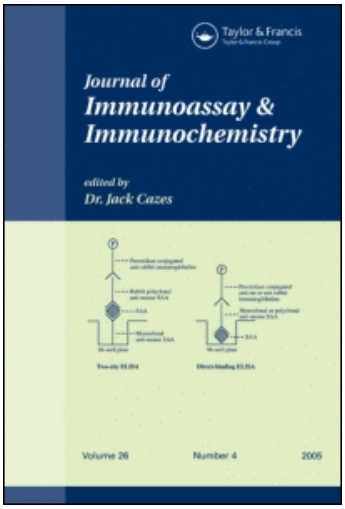

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Highly Specific and Sensitive Radioimmunoassay of Caerulein, An Analogue of Cholecystokinin-8

Goro Kominami[a]; Shusuke Mori[a]; Keiko Sagawa[a]; Ken'Ichi Igano[a]; Ken Inouye[a]; Masao Kono[a]
[a] Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka, Japan

To cite this Article Kominami, Goro , Mori, Shusuke , Sagawa, Keiko , Igano, Ken'Ichi , Inouye, Ken and Kono, Masao(1988) 'A Highly Specific and Sensitive Radioimmunoassay of Caerulein, An Analogue of Cholecystokinin-8', Journal of Immunoassay and Immunochemistry, 9: 3, 229 — 243
To link to this Article: DOI: 10.1080/01971528808053214
URL: http://dx.doi.org/10.1080/01971528808053214

JOURNAL OF IMMUNOASSAY, 9(3&4), 229-243 (1988)

A HIGHLY SPECIFIC AND SENSITIVE RADIOIMMUNOASSAY OF CAERULEIN, AN ANALOGUE OF CHOLECYSTOKININ-8

(KEY WORDS: Caerulein, Radioimmunoassay, ^{125}I-Label, Synthetic peptides, Antibody specificity)

Goro Kominami, Shusuke Mori, Keiko Sagawa, Ken'ichi Igano, Ken Inouye, and Masao Kono
Shionogi Research Laboratories, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka, 553 Japan

ABSTRACT

A highly specific and sensitive competitive radioimmunoassay was developed for caerulein (CLN), an analogue of cholecystokinin-8 (CCK-8), in plasma and brain. Antiserum was produced in rabbit by immunization with N^{δ}-[CLN-(1-6)]-ornithine amide conjugated with bovine serum albumin by the glutaraldehyde method. N^{α}-[CLN-(1-6)]-lysine amide was labelled with ^{125}I-Bolton & Hunter reagent and used as a labelled antigen after purification by high-performance liquid chromatography. This assay was highly specific for CLN, and cross reactivities for other related peptides, CCK-4, CCK-8, gastrin-I, and gastrin-(14-17), were not observed (<0.01%). The limits of determination in biological specimens after CLN administration were 11 pg/ml in human plasma and rat plasma and 80 pg/g in rat brain. This study showed that the slight structure difference between hapten and ^{125}I-labelled antigen is important to the assay performance.

INTRODUCTION

Caerulein (CLN), a decapeptide isolated from frog skin, is known to possess similar biological activities and pharmacological activities to cholecystokinin

(CCK), an important gastrointestinal hormone, which may also play an important role as a neurotransmitter or a neuromodulator (1).

Investigations on radioimmunoassays (RIAs) for CCKs, especially labelled-CCKs and/or highly specific antibodies against CCKs, have been performed (2-7) but those on CLN-specific RIA have not yet.

We developed a very sensitive and specific RIA for CLN in human plasma, rat plasma and rat brain. It can be used to study the pharmacological fate of CLN in humans and rats. We used synthetic CLN-(1-6) derivatives for the hapten and a labelled antigen to eliminate cross reactions from CCKs and gastrins whose C-terminal amino acid sequences Gly-Trp-Met-Asp-Phe-NH_2 are common to those of CLN (Fig. 1). .

