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Combined high-performance liquid chromatography and radioimmunoassay for ceruletide and its metabolites in dog plasma and urine

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Abstract: A combined high-performance liquid chromatographic (HPLC) and competitive radioimmunoassay (RIA) method for ceruletide (CLT), an analogue of cholecystokinin-8, was developed to investigate the behaviour of CLT in dogs. Dog plasma samples after administration of CLT were deproteinized and separated by reversed-phase HPLC. Fractions for the HPLC eluate were measured by a RIA and two immunoreactive components were found in dog plasma. One fraction was assumed to be unchanged CLT and the other was the (1–6) fragment peptide of CLT [CLT(1–6)] in accordance with the retention times. For the simultaneous determination by the combined method in dog plasma, the assay recoveries of both compounds were 100% and the values for assay precision (RSD intra-assay) were 8–19% for the CLT assay and 2–17% for the CLT(1–6) peptide assay. The lower limit of quantitation in dog plasma was estimated as 18 pg ml^{-1} for CLT and 14–20 pg ml^{-1} for the CLT(1–6) peptide. Diluted urine samples from dogs were directly injected into the HPLC and the immunoreactivities of the fractions were measured. More than 98% immunoreactivity was found at the retention time of CLT(1–6) peptide. Thus only the CLT(1–6) peptide was measured in dog urine by the RIA without HPLC separation. Urine samples could be assayed directly only after dilution 1:20. The assay recovery for urine was 100% and the precision (RSD) was estimated to be <10%. The lower limit of quantitation for dog urine was 70 pg ml^{-1}. The combined method of HPLC and immunoassay is extremely useful for peptide analyses.

Keywords: *Reversed-phase high-performance liquid chromatography; competitive radioimmunoassay; combined method; ceruletide; metabolite; peptide.*

Introduction

Investigation of the metabolism and pharmacokinetics of peptides is very difficult because a suitable analytical method is not generally available; sufficient sensitivity was not always attained because of some interference from biological matrices, especially after administration of a small dose of peptides. ^{3}H-, ^{14}C-, or ^{125}I-labelled peptides are useful for tracing the unchanged form but after metabolism or degradation information from the label rarely reflects the behaviour of the major metabolites, where labels are sometimes carried by very small peptides or amino acids. There are more problems with ^{125}I-labelled peptides [1]. Immunoassays are very useful for the determination of proteins and peptides but are not always capable of identifying compounds which have similar residues of epitopes.

Ceruletide (CLT, caerulein), an analogue of cholecystokinin-8 (CCK-8), is a decapeptide [Pyr-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2, Pyr: pyroglutamic acid] isolated from the skin of the Australian frog *Hyra caerulea* and has similar biological and pharmacological activities to those of CCK [2]. Clinical studies showed that 0.8 μg of CLT given intramuscularly effectively suppressed dyskinetic and choreic movement disorders [3]. However, its metabolic fate *in vivo* is not well known. For studying the behaviour *in vivo*, it is very important to develop sensitive and specific assays for the parent peptide and its major metabolites.

A competitive radioimmunoassay (RIA) for CLT was developed [4] using an antiserum against N^{δ}-[CLT(1–6)]-ornithine amide conjugated with bovine serum albumin and N^{α}-[CLT(1–6)]-lysine amide labelled with ^{125}I-Bolton and Hunter reagent. This RIA, specific for the N-terminal part of CLT, was very sensitive and enabled the detection of 0.78 pg of CLT in an assay tube. The high sensitivity was attained by taking advantage of the different bridge structures of the immunogen and the labelled antigen. However, the immunoassay represented the total immunoreactivity

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but not the individual concentrations of the parent CLT and its metabolites, which were produced by digestion by proteases in tissues and biological fluids [5].

To investigate the metabolic behaviour of CLT *in vivo*, an HPLC/RIA method which combines the high resolving power of high-performance liquid chromatography (HPLC) with the high sensitivity of RIA has been developed. Previously, the development of an HPLC/enzyme immunoassay for 1,4-benzodiazepine was reported and the extensive applicability of the HPLC/immunoassay method was discussed [6]. In the present study, the main metabolite of CLT in dog plasma and urine has been identified and an HPLC/RIA method for the simultaneous measurement of unchanged CLT and the metabolite has been developed.

