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Liquid chromatography–tandem mass spectrometry to determine the stability of collagen pentapeptide (KTTKS) in rat skin

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ABSTRACT

The objective of this study was to develop a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to determine the stability of collagen pentapeptide (KTTKS), which is a subfragment of collagen and has been proved to promote the extracellular release of collagen in skin fibroblast, in rat skin. The chromatographic condition was optimized on an Acclaim C-18 column (2.1 mm × 150 mm, 3 μm) under isocratic elution using a mobile phase consisting of deionized water and acetonitrile (87:13, v/v) mixture containing 5 mM pentafluoropropionic acid as an ion-pairing reagent. The quantitation of KTTKS was performed on a triple quadrupole mass spectrometer in multiple reaction monitoring mode. The calibration curve showed good linearity in the concentration range of 0.05–10.0 μg/mL ($r^2 > 0.999$). The intra- and inter-day precisions were 0.8–6.5% and 2.4–5.8%, respectively, and the intra- and inter-day accuracies were 96.3–102.7% and 92.8–98.5%, respectively. The developed LC–MS/MS method was successfully applied to investigate the degradation rate and sites of KTTKS in rat skin homogenate. KTTKS was found to be very susceptible to the peptide bond cleavage by aminopeptidases present in the skin.

1. Introduction

Collagen pentapeptide (Lys-Thr-Thr-Lys-Ser, KTTKS), a subfragment of type I collagen, has been known to stimulate the synthesis of extracellular matrix production in subconfluent fibroblasts [1]. Among several subfragments of the C-terminal propeptide of type I collagen (residues 197–241), KTTKS (residues 212–216 in type I collagen) was found to be the minimum sequence necessary to stimulate the production of collagen and fibronectin in a variety of mesenchymal cells [2]. Due to this stimulating effect of collagen synthesis, KTTKS has attracted great attention as a cosmeceutical peptide ingredient for providing anti-wrinkle and anti-aging effects [3–5].

Peptides are generally known to be poorly transported across biological membranes [6]. The poor transport of peptides often originates from the lack of stability due to enzymatic or chemical degradation as well as hydrophilicity of peptides [5]. In fact, peptides are rapidly degraded by enzymatic metabolism in the dermal layer of the skin [7–9]. Several endopeptidases, such as deaminases and esterases, and exopeptidases, such as aminopeptidases, are found in the stratum corneum, epidermis and dermis of the skin [10]. Therefore, the stability of peptide against several proteolytic enzymes in the skin is potentially significant to successful transdermal delivery of peptides.

Although KTTKS was demonstrated in the use for its anti-wrinkle and anti-aging effects, there has been no report on analytical methods of KTTKS and its stability in the skin. Chirita et al. recently reported a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to monitor palmitoyl-KTTKS contents in cosmetic formulation [11], but no method has been reported to investigate the stability of KTTKS for transdermal delivery. Therefore, the purpose of this study was to develop a LC–MS/MS method as a sensitive analytical method to monitor low content of KTTKS for stability study in skin tissue. The chromatographic condition was optimized by investigating the effect of anionic ion-pairing reagents in the mobile phase of water–acetonitrile mixture on the retention of KTTKS in reversed-phase column. The degradation rate and sites of KTTKS in rat skin homogenate were investigated by the validated LC–MS/MS method.

2. Materials and methods

2.1. Reagents and chemicals

GHK (Gly-His-Lys) used as an internal standard (IS) and KTTKS were obtained from Bachem (Torrance, CA, USA). Acetonitrile

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(HPLC grade) was purchased from J.T. Baker (Philipsburg, NJ, USA). Trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA), and nonafluorovaleric acid (NFVA) were obtained from TCI (Tokyo, Japan). Rats were supplied from Orient Bio Inc. (Gyeonggi, Korea). Animal experiment was approved by Kyungsung University Animal Care and Use Committee, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. All other chemicals were of analytical grade and used as obtained commercially.

2.2. Instrumentation and LC–MS/MS conditions

LC–MS/MS was performed using an Agilent Series 1200 LC instrument with 6410 Triple Quad LC–MS/MS system (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved on an Acclaim C_{18} column (150 mm × 2.1 mm i.d., 3 μm particle size) from Dionex Corp. (Sunnyvale, CA, USA) under isocratic elution using a mobile phase consisting of a deionized water and acetonitrile (87:13, v/v) mixture to which 5 mM TFA, PFPA, HFBA or NFVA as an ion-pairing reagent was added. The flow rate was 0.2 mL/min. The autosampler and column temperatures were maintained at 4 and 25 °C, respectively. The sample injection volume was 5 μL.