MATERIALS AND METHODS

Chemicals

CLN was obtained from Shionogi & Co. Ltd. (Osaka, Japan). CCK-8, CCK-4, and gastrin-(14-17) were from Peptide Institute (Osaka, Japan) and gastrin I from Sigma (St. Louis, MO). All chemicals were of analytical grade, unless otherwise specified.

Synthesis of Peptides

Z-Orn(Boc)-NH_2 (Z: benzyloxycarbonyl, Boc: *t*-butoxycarbonyl) derived from Z-Orn(Boc)-OH (8) was subjected to catalytic hydrogenolysis and the resulting H-Orn(Boc)-NH_2 was acylated with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (Aldrich, Milwaukee, WI) to give Fmoc-Orn(Boc)-NH_2. This was treated with 1

Caerulein; Pyr-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2

Cholecystokinin-33; (1.....20)-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2

Cholecystokinin-8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2

Gastrin I; Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2

Gastrin II; Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2

FIGURE 1. Amino acid sequences of caerulein and related peptides. Pyr: pyroglutamic acid.

mol/l hydrogen chloride in acetic acid to remove the Boc group yielding Fmoc-Orn-NH_2 (I). The azide derived from Pyr-Gln-Asp-Tyr-Thr-Gly-$NHNH_2$ (II) (1) was coupled with compound I to give Fmoc-Orn(Pyr-Gln-Asp-Tyr-Thr-Gly)-NH_2, which was sulfated with SO_3-pyridine complex (Aldrich, Milwaukee, WI) in 1 mol/l sodium carbonate buffer plus N,N-dimethylformamide (DMF) (2:1, pH 10) at 22-25°C. The purified product was treated with 1 mol/l diethylamine to give N^{δ}-[CLN-(1-6)]-ornithine amide (III). Amino acid ratios in the acid hydrolysate: Asp 1.00 (1), Thr 0.94 (1), Glu 1.99 (2), Gly 1.03 (1), Tyr 1.03 (1) and Orn 1.03 (1).

Boc-Lys(Z)-OH (Peptide Institute, Osaka, Japan) was converted, via a mixed anhydride (9), into Boc-Lys(Z)-NH_2, from which Boc-Lys-NH_2 was obtained by catalytic hydrogenolysis (H_2/Pd). It was then acylated with Fmoc-Cl to give Boc-Lys(Fmoc)-NH_2, which was treated with 1 mol/l hydrogen chloride in acetic acid, yielding Lys(Fmoc)-NH_2 (IV). The azide derived from compound II was coupled with IV to give Pyr-Gln-Asp-Tyr-Thr-Gly-Lys(Fmoc)-NH_2, which was sulfated in an aqueous DMF solution as given above. The product was treated with 1 mol/l diethylamine in DMF to remove the Fmoc group, yielding N^{α}[CLN-(1-6)]-lysine amide (V). Amino acid ratios in the acid hydrolysate: Asp 1.00 (1), Thr 0.99 (1), Glu 1.94 (2), Gly 1.00 (1), Tyr 1.04 (1), and Lys 1.00 (1).

Other related peptides in Table 1 were synthesized by the solution method as described previously (1). The synthetic peptides were satisfactory pure as assessed by reversed phase high-performance liquid chromatography (HPLC).

Preparation of Antisera

To a mixture of 25.3 mg of N^{δ}-[CLN-(1-6)]-ornithine amide (III) and 75.9 mg of bovine serum albumin (BSA) in 4 ml of 50 mmol/l phosphate buffer, pH 7.4, 1 ml

of 12.5% (w/w) glutaraldehyde was added drop by drop with stirring. The reaction mixture was stirred at 22-25°C for 2 h. After dialysis against water, the conjugate (VI) was lyophilized to afford 88.8 mg.

The conjugate in saline was emulsified with an equal volume of complete Freund's adjuvant to give a final concentration of 0.5 mg/ml. A half milliliter of the emulsion was injected intradermally into each of five New Zealand white rabbits at multiple sites on the back. Booster injections were given every 3 weeks and whole blood was collected 10 days after the 4th one.