Materials and Methods

Chemicals

CLT was obtained from Shionogi and Co. Ltd (Osaka, Japan). CLT and its fragment peptides, CLT(1–6), CLT(1–7), CLT(1–8), CLT(1–9) and CLT(1–10) (carboxyl form), were gifts from Dr K. Inouye of the authors' institute. All chemicals were of analytical grade, unless otherwise specified.

HPLC conditions

CLT and the related compounds were separated with a reversed-phase HPLC system, model LC-4A (Shimadzu, Kyoto, Japan) fitted with a syringe-loading sample injector (model 7125; Rheodyne, Cotati, CA, USA) with a 500-μl loop and a 200 × 4.6 mm i.d. column packed with 5-μm Nucleosil C_{18} (Machery-Nagel, Duren, Germany). Gradient elution was obtained with water containing 0.1% (v/v) trifluoroacetic acid (TFA; mobile phase A) and acetonitrile containing 0.1% (v/v) TFA (mobile phase B). The linear gradient profile adopted was: $t = 0$, %B = 0; $t = 30$ min, %B = 50, at a flow-rate of 1 ml min^{-1}. This was followed by a 10-min wash with acetonitrile (100%). Authentic peptide fragments were detected by UV absorption at 220 nm (model SPD-6A; Shimadzu, Kyoto, Japan).

Combined HPLC and RIA for urine and plasma samples

Plasma (300 μl) was mixed with 600 μl of ethanol and 600 μl of the supernatant, after centrifugation at 2000*g* for 5 min, was evaporated to dryness. The residue was redissolved in 200 μl of water and the solution was injected into the HPLC after the filtration. The urine sample after 20-fold dilution with water was filtered through a 0.45-μm filter, and 100 μl was injected into the HPLC.

Fractions of the eluate were collected every 1 or 2 min into 75 × 12 mm i.d. glass gubes, directly evaporated to dryness under vacuum at 50°C and redissolved in 500 μl of a RIA buffer comprising 1 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA 4 Na), 0.9% (w/v) sodium chloride, 0.01% (w/v) sodium azide and 0.5% (w/v) bovine serum albumin (crystallized and lyophilized; Sigma, St Louis, MO, USA) in phosphate buffer (pH 7.4; 0.01 M).

RIA was performed according to ref. 4. Concentrations were calculated by an absolute calibration method using the RIA standard curve, as described in ref. 6.

Direct RIA for urine samples

The development of the RIA has been reported in detail previously [4]. Urine samples were measured by the RIA after 20-fold dilution with 1 mM EDTA 4 Na, 0.01% (w/v) sodium azide and 0.5% (w/v) bovine serum albumin in phosphate buffer (pH 7.4; 0.2 M). Standard solutions of CLT(1–6) peptide for the urine assay were also prepared with this buffer. The diluted urine samples and the standard solutions (500 μl) were subjected to the RIA in duplicate as reported previously [4].

Plasma and urine samples

CLT was administered to dogs fasted for 17 h and blood and urine were collected regularly. Trasylol$^{(R)}$ (Bayer, Leverkusen, Germany), 1500 IU, and 1.5 mg of EDTA 2 Na were added to 3 ml of the blood or the urine just after the collection and mixed carefully. The plasma was separated from the blood by centrifugation at 2000*g* for 15 min. These plasma and urine samples were stored at −20°C.

Both CLT and CLT(1–6) peptide in the plasma and urine samples were stable at −20°C for more than 6 months and were also stable at room temperature for 6 h. The peptides were not degraded in the RIA buffer at room temperature within 24 h.