Agilent 6410 Triple Quad LC–MS/MS equipped with an electrospray ionization (ESI) source was employed for both quantification and identification of KTTKS in skin tissue. Optimization of the tuning parameters was performed by using KTTKS and GHK solutions at 5 μg/mL to maximize product ion intensities. Quantitation was performed by using multiple-reaction-monitoring (MRM) mode to monitor protonated precursor → product ion transition of *m/z* 564.4 → 129.1 for KTTKS and 341.2 → 195.1 for IS (GHK) (Fig. 1). The needle voltage was set at 4 kV. The source and gas temperatures were 100 and 350 °C, respectively, and the gas flow rate was 10 L/min. Nebulizer pressure was 40 psi. Nitrogen was used as curtain, nebulizer and collision gas. The fragmentor was set at 167 and 130 V for KTTKS and IS, respectively. The values of collision energy were 37 and 13 V for KTTKS and IS, respectively.

2.3. Method validation

The KTTKS standard stock solution of 1 mg/mL was prepared by dissolving a requisite amount in deionized water. This was further diluted in HEPES buffer (10 mM, pH 7.4) to obtain KTTKS calibration standards at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/mL. The GHK used as IS was dissolved in HEPES buffer to prepare a concentration of 10 μg/mL. The linearity of calibration curve was confirmed by plotting peak-area ratios of KTTKS to IS versus KTTKS concentrations with least-squares linear regression analysis. Intra- and inter-day precisions and accuracies of the LC–MS/MS method were determined by analyzing quality control (QC) samples of KTTKS at concentrations of 0.05, 0.1, 1 and 10 μg/mL. Intra-day precision was determined by repeating the analysis of each QC sample three times in a single day, and inter-day precision and accuracy were determined by repeating this analysis on three consecutive days. The concentration of each sample was determined by using calibration standards prepared on the same day.

2.4. Stability study of KTTKS in rat skin homogenates

Freshly excised rat skin was homogenized in HEPES buffer (10 mM, pH 7.4) using a homogenizer cooled by ice-cold sterilized water. The dorsal skin removed from the nape of the neck and back was used after the removal of the adherent fat and subcutaneous tissue. The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was collected and filtered using a 0.45 μm membrane filter. The filtrate was diluted with the HEPES buffer (10 mM, pH 7.4) to obtain the solutions with protein concentrations of 1, 2, and 4 mg/mL on the basis of bovine serum albumin (BSA) concentration at 280 nm. A 100 μL aliquot of KTTKS solution (100 μg/mL) was mixed with 1 mL of each skin homogenate solution (protein concentrations of 1, 2, and 4 mg/mL) to make the ratios of KTTKS:protein of skin homogenate of 1:100, 1:200 and 1:400 (w/w), respectively. At predetermined times after incubation at 37 °C, the resulting mixture was subjected to LC–MS/MS and residual KTTKS was measured. Peak identification was performed by positive ionization mode, the fragmentor set in the range of 0–200 (arbitrary units), and the product ion scan from *m/z* 0 to 600 was used. The degradation products of KTTKS in the skin were identified by comparing retention times and mass spectra of KTTKS incubated in rat skin homogenate with those of blank rat skin homogenate sample and KTTKS in HEPES buffer (10 mM, pH 7.4). The source and gas temperature, curtain gas flow, and nebulizer gas pressure were as reported for quantitation.

