plasma was 64%, and the limit of quantification was 5 ng/mL [14]. To the best of our knowledge, the sensitive, selective and reliable analytical methods of N-terminal thymosin β tetrapeptides in human plasma have not been reported. In this paper, we describe a sensitive, selective and reliable method for the quantification of Ac-SDKP from thymosin $\beta_4$ and minor Ac-ADKP from thymosin $\beta_{10}$ in human plasma that employs solid phase extraction (SPE), LC separation, and stable isotope dilution electrospray tandem mass spectrometry (SID-ESI-MS/MS) in multiple reaction-monitoring (MRM). This analytical method is applicable to monitoring Ac-SDKP and Ac-ADKP in human plasma with chronic renal failure such as hemodialysis whom was administered ACE inhibitors.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Synthetic Ac-SDKP tetrapeptide (peptide purity: 98.8%, molecular weight: 487) was obtained from Peptide Institute, Inc. (Osaka, Japan). L-Lysine-$^{13}C_6$, $^{15}N_2$ (isotopic purity: 98% $^{13}C$, 98% $^{15}N$), synthetic Ac-ADKP tetrapeptide (peptide purity: 96.0%, molecular weight: 471), and trifluoroacetic acid (TFA)-d (isotopic purity: 99.5%) were obtained from Sigma-Aldrich (MO, USA). HPLC-grade water, methanol, and formic acid (FA; 99%, LC/MS-grade) were obtained from Wako Chemical Co., Inc. (Osaka, Japan). *N*-*tert*-butoxycarbonyl(Boc)-$O^3$-benzyl(Bn)-L-serine, *N*-Boc-$O^4$-Bn-L-aspartic acid, *N*-[(9H-fluoren-9-ylmethoxy)carbonyl](Fmoc)-$O^4$-*tert*-butyl(tBu)-L-aspartic acid and $N^6$-Boc-$N^2$-Fmoc-L-lysine were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Proline attached to 2-chlorotrityl resin was obtained from HiPep Laboratories (Kyoto, Japan). L-Alanine-$d_4$ (isotopic purity: 98%), acetic anhydride-$d_6$ (isotopic purity: 98%), acetic acid-$d_4$ (isotopic purity: 99.5%), dimethylformamide (DMF)-$d_7$ (isotopic purity: 99.5%) and deuterium oxide ($D_2O$; isotopic purity: 99.9%) were obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA. USA). The pooled human plasma was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan).

The stock solutions (0.1 mg/mL) for Ac-SDKP and Ac-ADKP were prepared by dissolving the appropriate amount of standard in pure water. Mix standard solutions were prepared by diluting an aliquot of the stock solution in water/methanol (80/20, v/v).

We recruited subjects from September 2009 to March 2010 at Meiyo Clinic, Toyohashi City in Aichi, Japan. This study was conducted with all the subject's written informed consent and approved by the institutional ethical board for epidemiological studies. Blood samples were sampled from the healthy ($n = 7$), hemodialyzed ($n = 34$) and hemodialyzed subjects treated with ACE inhibitors ($n = 28$), and stored at −30 °C until use for 7 days.

### 2.2. Synthetic experiments of stable isotope tetrapeptides

The Ac-SDKP-$^{13}C_6$, $^{15}N_2$ was synthesized based on previous report [15]. First, $N^2$-Boc-$N^6$-benzyloxycarbonyl(Z)-L-lysine-$^{13}C_6$, $^{15}N_2$ was prepared by protection of ε-amino group of L-lysine-$^{13}C_6$, $^{15}N_2$ with benzyloxycarbonyl chloride in the presence of basic copper(II) carbonate, followed by the α-amino group protection with di-tert-butyl dicarbonate. The dipeptide BocK(Z)P-$^{13}C_6$, $^{15}N_2$ was obtained by coupling reaction between L-proline and $N^2$-Boc-$N^6$-Z-L-lysine-$^{13}C_6$, $^{15}N_2$ *N*-hydroxysuccinimide (HOSu) ester which was prepared with HOSu and *N*,*N*′-dicyclohexylcarbodiimide. After removal of the Boc group with TFA, the dipeptide K(Z)P-$^{13}C_6$, $^{15}N_2$ was purified with chromatorex ODS column (20 × 250 mm; Fuji Silysia Chemical Ltd., Aichi, Japan). The same HOSu ester methodology was also used for preparation of both the tripeptide D(Bn)K(Z)P-$^{13}C_6$, $^{15}N_2$ and the tetrapeptide S(Bn)D(Bn)K(Z)P-$^{13}C_6$, $^{15}N_2$. This peptide was acetylated with acetylimidazole and triethylamine in dimethylformamide (DMF). Finally, hydrogenolysis of the AcS(Bn)D(Bn)K(Z)P-$^{13}C_6$, $^{15}N_2$ with 10% Pd/C in methanol + water (9:1(v/v)) was carried out to yield AcSDKP-$^{13}C_6$, $^{15}N_2$. All peptides were purified by HPLC fractionation and confirmed by ESI-MS analysis. AcSDKP-$^{13}C_6$, $^{15}N_2$: ESI-MS ($H_2O$, positive mode); *m*/*z* 496 $[M+H]^+$.

