

Pharmacokinetic Disposition of Polyethylene Glycol-Modified Salmon Calcitonins in Rats

Sun Dong YOO,*^a Hun JUN,^a Beom Soo SHIN,^a Hye Suk LEE,^b Myung Ok PARK,^a Patrick P. DeLUCA,^c and Kang Choon LEE^a

College of Pharmacy, Sungkyunkwan University,^a 300 Chonchon-dong, Changan-gu, Suwon, Kyonggi-do, 440–746, Korea, College of Pharmacy, Wonkwang University,^b 344–2 Shinyong-dong, Iksan, Jeonlabook-do, 570–749, Korea, and College of Pharmacy, University of Kentucky,^c Lexington, KY 40536–0082, U.S.A.

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This study first reports the pharmacokinetic disposition of polyethylene glycol (PEG)-modified salmon calcitonin (sCT) based on the number of attached PEG molecules. PEG-modified sCT was prepared by covalent linkage with succinimidyl carbonate monomethoxy polyethylene glycol. Mono- and di-PEG-sCTs were separated by size exclusion and reverse phase HPLC, and radiiodinated by the chloramine-T method with Na¹²⁵I. ¹²⁵I-mono-PEG sCT, ¹²⁵I-di-PEG-sCT and unmodified ¹²⁵I-sCT were administered to rats by i.v. injection. Serial blood samples, urine and various tissue samples were taken for the determination of radioactivity. Di-PEG-sCT exhibited significantly reduced systemic clearance (2.3 vs. 11.1 ml/min/kg) and steady-state volume of distribution (229.9 vs. 603.1 ml/kg), while mono-PEG-sCT showed a prolonged elimination half-life (189.1 min vs. 59.8 min) compared with unmodified sCT. The extent of urinary excretion of the PEG-modified sCTs was higher than for the unmodified sCT, but all these chemicals were excreted in urine in small quantities (≤0.6%). There was a tendency toward reduced accumulation of PEGylated sCTs in tissues, with its reduction being inversely proportional to the molecular size. Accumulation of the total radioactivity of the unmodified and PEG-modified sCTs was highest in the liver, followed by kidneys, lungs, spleen, heart and thyroid. When expressed per tissue gram weight, however, the highest radioactivity was found in the kidneys. PEGylated sCTs may have greater therapeutic potential *via* reduced systemic clearance and prolonged elimination half-life over unmodified sCT.

Key words salmon calcitonin; PEGylation; pharmacokinetics; tissue distribution

Salmon calcitonin (sCT) is a therapeutic polypeptide hormone consisting of 32 amino acids (3432 Da), with an N-terminal disulfide bond between the 1 and 7 positions and a C-terminal proline amide residue. It is currently marketed either as a solution for intramuscular or subcutaneous injection, or as a nasal spray in the treatment of postmenopausal osteoporosis, symptomatic Paget's disease of the bone, and hypercalcemia due to a malignancy. As with other peptide therapeutics, sCT is subject to enzymatic degradation and has a short elimination half-life (approximately 1 h) in humans.^{1,2)} The mean absolute bioavailability of sCT ranges from 11.2–23.1% after subcutaneous injection to rats.³⁾ Animal studies suggest that sCT is rapidly metabolized to smaller inactive fragments primarily in the kidneys, but also in blood and other tissues.^{4–6)} Only small amounts of unchanged sCT and inactive metabolites are excreted in the urine.⁷⁾

Chemical modification of therapeutic polypeptides with polyethylene glycol (PEG) is a technique widely used to provide functional bioconjugates with increased resistance to proteolytic degradation, increased solubility, and reduced immunogenicity.^{8–11)} Clinically available PEGylated polypeptides are, however, limited, mainly due to restricted distribution to target tissues and reduced receptor binding affinity.¹²⁾ In addition, difficulty in physicochemical characterization due to heterogeneity with respect to the distribution in the number and position of the attached PEG molecules and inherent polydispersity of PEG itself is a barrier to the development of polypeptide therapeutics.