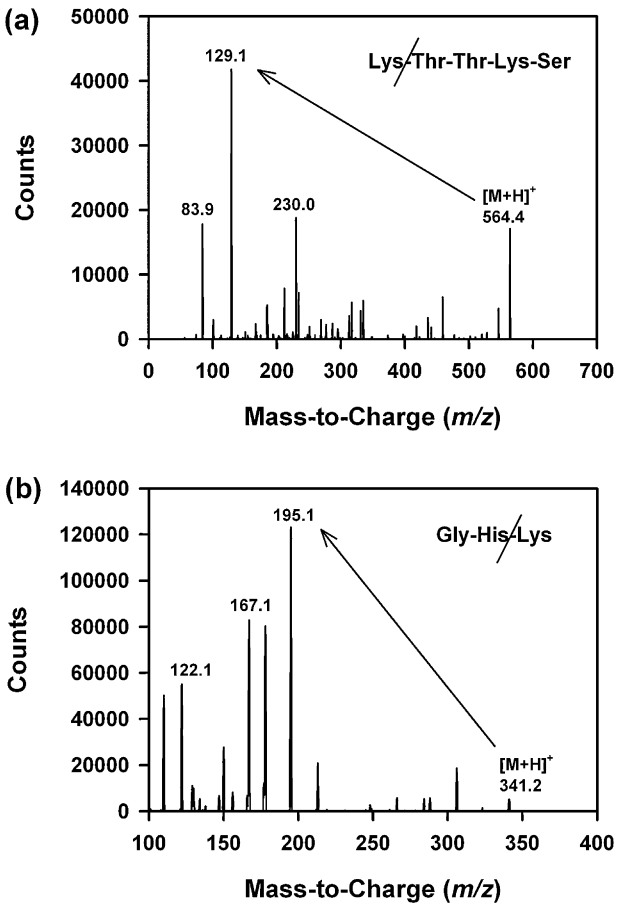

Fig. 1. Product ion mass spectra of KTTKS (a) and GHK used as IS (b). Protonated precursor ions of KTTKS and GHK were *m/z* 564.4 and 341.2, respectively. The diagonal line in the peptide sequence means breakage of amide bond for the generation of product ion.

3. Results and discussion

3.1. Effect of ion-pairing agent on peptide retentions in HPLC

KTTKS is a highly hydrophilic molecule due to its positively charged basic residues, such as primary amines in the two lysine residues and the N-terminal amines. For the separation of cationic molecules in reversed-phase HPLC, TFA has been widely used as an ion-pairing reagent [12]. The most common solvent

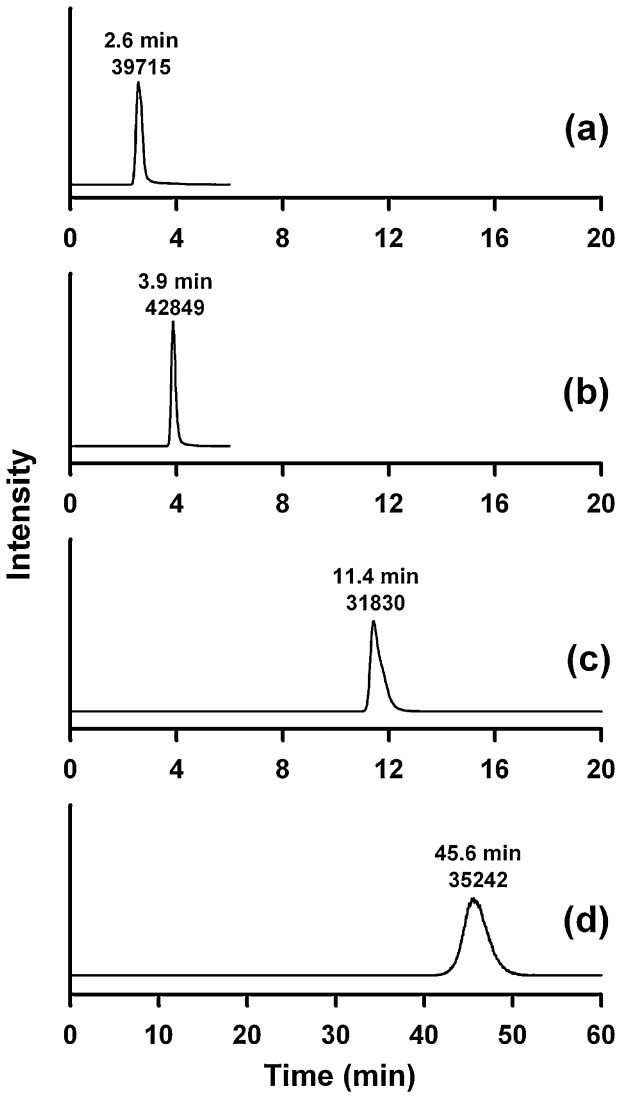

Fig. 2. Effect of ion-pairing reagents on the retention of KTTKS in a C-18 column. Each LC–MS/MS chromatogram of KTTKS (m/z 564.4 → 129.1) was obtained by using the mobile phase consisting of a deionized water and acetonitrile (87:13, v/v) mixture containing 5 mM TFA (a), PFPA (b), HFBA (c), and NFVA (d) as ion-pairing reagents.

