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Journal of Chromatography B, 775 (2002) 247–255

JOURNAL OF CHROMATOGRAPHY B

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Simple liquid chromatography–electrospray ionization mass spectrometry method for the routine determination of salmon calcitonin in serum

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Received 5 February 2002; received in revised form 16 April 2002; accepted 13 May 2002

Abstract

A simple liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method was developed for the quantification of salmon calcitonin (sCT) in serum. Serum samples from rats and dogs were deproteinized and freeze-dried. The residue was then reconstituted with 57% acetonitrile in water containing 0.1% trifluoroacetic acid and 0.005% benzalkonium chloride. A 20-μl aliquot of the reconstituted solution was injected onto a polymer based RP-C_{18} column, The outlet was connected to an ion-trap mass spectrometer equipped with an ESI source, and spectra were recorded in a positive-ion, selected-ion monitoring mode. The limit of quantification of the method was 10 ng/ml. Biexponential curves were observed for the temporal serum concentration of sCT following intravenous administration of sCT to rats (100 μg/kg) and dogs (250 μg/kg), resulting in reasonable pharmacokinetic parameters. The present method appears applicable to routine analysis of serum sCT in pharmacokinetic studies with good selectivity, accuracy and precision.

Keywords: Pharmacokinetics; Calcitonin

1. Introduction

Salmon calcitonin (sCT) is a polypeptide hormone composed of 32 amino acids with a molecular mass of about 3400. It is one of the most therapeutically effective peptides available for the treatment of postmenopausal osteoporosis, Paget's disease and hypercalcemia [1,2]. Various analytical methods for the quantitative determination of sCT, which employ a variety of techniques including high-performance liquid chromatography with ultraviolet detectors (HPLC–UV) [3,4], radioimmunoassay (RIA) [5,6], enzyme immunoassay [7], immunoradiometric assay [8] or immunofluorometric assay [9] are currently available. However, none of these can be used for the practical quantification of intact sCT in biological fluids. For example, HPLC–UV [3,4], a common tool for the separation of polypeptides [10,11], is not

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00316-1

suitable for the microanalysis of sCT due to its low UV absorption. RIA [5,6], the most common method to date for the quantification of sCT, lacks sufficient selectivity and precision, although it exhibits the highest sensitivity among the reported methods for the quantification of sCT. The reason for is that some degradation products from calcitonin (CT) or endogenous hormones in biological fluids may crossreact with reagents used in this method [12–15]. Moreover, the RIA requires ^{125}iodine-labeling, which is time-consuming (i.e. 3 days are generally required). Immunoassay methods [7–9] other than the RIA have similar disadvantages in terms of selectivity. Therefore, developments of alternate methods for quantifying intact sCT more rapidly are needed. A liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method is described here. The method involves deproteinization of serum samples, freeze-drying and reconstitution with 57% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 0.005% benzalkonium chloride (BC), followed by chromatographic separation using LC and detection by ESI-MS.

2. Experimental

2.1. Materials

Synthetic sCT (M_r 3431.9; purity >99%) was purchased from Hyundai Pharm. (Bucheon, Korea) and was stored in a deep freezer (−70 °C). HPLC grade acetonitrile was obtained from Fisher (Fair Lawn, NJ, USA). Analytical grade trifluoroacetic acid (TFA) and benzalkonium chloride (BC), ultra grade, were purchased from Sigma (St. Louis, MO, USA). HPLC grade water was doubly purified with a Milli-Q (Millipore, Molsheim, France) system and cellulose nitrate membrane filters (47 mm, 0.2 μm, Whatman, Maidstone, UK). All other reagents were of the highest grade commercially available.

2.2. Intravenous administration of sCT to rats and dogs

Six Sprague–Dawley male rats (270–280 g) and four male beagle dogs (10–11 kg) were used in the study. The animals were fasted overnight prior to/and during the study but had free access to water. Prior to the study, the animals were anesthetized by an intra-peritoneal injection of ketamine (Yuhan, Seoul, Korea) at a dose of 500 μl/kg.

