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Time-resolved fluoroimmunoassay for pituitary adenylate cyclase activating polypeptide 27 (PACAP27) using europium (III) ion chelate labeled streptavidin-biotin complex[1]

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) is a novel peptide hormone and has a variety of biological action. In studies of the physiological behaviour of endogenous PACAP, the determination of PACAP levels in biological materials require a highly sensitive and specific method. Therefore, we developed a sensitive time-resolved fluoroimmunoassay (TR-FIA) for PACAP27 which is the biologically important fragment of PACAP. Accordingly, we developed TR-FIA using a biotinylated PACAP27 (b-PACAP27) as a tracer and europium (III) chelate labeled streptavidin-biotinylated bovine serum albumin complex as a detection of biotin on solid phase. A measurable range of PACAP27 was 7.8–1000 pg ml^{-1} by the proposed TR-FIA. For measurement of biological samples, the samples were purified to eliminate substances which interfered with the TR-FIA. The mean recovery of PACAP27 using commercially reversed phase column was 74.7% ($n = 12$). The various tissues, extracts and plasma concentrations of rat could be measured by the proposed TR-FIA. © 1997 Elsevier Science B.V.

Keywords: Pituitary adenylate cyclase activating polypeptide 27 (PACAP27); Time-resolved fluoroimmunoassay; Europium (III) chelate labeled streptavidin; Rat tissues contents; Rat plasma concentration

1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP), originally isolated from ovine hypothalamus, is a new member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family [1]. PACAP has been demonstrated in two forms, one of 38 amino acid residues (PACAP38) [1] and the other corresponding to the *N*-terminal 27 amino acid residues of PACAP38 (PACAP27) [2]. The *N*-terminal 28 amino acid residues of PACAP38 have 68% homology with VIP, but its adenylate cyclase stimulating activity in rat pituitary cell cultures is 1000 times greater than VIP [1]. In studies of the physiological behaviour of a endogenous PACAP, the determination of PACAP levels in biological

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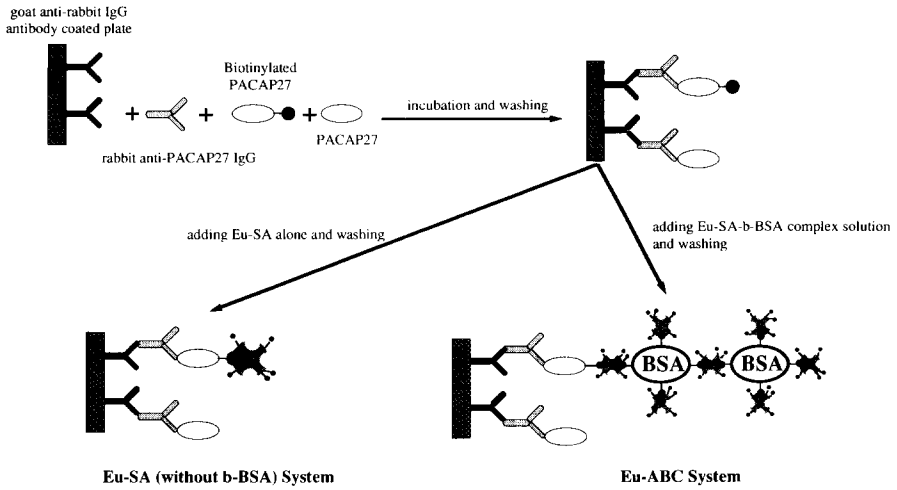

Scheme 1. Schematic illustration for immunoassay systems of the proposed method.

materials require a sensitive and specific method. Therefore, we attempted to develop a highly sensitive non-isotopic immunoassay for PACAP27, in order to eliminate the disadvantages of radioimmunoassay (RIA) associated with the use of radioisotopes. The immunoassay which we developed here, is based on competition between a free PACAP27 and a biotinylated PACAP27 (*b*-PACAP27) as a tracer for rabbit anti-PACP27 antibodies on a goat anti-rabbit IgG antibody coated microtiter plate. After separation of bound and free fractions, the *b*-PACAP27 bound on the microtiter plate was reacted with europium (III) ion chelate labeled streptavidin (Eu-SA)-biotinylated bovine serum albumin (*b*-BSA) complex (Eu-ABC) solution as a enhanced system or Eu-SA (without *b*-BSA) solution. The microtiter plate was washed and then the europium (III) ion was measured by time-resolved fluorometry [3,4] shown in Scheme 1. In addition, we attempted to measure biological samples by the proposed method.