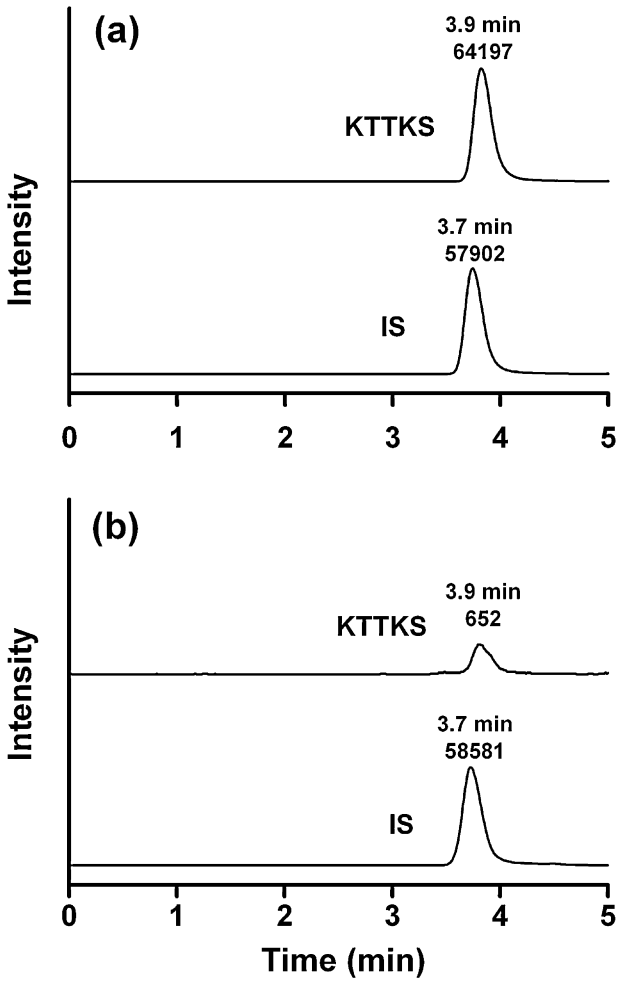

Fig. 3. LC–MS/MS chromatograms of KTTKS (m/z 564.4 → 129.1) and IS (m/z 341.2 → 195.1) spiked to rat skin homogenate at room temperature (a) and after incubation in rat skin homogenate (protein concentration at 4 mg/mL) for 5 min at 37 °C (b).

system in use for peptide separation is acetonitrile–water containing 0.05–0.20% TFA [13–15]. Initially, this solvent system was tried to analyze KTTKS on a C-18 reversed-phase column. However, the acetonitrile–water containing 0.1% TFA could not retain KTTKS on a C-18 column. Even when 0.2% TFA and the minimal amount of acetonitrile (~5%) were used, KTTKS was eluted together with the sample solvent. Therefore, the use of a more hydrophobic ion-pairing reagent was required.

Fig. 2 illustrates the effect of increasing hydrophobicity of ion-pairing reagents (TFA < PFPA < HFBA < NFVA) added to the mobile phase at a constant concentration (5 mM) on the elution behavior of KTTKS from a C-18 column. In progressing from the TFA to the NFVA system, the retention times of KTTKS were increased from 2.6 to 45.6 min, which may be attributed to the increased hydrophobicity of a complex between KTTKS and an ion-pairing reagent. In terms of speed, peak shape and peak efficiency, HFBA and NFVA were less efficient than PFPA, as shown in Fig. 2. Therefore, PFPA was chosen as an appropriate ion-pairing reagent for the analysis of KTTKS. In LC–MS/MS using the isocratic elution with water–acetonitrile (87:13, v/v) containing 5 mM PFPA, KTTKS and IS (GHK) were observed at 3.9 and 3.7 min, respectively (Fig. 3a).

3.2. Validation of LC–MS/MS

The LC–MS/MS method was validated by simultaneously monitoring the selected MS/MS transitions for KTTKS (m/z 564.3 → 129.1) and IS (m/z 341.2 → 195.0) in HEPES buffer (10 mM, pH 7.4). As KTTKS was easily degraded in rat skin homogenate, the validation could not be performed in rat skin homogenate. However, specificity could be checked by comparing the LC–MS/MS chromatograms of KTTKS and IS in HEPES buffer (10 mM, pH 7.4) with that of the blank sample of rat skin homogenate. No interference from rat skin homogenate was found at the same retention times of both KTTKS and IS. In HEPES buffer (10 mM, pH 7.4) at 37 °C, KTTKS was stable over for 7 days (data not shown).