The right vena cava of an anesthetized rat was exposed and cannulated with a polyethylene tubing (PE50, O.D. 0.965 mm, Becton Dickinson, Sparks, MD, USA). A solution of sCT in normal saline was administered intravenously through a catheter at a dose of 100 μg/kg body weight followed by a rinse of the catheter with 0.3 ml heparinized saline. At 5 min before the administration and at 2, 5, 10, 20, 30, 40 and 60 min after administration, 350 μl blood samples were removed through the catheter into polypropylene tubes that were kept at 4 °C (crushed ice).

In the study using dogs, the external jugular vein of the dog was cannulated with a two-lumen central venous catheter (Arrow, USA), and sCT was administered in a similar manner at a dose of 250 μg/kg. At 5 min before the administration and at 1, 3, 6, 10, 15, 20, 30, 45 and 60 min after the administration, 500 μl blood samples were removed through the catheter into polypropylene tubes that were kept at 4 °C (in crushed ice). The blood samples from rats and dogs were centrifuged immediately and the separated serum samples were kept at −70 °C until used for sCT analysis.

2.3. Preparation of standard solutions and serum samples

A stock solution of sCT was prepared by dissolving sCT in a mixed solvent of 57% acetonitrile and 0.1% TFA in water, which was then kept in a deep freezer (−70 °C). Aliquots of a stock solution containing specified amounts of sCT were placed in polypropylene test tubes and dried using a freeze-dryer (IlShin, Seoul, Korea) at −70 °C and 1.2 Pa. Polypropylene test tubes were used in order to minimize possible adsorption of sCT. A 150-μl volume of the blank serum from a rat or dog was added to each test tube followed by vortexing for 30 s to yield sCT concentrations of 5, 10, 20, 50, 100 and 200 ng/ml. Deproteinization was then performed by the addition of 300 μl of acetonitrile to each tube, followed by vortexing for 30 s and centrifuging at 1000 *g* for 20 min. The supernatant was freeze-dried,

and the residue reconstituted with 150 μl of a reconstitution solvent [i.e. 57% (v/v) acetonitrile, 0.1% (v/v) TFA and 0.005% (w/v) BC in water]. Serum samples from the i.v. administration study were deproteinized, freeze-dried and reconstituted in the same way (Scheme 1).