The Ac-ADKP-$d_7$ was synthesized by solid phase peptide synthesis methodology [16]. Coupling reaction of $N^6$-Boc-$N^2$-Fmoc-L-lysine and proline attached to 2-chlorotrityl resin was carried out with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and methylmorphorin in DMF. After removal of Fmoc protective group with piperidine, Fmoc-$O^4$-*t*Bu-L-aspartic acid was reacted under same condition and the Fmoc group was removed. Finally, *N*-acetyl-L-alanine-$d_7$, which was prepared by acetylation of L-alanine-$d_4$ with acetic anhydride-$d_6$ in the presence of acetic acid-$d_4$, was introduced with HBTU and methylmorphorin in DMF-$d_7$, followed by cleavage with TFA-d and $D_2O$. AcADKP-$d_7$ was purified with chromatorex ODS column (20 × 250 mm) and confirmed by ESI-MS analysis. AcADKP-$d_7$: ESI-MS ($H_2O$, positive mode); *m*/*z* 479 $[M+H]^+$.

### 2.3. LC–MS/MS analysis

LC analyses were performed with a Waters Alliance 2695 system (Waters, Milford, MA, USA). LC separation was achieved on an Atlantis T3 (2.1 × 150 mm, 3 μm: Waters, Milford, MA, USA) maintained at 30 °C and the mobile phase consisted of 0.1% FA in water (Solvent A) and 0.1% FA in methanol (Solvent B). LC linear gradient was as follows: 2% Solvent B at 0 min, 35% B at 10 min, 95% B at 10.1 min, 95% B at 15 min, and 2% B at 15.1 min with flow rate of 0.2 mL/min. The injection volume was 10 μL. The separated compounds were detected with a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The mass spectrometer was operated with an electrospray source in positive ionization mode. The electrospray ionization (ESI) source conditions were: capillary voltage of 2.8 kV, extractor of 4 V, RF lens of 0 V, source temperature of 110 °C and desolvation temperature of 400 °C. The cone and desolvation gas flows were 50 L/h and 900 L/h, respectively and were obtained from nitrogen source ($N_2$ Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). Argon was used as collision gas and was regulated at 0.35 mL/h and the multipliers were set to 650 V. The LH resolution 1, HM resolution 1, ion energy 1, LM resolution 2, HM resolution 2, and ion energy 2 were 12.0, 12.0, 0.5, 12.0, 12.0 and 0.8, respectively.

### 2.4. Sample preparation

Plasma samples were extracted and clean-up using solid phase extraction (SPE) cartridges (Bond Elut-C18: 100 mg/1 mL, Varian Co., CA. USA). Before extraction the SPE columns were preconditioned with 1 mL of methanol followed by the addition of 1 mL of 0.5% FA in water. The samples (0.5 mL) were added with 1 μL of stable isotope peptides solutions and 1.0 mL of 0.5% FA in