The eight point calibration curve obtained by least-squares linear regression showed good linearity in the concentration range of 0.05–10.0 μg/mL. The calibration curve had a slope of 0.3374, an intercept of 0.0038 and r^2 value of 0.9999. The lower limit of quantification (LLOQ) for KTTKS was 0.05 μg/mL (signal-to-noise ratio of 10), which was enough to measure the amount of KTTKS for the stability study in rat skin homogenate. Intra- and inter-day precision and accuracy were determined by analyzing three replicates at each of 0.05 (LLOQ), 0.1 (low QC), 1 (medium QC) and 10 μg/mL

Table 1
Validation of the LC–MS/MS method.

KTTKS added (μg/mL)	Intra-day			Inter-day		
	Amount detected (μg/mL)[a]	Accuracy (%)	Precision (%)	Amount detected (μg/mL)[a]	Accuracy (%)	Precision (%)
0.05	0.048 ± 0.003	96.3	6.5	0.049 ± 0.003	98.5	5.8
0.1	0.103 ± 0.005	102.7	4.9	0.093 ± 0.005	92.8	5.3
1	1.006 ± 0.019	100.6	1.9	0.941 ± 0.037	94.1	4.0
10	9.911 ± 0.077	99.1	0.8	9.847 ± 0.075	98.5	2.4

[a] The values represent mean ± standard deviation.

(high QC) of KTTKS. Precision was expressed as a relative standard deviation (RSD) and accuracy was expressed as a mean percentage of analyte recovered in the assay. At LLOQ, intra- and inter-day precisions were 6.5% and 5.8%, respectively, and intra- and inter-day accuracies were 96.3% and 98.5%, respectively. Overall, the intra- and inter-day precisions were 0.8–6.5% and 2.4–5.8%, respectively, and the intra- and inter-day accuracies were 96.3–102.7% and 92.8–98.5%, respectively (Table 1).

3.3. Stability study of KTTKS in rat skin homogenate

The validated LC–MS/MS method was applied to a stability study of KTTKS in rat skin homogenate. The protein concentration of rat skin homogenate was fixed in 1, 2 and 4 mg/mL on the basis of BSA concentration at 280 nm through dilution by HEPES buffer (10 mM, pH 7.4). Fig. 3 shows the representative LC–MS/MS chromatograms of KTTKS spiked to rat skin homogenate at room temperature and after incubation in rat skin homogenate (protein concentration at 4 mg/mL) for 5 min at 37 °C. The degradation of KTTKS was dependent on the protein concentration of rat skin homogenate. Fig. 4 illustrates the dependency of KTTKS degradation on the protein concentration of rat skin homogenate at 37 °C. The half-lives of KTTKS at 1, 2 and 4 mg/mL protein concentrations in rat skin homogenate were 2.8, 1.7, and 0.8 min, respectively.

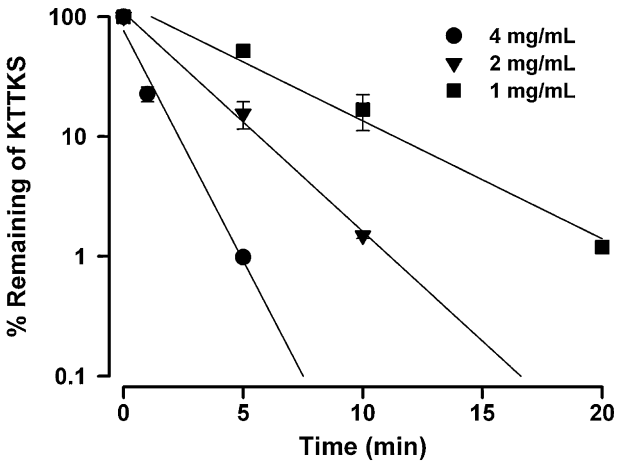

Fig. 4. Degradation kinetics of KTTKS in rat skin homogenates with different protein concentrations (1, 2 and 4 mg/mL) at 37 °C ($n = 3$).

3.4. Identification of degradation products of KTTKS in skin

As shown in Fig. 4, KTTKS was extremely susceptible to the degradation in skin homogenate. To find the degradation products, a blank sample of rat skin homogenate and KTTKS samples used in the stability study were analyzed by LC–MS in positive scan mode.