We previously reported a chemical modification of sCT by covalent linkage with PEG, and further isolated positional isomers of PEGylated sCTs.^{13,14)} PEG may bind to sCT at ly-

sine 11, lysine 18 and N-terminus (cysteine 1) positions, yielding mono-, di-, and tri-PEGylated sCTs depending on the number of attached PEG molecules per molecule of sCT.¹⁴⁾ The formation of mono-PEG-sCT appears to be favored over that of di-PEG-sCT, while the formation of tri-PEG-sCT is minimal. PEGylated sCTs exhibit substantially improved stability in rat liver and kidney homogenates over unmodified sCT, yet they retain biological activity similar to that of unmodified sCT, as examined by the adenosine cyclic 3',5'-phosphate (cAMP) assay.^{13,14)}

The purpose of this study was to characterize the pharmacokinetic disposition of mono- and di-PEG-sCTs and their tissue distribution after i.v. injection to rats. Our results showed that the systemic clearance and the steady-state volume of distribution were significantly reduced while the AUC was increased for di-PEG-sCT, and the elimination half-life was significantly prolonged for mono-PEG-sCT compared with unmodified sCT.

Materials and Methods

Chemicals Salmon calcitonin (synthetic cyclic sCT) and Na¹²⁵I were purchased from BACHEM (Torrance, CA, U.S.A.) and Dupont NEN (Boston, MA, U.S.A.), respectively. Succinimidyl carbonate monomethoxy polyethylene glycol (SC-mPEG, M.W. 5000) was purchased from Shearwater Polymers, Inc., (Huntsville, AL, U.S.A.). Chloramine T was purchased from Shinyo Pure Chemicals Co. (Osaka, Japan), and ketamine and xylazine from Sigma Chem. Co. (St. Louis, MO, U.S.A.). HPLC grade acetonitrile and trifluoroacetic acid were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.) and Acros (Springfield, NJ, U.S.A.), respectively. Other chemicals used in the study were of analytical grade.

Preparation and Purification of SC-mPEG-Modified sCT SC-mPEG (40 μg in 0.2 ml of 0.1 M phosphate buffer, pH 8.0) was added to 0.2 ml of sCT solution (2 mg/ml in 0.1 M phosphate buffer, pH 8.0) (SC-mPEG : sCT molar ratio, 1 : 3). The mixture was shaken gently at room temperature for 20 min and the reaction was stopped by the addition of an excess amount of

* To whom correspondence should be addressed. e-mail: sdyoo@skku.ac.kr

1.0 M glycine solution. The reaction mixture was then subjected to size-exclusion chromatography on a Superose HR 12/30 column (Pharmacia LKB, Uppsala, Sweden) eluted with 10 mM phosphate buffer (pH 7.4) at a flow rate of 0.4 ml/min. In fluorescence measurements, the excitation and emission wavelengths were set at 280 and 315 nm, respectively (Hitachi F4010 spectrofluorometer, Tokyo, Japan). Fractions corresponding to mono- and di-PEGylated sCT were collected and concentrated to 100 µg/ml using Centricon-10 concentrators (Amicon, Beverly, MA) and were kept at 4 °C until use.

HPLC Conditions for sCT and PEGylated sCT The HPLC system consisted of 2 Gilson pumps (Model 307), a UV-visible detector (Model 118) and an auto-injector (Model 234) (Gilson, Villiers-le-Bel, France). The analytical column used was a LiChrosphere 100 RP-18 cartridge (4.0 × 125 mm, 5 µm) with a guard column (4.0 × 4 mm, 5 µm) (Merck, Darmstadt, Germany). A linear gradient elution was carried out at a flow rate of 1.2 ml/min at a solvent A (0.1% trifluoroacetic acid in distilled water):solvent B (0.1% trifluoroacetic acid in acetonitrile) ratio of 70:30% to 100% B over 20 min. Additional elution was allowed for 10 min with 30% B between injections. UV responses were measured at 215 nm after the injection of a portion (40 µl) of sCT and PEGylated sCT samples by using an auto-injector.

