Isolation, Characterization, and Stability of Positional Isomers of Mono-PEGylated Salmon Calcitonins

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Purpose. To separate and characterize the different positional isomers of mono-PEGylated salmon calcitonins (mono-PEG-sCTs) and to evaluate the effects of the PEGylation site on the stability of different mono-PEG-sCTs in rat kidney homogenate.

Methods. Mono-PEG-sCTs were prepared using succinimidyl carbonate monomethoxy polyethylene glycol (5,000 Da) and separated by gel-filtration HPLC followed by reversed-phase HPLC. To characterize PEGylated sCTs, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and reversed-phase HPLC of the trypsin digested samples were performed. Mono-PEG-sCTs and sCT in rat kidney homogenates were measured by column-switching reversed-phase HPLC with on-line detection of the radioiodinated samples using a flow-through radioisotope detector.

Results. Three different mono-PEGylated sCTs were separated by reversed-phase gradient HPLC. From the MALDI-TOF MS analysis, the average molecular weight of mono-PEG-sCTs was confirmed as around 8650 Da. The presence of PEG moiety in the mono-PEG-sCTs was also manifested by the fact that the distance between two adjacent mass spectum lines was 44 Da which corresponds to PEG monomer unit. Tryptic digestion analysis demonstrated that these mono-PEG-sCTs are 3 positional isomers of N-terminus, Lys^{18}- and Lys^{11}-residue modified mono-PEGylated sCTs. The degradation half-life of these 3 positional isomers in rat kidney homogenates significantly increased in order of the N-terminus (125.5 min), Lys^{11}- (157.3 min), and Lys^{18}-residue modified mono-PEGylated sCT (281.5 min) over the native sCT (4.8 min).

Conclusion. Three positional isomers of mono-PEGylated sCTs were purified and characterized. Of these, the resistance to proteolytic degradation was highest for the Lys^{18}-residue modified mono-PEG-sCT. These studies demonstrate that the in vivo stability of PEGylated sCTs is highly dependent on the site of PEG molecule attachment.

KEY WORDS: calcitonin; polyethylene glycol; PEGylation; peptide; tryptic digestion; stability; HPLC.

INTRODUCTION

Salmon calcitonin (sCT) is a single polypeptide chain calcitropic hormone consisting of 32 amino acids (3,432 Da) with an N-terminal disulfide bridge between positions 1 and 7, and a C-terminal proline amide residue (Fig. 1). It is currently formulated as either a sterile solution for intramuscular or subcutaneous injection, or as a nasal spray in the management of several bone-related diseases including Pagets disease, hypercalcemia and osteoporosis as well as management of severe pain resulting from vertebral crush fractures (1–3). These cases generally require long-term therapy through a daily or alternate daily treatment with calcitonin. As a polypeptide, sCT is subject to chemical and enzymatic degradation and has a short biological half-life in the body, approximately 14 min (4–10).

To overcome the rapid clearance of sCT from the blood circulation and its *in vivo* instability, the modification of sCT with polyethylene glycol (PEG) is considered to be a novel approach. The chemical modification of therapeutic polypeptides with PEG is a technique widely investigated to obtain functionalized bioconjugates of increased resistance to proteolytic degradation, increased solubility, reduced immunogenicity and antigenicity, and low toxicity (11–15). However, clinically available PEGylated polypeptides are limited, mainly due to the increased molecular size by PEGylation, restricted distribution from blood to target tissues and decreased receptor-binding affinity (16). In addition, difficulty in the physicochemical characterization of PEGylated polypeptides is another major barrier to clinical application. The physicochemical characterization of PEGylated polypeptides is rampered because of the heterogeneity with respect to distribution in both the number and position of the attached PEG molecules and inherent polydispersity of PEG itself. Small structural changes in polypeptides can influence the physicochemical properties, biological activity and potency. Therefore, the preparation and separation of chemically defined PEGylated polypeptides, including their characterization, are important in assuring product consistency (17–20). Many studies have been published about site-specific conjugation of polypeptides at glycosylated site, cysteine residue, NH_2 terminus, side chain of Lys and Asp, and COOH terminus (21–24). However, few studies on the separation of PEGylated polypeptides mixtures according to attachment site to characterize their stability and biological properties has been published (25).

