Simple enzyme immunoassay for the measurement of immunoreactive chromogranin A in human plasma, urine and saliva

SHINGO NAGASAWA¹, YASUKO NISHIKAWA², LI JUN¹, YOKO FUTAI¹, TOMIO KANNO¹, KAZUAKI IGUCHI², TOHRU MOCHIZUKI², MINORU HOSHINO², CHIZUKO YANAIHARA³ and NOBORU YANAIHARA¹

¹Yanaihara Institute Inc., Awakura, Fujinomiya-shi, 418-0011, ²Department of Pharmaceutical Sciences, University of Shizuoka, Yada, Shizuoka-shi, 422-8002, and ³Department of Pharmacy, Hyogo Medical University, Nishinomiya-shi, 663-8131, Japan

ABSTRACT

A simple and sensitive enzyme immunoassay (EIA) was developed for determining human chromogranin A (CgA) -like immunoreactivity (IR) with use of synthetic human CgA (344-374) as antigen. In the development of the present EIA system, purposely designed N^α-biotinylated glycylglycyl-human CgA (344-374), which was synthesized, was proved to be a useful biotinylated derivative. Typical standard curves ranged from 13.7 fmol/mL to 10 pmol/mL of CgA-like IR. In the present EIA, the curves for dilution of human plasma, urine, and saliva paralleled the standard curve for synthetic human CgA (344-374). The intra-assay coefficient of variation for measurement of human plasma CgA was from 2.6% to 8.5%, indicating that reliable determinations can be performed for human plasma. The levels of CgA-like IR were 0.34 ± 0.08 pmol/mL (mean $\pm$ SD, n=10) in fasting normal plasma, 0.18 ± 0.05 pmol/mL (n=8) in urine, and 0.11 ± 0.05 pmol/mL (n=8) in saliva in the morning are comparable with the respective values in RIA reported in the previous papers.

Chromogranin A (CgA) is an acidic glycoprotein (3, 8, 18, 19, 20), which is localized in secretory granules of a wide variety of endocrine cells and neurons (7, 16, 17, 23) including chromaffin cells and sympathetic neurons. This protein has been shown to be co-released with catecholamines by exocytosis (1, 2, 10, 18). We have previously developed a region-specific radioimmunoassay (RIA) for human CgA using synthetic CgA (344-374) (15), based on the amino acid sequence reported by Konecki *et al.* (11) and Mouland *et al.* (13), which was extremely useful for the measurement of CgA in the plasma, urine, and saliva in human. On the other hand, synthetic human CgA (344-374) based on the amino acid sequence deduced by Helman *et al.* (9) did not show any parallelism to the standard curve, sug-

gesting that the 344-374 sequence of human CgA deduced by Konecki *et al.* (11) and Mouland *et al.* (13) is the correct one.

The present study describes the development of simple enzyme immunoassay (EIA) for human CgA using synthetic human CgA (344-374), EEEEDNRDSSMKLSFRARAYGFRGPGPQLRR. In the study, we have designed novel human CgA analogue, N^α-biotinylated glycylglycyl-human CgA (344-374) as biotinylated derivative, in which glycylglycyl sequence was inserted as spacer between biotin moiety and human CgA (344-374).

N^α-biotinylated glycylglycyl-human (344-374) was synthesized by solid phase methodology with Fmoc-strategy using an automated peptide synthesizer (Perseptive model 9050 plus, USA), according to the method as described previously (15). The peptide chain was elongated on polyethylene glycol polystyrene resin (0.18 g). After construction of protected human CgA (344-374)

Correspondence to: Dr Noboru Yanaihara at the above address.

Tel: +81-544-22-2771; Fax: +81-544-22-2770

-resin, two Fmoc-glycins were introduced successively to produce protected glycyglycyl-human CgA (344–374)-resin, and after removal of Fmoc moiety, N-hydroxysuccinimideester of biotin (4 equimol, Pierce, USA) was treated with the resulting protected peptide resin to give the desired N $^{\alpha}$ -biotinylated peptide resin.

The N $^{\alpha}$ -biotinylated peptide resin (0.30 g) was treated with trifluoroacetic acid (24 mL) in the presence of *m*-crezol (0.6 mL), 1, 2-ethanedithiol (1.8 mL) and thioanisole (3.6 mL) at 25°C for 120 min. The resin was removed by filtration, anhydrous ethylether was added to the filtrate to give the crude product (39 mg).