Radioiodination of sCT and PEGylated sCT Radioiodinated sCT and PEGylated sCT were prepared as described previously.¹⁵ A portion (15 µl) of a chloramine T solution (50 µmole/ml in 0.1 M phosphate buffer, pH 7.4) and 50 µl of sCT (200 µg/ml) in 0.1 M phosphate buffer (pH 7.4) were added to 0.2 mCi Na¹²⁵I (1 mCi/100 µl 0.1 M phosphate buffer, pH 7.4). After reaction for 30 s, the reaction mixture was quickly transferred to a Sephadex G-25 desalting column. The loaded mixture was eluted with 0.1 M phosphate buffer (pH 7.4), and fractions were collected in test tubes every 1 min over a 60 min period. The radioactivity of these collected samples was measured by gamma counting (Cobra™ Series Auto-Gamma Counting System, Packard Instrument Co., Groningen, the Netherlands).

HPLC Conditions for Radioiodinated sCT and PEGylated sCT The HPLC system consisted of two Waters pumps (Model 510), a 6-port switching valve, a UV visible detector (Model 440) (Milford, MA, U.S.A.) and a Ramona 2000 flow-through radioisotope detector (Raytest, Straubenhardt, Germany). The columns and elution conditions were identical to those described above. A portion (100 µl) of the radioisotope samples was injected onto the switching valve. The UV detection was carried out at 215 nm for the eluted samples to determine the concentration of PEG-modified and unmodified sCTs. The radioactivity of the effluents was determined using a flow-through radioactive detector. Data handling was performed by means of an integration computer program Winnie 2.01 (Raytest, Straubenhardt, Germany).

Animals Male Sprague Dawley rats (7–9 weeks of age, 200–250 g) were obtained from Hanlim, Inc. (Suwon, Korea). The rats were kept in plastic rat cages and were housed in an animal facility (temperature 23 ± 2 °C) with a light/dark cycle of 12/12 h and a relative humidity of 50 ± 10%. The animals were fed a standard rat diet (DaeJong Co., Seoul, Korea) and had free access to water. At least one week of acclimatization was allowed prior to experimentation.

I.v. Injection Study Rats were anesthetized with i.m. injection of ketamine and xylazine (90/10 mg/kg), and were cannulated with PE tubing (0.58 mm i.d. and 0.96 mm o.d., Natsume Co., Tokyo, Japan) in the right jugular and left femoral veins. After at least two days of recovery, the rats were administered ¹²⁵I-sCT, ¹²⁵I-mono-PEG-sCT or ¹²⁵I-di-PEG-sCT by i.v. bolus injection (5 µg) into the femoral venous catheter (injection volume 0.5 ml) in three groups of rats (*n* = 4 each). The animals were unrestrained and conscious and kept in metabolic cages during the dosing and sampling period. The average specific radioactivity of administered ¹²⁵I-sCT, ¹²⁵I-mono-PEG-sCT and ¹²⁵I-di-PEG-sCT was in the range of approximately 6–8 × 10⁶ cpm. Serial blood samples (approximately 0.3 ml each) were withdrawn from the jugular vein at 0, 2, 5, 10, 15, 30 and 45 min, and at 1, 1.5, 2, 4 and 6 h into Eppendorf tubes which were then placed in an ice bath. Equal volumes of isotonic saline were replaced after each sampling. Serum samples were harvested immediately by centrifugation at 10000 rpm for 10 min. A portion (200 µl) of 20% TCA solution was added to a 100 µl of serum samples. The mixture was mixed on a vortex mixer for 10 s and centrifuged at 10000 rpm for 10 min. The radioactivity of the resulting supernatants and precipitates was measured by gamma counting. In addition, the rats were sacrificed at 6 h after i.v. injection, and the liver, kidneys, thyroid, spleen, heart, lungs and muscle were excised, blot dried and weighed. The radioactivity was determined for these tissue samples by gamma counting. Urine samples were also collected for 6 h in metabolic cages. A portion (200 µl) of the 20% TCA solution was added to the urine samples, mixed on a vortex

mixer and centrifuged at 10000 rpm for 10 min. The radioactivity of the resulting supernatants and precipitates was then measured by gamma counting.