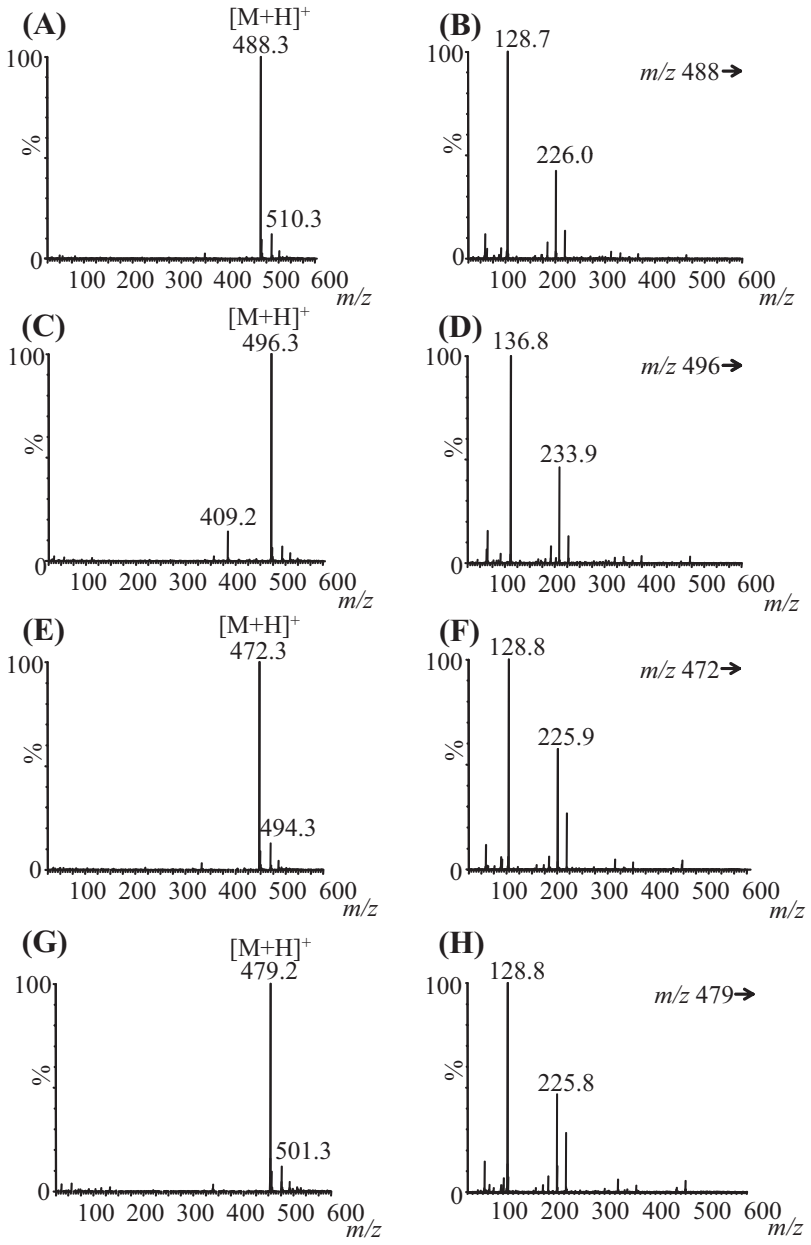


**Fig. 1.** Mass spectra of Ac-SDKP, Ac-SDKP-$^{13}C_6$, $^{15}N_2$, Ac-ADKP and Ac-ADKP-$d_7$. Condition: Waters Micromass Quattro Premier triple quadrupole mass spectrometer with an electrospray source in positive ionization mode ($m/z$ 50–400). (A) ESI-MS spectrum of Ac-SDKP. (B) ESI-MS spectrum of Ac-SDKP from precursor ion of $m/z$ 488. (C) ESI-MS spectrum of Ac-SDKP-$^{13}C_6$, $^{15}N_2$. (D) ESI-MS spectrum of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ from precursor ion of $m/z$ 496. (E) ESI-MS spectrum of Ac-ADKP. (F) ESI-MS spectrum of Ac-ADKP from precursor ion of $m/z$ 472. (G) ESI-MS spectrum of Ac-ADKP-$d_7$. ESI-MS spectrum of Ac-ADKP-$d_7$ from precursor ion of $m/z$ 479.

water. The samples were passed through the SPE cartridges which were then washed with 1 mL of 0.5% FA in water. The retained compounds were eluted using 1 mL of methanol at low flow rate. These elutes were dried under a stream of nitrogen at 30 °C. These samples were resoluble in 0.1 mL of water/methanol (80/20, v/v), and then centrifuged at 13000 rpm for 5 min. These solutions were then subjected to LC-ESI-MS/MS.

### 2.5. Analytical validation

In this study, FDA guidance for industry (Bioanalytical Method Validation) was modified for the analytical validation of en endogenous peptides in human plasma samples using stable isotope labeled internal standards [17]. Mostly, stable isotopes labeled internal standards are used to compensate for sample-to-sample differences occurring during SPE preparation and LC–MS/MS analysis such as absolute recovery, injection volume and matrix effects. Thus, the calibration curves were used with the analyte to stable isotope internal standard's peak area ratios by weighted ($1/x^2$) least-squares linear regression on consecutive days.