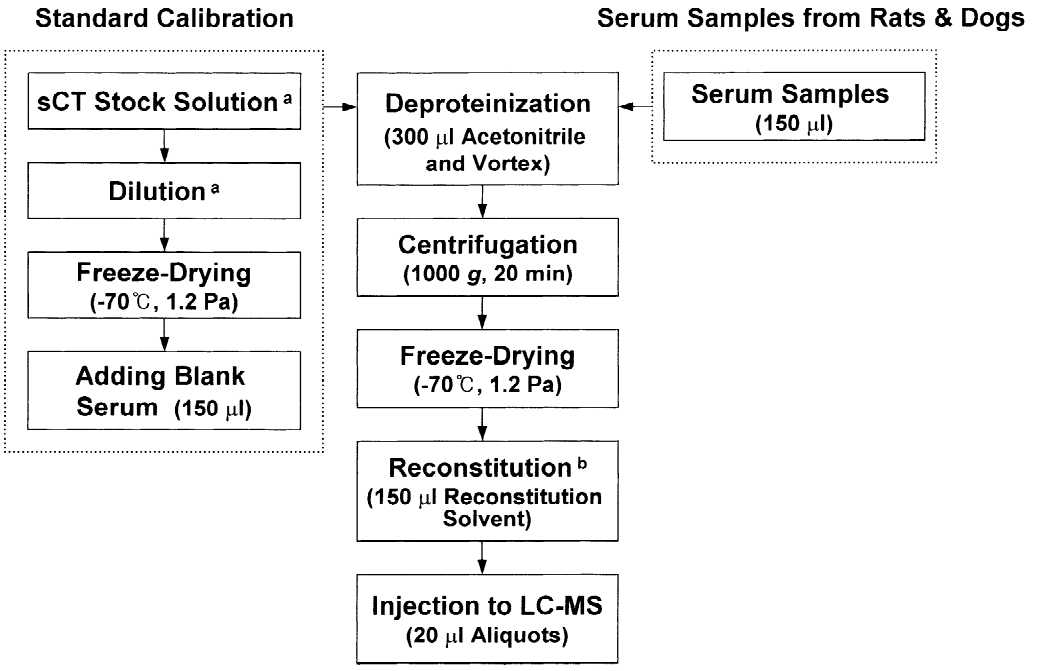
2.4. Column liquid chromatography

The HPLC system consisted of a binary pump, an autosampler equipped with a 100-μl loop allowing injections of various sample volumes, a diode array detection system (HP1100 series, Hewlett-Packard, Avondale, CA, USA) and an HP CHEMSTATION software for LC (Hewlett-Packard). Aliquots (20-μl) of the reconstituted solutions were injected onto the LC column. Separation of the sCT was performed on a C_{18} reversed-phase polymer column (2.1×150 mm I.D., 5 μm particle size, 300 Å pore diameter, Vydac, Hesperia, CA, USA) at ambient temperature under the flow (0.2 ml/min) of a mobile phase with gradient elution. The polymer-based column was used in order to minimize possible adsorption of sCT. The eluents were composed of acetonitrile containing 0.1% TFA (Eluent A) and water containing 0.1% TFA (Eluent B). The gradient program is listed in Table 1.

Table 1
Mobile phase composition over time

Time (min)	Eluent A[a] (%)	Eluent B[b] (%)
0	27	73
10	57	43
14	27	73
20	27	73

[a] Eluent A was composed of acetonitrile containing 0.1% TFA.
[b] Eluent B was composed of water containing 0.1% TFA.



[a] In preparing and diluting the stock solution, an aqueous solution containing 57% acetonitrile and 0.1% trifluoroacetic acid (TFA) was used.
[b] Reconstitution solvents were composed of an aqueous solution containing 57% acetonitrile, 0.1% TFA and 0.005% benzalkonium chloride.

Scheme 1. Preparation of standard calibration curves and assay of sCT in serum samples.

2.5. Mass spectrometry

The outlet from the LC column was connected directly to the mass analyzer. Mass spectra were recorded on a LCQ ion-trap mass spectrometer system (Finnigan MAT, San Jose, CA, USA) equipped with an ESI source. The mass spectrometer was calibrated in the positive ion mode using a mixture of caffeine, MRFA and Ultramark 1621 (Sigma). Nitrogen (>99.999%) was used as the sheath and auxiliary gas and helium (>99.999%) served as the damping gas. The MS instrument was operated in a positive-ion, selected-ion monitoring (SIM) mode at m/z 1144.9 with XCALIBUR software (Finnigan MAT) under the following MS tuning conditions: capillary temperature of 225 °C; sheath gas flow of 70 arbitrary units; auxiliary gas flow of ten arbitrary units; source voltage of +4.5 kV; capillary voltage of +32 V; inter-Octapole lens voltage of −44 V. The area of the mass peak of sCT in SIM chromatograms was plotted against the concentration of sCT in standard solutions, and a calibration curve was then obtained from the linear least-square regression analysis of the plot.

2.6. Assay validation

The stability of sCT during the assay was assessed by measuring the peak area of sCT using the presently established assay method. Inter-day variation was then assessed by analyzing three or four sets of standard sCT solutions on 3 successive days. The intra-day variation was evaluated from data generated from the determinations of three batches of the standard solutions in 1 day. The precision of the assay was calculated from the percent coefficient of variance of the mean. The accuracy of the assay was calculated as relative errors of the mean between the expected (i.e. theoretical concentration) and calculated concentrations (i.e. obtained from the standard calibration curves).