Pharmacokinetic analysis

The pharmacokinetic parameters of CLT were estimated by fitting to a one-compartment model for the plasma level after intravenous administration or to that of a first-order absorption process for the plasma level after intramuscular administration. The program NONLIN-74 [7] was used for the calculations. The half-lives for absorption ($t_{1/2,abs}$) and elimination ($t_{1/2}$), the time (t_{max}) at which the maximal concentration (C_{max}) was attained, the area under the concentration–time curve (AUC) and the bioavailability (F) were calculated according to the methods of Gibaldi and Perrier [8].

Results and Discussion

HPLC separation of related compounds of CLT

Immunoreactivities of CLT were found in both plasma and urine from dogs, and those in urine suggested metabolite formation from the results from the rat study [5]. The RIA used in this study was highly specific for the (1–6) residue of CLT and did not react with N-terminal modified and/or des SO_3 peptides [4]. Thus, some candidates were selected for the metabolite, i.e. CLT(1–6), CLT(1–7), CLT(1–8), CLT(1–9) and CLT(1–10) (carboxyl form), which have both immunoreactivity for the RIA and the possibility of metabolites from CLT.

To identify the immunoreactive metabolites by their retention time (t_R) on a chromatogram, authentic compounds of the candidates were separated by reversed-phase HPLC. After several experiments, good separation was attained with the HPLC conditions described under Materials and Methods. The t_R values of CLT(1–6), CLT(1–7), CLT(1–8), CLT(1–9), CLT(1–10) and CLT were 12.4, 19.5, 21.8, 20.8, 25.6 and 24.8 min, respectively.

Identification of metabolites in dog plasma

Dog plasma samples after the administration of CLT were deproteinized by addition of ethanol and separated by reversed-phase HPLC. Fractions from the HPLC eluate were measured by the RIA (Fig. 1). The chromatogram at 0 h, just before the administration, suggested that this HPLC/RIA method was so specific that no peaks from plasma components were observed. Two immunoreactive components were found in dog plasma after the administration of CLT; a large peak (t_R = 13 min), which was observed for 2 h after the administration, was assumed to be CLT(1–6) peptide and the other (t_R = 25 min) was the unchanged CLT, from a comparison of the t_R values with those of standard compounds. Another very small shoulder near t_R = 21–23 min, although unidentified, could be due to the CLT(1–7), (1–8) or (1–9) peptide. The CLT(1–6) peptide was thought to be one of the main metabolites.

Quantitative analysis of CLT and its (1–6) peptide in dog plasma by HPLC/RIA

An HPLC/RIA was developed in order to determine the CLT and CLT(1–6) peptide in dog plasma simultaneously as described under Materials and Methods. The control plasma samples, prepared by the addition of known

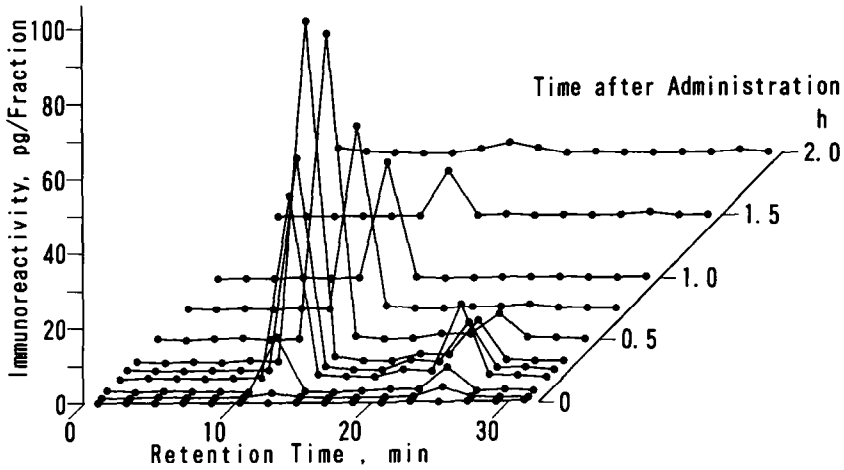

Figure 1
HPLC/RIA chromatogram of dog plasma after intramuscular administration of CLT, 0.84 μg kg^{-1}.