Synthesis of ^{125}I-Labelled Caerulein

N^{α}-[CLN-(1-6)]-lysine amide (V), 2 µg, in 20 µl of 0.1 mol/l borate buffer, pH 8.5, was added to the reaction vessel containing 0.5 mCi of dry ^{125}I-Bolton & Hunter reagent (BHR) (NEN, Boston, MA) (10) and the mixture was stirred at 4°C for 24 h. The product was purified by HPLC, Shimadzu Model LC-6A system (Kyoto, Japan), using an octadecylsilanol silica (ODS) column [Nucleosil C_{18}, particle size 5 µm (Machery Nagel, Duren, F.R.G.), column size 150 mm x 4 mm I.D.], 50 mmol/l phosphoric acid, pH 3.0, containing 50 mmol/l sodium sulfate and 5 mmol/l sodium 1-butanesulfonate: acetonitrile (80:20) as the mobile phase, and a flow rate of 1 ml/min. The second radioactive peak of N^{α}-[CLN-(1-6)]-Lys-BHR (VII) was collected and stored.

Pretreatment of Plasma Samples and Brain Homogenates for RIA

Ethanol, 500 µl, and 250 µl of human or rat plasma were mixed well and centrifuged at 2,000 x *g* for 5 min. A 500-µl portion of the supernatant was evaporated and the residue was redissolved in 1.2 ml of the assay buffer. The

standard solutions of CLN for human plasma samples were made with the assay buffer described below. For rat plasma samples, the standard solutions were made with the buffer containing a control rat plasma extract.

The CLN in rat brain was also measured using the RIA. A half of a rat brain (ca. 1 g) was homogenized with 2 ml of saline and diluted to 10 ml with saline. Ethanol, 10 ml, was added to a 1-ml portion of the homogenate and the mixture was centrifuged. Whole supernatant was evaporated and the residue was redissolved in 1 ml of water. The solution was washed twice with 10 ml of ether and 100 µl of the water phase was pipetted into the assay tube containing 400 µl of the assay buffer.

Radioimmunoassay

RIA was performed in 0.01 mol/l phosphate buffer, pH 7.4, containing 0.5% (w/v) BSA, 1 mmol/l ethylenediaminetetraacetic acid, 0.9% (w/v) sodium chloride, and 0.01% (w/v) sodium azide as an assay buffer.

A 100-µl portion of the diluted antiserum (1:100,000) and 200 µl of the labelled antigen (50,000 cpm/ml) were added to an assay tube containing 500 µl of the sample or standard solution (0.78-200 pg CLN/tube). The mixture was incubated at 4°C for 16 h and then 100 µl suspension of immobilized anti-rabbit second antibody (1 mg/ml), Immunobead® (Bio-Rad, Richmond, CA), was added. After further incubation at 4°C for 3 h, the mixture was centrifuged at 2,000 x g for 5 min. The supernatant was aspirated off and the radioactivity of the residue was counted with a gamma counter, Aloka ARC-600 (Tokyo, Japan).

RESULTS AND DISCUSSION

Strategies of RIA and Peptide Synthesis

As for iodine labelling of CLN, a single tyrosine residue of CLN is sulfated, so that the usual methods of oxidative iodination (chloramine T, iodogen or glucose oxidase method) can not be employed. The Bolton & Hunter method (10) can not be used either, because of the absence of a free amino group. To solve these problems and to develop a CLN-specific RIA, we synthesized two derivatives of CLN-(1-6), in which a lysine or ornithine amide was attached to the C-terminal of the hexapeptide to give CLN-(1-6)-$NH(CH_2)_3$-$CH(CONH_2)NH_2$ (III) and CLN-(1-6)-$NHCH(CONH_2)(CH_2)_4NH_2$ (V), respectively. BHR may be more reactive to a ε-amino group than to an α-amino group. Therefore, compound V was chosen for coupling with BHR to give CLN-(1-6)-$NHCH(CONH_2)(CH_2)_4$NH-BHR (VII) as a labelled antigen.