Data Analysis Serum concentration vs. time data obtained after i.v. administration were analyzed by fitting a bi-exponential equation to these time profiles using the nonlinear least squares regression program WinNonlin (Scientific Consulting, Inc., Cary, NC, U.S.A.). Area under the concentration vs. time curve (*AUC*) and the area under the first moment of the concentration vs. time curve (*AUMC*) were determined from the coefficients and exponents of these fitted relationships.¹⁶ Pharmacokinetic parameters were calculated from the following equations: systemic clearance (*Cl*) = dose/*AUC*, volume of distribution at steady-state (*V_{ss}*) = dose · *AUMC*/*AUC*², distribution half-life (*t_{1/2,λ₁}*) = 0.693/λ₁, and elimination half-life (*t_{1/2,λ₂}*) = 0.693/λ₂. Pharmacokinetic parameter values were expressed as the mean ± S.D.

Results

Conjugation of sCT with SC-mPEG (M.W. 5000) produced a heterogeneous mixture of PEG-modified and unmodified sCT species. The mixture was subjected to size-exclusion chromatography, and unmodified sCT, mono-PEG-sCT and di-PEG-sCT were separated from each other. Their retention times were 44.5, 36.8 and 33.4 min, respectively. Tri-PEG-sCT was eluted as a minor shoulder peak. The percentage of the peak area counts was 11.9% for sCT, 42.9% for mono-PEG-sCT and 33.4% for di-PEG-sCT. HPLC analysis of the radioiodinated sCT, mono-sCT and di-sCT prepared by the chloramine-T method showed that the free to labeled ¹²⁵I ratio was 21.5 ± 11.5% for sCT, 85.5 ± 2.5% for mono-PEG-sCT and 66.4 ± 8.6% for di-PEG-sCT.

Figure 1 shows average decay curves of the total radioactivity and the radioactivities corresponding to intact and degradation species after i.v. injection of sCT, mono-PEG-sCT and di-PEG-sCT to rats (*n* = 4 each). The average serum concentration vs. time curves of the unmodified sCT, mono-PEG-sCT and di-PEG-sCT are shown in Fig. 2. Serum levels of the unmodified and PEGylated sCTs declined bi-exponentially, with the mean initial distribution half-lives ranging from 3.6–6.2 min and the terminal elimination half-lives ranging from 59.8–189.1 min (Table 1). The systemic clearance of mono-PEG-sCT was not significantly altered over unmodified sCT, although it was reduced (5.1 vs. 11.1 ml/min/kg). Also, the steady-state volume of distribution (821.8 vs. 603.1 ml/kg) and *AUC* (5670 vs. 3288 ng·min/ml) of mono-PEG-sCT were not significantly altered over unmodified sCT. In contrast, di-PEG-sCT showed significantly reduced systemic clearance (2.3 ml/min/kg) and steady-state volume of distribution (229.9 ml/kg), and increased *AUC* (11008 ng·min/ml) over unmodified and mono-PEGylated sCT. The extent of urinary excretion of intact and degradation species after i.v. injection of unmodified and PEG-modified sCTs is shown in Fig. 3. PEG-modified and unmodified sCTs were excreted intact in urine in small quantities (0.1–0.6%). The urinary excretion of degradation products was also low (range 1.5–2.5%). The total radioactivity found in various tissues is shown in Table 2. The highest radioactivity was found in the liver, followed by the kidneys, lungs, spleen, heart and thyroid for unmodified and PEG-modified sCTs. Figure 4 shows the total radioactivity in various tissues normalized by gram tissue weight. The weight-normalized total radioactivity was highest in the kidneys for both unmodified and PEG-modified sCTs. Nevertheless, the accumulation of mono- and di-PEG-sCTs in the kidneys was significantly reduced over unmodified sCT. The accumulation of di-PEG-sCT was also significantly reduced in the liver and