amounts of authentic compounds, were measured by this method using a RIA standard curve of CLT and the recoveries were assessed by peak–area measurements after correction for cross reactivities for CLT (100%) and CLT(1–6) peptide (133%). The relationships between added (x pg ml^{-1}) and measured (y pg ml^{-1}) concentrations were linear. For CLT, $y = 0.980\ (\pm 0.033)x + 22.9\ (\pm 34.5)$, $n = 21$ (range, 0–2000 pg ml^{-1}); and for CLT(1–6) peptide, $y = 1.020\ (\pm 0.021)x + 5.14\ (\pm 17.53)$, $n = 20$ (range, 0–1500 pg ml^{-1}). The differences between the added and the measured values were not significant ($P < 0.05$) by regression analyses for both the CLT and CLT(1–6) peptide assays. Thus, no corrections of recoveries were necessary. The assay precision (RSD intra-assay) was good, 8–19% for the CLT and 2–17% for the CLT(1–6) peptide as shown in Table 1. The lower limits of quantitation in dog plasma were estimated as 18 pg ml^{-1} for CLT and 14 pg ml^{-1} for the CLT(1–6) peptide by the HPLC/RIA. The lower limit of quantitation, i.e. the lowest concentration of analytes where the assay precision (RSD) did not exceed 20%, was obtained by extrapolation of precision profiles of the RIA standard curves.

Only the CLT(1–6) peptide was found in dog plasma after CLT had been completely metabolized. These dog plasma samples were measured by the RIA without HPLC separation after being deproteinized with ethanol. Recovery of the RIA [$y = 1.033\ (\pm 0.018)x - 18.2\ (\pm 14.4)$, $n = 27$ (range 0–1500 pg ml^{-1})] was found to be complete using the control dog plasma samples; the intra- and inter-assay precision (RSD) values were 7–10 and 8–16%, respectively, as shown in Table 2. The lower limit of quantitation in dog plasma was estimated as 20 pg ml^{-1} for the CLT(1–6) peptide by the RIA.

Identification of metabolites in dog urine

Diluted urine samples from dogs after administration of CLT were directly injected into the HPLC and the immunoreactivities of the fractions were measured by the RIA. As shown in Fig. 2, more than 98% immunoreactivity was found at the retention time of the CLT(1–6) peptide (t_R = 13 min). The results were in good agreement with those from rat urine using ^{14}C-labelled CLT [5].

Quantitative analysis of CLT(1–6) peptide in dog urine

A direct RIA method without HPLC was developed for CLT(1–6) peptide in dog urine because most of the immunoreactivity was from the CLT(1–6) peptide. The assay buffer was changed from a 0.01 M phosphate buffer-based solution [4] to a 0.2 M phosphate buffer-based solution and 20-fold diluted urine samples were used before the RIA to eliminate the effects from dog urine components, especially individual differences in urine effect. Four kinds of dog urine were used for the recovery test and all recoveries were excellent. No significant difference ($P < 0.05$) was observed between added (x pg ml^{-1}) and found (y pg ml^{-1}) in each urine and the overall correlation was $y = 0.976\ (\pm 0.020)\ x - 17.6$

Table 1
Intra-assay precision of HPLC/RIA for CLT and CLT(1–6) peptide in dog plasma

CLT (pg ml^{-1})				CLT(1–6) peptide (pg ml^{-1})			
Spiked	Mean ± SD	%RSD	n	Spiked	Mean ± SD	%RSD	n
399	394 ± 74.0	19	4	311	354 ± 47.7	13	4
992	910 ± 73.7	8.1	3	928	944 ± 15.4	1.6	4
1970	1860 ± 248	13	3	1540	1590 ± 270	17	3

Table 2
Assay precision of RIA for CLT(1–6) peptide in dog plasma

Spiked (pg ml^{-1})	Intra-assay			Inter-assay		
	Mean ± SD (pg ml^{-1})	%RSD	n	Mean ± SD (pg ml^{-1})	%RSD	n
312	321 ± 28.9	9.0	4	337 ± 28.4	8.4	3
727	693 ± 72.2	10.4	4	630 ± 101	16.0	3
1559	1580 ± 107	6.8	4	1639 ± 159	9.7	3