Ac-SDKP and Ac-ADKP were quantified by a 8-point internal calibration with stable isotope peptides. Calibration range was from 0.5 to 100 ng/mL for standard solutions. The acceptance criterion for a calibration curve was a correlation coefficient ($r^2$) of 0.99 or better. Spiked levels for quality control (QC) in pooled human plasma were selected of six replicates of QC at three concentration levels. For the determining of intra-day accuracy, a replicate ($n = 6$) analytes of plasma samples were performed on the same day. The inter-day accuracy were expressed as the recovery and relative standard deviation (RSD, %), and determined with two times per day for three days ($n = 6$). For lower limit of quantification (LLOQ), the analyte response at the LLOQ was based on signal per noise

(control) = 10 times. Process sample stability was evaluated with QC samples after 24 h in autosampler at 4 °C. Bench-top stability was evaluated for 6 h at room temperature. The freeze–thaw stability was determined by comparing the freeze–thaw QC that had been frozen and thawed three times at −30 °C with normal QC samples. Long-term stability was evaluated by analyzing QC samples stored at −30 °C for 30 days.

The matrix effects were evaluated by comparing the MS/MS responses of each stable isotope peptides in samples. The human plasma samples ($n$ = 3) were used for the evaluation of matrix effects. The validation data obtained in the above manner enabled us to determine the matrix effect value for the SPE procedure by comparing the absolute peak areas for the target stable isotope peptides. By calculating the peak areas obtained using a standard solution, and the corresponding peak areas for the standards spiked after extraction, this vale was calculated as follows: matrix effect value = (stable isotope peptides spiked after extraction/stable isotope peptides standard) × 100. A value greater than 100% indicates ionization enhancement and a value less than 100% indicates ionization suppression.

## 3. Results and discussion

### 3.1. LC–MS/MS analysis of Ac-SDKP and Ac-ADKP

To determine Ac-SDKP and Ac-ADKP using single reaction monitoring (SRM) mode, full scan and product ion spectra of the analytes are investigated under LC separation. They can be detected under electrospray ionization (ESI)-MS conditions based on LC conditions. Junot et al. reported that the ESI-MS with positive mode was used for the single ion monitoring (SIM) mode of Ac-SDKP $[M+H]^+$ $m/z$ 488 [14]. Therefore, ESI interface with FA base mobile phase and reversed phase column (Atlantis T3, Waters) were selected as ionization source and separation of Ac-SDKP and Ac-ADKP in our experiment. The protonated molecules of $[M+H]^+$ (Ac-SDKP: $m/z$ 488 and Ac-ADKP: $m/z$ 472) was detected in positive mode (Fig. 1(A) and (E)). When collision energy was used in product ions of $[M+H]^+$, the major fragment ions of Ac-SDKP at $m/z$ 488 → 129/226 and Ac-ADKP at $m/z$ 472 → 129/226 were observed, respectively (Fig. 1(B) and (F)). The cone voltage (V) and collision energy (eV) in MS/MS condition were then investigated to achieve highly sensitive and selective detection of Ac-SDKP and Ac-ADKP (Table 1). Stable isotope peptides of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were synthesized based on above procedures. However, these peptides of isotopically purity were not known. Therefore, this isotopically purity was investigated by infusion with MS/MS system. We make a comparison between the standard peptide of main peak of $[M+H]^+$ in MS and MS/MS spectra. These MS spectra of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were shown in Fig. 1(C) and (G). The major fragment ions of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ at $m/z$ 496 → 137 and Ac-ADKP-$d_7$ at $m/z$ 479 → 129 were observed, respectively (Fig. 1(D) and (H), and Table 1). Then, these isotopically peptides were analyzed by LC–MS/MS for the investigation of background noise in MRM mode. The peak responses of Ac-SDKP and Ac-ADKP were not detected. Therefore, these internal standards are useful for the determination of Ac-SDKP and Ac-ADKP with isotopically peptides. Standard solutions of Ac-SDKP and Ac-ADKP were prepared in water/methanol (80/20, v/v) and added to a fixed concentration of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ for a calibration curve covering the concentration range from 0.5 to 100 ng/mL for human plasma sample. Quantitative analysis was performed using MRM mode in order to maximize sensitivity of quantitative ion, and ratio of analyte/internal standard. Concentrations were calculated relative to isotopically standards that were added to the samples prior to extraction giving a final concentration of 50 ng/mL. Eight-point calibrations were performed daily for all analytes with internal standards. The MRM chromatograms of Ac-SDKP and Ac-ADKP were shown in Fig. 2(A).