In the previous study, we reported on the preparation and primary characterization of PEGylated sCTs (26). In this study, we separated 3 different positional isomers of mono-PEGylated sCTs from each other and these species were identified and characterized by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), tryptic digestion, radioiodination, column-switching HPLC with flow-through radioisotope detection. Increased biological stability of the derivatives was also established.

MATERIALS AND METHODS

Materials

Salmon calcitonin (synthetic cyclic sCT) and succinimidyl carbonate monomethoxypolyethylene glycol (5,000 Da, SC-mPEG) were purchased from Bachem (Torrance, CA) and Shearwater Polymers (Huntsville, AL), respectively. Chloramine-T, pentafluoropropionic acid (PFPA), trifluoroacetic acid (TFA), and trypsin were purchased from Sigma (St. Louis,

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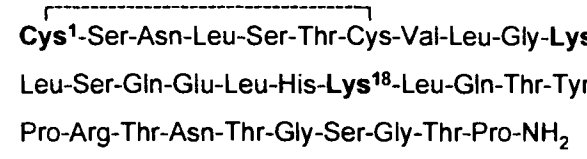

Fig. 1. Primary structure of salmon calcitonin. Possible PEGylated sites are Cys^1, Lys^{11} and Lys^{18}.

MO). $Na^{125}I$ and acetonitrile (HPLC grade) were purchased from Dupont NEN (Philipsburg, NJ) and Baker (Boston, MA), respectively. All other chemicals were of analytical grade and were obtained commercially. Male Sprague-Dawley rats (250 ~ 300 g) were purchased from the Experimental Animal Lab of Korean FDA (Seoul, Korea).

PEGylation and Separation of Mono-PEG-sCTs

PEGylated sCTs were prepared using the method previously described (26). In brief, SC-mPEG (1.46 mg) was added to 0.2 ml of 0.1 M phosphate buffer solution (PBS, pH 8.0) containing sCT (5 mg/ml). The mixture was shaken gently at ambient temperature for 60 min and was subjected to size-exclusion HPLC. The HPLC fraction of mono-PEG-sCT mixture was concentrated on a Centricon-10 (Amicon, Beverly, MA). The concentrated mono-PEG-sCT mixture was further purified to three different fractions using the reversed-phase HPLC method (see further for details). The peak effluents corresponding to three individual mono-PEG-sCT fractions were then collected separately in reservoir vials pre-filled with 1 ml of 0.1 M PBS (pH 7.0). The effluent was flushed off with nitrogen and stored at 4°C.

MALDI-TOF Mass Spectrometry

Mass spectra were recorded on MALDI-TOF MS (Voyager-RP, PerSeptive Biosystems, Cambridge, MA) operating in the positive-ion linear mode of detection. The ion acceleration potential was +25 kV and the length of flight tube was 1.3 m. A nitrogen laser was set to deliver 337 nm wavelength pulses onto the sample. A saturated solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% acetonitrile was used as a matrix solution and a mixture of peptide mass standards of angiotensin I, ACTH (clip 1–17), ACTH (clip 18–39), ACTH (clip 7–38) and bovine insulin was adapted for the external calibration.

Radioiodination

Radiolabeling of sCT and mono-PEG-sCTs for stability studies was performed by the Chloramine-T method. In brief, aliquots (80 μl each) of sCT and mono-PEG-sCTs in 0.1 M PBS (approx. 20 μg/ml, pH7.0) were mixed with 10 μl of $Na^{125}I$ (0.2 mCi) and 10 μl of Chloramine-T in 0.1 M PBS (2.5 μmol/ml, pH 7.0). After reaction for 20 seconds at ambient temperature, the reaction mixture was injected onto the pre-column (30 × 4.0 mm) tap-filled with Sepak C-18 (Waters) followed by the column-switching desalting HPLC. The free iodine and reagent were washed with 0.1% TFA in water and the fractions of ^{125}I-labeled sCT and mono-PEG-sCTs retained in the pre-column were collected directly by the isocratic elution with acetonitrile using the flow-through radioisotope detector. The specific radioactivity of sample solutions was adjusted to 1×10^6 cpm/100 μl.