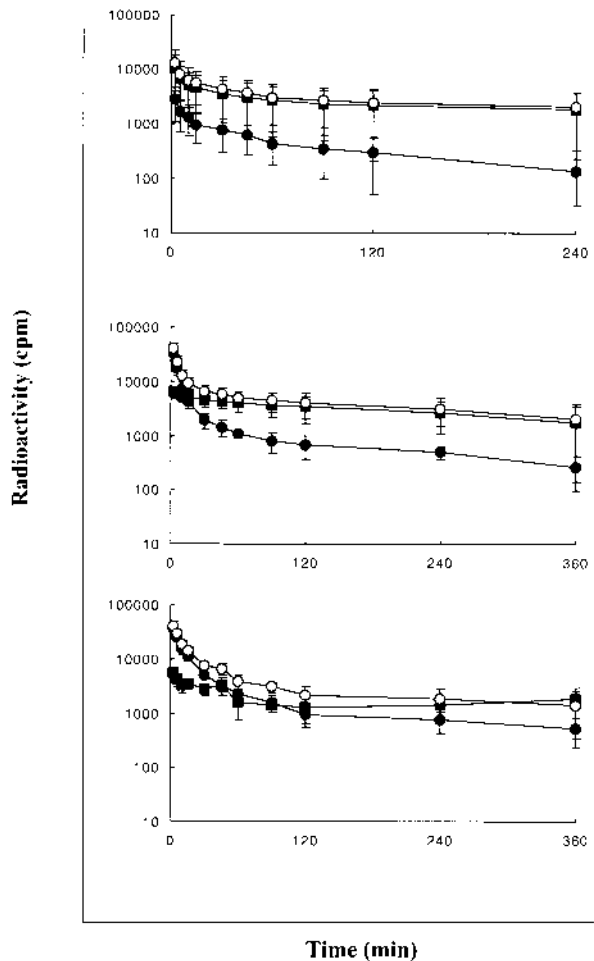


Fig. 1. Average Decay Curves of the Total Radioactivity (○) and the Radioactivities Corresponding to the Intact (●) and Degradation (■) Species Found in the Serum after I.v. Injection of sCT (Top Panel), Mono-PEG-sCT (Middle Panel) and Di-PEG-sCT (Bottom Panel) to Rats
n=4 each.

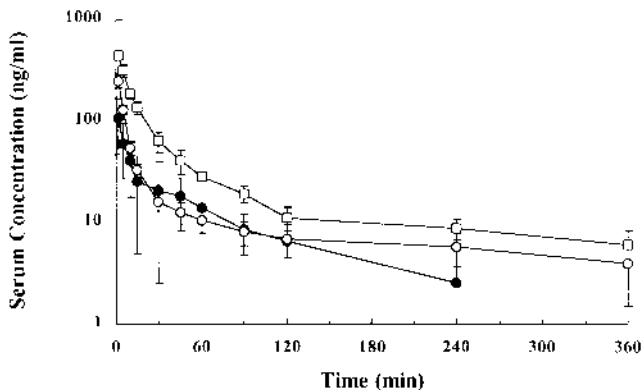


Fig. 2. Average Serum Concentration vs. Time Curves of sCT (●), Mono-PEG-sCT (○) and Di-PEG-sCT (□) in Rats after Their Respective I.v. Injections
n=4 each.

spleen.