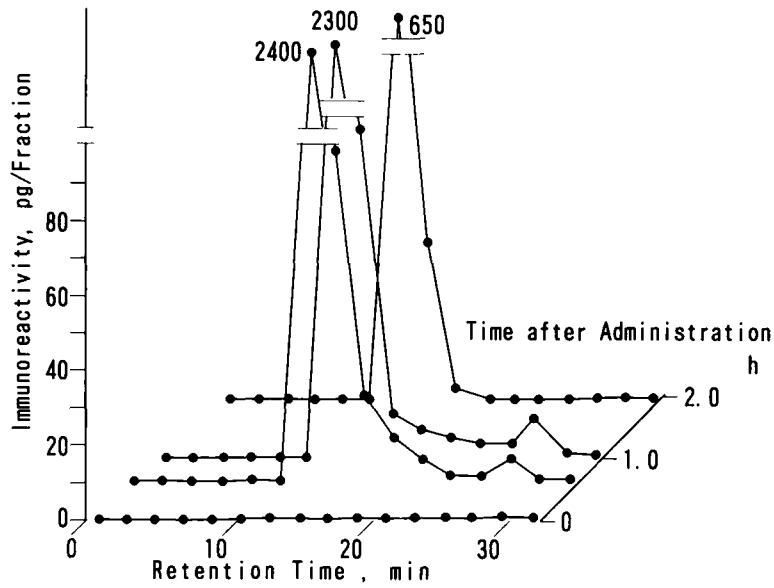

Figure 2
HPLC/RIA chromatogram of dog urine after intramuscular administration of CLT, 18.2 μg kg^{-1}.

Table 3
Assay precision of RIA for CLT(1–6) peptide in human urine

	Intra-assay			Inter-assay		
Sample	Mean ± SD (pg ml^{-1})	%RSD	*n*	Mean ± SD (pg ml^{-1})	%RSD	*n*
Low	109 ± 6.4	5.9	5	99.4 ± 2.3	2.3	5
Medium	563 ± 34.3	6.1	5	528 ± 5.0	0.9	5
High	3190 ± 281	8.8	5	3365 ± 280	8.3	5

(±36.3), n = 12 (range, 0–3600 pg ml^{-1}). The precision values of the RIA shown in Table 3 were estimated using human urine which could be assayed by an identical assay procedure and the same standard curve. The limit of quantitation was estimated to be approximately 70 pg ml^{-1}.

Measurement of dog plasma and urine samples

Urine and blood samples from dogs after administration of CLT were measured by the assays described above. Figures 3–5 show that the behaviour of the drug was traced successfully by these assays.

Pharmacokinetic parameters of CLT

Table 4 shows some pharmacokinetic parameters of CLT. The CLT level in plasma after intravenous (i.v.) administration was rapidly decreased and the half-life ($t_{1/2}$) was about 2 min. On the other hand, the CLT level attained a maximum at 4–8 min after intramuscular (i.m.) administration and CLT was eliminated with a longer $t_{1/2}$ than that after i.v. administration. The absorption half-life ($t_{1/2,abs}$) for i.m. administration was almost equal to the elimination half-life ($t_{1/2}$) for i.v. administration. Therefore, this plasma concentration curve may indicate a flip-flop phenomenon. The bioavailabilities of CLT after i.m. administration of 0.844 and 18.2 μg kg^{-1} were both about 20% which suggests that part of the CLT was metabolized in the muscle at the injection site prior to absorption into the circulation. Further investigation of the pharmacokinetics of the metabolite is now in progress and the details will be published elsewhere.