It is often the case that antibodies which recognize the structure of a bridge between the hapten and the carrier are raised and interference of the RIA arises when the same structure is present in the labelled antigen (11,12). BSA was coupled to the α-amino group of compound III to give CLN-(1-6)-$NH(CH_2)_3CH(CONH_2)$NH-BSA conjugate (VI) and used for an immunogen with a different structure of the bridge from the labelled antigen.

Preparation of Labelled Antigen

N^{α}-[CLN-(1-6)]-lysine amide (V) was made to react with BHR under the usual conditions (10). The labelled compound was purified by reversed phase HPLC using an ODS column. The chromatogram is shown in Fig. 2. Retention

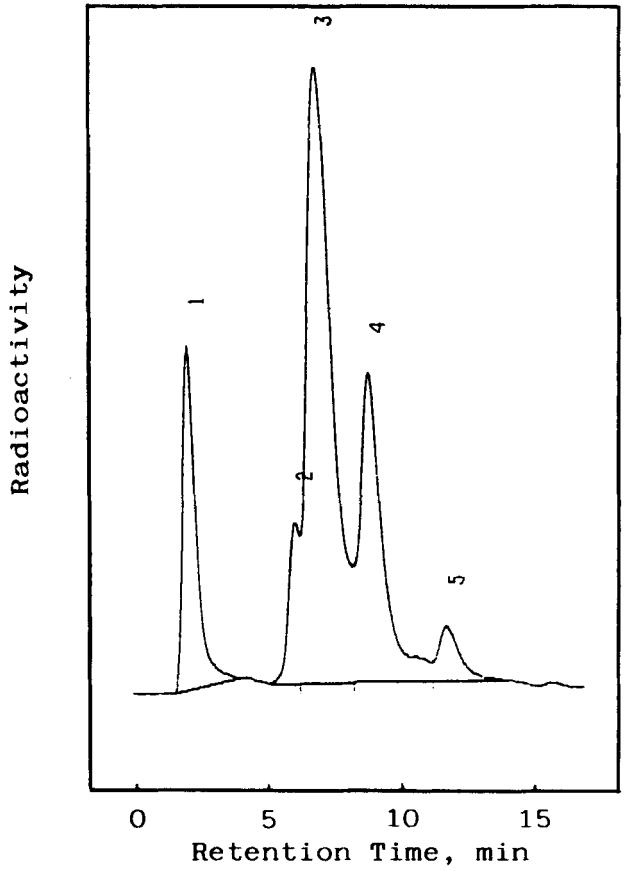

FIGURE 2. Purification of ^{125}I-labelled caerulein by reversed phase HPLC.

time (t_R) of the labelled antigen (VII) was 7 min. The separation from the unlabelled antigen (V) (t_R = 3 min) was complete; conventional liquid chromatography did not allow their separation. The specific radioactivity of the labelled compound was 1.54 x 10^6 Ci/mol as determined by the self-displacement RIA method (13). This high specific activity proves that HPLC is an excellent tool for purification of a labelled antigen (14).