Discussion

To our knowledge, this study first examined the pharmaco-

Table 1. Pharmacokinetic Parameters (Mean±S.D.) of sCT, Mono-PEG-sCT and Di-PEG-sCT Obtained after Their Respective I.v. Injections (5 μg) in the Rat (n=4 Each)

Parameter	sCT	Mono-PEG-sCT	Di-PEG-sCT
Weight (g)	190±6	193±5	190±8
$t_{1/2,\lambda_1}$ (min)	3.6±3.0	3.3±0.7	6.2±0.9
$t_{1/2,\lambda_2}$ (min)	59.8±45.2	189.1±119.6 ^{a)}	107.2±32.8
AUC (ng·min/ml)	3288±1553	5670±2183	11008±224 ^{b)}
Cl (ml/min/kg)	11.1±9.0	5.1±1.8	2.3±0.2 ^{b)}
V_{ss} (ml/kg)	603.1±332.3	821.8±324.3	229.9±68.3 ^{b)}

a) $p < 0.05$ over unmodified sCT. b) $p < 0.05$ over unmodified and mono-PEGylated sCT.

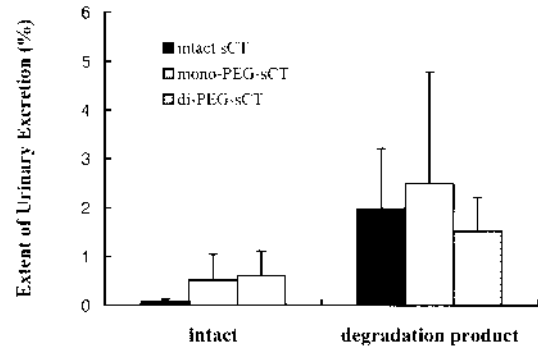


Fig. 3. Extent of Urinary Excretion (%) of the Unmodified sCT, Mono-PEG-sCT and Di-PEG-sCT, as well as Their Degradation Products Determined after I.v. Injection to Rats
n=4 each.

Table 2. The Total Radioactivity (Mean±S.D.) Found in Various Organs Determined at 6 h after I.v. Injection of sCT, Mono-PEG-sCT and Di-PEG-sCT in the Rat (n=4 Each)

Tissue	Radioactivity (cpm) found in whole organ		
	sCT	Mono-PEG-sCT	Di-PEG-sCT
Liver	99776±34685	73271±57572	46342±13377 ^{b)}
Kidney	67657±31261	44376±22806 ^{a)}	21704±3441 ^{a)}
Lung	12503±10764	10745±6357	9071±2728
Spleen	6985±4507	7846±3566	4875±2418
Heart	6829±6281	6935±2472	4265±808
Thyroid	1284±1303	3115±3195	3145±458

a) $p < 0.05$ over unmodified sCT. b) $p < 0.05$ over unmodified and mono-PEGylated sCT.

kinetics of PEGylated sCT based on the number of attached PEG molecules. sCT contains three potential sites for PEGylation at the primary amine moiety of the N-terminus (cysteine 1) and two lysine residues (lysine 11 and 18). Mono-PEG-sCT is, therefore, a mixture consisting of the N-terminus-, lysine 11- and lysine 18-modified sCTs, whereas di-PEG-sCT is a mixture consisting of the N-terminus- and lysine 11-, N-terminus- and lysine 18-, and lysine 11- and lysine 18-modified sCTs. Tri-PEG-sCT is sCT PEGylated at all three available sites, and is formed as a minor product. The administered dose of sCT was within a linear i.v. dose range (1–10 μg) reported previously in rats.³⁾ Consistent with previous findings,^{3,17)} serum levels of unmodified sCT declined bi-exponentially. Also, serum levels of mono- and di-PEGylated sCTs declined bi-exponentially. The pharmacokinetic parameters of unmodified sCT determined in this study (ter-