LC separation, and SID-ESI-MS/MS in MRM mode was found to be a sensitive and selective technique for determining Ac-SDKP

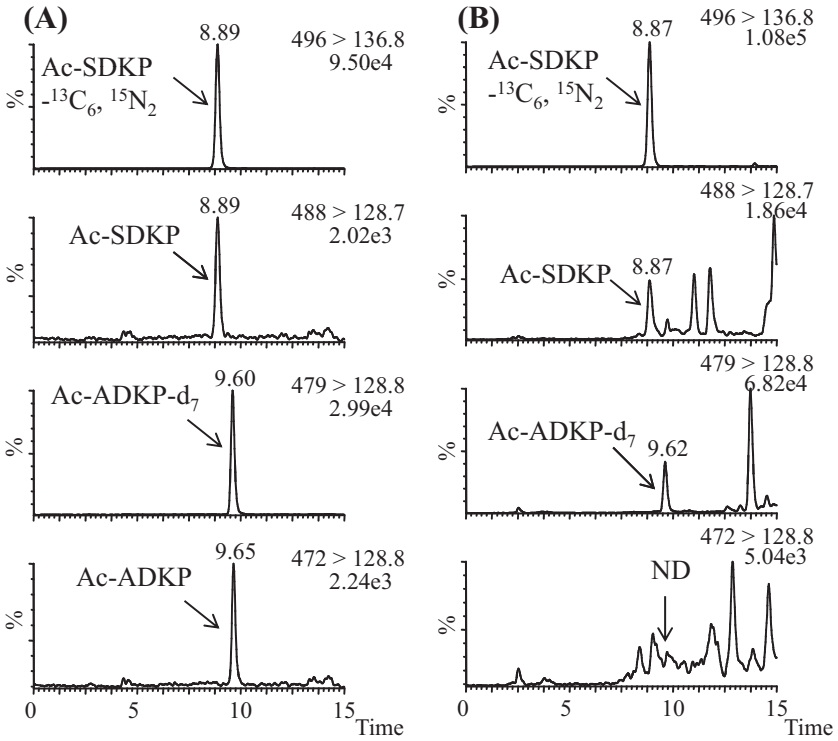


**Fig. 2.** MRM chromatograms of Ac-SDKP and Ac-ADKP. Standard solution (A): 1 ng/mL of Ac-SDKP and Ac-ADKP, and internal standards. Plasma sample (B): 1.3 ng/mL of Ac-SDKP and ND (<0.1 ng/mL) of Ac-ADKP, and internal standards.

**Table 1**
MS/MS condition of analytes.

| Analytes | Precursor ion (*m*/*z*) | Cone voltage (V) | Product ions (*m*/*z*) | Collision energy (eV) |
|---|---|---|---|---|
| Ac-SDKP | 488 | 37 | 129*, 226 | 27 |
| Ac-SDKP-$^{13}C_6$, $^{15}N_2$ | 496 | 37 | 137 | 27 |
| Ac-ADKP | 472 | 37 | 129*, 226 | 25 |
| Ac-ADKP-$d_7$ | 479 | 37 | 129 | 25 |

* Quantitative ion.

and Ac-ADKP at trace levels. The method was proposed to evaluating a measurement of endogenous Ac-SDKP and Ac-ADKP levels in human plasma samples. The matrix effect and the possibility of ion suppression of specific Ac-SDKP and Ac-ADKP in biological samples would be observed by sample concentration. The purpose was to examine whether SPE is useful preparation for the trace analysis of Ac-SDKP and Ac-ADKP in human plasma samples by LC–MS/MS in the absence of the matrix effect. Then, we evaluated the matrix effect of stable isotope tetrapeptides such as Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ subjected to SPE that decreased ionization suppression for LC–MS/MS. For this purpose, sample extracts were spiked at 50 ng/mL, and an internal standard solution at the same concentration was prepared. The matrix effect values of the SPE concentration at 10 times was lower (<60%) than others. This sample showed that extract concentration at 5 times is sufficient to remove or minimize the ion suppression effect. The matrix effect values of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were 99.8 ± 0.8% and 97.2 ± 2.9% ($n$ = 3), respectively. Moreover, SPE with the reversed-phase $C_{18}$ mode cartridge was examined in the recovery test of absolute quantity Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ in human plasma samples. The recoveries of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were performed using pooled human plasma samples (50 ng/mL concentration level) spiked with an internal standard solution. The extraction using the SPE cartridge was performed according to the above-described method. The absolute recovery values of Ac-SDKP-$^{13}C_6$, $^{15}N_2$ and Ac-ADKP-$d_7$ were 86.0 ± 7.4% and 105.0 ± 5.1% ($n$ = 3), respectively. Based on these results, reversed-phase $C_{18}$ mode is sufficiency for the recovery of Ac-SDKP and Ac-ADKP in human plasma and removing the high concentrations of contaminating materials present in biological samples. There-