2. Material and methods

2.1. Materials

Synthetic PACAP27, PACAP38 and other polypeptides were purchased from the Peptide Institute, (Osaka, Japan), rabbit anti-PACAP27 (human, ovine, rat) antiserum was purchased from Peninsula laboratories (Belmont, CA), streptavidin, aprotinin and Biotinamidocaproate *N*-hydroxysuccinimde ester (NHS-LC-biotin) were purchased from Sigma (St. Louis, MO), Block Ace was purchased from Dainipponseiyaku Co. (Osaka, Japan), N^1-(*p*-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetic acid europium (III) ion chelate (DTTA-Eu) was purchased from Wallac Oy (Turku, Finland). An affinity purified goat anti-rabbit IgG antibody and TSK-gel ODS 120T column were obtained from Tosoh (Tokyo, Japan).

2.2. Solutions

Immunoreaction buffer: 0.1 M Tris–HCl buffered saline (TBS) (pH 7.0) containing 1 mg

ml^{-1} EDTA, 0.05% Tween 20, 10% Block Ace, 200 KIU (kallikrein inhibitor unit) ml^{-1} aprotinin and 0.05% NaN_3. Washing buffer: 50 mM TBS (pH 7.0) containing 0.05% Tween 20. Assay buffer: 0.1 M Tris-HCl buffer (pH 7.75) containing 0.15 M NaCl, 0.05% NaN_3, 20 μM diethylenetriaminepentaacetic acid (DTPA), 0.5% bovine serum albumin (BSA), 0.05% bovine γ-globulin and 0.01% Tween 40. Enhancement solution: 0.1 M acetate-phathalate buffer (pH 3.2) containing 0.1% Triton X-100, 15 μM 2-naphthoyltrifuluoroacetone and 50 μM tri-*n*-octylphosphine oxide. Assay buffer and Enhancement solution were purchased from Wallac Oy (Turku, Finland).

2.3. Preparation of biotinylated PACAP27 (b-PACAP27)

Synthetic PACAP27 was biotinylated and purified according to the slightly modified method of Newman et al. [5]. Briefly, PACAP27 (0.1 mg) was dissolved in 400 μl of 0.25 M carbonate buffer (pH 9.0), 10 μl of NHS-LC-biotin (0.014 mg) solution in DMSO was added and incubated at room temperature for 1 h. Then, the reaction was stopped by adding 100 μl of 1M Tris–HCl buffer (pH 8.0). After reaction for 10 min at room temperature, the reaction mixture was purified by reversed phase HPLC column (TSK-gel ODS 120 T, 7.8 mm i.d. × 300 mm) using 0.1% trifluoroacetic acid/CH_3CN gradient system. The fraction of *b*-PACAP27 having high immunological and biotin binding activities was collected, dried and reconstituted by immunoreaction buffer for use.

2.4. Preparation of europium (III) chelate labeled streptavidin (Eu-SA) and biotinylated BSA (b-BSA)

A streptavidin (1 mg) was dissolved in 900 μl of 0.1 M carbonate buffer (pH 9.5) and reacted with 100 μl of DTTA-Eu (2 mg ml^{-1}) for 16 h at room temperature. After incubation, the reaction was stopped by adding 100 μl of 1 M Tris-HCl buffer (pH 8.0). The europium (III) chelate labeled streptavidin (Eu-SA) was purified on a Sephadex G-25 (Pharmacia BioProcess Technology AB, Uppsala, Sweden) column (1.4 i.d. × 40 cm) equilibrated and eluted with 0.05 M Tris-HCl buffered saline (pH 7.0) containing 0.05% NaN_3. We collected 0.5 ml fractions and added 10 μl of 7.5% BSA solution. The fractions of Eu-SA having with high biotin binding activity and time-resolved fluorescence intensity were stored at −20°C prior to use.

A bovine serum albumin (BSA) was biotinylated by mixing 1 ml of BSA solution (1 mg ml^{-1}) in 0.1 M carbonate buffer (pH 9.5) and adding 50 μl of NHS-LC-biotin DMSO solution (8.26 mg ml^{-1}) for standing 16 h at room temperature. After incubation, the reaction was stopped by adding 100 μl of 1 M Tris-HCl buffer (pH 8.0). The excess biotin derivative was removed by passing the reaction mixture through the same column as for purification of Eu-SA. The biotinylated BSA fractions were stored at −20°C before use.

2.5. Preparation of goat anti-rabbit IgG antibody coated plate

The wells of microtiter plates were coated with 200 μl of an affinity purified goat anti-rabbit IgG antibody (20 μg ml^{-1}) in 50 mM sodium bicarbonate buffer (pH 9.5). The plates were allowed to stand at room temperature for 16 h. The IgG solution was removed and the wells were then post-coated by adding 250 μl of a 1% BSA solution containing 0.05% NaN_3 and 5 mM DTPA. The plates were stored at 4°C prior to use and remained stable for longer than 6 months.