2.7. Pharmacokinetic analysis

Noncompartmental pharmacokinetic parameters following i.v. administration of sCT, such as the area under the serum concentration versus time curve from time 0 to time infinity (AUC), total clearance (CL), serum half-life ($t_{1/2}$), volume of distribution (V_{ss}) and mean residence time (MRT) of sCT, were calculated according to standard methods using serum sCT concentration–time data. For example, the AUC_{0-60} was calculated by the linear trapezoidal method using data from time 0 to 60 min, and the AUC was then estimated based on the relationship, $AUC = AUC_{0-60} + C_{60}/\beta$, where C_{60} is the serum concentration of sCT at 60 min after the administration of sCT, and β is the rate constant of the terminal disposition phase. The β value was obtained by fitting the i.v. serum concentration data using WINNONLIN® software (Version 3.1, Pharsight Cary, NC, USA). The results are expressed as the mean±SD (standard deviation) of six (in the rat study) and four (in the case of the dog study) experiments.

3. Results and discussion

3.1. Full scan spectra and SIM chromatograms of sCT

Intense multicharged ions with the triply protonated molecule $[M+3H]^{3+}$ and doubly protonated molecule $[M+2H]^{2+}$ appeared in the full scan spectra under the positive ESI at m/z 1144.9 and 1716.8, respectively, corresponding precisely to the molecular mass of sCT (Fig. 1). Under the described conditions, the sCT peak had a retention time of 10.8 min in the SIM mode chromatograms at m/z 1144.9, and no interference by the blank serum or reconstitution solvent was observed (Fig. 2).

3.2. Effect of reconstitution solvent on the mass peak of sCT

The effect of the reconstitution solvent used in the preparation of samples for LC injection, on the peak area of sCT was examined (Fig. 3). sCT is reported to be more stable in solvents of acidic pH compared to neutral or alkaline pH [16]. Consistent with this report, a higher peak area was obtained for the reconstitution solution of pH 3.4 (Fig. 3C) compared to pH 7.4 (Fig. 3D). More stable and higher peaks could be obtained when a mixed solvent of 57% acetonitrile and 0.1% TFA in water was used (Fig.