**Table 2**
Analytical validation for the determination of Ac-SDKP and Ac-ADKP in human plasma.

| Analytes | Stability | *n* | Control concentration (ng/mL) | Spiked levels (ng/mL) | Detection levels (ng/mL) | Recovery [RSD] % | |
|---|---|---|---|---|---|---|---|
| Ac-SDKP | Intra-day assay | 6 | 0.4 | 1.0 | 1.3 | 94.7 | [4.9] |
| | | 6 | 0.4 | 5.0 | 5.1 | 94.8 | [1.5] |
| | | 6 | 0.4 | 10.0 | 11.3 | 105.6 | [0.4] |
| | | 6 | 0.4 | 50.0 | 52.9 | 104.3 | [0.7] |
| | Inter-day assay | 6 | 0.4 | 1.0 | 1.4 | 99.7 | [3.5] |
| | | 6 | 0.4 | 5.0 | 5.2 | 97.3 | [2.2] |
| | | 6 | 0.4 | 10.0 | 11.4 | 106.3 | [0.6] |
| | | 6 | 0.4 | 50.0 | 51.1 | 100.9 | [2.8] |
| | Process/wet extract[a] | 6 | 0.6 | 1.0 | 1.6 | 99.3 | [7.1] |
| | | 6 | 0.6 | 5.0 | 5.5 | 98.4 | [4.7] |
| | Bench-top[b] | 6 | 0.5 | 1.0 | 1.5 | 106.7 | [2.6] |
| | | 6 | 0.5 | 5.0 | 5.2 | 94.0 | [0.9] |
| | Freeze and thaw[c] | 6 | 0.6 | 1.0 | 1.7 | 107.0 | [7.5] |
| | | 6 | 0.6 | 5.0 | 5.8 | 103.6 | [3.7] |
| | Long-term[d] | 6 | 0.4 | 1.0 | 1.3 | 97.7 | [5.9] |
| | | 6 | 0.4 | 5.0 | 5.2 | 97.5 | [3.5] |
| Ac-ADKP | Intra-day assay | 6 | 0.1 | 1.0 | 1.1 | 97.7 | [3.5] |
| | | 6 | 0.1 | 5.0 | 5.2 | 101.1 | [2.3] |
| | | 6 | 0.2 | 10.0 | 10.4 | 102.1 | [0.2] |
| | | 6 | 0.2 | 50.0 | 53.6 | 106.7 | [1.2] |
| | Inter-day assay | 6 | 0.1 | 1.0 | 1.1 | 98.7 | [3.1] |
| | | 6 | 0.1 | 5.0 | 5.2 | 100.9 | [2.1] |
| | | 6 | 0.2 | 10.0 | 10.6 | 101.6 | [1.1] |
| | | 6 | 0.2 | 50.0 | 54.0 | 106.7 | [1.9] |
| | Process/wet extract[a] | 6 | 0.2 | 1.0 | 1.2 | 98.7 | [4.9] |
| | | 6 | 0.2 | 5.0 | 5.1 | 98.9 | [3.9] |
| | Bench-top[b] | 6 | 0.2 | 1.0 | 0.7 | 52.7 | [3.9] |
| | | 6 | 0.2 | 5.0 | 2.8 | 52.1 | [4.1] |
| | Freeze and thaw[c] | 6 | 0.2 | 1.0 | 1.2 | 99.0 | [3.6] |
| | | 6 | 0.2 | 5.0 | 5.4 | 104.1 | [6.6] |
| | Long-term[d] | 6 | 0.1 | 1.0 | 0.7 | 57.0 | [8.2] |
| | | 6 | 0.1 | 5.0 | 3.6 | 70.5 | [4.4] |

[a] After 24 h in autosampler at 4 °C.
[b] For 6 h at room temperature.
[c] After three freeze and thaw cycles at −30 °C.
[d] For 30 days at −30 °C.

fore, SPE pre-concentration procedure with reversed-phase $C_{18}$ cartridge has the ability to cleanup Ac-SDKP and Ac-ADKP in human plasma for LC–MS/MS quantification.