PEGylation Site Mapping

Tryptic digestion of sCT and mono-PEG-sCTs was performed by adding 20 μl of trypsin (5 mg/ml in water) to 100 μl of a sample (approx. 20 μg/ml in 0.1 M PB, pH 7.0) and proteolytic digestion was carried out for 5 hr at 39°C as described previously (27). Digested products were stored at −20°C and a 100 μl of the tryptically digested mixture was analyzed by RP-HPLC. The chromatograms were subtracted from that of the blank [20 μl of trypsin (5 mg/ml in water) in 100 μl of 0.1 M PB, pH 7.0]. Amino acid composition of the fragments of digested sCT and three different fractions of mono-PEG-sCT was determined using the PITC derivatizing method as described previously(27).

Degradation in Rat Kidney Homogenate

Rats were fasted overnight prior to sacrifice. The kidneys were quickly harvested and cleaned with 10 mM saline PBS (pH 7.4). A 2 g kidney specimen was homogenized with 8 ml ice-cold saline PBS and the homogenate centrifuged at 20,000 g for 30 min at 4°C. The total protein concentration in the transferred supernatant was adjusted to approx. 10 mg/ml as total protein with saline PBS. The supernatant was stored at −20°C until analysis.

A 10 μl aliquot of a radioiodinated sample was mixed and incubated at 37.5°C with 100 μl of rat kidney supernatant, which was pre-incubated for 10 min. After various time intervals (sCT: 0, 1, 3, 5, 10 min, mono-PEG-sCTs: 0, 10, 20, 30, 45, 60 min), the incubation was stopped. After centrifugation for 1 min, 100 μl of supernatant was quickly injected onto the pre-column followed by the column-switching HPLC procedure. Each sample was prepared immediately prior to injection.

High Performance Liquid Chromatography

The HPLC system consisted of three model 307 pumps (two pumps for gradient elution of mobile phase, one pump for isocratic elution of washing solvent for the pre-column), a model 234 autosampler, a model 118 UV-visible detector and a model 712 system controller with a 506C interface module (Gilson, Villiers-le-Bel, France). A model F4010 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with flow cell for HPLC and a Ramona 2000 flow-through radio-isotope detector (Raytest, Straubenhardt, Germany) equipped with a 100 μl gamma scintillator flow cell were connected to UV detector. The UV responses were measured at 215 nm, whereas fluorescence was monitored at λ_{ex} = 280 nm and λ_{em} = 315 nm.

Size-Exclusion HPLC

A 200 μl of sample was injected on Superose12 HR 10/30 (Pharmacia, Sweden) at a flow rate of 0.4 ml/min with 10 mM saline PBS (pH 7.0).

Reversed-Phase HPLC for Mono-PEG-sCTs Separation

A 100 μl aliquot of the isolated mono-PEG-sCT mixture was injected on a LiChrospher 100 RP-18 (4.0 × 125 mm, 5

μm, Merck) cartridge with guard column (4.0 × 4.0 mm, 5 μm, Merck). Gradient elution was carried out at a flow-rate of 1.0 ml/min with solvent A (0.1% PFPA in water) and solvent B (0.1% PFPA in acetonitrile). A gradient condition was employed: 36% B to 42% B over 20 min.

Reversed-Phase HPLC for Tryptic Digested Fractions

The analysis was conducted with Inertsil ODS-2 (4.6 × 150 mm, 5 μm, GL Science) at 40°C. Gradient elution was carried out at a flow-rate of 1.2 ml/min with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The following gradient profile was chosen: 0% B for 8min, 0% B to 55% B over 16 min, 55% B to 100% B over 5 min. After an additional elution for 2 min with 100% B followed by 10 min with 100% A, the system was ready for the next injection.