The crude peptide was purified by reverse phase HPLC on a column of YMC-Pack D-ODS-5 (2.0 $\times$ 25.0 cm) using 0.01 NHCl/CH₃CN (82/18–74/26 v/v, 30 min) as eluent at a flow rate of 7 mL/min to yield the desired N $^{\alpha}$ -biotinylated glycyglycyl-human CgA (344–374) (6 mg).

Purity of the peptide was assessed by analytical HPLC on a column of YMC Pack R-ODS-5 (0.46 $\times$ 25.0 cm) using 0.01 NHCl/CH₃CN (95/5–40/60, v/v, 30 min) as eluent at a flow rate of 1 mL/min and FAB-MS.

In the development of EIA for human CgA, we used synthetic human CgA (344–374) as standard antigen, anti-human CgA (344–374) serum RY 76 as antiserum and N $^{\alpha}$ -biotinylated glycyglycyl human CgA (344–374) as labeled antigen, respectively.

Goat anti-rabbit IgG (1:200 dilution, ICN Pharmaceuticals, CA, USA) diluted with 0.01 M phosphate buffer was adsorbed onto 96 wells microtiter plates (Maxisorb, framed NUNC, Denmark) overnight at room temperature. The plate was then kept with 25% Block Ace (0.3 mL, Dainihon Pharmaceutical Co. Ltd., Osaka, Japan) at room temperature for 3 h and stored at 4°C before use. All the following experiments were carried at room temperature.

Standard dilution in the present EIA was 0.01 M phosphate buffer (pH 7.4, 50 mL) containing 0.5% (w/v) bovine serum albumin and 0.15 M NaCl and 0.025 ethylenediamine tetraacetate (EDTA). The standard antigen (13.7 fmol/mL–10 pmol/mL) and N $^{\alpha}$ -biotinylated glycyglycyl-human CgA (344–374) (5 ng/mL) were dissolved in standard diluent, respectively. To the wells, standard diluent (50 μ L), standard antigen solution or unknown sample (25 μ L), N $^{\alpha}$ -biotinylated antigen (50 μ L) and rabbit anti-human CgA (344–374) serum RY76 (1:2,500

dilution) (100 μ L) were added in duplicates into wells. The wells were incubated for 3 h under shaking and then washed three times with washing solution (18% NaCl solution containing 1% Tween 20).

Streptoavidin-horseradish peroxidase solution (1:10,000 dilution; Carbiochem, U.S.A.) in standard diluent containing 0.1% Tween 20 (100 μ L), was added to the wells. The wells were incubated for 2 h under shaking. The wells were then washed four times with washing solution (320 μ L each).

O-Phenylenediamine (OPD) solution (100 μ L/well), which was prepared by dissolving OPD (10 mg, Sigma, U.S.A.) in 0.1 M citrate-phosphate buffer (pH 5.0) containing 0.015% H₂O₂ (11 mL), was added. After 30 min under shaking, 1NH₂SO₄ (100 μ L) was added to stop the reaction. The absorbance of each well at 490 nm was measured by a micro-plate reader (BioRad Model 450, U. S. A.).

Fig. 1 shows the standard curve ranging from 13.7 fmol/mL to 10 pmol/mL of CgA-like IR in the present EIA.

Crossreactivities of synthetic human CgA (344–374) having amino acid sequence reported by Helman *et al.* (9) and rat CgA (359–389) (14) were 12.0% and 32.4%, respectively, when compared with that of the standard curve, while the shorter fragments, such as human CgA (360–374) and rat CgA (375–389) did not crossreact in the concentration used, suggesting that the major antigenic determinants reside in the middle part of human CgA (344–374). The crossreaction at 50% B/Bo was used as the crossreactivity. It is noteworthy that the 344–374 sequence of human CgA, which was used as antigen in the present

Fig. 1 Standard curve of human CgA EIA.