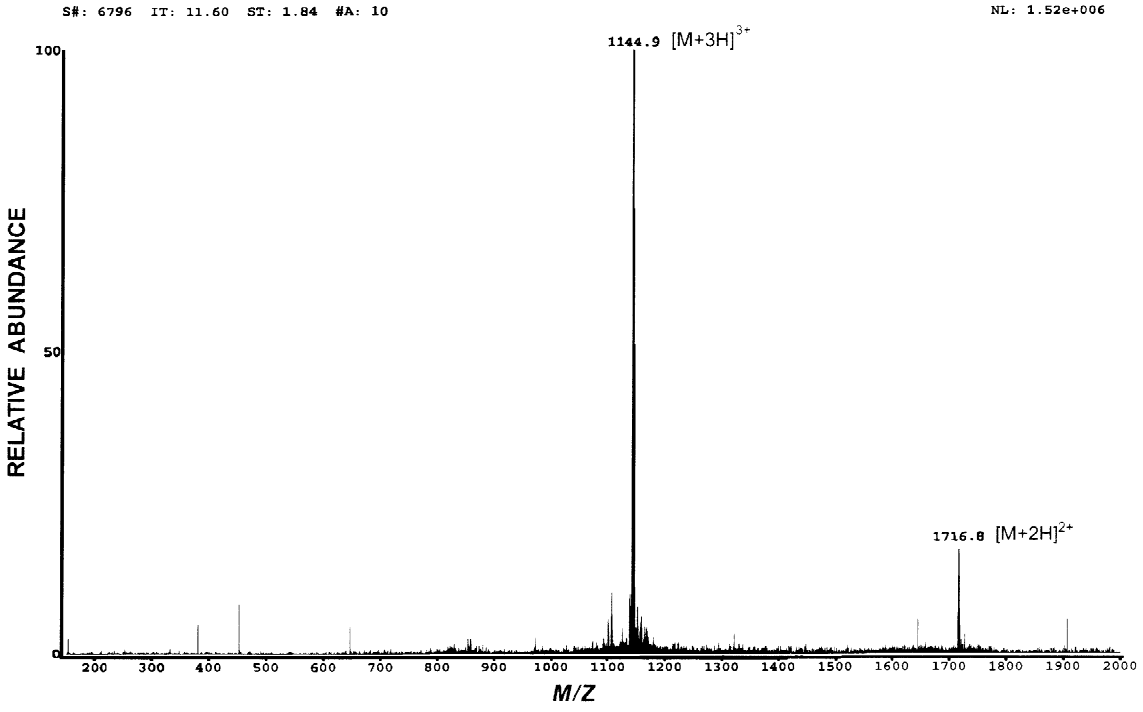


Fig. 1. Full scan spectra of sCT under the positive ESI mode.

3B). In general, excipients such as Tween 80, Cremophor EL, Triton X-100, sodium lauryl sulfate, benzetonium chloride and BC are included in formulations for peptides and proteins. Therefore, the effect of these excipients, at the concentration of 0.005%, on the peak area of sCT was examined. Most interestingly, the largest peak area with the best stability was obtained when BC was added to the mixed solvent (Fig. 3A). The effect of BC increased with increasing concentration, reaching a maximum at 0.005% (Fig. 4). This effect was not observed for other excipients examined, indicating that such an effect is specific to BC. The mechanism of increased peak area by BC was not pursued in the present study. However, it does not appear to be associated with surface activation, since such an increase was not observed for the other excipients, which are typical surface active agents. At higher concentrations of BC, however, the MS capillary was often blocked on repeated running, consistent with the decline of the peak area at BC concentrations higher than 0.005% (Fig. 4). Because of this problem, BC was not added to the mobile phase. In summary, based on the above observations, the composition of the reconstitution solvent was set at 57% acetonitrile, 0.1% TFA and 0.005% BC in water.

3.3. Validation of the assay

sCT is well known to be unstable in aqueous solvents at ambient temperature. However, it was fairly stable in the stock solution (Scheme 1) at $-70\,^{\circ}C$ based on the fact that the peak area of sCT obtained was unchanged for 1 month under the given condition by the present method. In addition, sCT in freeze-dried serum samples and in the reconstituted solution (Scheme 1) was stable at $-70\,^{\circ}C$ and ambient temperature at least for 1 month and up to 2 h, respectively. These observations indicate that sCT is stable enough during the assay procedure in the present protocol (Scheme 1).

Calibration curves of sCT in serum solutions exhibited a good linearity with squared correlation coefficients (r^2) of >0.9999 for the concentration range of 10–200 ng/ml ($y = 126473x + 20866$ for the rat study and $y = 155229x + 108607$ for the dog