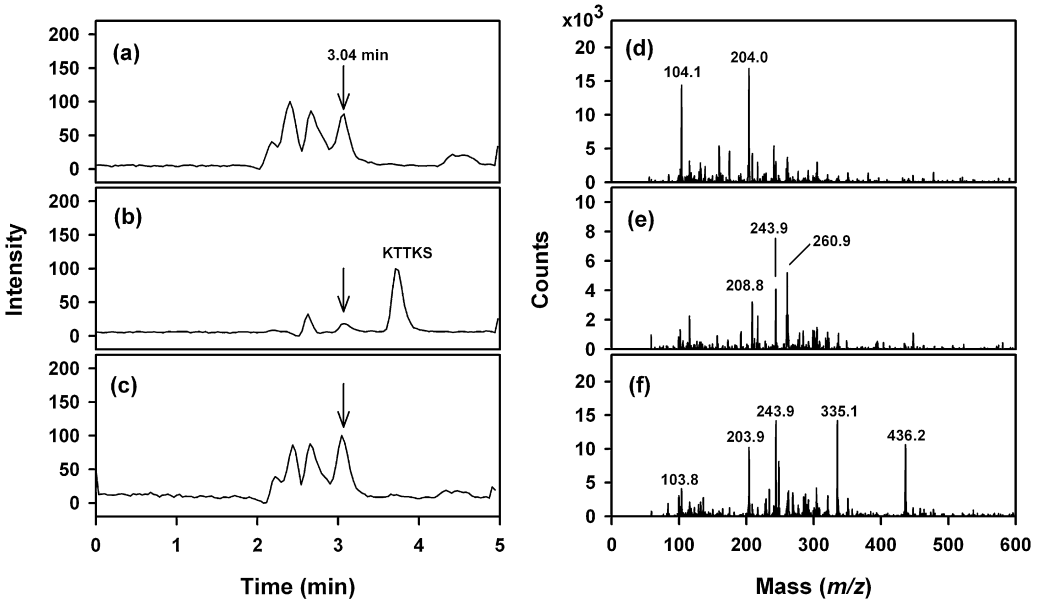

Fig. 5. Identification of the degradation products of KTTKS in rat skin homogenate. Left shows total ion chromatograms of rat skin blank homogenate (a), KTTKS in HEPES buffer (10 mM, pH 7.4) (b), and KTTKS incubated in rat skin homogenate for 5 min at 37 °C (c). Right panels show mass spectra at 3.04 min in total ion chromatograms of rat skin blank homogenate (d), KTTKS in HEPES buffer (10 mM, pH 7.4) (e), and KTTKS incubated in rat skin homogenate for 5 min at 37 °C (f).

Table 2
Protonated mass list of KTTKS sequences and identification of degradation products of KTTKS in rat skin homogenate.

Sequence	Mass $[M+H]^+$	Observed after incubation of KTTKS in rat skin homogenate
K	147.11	–
KT	248.16	–
KTT	349.21	–
KTTK	477.30	–
KTTKS	564.34	–
TTKS	436.24	Found
TKS	335.19	Found
KS	234.15	–
S	106.05	–

Fig. 5 shows the LC–MS total ion chromatograms and mass spectra of blank rat skin homogenate, KTTKS in HEPES buffer (10 mM, pH 7.4), and KTTKS incubated in rat skin homogenate (protein concentration at 4 mg/mL) for 5 min. When the total ion chromatograms of three samples were compared, the difference was found at 3.04 min in the chromatograms. As shown in Fig. 5f, in the mass spectrum of KTTKS incubated in rat skin homogenate, two distinct mass peaks of m/z 335.1 and 436.2 were observed in comparison with the samples of blank rat skin homogenate and KTTKS in HEPES buffer (10 mM, pH 7.4). Based on the mass of KTTKS sequence, m/z 335.1 and 436.2 peaks corresponded to the positive ions of TKS and TTKS, respectively (Table 2). At the initial stage after incubation, the m/z 436.2 peak was higher than the m/z 335.1 peak, and the m/z 335.1 peak was subsequently increased. This result indicates that KTTKS is mostly degraded by aminopeptidases of the skin in a stepwise manner.

4. Conclusions

The LC–MS/MS method for the stability study of KTTKS in skin homogenate was developed and validated. The developed method was successfully applied to investigate the degradation rate and sites of KTTKS in rat skin. KTTKS was found to be very susceptible to the peptide bond cleavage by aminopeptidases present in the skin. The enzymatic degradation at the skin site would be one of major barriers to the transdermal delivery of KTTKS. Therefore, the strategies for protecting KTTKS from the degradation from aminopeptidases would be necessary for the effective delivery of KTTKS to the skin.

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