The results are plotted as binding (B/Bo,%) versus concentration of added human CgA (344–374) standard.

study, contains a part of human parastatin (6), which corresponds to the 356–428 sequence of human CgA, and synthetic human CgA (352–372) which was reported as catestatin (12). The level of human CgA-like immunoreactivity in fasting normal human plasma were 0.34 ± 0.08 pmol/mL (mean $\pm$ SD, $n=10$). The range of the intra- and inter-assay coefficients of variation were from 2.6% to 8.5% and from 6.4% to 14.7%, respectively. The levels of CgA-like IR in human urine and saliva in the morning were 0.18 ± 0.05 pmol/mL (mean $\pm$ SD, $n=8$) and 0.11 ± 0.05 pmol/mL (mean $\pm$ SD, $n=8$), respectively.

Fig. 2 shows the elution profile of CgA-like IR in human plasma.

Levels of CgA-like IR in plasma of patients with humoral hypercalcemia of malignancy (HHM) and pheochromocytoma showed 3.28 pmol/mL ($n=1$) and 3.40 pmol/mL ($n=1$), respectively. These levels were markedly higher than those in the plasma of human healthy volunteers. Recovery of synthetic human CgA (344–374) added to plasma ranged from 85.4% to 99.3%.

CgA has been known to be a useful marker of neuroendocrine tumors in humans (15, 21, 22, 24). The present study provides a simple EIA method for determination of CgA-like IR in human biologic fluid. The detection limit of the present EIA was comparable to that of RIA using same synthetic human CgA (344–374) as antigen

Fig. 2 Elution profile of CgA-like immunoreactivity in human plasma.

Column: Superdex 75HR (10/30), eluent: 0.14 M NaCl containing 10 mM Na-phosphate (pH 7.4)(0.5 mL/fraction).

Fig. 3 Correlation between levels of CgA-like immunoreactivity measured by EIA and RIA in human plasma ($r^2=0.935$).

as reported previously (15). We found a significant positive correlation between levels of CgA-like IR measured by EIA and RIA using same antigen (Fig. 3). Although several enzyme-linked immunoassays have been reported (4, 5, 21, 22), the present study provided a simple and sensitive EIA method for measurement of human CgA-like IR with use of synthetic N $^{\alpha}$ -biotinylated glycylglycyl-human CgA (344–374) and anti-synthetic human CgA (344–374) serum, RY 76.

In conclusion, we have found that the present EIA developed using synthetic human CgA (344–374) and its derivative is sensitive and reproducible and will be a useful tool in the determination of CgA in plasma, saliva, and urine in humans.

Received 10 October 1998; and accepted 16 November 1998.

REFERENCES

1. BANKS P. and HELLE K. (1965) The release of protein from the stimulated adrenal medulla. *Biochem. J.* **97**, 40c–41c.
2. BARBOSA J. A., GILL B. M., TAIKIYUDDIN M. A. and O'CONNOR D. T. (1991) Chromogranin A: posttranslational modifications in secretory granules. *Endocrinology* **128**, 1174–1190.
3. BLASCHKO H., COMLINE R. S., SCHNEIDER F. H., SILVER M. and SMITH A. D. (1967) Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature* **215**, 58–59.
4. BOOMSMA F., BHAGGOE U. M., Man in't VELD A. J. and SCHALEKAMP M. A. D. H. (1995) Sensitivity and specificity of a new ELISA method for determination of chromogranin A in the diagnosis of pheochromocytoma and neuroblastoma. *Clin. Chim. Acta*