Column-Switching Reversed-Phase HPLC for Radioiodinated Samples in Biological Fluids

This HPLC analysis was performed according to the 6-port column-switching system described previously with slight modification (6,26). Briefly, the pre-column was tap-filled with Sepak C-18 (30 × 4.0 mm, Waters). The analytical column was a LiChrospher 100 RP-8 (4.0 × 125 mm, 5 μm, Merck) cartridge fitted with a guard column (4.0 × 4.0 mm, 5 μm, Merck). A 100 μl aliquot of the biological sample was injected onto the pre-column. Polar interfering components in the samples were washed out with 0.1% TFA for 5 min at a flow-rate of 0.8 ml/min. The retained components were eluted in the back-flush mode from the pre-column to analytical column by linear gradient elution of the mobile phase at a flow rate of 1 ml/min with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). A linear gradient profile was chosen: 30% B to 100% B over 20 min after column-switching. The radioactivity of the column effluent was measured by on-line detection using a flow-through radio-isotope detector.

RESULTS AND DISCUSSION

sCT contains three potential sites for PEGylation, i.e., the primary amine moiety of the N-terminus (Cys^1), and two lysine residues (Lys^{11} and Lys^{18}). Covalent attachment of PEG to sCT produces a heterogeneous mixture of 7 possible PEG modified sCTs: 3 mono-PEG-sCTs (N-terminus-; Lys^{11}-; Lys^{18}-), 3 di-PEG-sCTs (N-terminus- and Lys^{11}-; N-terminus- and Lys^{18}-; Lys^{11}- and Lys^{18}-) and 1 tri-PEG-sCT (N-terminus-, Lys^{11}-, and Lys^{18}-). In the present study, PEGylated sCTs were prepared and separated into mono-, di- and tri-PEGylated sCTs using the size-exclusion HPLC as described previously (26) and the fraction corresponding to the mono-PEG-sCT mixture was further separated by reversed-phase HPLC into three peaks (Fig. 2). These mono-PEGylated sCT species were well separated despite the inherent m.w. distribution of the polymer itself (17,19). These mono-PEG-sCTs were tentatively named fragment M1 (Rt = 10.0 min), M2 (Rt = 12.0 min), and M3 (Rt = 14.2 min).

The presence of the PEG molecule in the mono-PEG-sCTs was identified from the corresponding mass spectra detected by MALDI-TOF MS. As shown in Fig. 3, each mono-PEG-sCT appeared as a group of peaks with adjacent peaks separated by 44 Da. This is due to PEG being a polydisperse mixture of

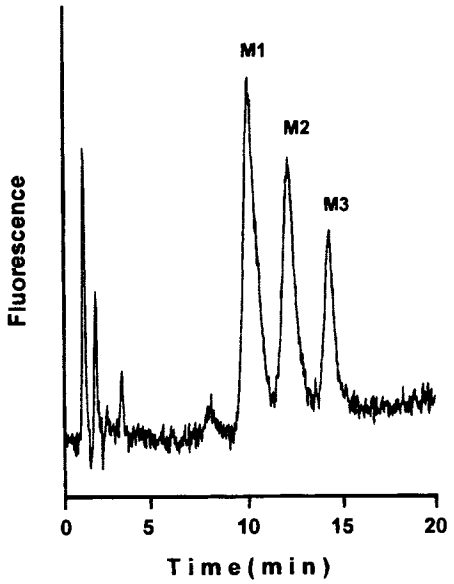

Fig. 2. Reversed-phase gradient HPLC separation of 3 positional isomers of mono-PEGylated sCTs. Fraction of mono-PEGylated sCTs from size-exclusion HPLC was injected on a C_{18} column and monitored by fluorescence detector.

PEG chains of different lengths, with each component differing from others by n × 44 Da (n = integer), where 44 Da is the molecular weight of PEG monomer unit (25). The average molecular weight of mono-PEG-sCTs was found to be approximately 8650 Da.