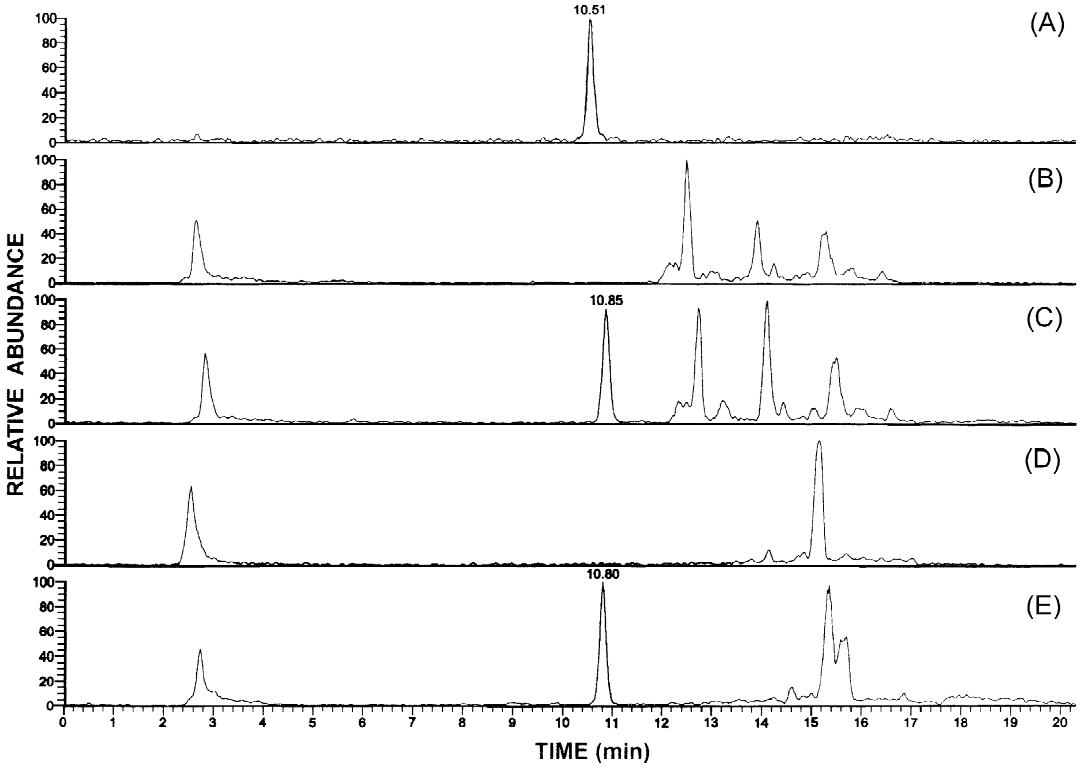


Fig. 2. SIM chromatograms at m/z 1144.9 for various samples. (A) sCT (100 ng/ml), (B) blank rat serum, (C) rat serum spiked with sCT (100 ng/ml), (D) blank dog serum, (E) dog serum spiked with sCT (100 ng/ml). All samples were prepared by reconstituting with a mixed solvent of 57% acetonitrile, 0.1% TFA and 0.005% BC in water.

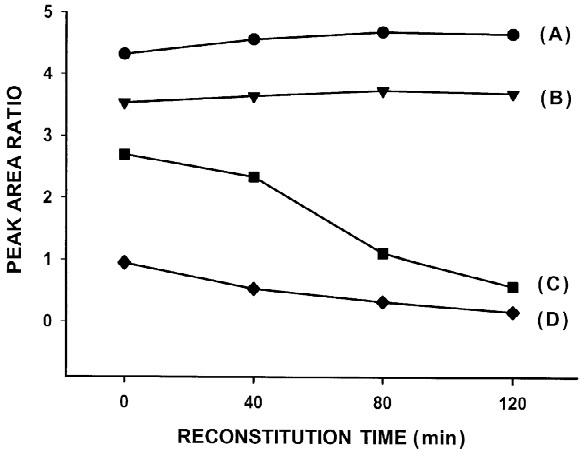


Fig. 3. Effect of various solvents used in the reconstitution process on the peak area of sCT (50 ng/ml) and its stability under the given assay conditions. (A) mixed solvent of 57% acetonitrile, 0.1% TFA and 0.005% BC in water, (B) mixed solvent of 57% acetonitrile and 0.1% TFA in water, (C) water (pH 3.4), (D) water (pH 7.4).

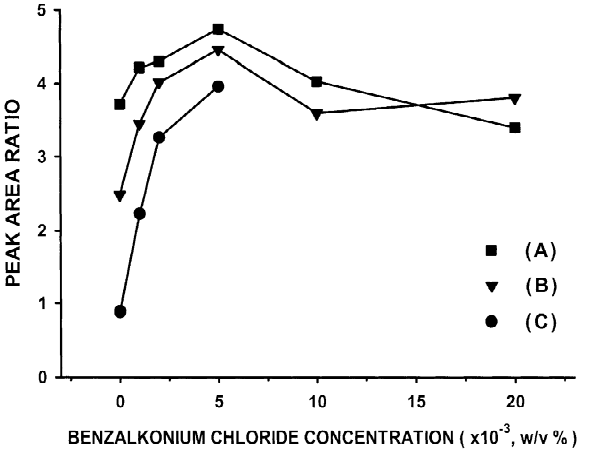


Fig. 4. Effect of benzalkonium chloride in the reconstitution solvents on the relative peak area of sCT. (A) mixed solvent of 57% acetonitrile and 0.1% TFA in water, (B) water (pH 3.4), (C) water (pH 7.4).