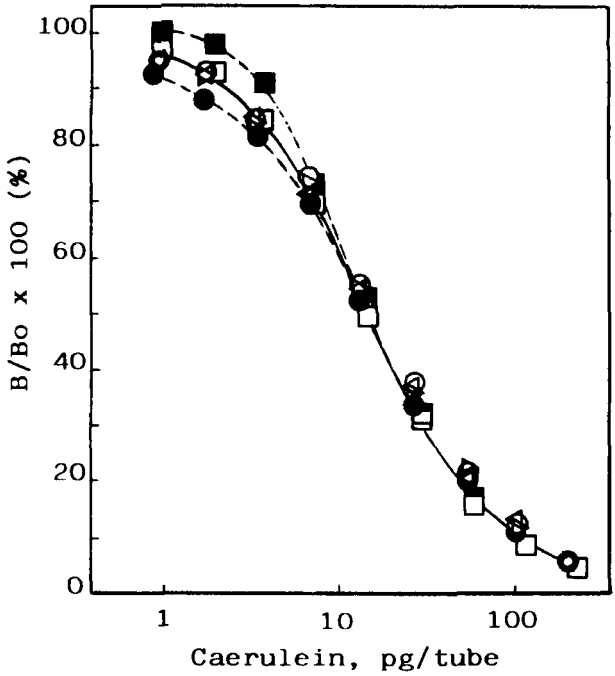

FIGURE 3. Standard displacement curves of the caerulein-specific RIA in various media for standard solution. Assay buffer only (○) and the assay buffer containing ethanol extract of: human plasma (two samples; ▷, ◁), rat plasma (●), rat brain homogenate (■), or rat brain homogenate with additional ether-washing (□). For the standard curve of the assay buffer only (○), Bo/T×100=48 (%), non specific binding/T×100 = 1 (%), concentration of 90% intercept = 2.0 pg/tube, and concentration of 50% intercept = 15.6 pg/tube.

Preparation of Immunogen and Antiserum

In trying to raise CLN-specific antiserum, N^{δ}-[CLN-(1-6)]-ornithine amide-BSA conjugate (VI) as immunogen was prepared by the glutaraldehyde method and was found to contain 14.2 mol hapten per mol BSA as estimated by amino acid analysis. The ornithine residue was useful for the measurement of the hapten content since BSA does not contain ornithine.

We obtained five similar antisera and chose antiserum S-71 for the RIA.

TABLE 1

Cross Reactivities of Caerulein and Related Peptides

Peptide	Cross reactivity*
Caerulein (CLN)	100
Glu^2-CLN	0.2
Des(SO_3H)-CLN	<0.01
Des(SO_3H)-Glu^2-CLN	<0.01
CLN-(1-10)-OH	260
CLN-(1-9)	123
CLN-(1-8)	135
CLN-(1-7)	101
CLN-(1-6)	111
N^{δ}-(1-6)-Orn-NH_2	43
N^{α}-CLN-(1-6)-Lys-NH_2	37
CLN-(4-10)	<0.01
CLN-(5-10)	<0.01
Cholecystokinin-8	<0.01
Cholecystokinin-4	<0.01
Gastrin I	<0.01
Gastrin-(14-17)	<0.01

* Calculated from peptide concentration yielding 50% displacement of the ^{125}I-labelled caerulein binding on a weight basis.

Radioimmunoassay

The standard displacement curve of the competitive RIA set up in the present study is shown in Fig. 3. Cross reactivities against several CLN-related peptides were estimated. As shown in Table 1, the peptides which have the CLN-(1-6) sequence fully cross reacted but the N-terminal-modified or non-sulfated peptides did not, especially CCK-8, CCK-4, gastrin I, and gastrin-(14-17) (Fig. 1).

TABLE 2

Assay Variations of the Caerulein (CLN)-specific RIA for Human Plasma Samples

Concentration (ng/ml)		Coefficient of variation (%)
CLN added	CLN found*	
(Intra-assay variations)		
20	20.4 ± 1.7	7.5
100	98.8 ± 4.3	4.3
500	507 ± 28	5.7
(Inter-assay variations)		
20	22.5 ± 2.4	10.7
100	99.5 ± 4.8	4.9
500	503 ± 42	8.5

* Mean ± S.D., N = 5 each.

The results suggested that the specificity against CLN is excellent, just as we had expected by using the N-terminal part, CLN-(1-6). The difference in cross reactivity between CLN and CLN-(1-10)-OH may be caused by their different electric properties. The association constant of 1.82×10^{-11} l/mol, calculated from Scatchard plots, suggests that affinity of the antibody is very high.