In the past, it has been very difficult to measure precisely a very small amount of peptides like CLT in biological fluids because there are no specific or sensitive methods to detect a particular peptide. In this study, very sensitive and specific assays for CLT and its metabolite have been developed by taking advantage of both the high resolving power of HPLC and the high sensitivity of RIA. Using the series of assays described here, the main metabolite of CLT in dog plasma and urine was identified; then unchanged CLT and the CLT(1–6) peptide were measured simul-

Table 4
Pharmacokinetic parameters of CLT after intravenous (i.v.) or intramuscular (i.m.) administration of CLT in dogs

Dose ($\mu g\ kg^{-1}$)	$t_{0,abs}$ (min)	$t_{1/2}$ (min)	t_{max} (min)	C_{max} ($pg\ ml^{-1}$)	AUC ($pg\ h\ ml^{-1}$)	F (%)
1.67		1.76			587	
i.v.		±0.37			±78	
0.844	2.18	11.5	8.13	164	63.7	21.4
i.m.	±1.35	±2.9	±1.69	±120	±39.4	±12.4
18.2	1.29	7.57	4.49	5640	1240	20.0
i.m.	±1.66	±4.35	±2.74	±3450	±380	±8.6

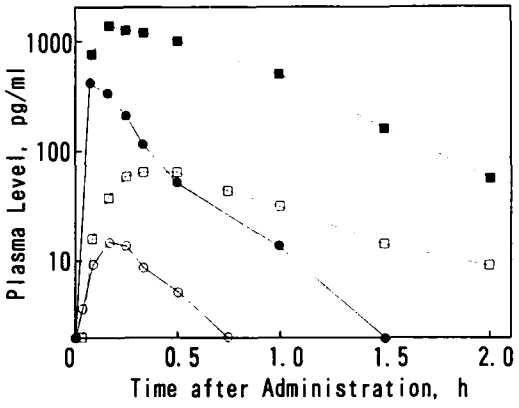

Figure 3
Plasma levels of CLT (○,●) and CLT(1–6) peptide (□,■) after intramuscular administration of CLT in dogs. Dose: 0.844 $\mu g\ kg^{-1}$ (open symbols) and 18.2 $\mu g\ kg^{-1}$ (closed symbols) of CLT.

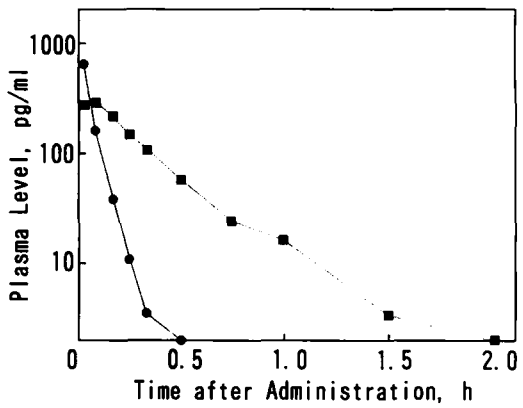

Figure 4
Plasma levels of CLT (●) and CLT(1–6) peptide (■) after intravenous administration of CLT in dogs. Dose: 1.67 $\mu g\ kg^{-1}$ of CLT.

taneously in these samples even after administration of only 0.84 $\mu g\ kg^{-1}$. This combined technique of HPLC and immunoassay is extremely useful for peptide analyses.

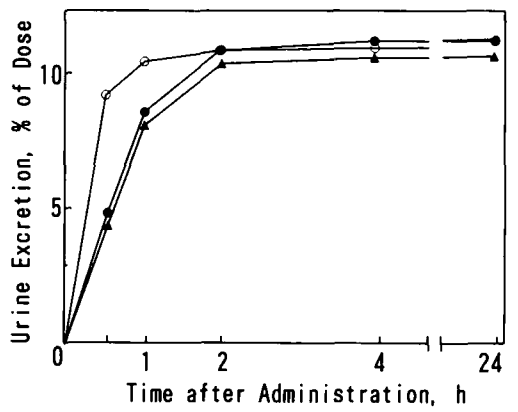

Figure 5
Cumulative excretion of CLT(1–6) peptide in urine after administration of CLT in dogs. Intramuscular administration, 0.844 $\mu g\ kg^{-1}$ (●) and 18.2 $\mu g\ kg^{-1}$ (▲), and intravenous administration, 1.67 $\mu g\ kg^{-1}$ (○).

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