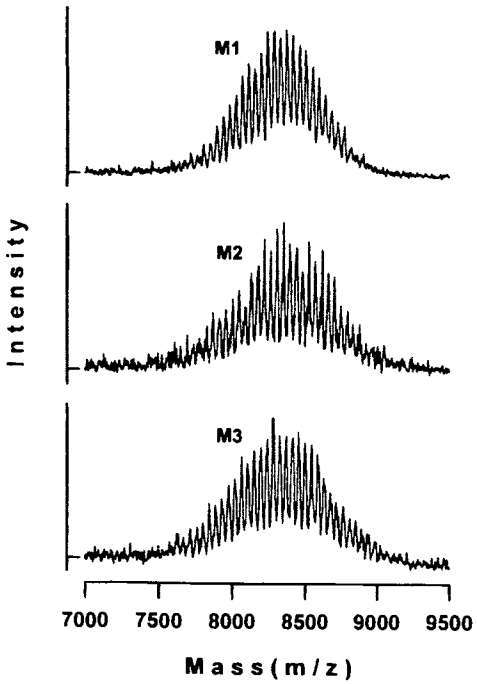

Fig. 3. MALDI-TOF mass spectra of 3 positional isomers of mono-PEGylated sCTs. The ion acceleration potential was set +25 kV under the positive-ion linear mode of detection and a saturated solution of α-cyano-4-hydroxycinamic acid in 50% acetonitrile was used as a matrix solution.

The conjugation sites of the purified mono-PEG-sCTs were determined by tryptic digestion followed by reversed-phase HPLC separation. PEGylation of sCT increased the resistance of the polypetide to proteolytic degradation, probably resulting from the steric hindrance of the PEG strands, shrouding the polypeptide and preventing proteolytic attack. Trypsin digestion cleaves the polypeptide at the carboxy-side of lysine and arginine and PEG covalently attached to the lysine residue of sCT should have prevented the cleavage. The PEGylation of sCT resulted in differences in the chromatographic profiles, e.g., migration of the PEGylated fragments to the more hydrophobic region (23,28–29).

sCT contains one arginine (24 position) and two lysine (11, 18 position) residues that may be cleaved by trypsin. Tryptic digestion of the unmodified sCT results in four possible fragments of **1** (Cys^{1}-Lys^{11}), **2** (Leu^{12}-Lys^{18}), **3** (Leu^{19}-Arg^{24}), and **4** (Thr^{25}-Pro^{32}). The amino acid composition of each of the tryptic fragments was previously examined (26). These fragments were found to be eluted in the order of **4**, **2**, **3**, and **1** by reversed-phase HPLC and are labeled as peaks **a**, **b**, **c**, and **d**, respectively (Fig. 4). In addition new peaks appeared beyond 25 min, presumably PEGylated sCT fragments. The tryptic digestion of M1, M2 and M3 fractions showed significantly different fragmentation patterns as compared to the native sCT. As summarized in Table 1, the size of peak **d** was significantly reduced for M1, peaks **b** and **c** reduced for M2, and peaks **b** and **d** reduced for M3. This means that each reduced peak fraction participated in the PEGylation and also indicates that these mono-PEG-sCTs are 3 positional isomers of N-terminus residue mono-PEGylated sCT (M1), Lys^{18}- residue mono-PEGylated sCT (M2), and Lys^{11}-residue mono-PEGylated sCT (M3) in elution order of reversed-phase HPLC.

Table 1. Identification of 3 Positional Isomers of Mono-PEG-sCT by Tryptic Digestion

	Peak height[a]				
	a	b	c	d	Identity
Native sCT	+	+	+	+	
M1	+	+	+	−	N-terminus mono-PEG-sCT
M2	+	−	−	+	Lys^{18}-mono-PEG-sCT
M3	+	−	+	−	Lys^{11}-mono-PEG-sCT

[a] Each of the four peaks was identified by amino acid analysis (12). Peak, a: Thr^{25}-Pro^{32}, b: Leu^{12}-Lys^{18}, c: Leu^{19}-Arg^{24}, d: Cys^{1}-Lys^{11}. Symbol, (+): comparable peak height found for sCT, (−): reduced or negligible peak.