### 3.2. Analytical validations

The lower limit of quantification (LLOQ) in human plasma sample was detected as the concentration of the lowest calibration by SPE pre-concentration. The LLOQ was 0.1 ng/mL based on signal per noise (plasma control sample) = 10. The results for intra-day and inter-day precision for Ac-SDKP and Ac-ADKP in QC samples are summarized in Table 2. The intra-day recovery and precision were from 94.7% to 105.6% and 0.4–4.9%, respectively. The inter-day recovery and precision were from 97.3% to 106.3% and 0.6–3.5%, respectively. The results of stability studies were shown in Table 2. QC samples were subjected to long-term storage (−30 °C), and to freeze–thaw stability studies. All stability tests were conducted at two concentration levels (1.0 and 5.0 ng/mL) with six determinations for each. The bench-top stability results (52.1% and 52.7%) indicated that Ac-ADKP in human plasma is not stable for at least for 6 h at room temperature. Thus, the time course of decreased Ac-ADKP was investigated for 2 hr at room temperature. In the results, the recoveries of Ac-SDKP for 1.0 and 5.0 ng/mL indicated 100.7 ± 0.2% and 98.9 ± 0.2% ($n$ = 3), respectively. The bench-top stability for 2 h indicated that Ac-ADKP level in human plasma is not decreased. Using this result, SPE preparation of human plasma should be completed for 2 h in room temperature. In addition, long-term stability results (57.0% and 70.5%) indicated that Ac-ADKP level has been decreased for 30 days at −30 °C. Thus, the stability of Ac-ADKP at −30 °C was investigated for the storage time until the analysis. In this result, the stability of Ac-ADKP (>90%, recovery) is stable for 7 days. The choice of the internal standard is a critical aspect of quantitative method because it influences precision and accuracy. Based on these validation data, this reliability of this analytical procedure would be useful to monitoring the clinical validation for the performance of analytical methods and interpretation of results.

### 3.3. Clinical application

Previous study was reported that levels of Ac-SDKP in anemic, nonanemic, and healthy control subjects were ranged from 0.5 to 4.5 nM (0.24–2.2 ng/mL) using EIA assay [18]. Thus, high sensitive and specific analytical method would be developed for the determination of trace Ac-SDKP in human plasma. Our analytical procedure with SPE and LC–MS/MS is also suitable to quantify levels of Ac-SDKP and Ac-ADKP at 0.1 ng/mL (LLOQ) in human plasma samples. We measured plasma Ac-SDKP concentrations in healthy ($n$ = 7), hemodialyzed ($n$ = 34) and hemodialyzed subjects treated with ACE inhibitors ($n$ = 28), respectively. These chromatograms of Ac-SDKP and minor Ac-ADKP in human plasma samples were shown in Fig. 2(B). These data were shown in Fig. 3.

Plasma Ac-SDKP levels in hypertensive patients with ACE inhibitors were measured by competitive EIA [5]. The ranges for plasma Ac-SDKP levels in the ACE inhibitor (captopril) group and the non-ACE inhibitor group were indicated that it is not overlap of 1.48–14.5 pmol/mL (0.7–7.1 ng/mL) versus 0.36–1.22 pmol/mL (0.2–0.6 ng/mL), respectively [5]. This study indicated that plasma Ac-SDKP levels are significantly correlated to creatinine clearance in the ACE inhibitor group. Thus, among diagnosed interventions for hemodialysis-induced hypotension, it is important problem that the monitoring approach would be to target the event in the generation of endogenous Ac-SDKP in chronic renal failure such as hemodialysis whom was administered ACE inhibitors. For patients with chronic renal failure, plasma Ac-SDKP levels in hemodialysis and non-hemodialysis with ACE inhibitors were measured by competitive EIA [19]. Plasma Ac-SDKP levels were significantly greater in hemodialysis of 10.3 ± 3.9 pmol/mL (5.0 ± 1.9 ng/mL) and non-hemodialysis of 3.1 ± 1.8 pmol/mL (1.5 ± 0.9 ng/mL) patients not administered ACE inhibitors than controls of 1.8 ± 0.2 pmol/mL (0.9 ± 0.1 ng/mL) [19]. Ac-SDKP levels in ACE inhibitor-treated hemodialysis patients were 43.5 ± 32 pmol/mL (21.2 ± 15.6 ng/mL), and high concentration about 40 ng/mL [19]. This report is a solitary experiment for the data of plasma Ac-SDKP levels in hemodialysis patients. These Ac-SDKP levels in human plasma samples were measured using only EIA methods. The immunoassay should be compared with a validated reference method such as LC–MS, LC–MS/MS, and GC–MS using incurred samples and predetermined criteria for agreement of accuracy. Moreover, the validated LC–MS/MS method of Ac-SDKP and minor Ac-ADKP in human plasma has not been applied for the hemodialysis patients administered ACE inhibitors. Based on our results, minor Ac-ADKP in all human plasma was trace levels from ND (<0.1 ng/mL) to 8.0 ng/mL.