The excellent minimal detectable dose ($p < 0.05$) of 0.78 pg/tube, i.e. 590 amol/tube, was attained by using the ^{125}I-labelled antigen which had high specific activity and the antiserum of best affinity.

For the reaction conditions, 16 h incubation at 4°C was enough for the first immunoreaction. Immunobead® was adopted for the B/F separation because of its

lower non-specific binding of 1%, especially for biological samples, than that by the polyethylene glycol method, 5%.

Pretreatment of biological Samples

Using this RIA, we tried to measure the CLN concentration in human plasma, rat plasma and rat brain. Direct RIA without any pretreatment was affected by some components of plasma or tissue at concentrations of more than 5% (1:20 dilution). For a more sensitive assay, we developed a pretreatment with ethanol extraction as described in the experimental section. As shown in Fig. 3, there was no interference from the human plasma extract but there was some from the rat plasma extract. For rat brain samples, interference from the ethanol extract was canceled by further washing with ether.

We used a standard solutions with the assay buffer containing a plasma extract from control rats for the rat plasma assay but used only the solutions with the assay buffer for the human plasma assay and the rat brain assay.

RIA Performance for Biological Samples

The sensitivity ($p < 0.05$) were 11 pg/ml for human plasma and rat plasma and 80 pg/g for rat brain. The assay precisions for human plasma were fine as shown in Table 2 and the recovery was complete based on the correlation between the CLN added to human plasma (x pg/ml) and the measured CLN (y ng/ml), $y = 1.02x + 6.5$ ($n=29$). Similar values were obtained for rat plasma and brain samples (data not shown).

An excellent specific and sensitive RIA for CLN was established as shown above. By the use of our CLN-specific RIA, we could observe elimination profiles of

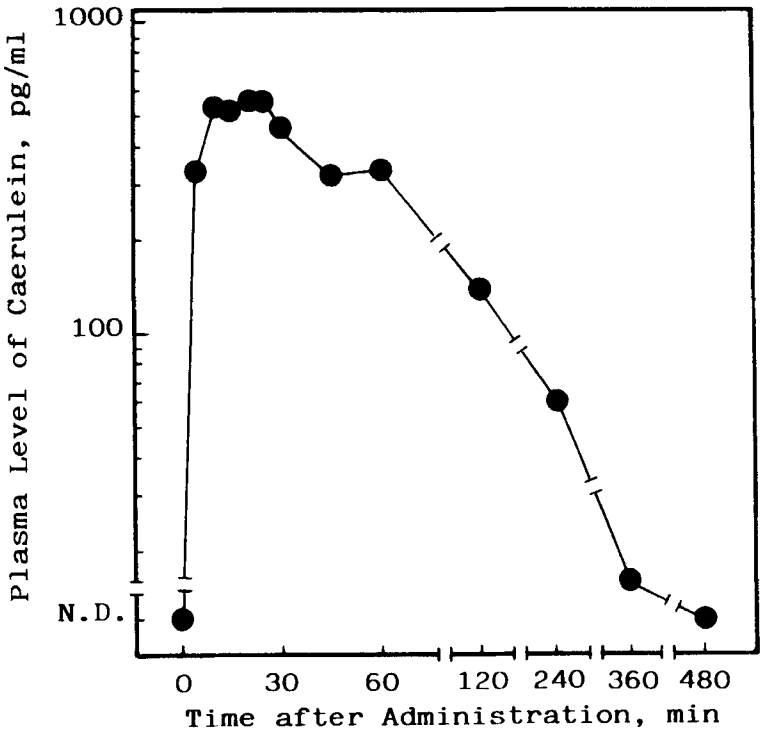

FIGURE 4. Elimination profile of caerulein in human plasma, determined by the RIA. Caerulein, 0.8 μg/kg, was injected intramuscularly to a healthy male volunteer, 34 years old, fasted for 16 h.