Table 2
Precision and accuracy of the sCT assay in rat and dog serum samples

Concentration (ng/ml)	Rat serum				Dog serum			
	Intra-day ($n=4$)		Inter-day ($n=3$)		Intra-day ($n=3$)		Inter-day ($n=4$)	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
5	21.8	22.3	17.6	25.7	17.0	33.1	19.4	24.5
10	6.3	10.9	12.8	10.2	7.0	12.8	5.7	5.0
20	12.3	6.5	4.9	8.1	2.2	2.2	4.4	2.1
50	5.7	2.8	2.9	4.8	9.3	5.4	6.3	5.2
100	2.0	4.1	3.9	6.2	5.9	4.1	7.5	3.3
200	7.6	0.7	8.0	1.0	10.0	1.1	5.3	0.7

study). Validation of the assay method was performed according to the standard method, and the results are listed in Table 2. No significant differences were observed in the lower limit of detection (LOD), precision, accuracy and limit of quantification (LOQ) between the serum of rats and dogs. The LOD was 5 ng/ml based on a signal-to-noise ratio of 3. For sCT concentrations of over 10 ng/ml, the intra- and inter-day variations of the precision were less than 12.3 and 12.8%, respectively, while those for accuracy were less than 12.8 and 10.2%, respectively, regardless of animal species. However, for a concentration of 5 ng/ml, some of the intra- and inter-day variations in the precision and accuracy exceeded the generally accepted limit (20%). Thus, 10 ng/ml was assigned as the LOQ of the present method.

3.4. Comparison with previous assay methods in studying pharmacokinetics of sCT in vivo

3.4.1. Radioactivity and RIA methods

The applicability of the presently developed method to routine pharmacokinetic study was examined by measuring temporal serum sCT concentrations following an i.v. administration of sCT to rats (100 μg/kg) and dogs (250 μg/kg). The temporal profiles of serum sCT are shown in Fig. 5 for rats and dogs. A biexponential decline was observed for the profiles. The relevant pharmacokinetic parameters for the profiles are listed in Table 3. Pharmacokinetic parameters for sCT obtained by a radioactivity method [17] and RIA methods [18–20] are also listed in Table 3, to serve as a comparison of the various assay methods available. The parameters for

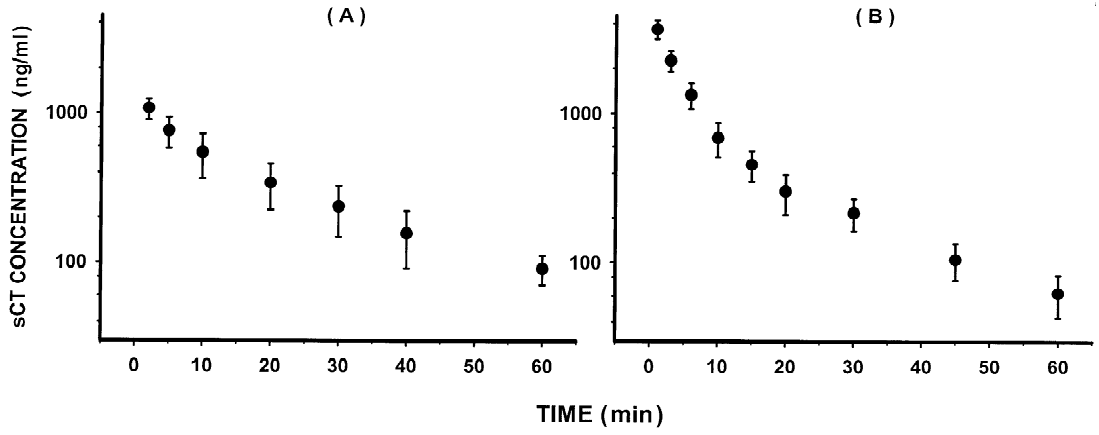


Fig. 5. Temporal profiles of mean (±SD) serum concentrations of sCT following i.v. administration of sCT to rats (100 μg/kg, $n=6$) and dogs (250 μg/kg, $n=4$).