The effect of the PEGylation site on the stability of sCT was examined in rat kidney homogenates, the main degradation tissue for sCT *in vivo* (7–9). For sCT assay, the conventional RIA assay was difficult to adopt due to the reduced immunoreactivity of PEGylated sCTs (30). All homogenate samples were assayed by the column-switching reversed phase HPLC connected with a flow-through radioisotope detector (Fig. 5). The degradation kinetics of mono-PEGylated sCTs is shown in Fig. 6. sCT was rapidly degraded, with only approximately 20% of sCT remaining after 10 min of incubation. The degradation of mono-PEGylated sCTs was, however, dramatically reduced, with >70% remaining after 60 min of incubation. Mono-PEGylation increased the degradation half-life of sCT, and the degradation half-life of these 3 positional isomers in rat kidney

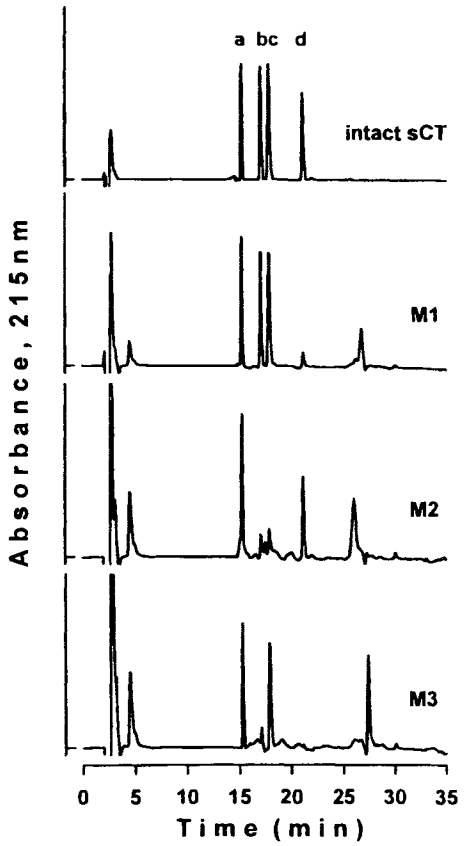

Fig. 4. Reversed-phase gradient HPLC of trypsin digested sCT and 3 isomers of mono-PEGylated sCTs. Tryptic digestion was carried out for 5 hr at 39°C, M1: N-terminus-mono-PEG-sCT, M2: Lys^{18}-mono-PEG-sCT, M3: Lys^{11}-mono-PEG-sCT.

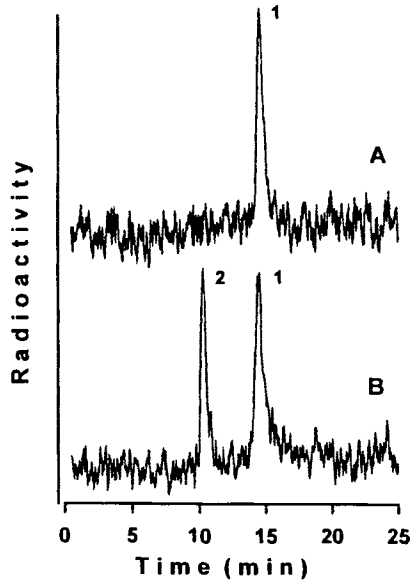

Fig. 5. Chromatogram of radio-iodinated mono-PEGylated sCT (N-terminal) incubated in rat kidney homogenate at 37°C, measured by flow-through radio-isotope detector. A: Initial, B: after 60 min., 1: mono-PEG-sCT, 2: degradation product.