CLN from human plasma after its administration (Fig. 4). Further investigation for its pharmacokinetics is now in progress and the details will be shown elsewhere. We hope that this RIA can aid in the development of more effective use of CLN.

REFERENCES

1. Fujimoto, M., Igano, K., Watanabe, K., Irie, I., Inoue, K. and Okabayashi, T. Effects of caerulein-related peptides on cholecystokinin receptor binding in brain and pancreas. Biochem. Pharmacol. 1985; 34: 1103-7.

2. Rehfeld, J. F. Immunochemical studies on cholecystokinin. J. Biol. Chem. 1978; 253: 4016-21.

3. Hashimura, E., Shimizu, F., Nishino, T., Imagawa, K., Tateishi, K. and Hamaoka, T. Production of rabbit antibody specific for amino-terminal residues of cholecystokinin octapeptide(CCK-8) by selective supression of cross-reactive antibody response. J. Immunol. Methods 1982; 55: 375-82.

4. Bacarese-Hamilton, A. J., Adrian, T. E. and Bloom, S. R. Measurement and characterisation of human cholecystokinin-like immunoreactivity (CCK-LI) in tissues by radioimmunoassay. Clin. Chim. Acta 1984; 144: 213-24.

5. Fourmy, D., Pradayrol, L., Vaysse, N., Susini, C. and Ribet, A. ^{125}I-(Thr_{34},Nle_{37})-CCK_{31-39} a non oxidisable tracer for the characterization of CCK receptor on pancreatic acini and radio-immunoassay of C-terminal CCK peptides. J. Immunoassay 1984; 5: 99-120.

6. Cantor, P. and Rehfeld, J. F. Radioimmunoassay of cholecystokinin: comparison of different tracers. J. Immunol. Methods 1985; 82: 47-55.

7. Turkelson, C. M., Dale, W. E., Reidelberger, R. and Solomon, T. E. Development of cholecystokinin radioimmunoassay using synthetic CCK-10 as immunogen. Regulatory Peptides 1986; 15: 205-17.

8. Inouye, K., Watanabe, K., Namba, K., Tanaka, A. and Otsuka, H. Synthesis of 15-ornithine analogus of corticotropin (ACTH)-octadecapeptide amide. In: Kaneko, T., ed. Peptide Chemistry 1970. Proc. 8th Symp. Pept. Chem.: Protein Research Foundation, 1971; 95-100.

9. Otsuka, H., Inouye, K., Kanayama, M. and Shinozaki, F. The syntheses of peptides related to the N-terminal structure of corticotropine. VII. The synthesis of peptide derivatives corresponding to positions 11 to 18 in the corticotropin molecule. Bull. Chem. Soc. Jpn. (Tokyo) 1966; 39: 882-8.

10. Bolton, A. E. and Hunter, W. M. The labelling of proteins to high specific radioactivity by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 1973; 133: 529-39.

11. van Weemen, B. K. and Schuurs, A. H. W. M. The influence of heterologous combinations of antiserum and enzyme-labeled estrogen on the characteristics of estrogen enzyme-immunoassays. Immunochemistry 1975; 12: 667-70.

12. Kominami, G., Matsumoto, H., Nakamura, M. and Kono, M. An enzyme immunoassay for a metabolite of a new sleep inducer (450191-S), a ring-opened derivative of 1,4-benzodiazepine, in human serum. J. Immunoassay 1987; 8: 267-81.

13. Chervu, L. R. and Marty, D. R. K. Radiolabeling of antigens. In: Freeman, L. M. and Blaufox, M. D., eds. Radioimmunoassay. New York: Grune & Stratton, 1975; 33-48.

14. Fourmy, D., Pradayrol, L., Antoniotti, H., Esteve, J. P. and Ribet, A. Purification of radio-iodinated cholecystokinin peptides by reverse-phase HPLC. J. Liq. Chromtgr. 1982; 5: 757-66.