Table 3
Pharmacokinetic parameters (mean±SD or range) for sCT following i.v. administration by various assay methods

Assay	Present method (LC–ESI-MS)		Radioactivity detection	RIA		
	Sprague–Dawley Male rat ($n=6$)	Beagle dog ($n=4$)	Sprague–Dawley male rat	Wistar female rat	Sabra male rat	Beagle dog
Dose (μg/kg)	100	250	20[a]	5–50 [a]	0.16	7.4–74
$t_{1/2}$ (min)	20.1±3.3	16.9±3.1	59.8±45.2	32.0–47.6	102±14	37.6–57.3
CL (ml/min/kg)	4.6±1.2	7.8±0.7	11.1±9.0	11.2–14.7	0.35±0.05[a]	12.6–13.6
V_{ss} (ml/kg)	116.2±27.1	115.3±16.7	603.1±332.3	650–750	56.0±3.5[a]	336.9–372.2
MRT (min)	25.4±1.6	14.8±2.1	NS[b]	46.2–68.7	147±20	25.4–29.3
Reference			[17]	[18]	[19]	[20]

[a] Recalculated assuming a 200 g body weight.
[b] NS, data not shown in the literature.

sCT varied depending on the assay methods employed. For example, longer $t_{1/2}$ [17–20], larger [17,18,20] or smaller [19] CL and V_{ss}, and larger MRT values [18–20], compared to the present method, were reported for the previous methods. The discrepancy might be associated with the poor selectivity of the previous methods [17–20] compared to the present method. The reported methods are likely to overestimate sCT because metabolites or immunoreactive peptides derived from CT or endogenous hormones may be cross-reactive in these methods. On the contrary, only intact sCT is detected in the present LC–ESI-MS method. Thus, the variation among the reported assay methods, and the discrepancy between the reported methods and the present method, in terms of the pharmacokinetic parameters of sCT, appears to support the validity of the present assay method. Therefore, despite the comparable or slightly lower sensitivity of the present method (10 ng/ml) compared to previous methods [i.e. ~0.1 ng/ml, 18–20], the method here appears to represent the only assay method capable of measuring intact sCT in serum.

3.4.2. LC–MS methods

Several reports have utilized LC–MS techniques in the analysis of degradation products of sCT in commercial preparations [21], in vitro metabolites of human calcitonin (hCT) in nasal mucosa [22] and in vitro metabolites of sCT and hCT in the liver and kidney [23]. For the assay of intact sCT and hCT, only Kobayashi et al. [23,24] reported a micro LC–ESI-MS method that is capable of quantitating CTs spiked in rat plasma at ng/ml levels. However, their method [23,24] appears to lack applicability to the routine analysis of sCT, because an immobilized antibody column, which is commercially unavailable, and rat calcitonin, are required in the treatment of serum samples and as an internal standard, respectively. Probably due to that reason, no report that applies their method to routine pharmacokinetic studies has appeared to date.

4. Conclusion

LC–ESI-MS is proposed as a simple method to quantify intact sCT in serum samples at the 10 ng/ml level. The method involves the deproteinization of serum samples, freeze-drying, reconstitution with a mixed solvent, injection of the reconstituted solution on to a polymer based LC column, and ESI mass spectrometry. The use of 57% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and 0.005% benzalkonium chloride (BC) as the reconstitution solvent was critical for obtaining maximum sensitivity and stability of sCT. Polypropylene tubes, vials and a polymer based LC column are used to minimize the adsorption of sCT during the analysis. The present LC–ESI-MS method appears to be applicable to the routine analysis of serum sCT in pharmacokinetic studies with favorable selectivity, accuracy and precision.

Acknowledgements

This study was supported by a grant from the Critical National Technology Program, Ministry of Science and Technology (M10115000003-01A130000311), Republic of Korea.

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