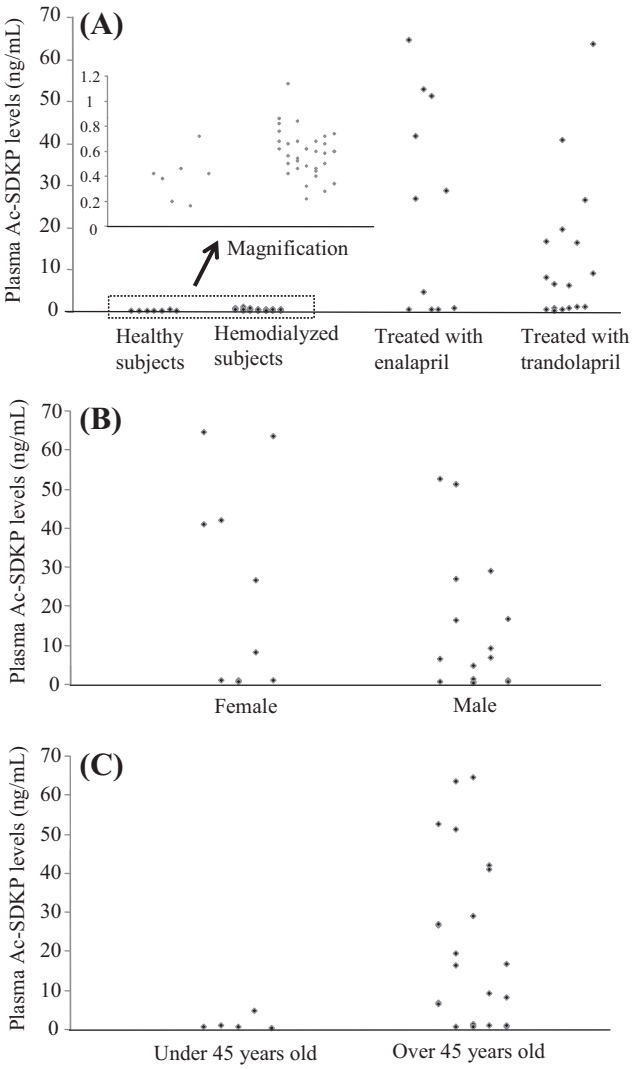


**Fig. 3.** Plasma Ac-SDKP levels in healthy, hemodialyzed and hemodialyzed subjects treated with ACE inhibitors. (A) Healthy ($n$ = 7), hemodialyzed ($n$ = 34), hemodialyzed subjects treated enalapril ($n$ = 10) and trandolapril ($n$ = 18). Detected levels: healthy subjects (0.16–0.72 ng/mL), hemodialyzed subjects (0.11–1.1 ng/mL), hemodialyzed subjects with enalapril (0.56–64.7 ng/mL), and hemodialyzed subjects with trandolapril (0.34–63.5 ng/mL). (B) Comparing sexes in hemodialyzed subjects treated with ACE inhibitors ($n$ = 28). Detected levels: Female (0.6–64.7 ng/mL), and male (0.34–52.7 ng/mL). (C) Comparing ages in hemodialyzed subjects treated with ACE inhibitors ($n$ = 28). Detected levels: Under 45 years old (0.34–4.7 ng/mL), and over 45 years old (0.6–64.7 ng/mL).

It is possible that very trace levels of Ac-ADKP in some kind of disorder would be increased such as a hemodialyzed subjects treated with ACE inhibitors. However, it is difficult to evaluate the minor Ac-ADKP levels in human plasma for other biomarkers. On the other hands, our result of Ac-SDKP was summarized using the information of ACE inhibitors, sexes, and ages (Fig. 3). We found that plasma Ac-SDKP levels were significantly higher in hemodialyzed subjects treated with ACE inhibitors of enalapril ($27.2 \pm 24.7$ ng/mL) and trandolapril ($11.6 \pm 16.8$ ng/mL) than healthy ($0.4 \pm 0.2$ ng/mL) and hemodialyzed subjects ($0.6 \pm 0.2$ ng/mL), respectively. After treatment with ACE inhibitors, plasma Ac-SDKP levels were increased to 45 (enalapril) and 20 (trandolapril) -fold compared with hemodialyzed subjects. Then, plasma Ac-SDKP levels in hemodialyzed subjects treated with ACE inhibitors were compared with sex and age (Fig. 3(B) and (C)). Plasma Ac-SDKP levels were significantly higher in hemodialyzed subjects treated with ACE inhibitors over 45 years old than under 45 years old. Based on significantly data of Ac-SDKP levels, future work would be tried to investigate the various blood markers, symptomatic state, and adverse effects in large scale of hemodialyzed subjects.

In this data, we show for the first time that Ac-SDKP levels in healthy and dialyzed subjects were compared by reliable LC–MS/MS method. Almost all previously analytical assays using EIA are able to indirectly detect Ac-SDKP levels in biological samples. In the present study, we focused on the relatively simple, high sensitive and specific analytical method for the quantitative determination of Ac-SDKP and minor Ac-ADKP in human plasma samples using SPE and LC–MS/MS in MRM mode.

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