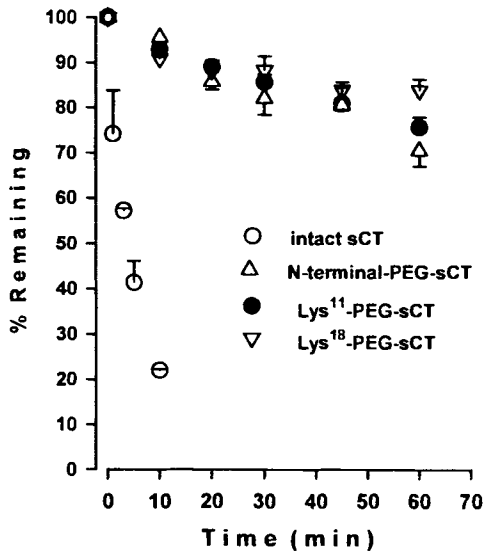

Fig. 6. Stability of mono-PEGylated sCTs in rat kidney homogenates. Radio-iodinated sCT and 3 isomers of mono-PEGylated sCTs were incubated at 37.5°C with rat kidney homogenates for various time intervals and measured by flow-through radio-isotope detector.

homogenates was increased in order of the N-terminus (125.5 min), Lys^{11}-(157.3 min), and Lys^{18}-residue modified mono-PEGylated sCT (281.5 min) over the native sCT (4.8 min). Lys^{18}-modified mono-PEG-sCT showed an approximately 2-fold longer half-life than both N-terminus and Lys^{11}-modified mono-PEG-sCT, as shown in Table 2. These results provide the evidence that the PEG attachment site to sCT can significantly influence the in vivo stability of sCT.

Another important aspect of the present study is related to the degradation pathway of sCT. There have been many studies reporting the chemical degradation of sCT. The degradation reaction of sCT follows first-order kinetics and its maximum stability is achieved by adjusting the pH to 3.3 (5). In aqueous solution, sCT undergoes hydrolysis, deamidation, and sulfide exchange leading to a trisulfide derivative (10). However, these degradation mechanisms are non-enzymatic and the literature data concerning the stability of sCT in biological fluids is limited. The metabolism of sCT by bovine nasal mucosal enzymes was examined recently (9). sCT was shown to be cleaved at the Lys^{18}-Leu^{19} bond in the nasal mucosa. The cleavage at Lys^{18}-Leu^{19} is indicative of tryptic endopeptidase activity. In our study, Lys^{18}-modified mono-PEG-sCT was found to be most stable in rat kidney homogenate. PEG strands appeared to protect the PEGylated polypeptide from attack by various peptidases and proteases due to their steric hindrance. If the crucial amino acid moiety for degradation is modified with PEG, these PEGylated polypeptides will show an enhanced stability over other PEGylated peptides modified at different sites. The increased half-life of Lys^{18}-mono-PEG-sCT over N-terminus- and Lys^{11}-mono-PEG-sCT suggests that the Lys^{18}-Leu^{19} bond is important for the stability of sCT in biological fluids and this result is fully consistent with the observation described previously (9).

Table 2. Stability of 3 Positional Isomers of Mono-PEG-sCT in Rat Kidney Homogenates

Mono-PEGylated sCTs	$t_{1/2}$ (fold increase[a])
sCT	4.8 min (1.0)
N-terminal-mono-PEG-sCT	125.5 min (26.1)
Lys^{11}-mono-PEG-sCT	157.3 min (32.8)
Lys^{18}-mono-PEG-sCT	281.5 min (58.6)

[a] Stability increases relative to native sCT.

In conclusion, three positional isomers of mono-PEGylated sCTs were separated by reversed-phase gradient HPLC and their molecular weights and PEGylation sites were identified. The degradation half-life of these three positional isomers in rat kidney homogenates increased in order of the N-terminus (125.5 min), Lys^{11}- (157.3 min), and Lys^{18}-residue modified mono-PEGylated sCT (281.5 min) over the native sCT (4.8 min). These findings provide evidence that the site of PEGylation is an important determinant in the in vivo stability of PEGylated sCTs.

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