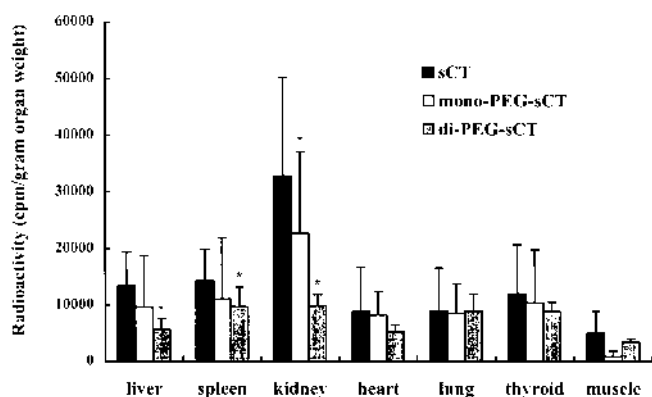


Fig. 4. Total Radioactivity in Tissues (cpm per Gram Organ Weight) Determined at 6 h after I.v. Injection of sCT, Mono-PEG-sCT and Di-PEG-sCT to Rats

$n=4$ each.

minimal elimination half-life of 59.8 min, systemic clearance of 11.1 ml/min/kg, and steady-state volume of distribution of 603.1 ml/kg) are comparable to those previously reported in rats.³⁾ Attachment of one PEG molecule to sCT did not significantly alter the systemic clearance or steady-state volume of distribution. On the other hand, attachment of two PEG molecules to sCT resulted in significantly reduced systemic clearance and steady-state volume of distribution. The reduced systemic clearance of di-PEG-sCT is consistent with its improved stability in rat kidney homogenates over that of unmodified sCT.¹³⁾ The elimination half-life of mono-PEG-sCT was prolonged as the systemic clearance was lowered and the volume of distribution was greater compared to those of unmodified sCT. However, the elimination half-life of di-PEG-sCT was unaltered as its steady-state volume of distribution and systemic clearance were simultaneously reduced. These observations are somewhat different from those of other PEGylated peptides, *e.g.*, recombinant methioninase,¹⁸⁾ tumor necrosis factor-alpha,¹⁹⁾ recombinant human granulocyte colony-stimulating factor,²⁰⁾ ribonuclease,²¹⁾ recombinant human interferon gamma,²²⁾ and recombinant human interleukin-2,²³⁾ in that their systemic clearances were reduced and elimination half-lives were increased simultaneously. Hence, the distribution volume of these peptides appears to be minimally affected by PEG modification.²³⁾ The molecular masses of these latter peptides are relatively large (>14 kDa) compared to that of sCT (3432 Da). The molecular masses of mono- and di-PEG-sCT were increased to approximately 8410 and 13230 Da, respectively, as measured by MALDI-TOF mass spectrometry. Whether a molecular size threshold exists beyond which the tissue distribution of PEG-modified peptides is minimally affected deserves further examination.

The kidney is known to be a major biotransformation site of sCT, accounting for two-thirds of its metabolism.^{24,25)} The removal of calcitonin from renal circulation appears to occur primarily by a glomerular filtration-independent pathway, with the urinary excretion being minimal.²⁵⁾ In our study, the urinary excretion of unmodified and PEG-modified sCTs was also low. Nevertheless, their total radioactivities were highest in the kidney when expressed per gram weight. When compared with unmodified sCT, mono- and di-PEGylated sCTs were accumulated in the kidney to a significantly lesser ex-

tent (Table 2, Fig. 4). Similarly, the accumulation of di-PEGylated sCT into the liver and spleen was significantly reduced. These reductions occurred despite the serum levels of PEGylated sCTs being higher than those of unmodified sCT at the time the rats were sacrificed (Fig 1). Therefore, PEG modification appears to have altered the tissue distribution characteristics of sCT depending on the degree of increase in the molecular mass.

In conclusion, PEGylated sCT showed altered pharmacokinetics depending on the number of attached PEG molecules. Given the similar biological activity of mono- and di-PEG-sCTs to that of unmodified sCT,¹³⁾ PEGylated sCTs may have greater therapeutic potential *via* prolonged elimination half-life and reduced systemic clearance.

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