- 239, 57-63.
5. DILLEN L., DE BLOCK J., VANLEAR L. and DE POTTER W. (1989) Enzyme-linked immunosorbent assay for chromogranin A. *Clin. Chem.* **35**, 1934-1938.
6. FASCIOTTO B. H., TRAUSS C. A., GREELEY G. H. Jr. and COHN D. V. (1993) Parastatin (porcine chromogranin A (347-419), a novel chromogranin A-derived peptide, inhibits parathyroid cell secretion. *Endocrinology* **133**, 461-466.
7. FISCHER-COLBRIE R., LASSMANN H., HAGN C. and WINKLER H. (1985) Immunological studies on the distribution of chromogranin A and B in endocrine and nervous tissues. *Neuroscience* **16**, 547-555.
8. HELLE K. B. (1966) Some chemical and physical properties of the soluble protein fraction of bovine adrenal chromaffin granules. *Mol. Pharmacol.* **2**, 298-310.
9. HELMAN L. J., AHN T. G., LEVINE M. A., ALLISON A., COHEN P. S., COOPER M. J., COHEN D. V. and ISRAEL M. A. (1988) Molecular cloning and primary structure of human chromogranin A (secretory protein I) cDNA. *J. Biol. Chem.* **263**, 11559-11563.
10. KIRSCHNER N., SAGE H. J., SMITH W. J. and KIRSCHNER A. G. (1966) Release of catecholamines and specific protein from adrenal glands. *Science* **154**, 529-531.
11. KONNECKI D. S., BENEDUM U. M., GERDES H.-H., and HUTTNER W. B. (1987) The primary structure of human chromogranin A and pancreastatin. *J. Biol. Chem.* **262**, 17025-17030.
12. MAHATA S. K., MAHATA M., YOO S. H., TAUPENOT L., WU H., ARODA V. R., LIVSEY C. V., TAULANE J. P., GOODMAN M., PARMER R. J. and O'CONNOR D. T. (1996) A novel catecholamine release-inhibitory peptide from chromogranin A: autocrine control of nicotinic cholinergic-stimulated exocytosis. *Pharmacology* **42**, 260-264.
13. MOULAND A. J., BEVAN S., WHITE J. H. and HENDY G. N. (1994) Human chromogranin A gene. Molecular cloning, structural analysis, and neuroendocrine cell-specific expression. *J. Biol. Chem.* **269**, 6918-6926.
14. NAGASAWA N., NISHIKAWA Y., OHSHIMA K., IGUCHI K., MOCHIZUKI T., GREELEY G. H. Jr. and YANAIHARA N. (1995) Distribution of chromogranin A-like peptides in the rat. *Biomed. Res.* **16**, 83-90.
15. NISHIKAWA Y., LI J., FUTAI Y., YANAIHARA N., IGUCHI K., MOCHIZUKI T., HOSHINO M. and YANAIHARA C. (1998) Region-specific radioimmunoassay for human chromogranin A. *Biomed. Res.* **19**, 245-251.
16. O'CONNOR D. T. (1983) Chromogranin: widespread immunoreactivity in polypeptide hormone producing tissues and in serum. *Regul. Pept.* **6**, 263-280.
17. O'CONNOR D. T., BURTON D. and DEFTOS L. J. (1983) Chromogranin A: immunohistology reveals its universal occurrence in normal polypeptide hormone producing endocrine glands. *Life Sci.* **33**, 1657-1663.
18. SCHNEIDER F. H., SMITH A. D. and WINKLER H. (1967) Secretion from the adrenal medulla: biochemical evidence for exocytosis. *Br. J. Pharmac. Chemother.* **31**, 94-104.
19. SMITH A. D. and WINKLER H. (1967) Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J.* **103**, 483-492.
20. SMITH W. J. and KIRSCHNER N. (1967) A specific soluble protein from the catecholamine storage vesicles of bovine adrenal medulla. Purification and chemical characterization. *Mol. Pharmacol.* **3**, 52-62.
21. SYVERSEN U., JACOBSEN M. B., O'CONNOR D. T., RØNNING K. and WALDUM H. L. (1994) Immunoassay for measurement of chromogranin A and pancreastatin-like immunoreactivity in humans: correspondence in patients with neuroendocrine neoplasia. *Neuropeptides* **26**, 201-206.
22. SYVERSEN U., MIGNON M., BONFIL S. S., KIRSTENSEN A. and WALDUM H. L. (1993) Chromogranin A and pancreastatin-like immunoreactivity in serum of gastrinoma patients. *Acta Oncol.* **32**, 161-165.
23. WILSON B. S. and LLOYD R. V. (1984) Detection of chromogranin in neuroendocrine cells with a monoclonal antibody. *Am. J. Pathol.* **115**, 458-468.
24. YANAIHARA N., NISHIKAWA Y., HOSHINO M., MOCHIZUKI T., IGUCHI K., NAGASAWA S., LI J., FUTAI Y., KANNO T., YANAIHARA H., MURAI M. and YANAIHARA C. (1998) Evaluation of region-specific radioimmunoassays for rat and human chromogranin A: measurement of immunoreactivity in plasma, urine and saliva. In *The Adrenal Chromaffin Cell: Archetype and Exemplar of Cellular Signalling in Secretory Control* (ed. KANNO T., NAKAZATO Y. and KUMAKURA K.) Hokkaido University Press, Sapporo, Japan, pp. 305-313.