2.6. Time-resolved fluoroimmunoassay (TR-FIA)

An aliquot of 50 μl of the standard PACAP27 or the sample solution, 50 μl of the rabbit anti-PACAP27 antiserum solution and 50 μl of immunoreaction buffer were added to a goat anti-rabbit IgG antibody coated microtiter plate. After incubation for 16 h at 4°C, 50 μl of *b*-PACAP27 was added and ioncubated at 4°C overnight. Then the plate was washed three times using the washing buffer, a *b*-PACAP27 bound on the microtiter plate was reacted with Eu-SA-*b*-BSA complex (Eu-ABC) solution. The Eu-ABC solution was prepared that 5 μl of Eu-SA (5.2

μM) solution added to 400 μl of b-BSA solution (9.37 nM) and incubated for 30 min at room temperature. We pipetted 100 μl of diluted Eu-ABC solution (1:50) to the microtiter plate which terminated immunoreaction. The microtiter plate was incubated at room temperature for 30 min and washed. Then, the europium (III) ion bound to wells of the microtiter plate was dissociated by adding 200 μl of the enhancement solution. After shaking for 5 min, the time-resolved fluorescence was measured with a Model 1230 Arcus fluorometer (Wallac Oy, Turku, Finland).

2.7. Extraction of PACAP27 from rat tissues

Extraction of PACAP27 from rat tissues was carried out according to the slightly modified method reported by Arimura et al. [6]. Various tissues were collected from SD strain rats of either sex, 300–400 g body weight (Saitama Experimental Animals Supply Co., Ltd., Saitama, Japan) after perfused with saline containing 1 mg ml^{-1} EDTA and 200 KIU ml^{-1} aprotinin. Whole or 250 mg aliquot of each of tissues were placed into a polypropylene tube, and added 2.5 ml of cold distilled water. Then, the tissues were boiled for 10 min on oil bath, cooled on ice bath, and 75 μl of acetic acid was added to the each tube. The tissues were then homogenized by Polytron homogenizer (Knematica, Switzerland), added 2.5 ml of distilled water and incubated at 4°C overnight. The extract was centrifuged at 3000 rpm for 30 min at 4°C and filtrated by disposable syringe filter unit (0.45 μm, cellulose acetate membrane, Toyo Roshi Kaisha, Ltd., Japan). The extract was pretreated by using Sep-pak C18 cartridge (Millipore, MA, USA) before assay.

2.8. Pretreatment of samples

Tissue extract (4 ml) or 4 ml of diluted plasma (1 ml of plasma was added 3 ml to 0.2% trifluoroacetic acid (TFA)) were applied to Sep-pak C18 cartridge, which was pretreated with 0.2% TFA in 60% CH_3CN, methanol, distilled water and 0.2% TFA. After washing the column with 0.2% TFA and distilled water, the PACAP in the cartridge was eluted with 3 ml of 0.2% TFA in 60% CH_3CN. The eluate was placed in a polypropylene tube and evaporated under a stream of nitrogen at 40°C. The residue was dissolved in 1 ml of assay buffer and measured with the proposed TRA-FIA.

3. Results and discussion

Recently, TR-FIA was developed and widely used for measurement of various hormones and drugs in serum or blood. In TR-FIA [3,4], an europium (III) ion chelate is used as a label which has large Stokes shift, narrow emission bands and long fluorescence decay time (over 10^4 times longer than the average background fluorescence). Therefore, the use of the europium (III) ion as a label in TR-FIA should remarkably reduce the background fluorescence arising from serum protein and plastic which has limited the sensitivity of conventional fluoroimmunoassay, and make highly sensitive measurements possible. We have also reported the determination of cholecystokinin [7] and secretin [8,9] in biological samples by using europium (III) labels.

Since the late 1970s, the exceptional affinity ($KA = 10^{15}$ M^{-1}) of the biotin-avidin system has been employed in immunoassay systems [10]. After *N*-Hydroxysuccinimide ester derivatives of biotin became commercially available, these were easily coupled to ε-amino groups of proteins such as antibodies or peptide-hormones involved in the immunoassay reaction. Biotinylated tracers, in contrast to ^{125}I-labeled tracers, offer the advantage of virtually unlimited stability and their specific activity is not affected by storage time. In conventional enzyme immunoassay, a tracer was an antigen or antibody conjugated with various enzyme. When an enzyme conjugate is prepared, a homo- or heteropolymer may be formed and interfere with highly sensitive assay by non-specific binding. However, we used *b*-PACAP27 as a tracer which was lower molecular weight, easy to prepare and displays low non-specific binding. Through the *b*-PACAP27 was stored at −20°C prior to use, it is possible that it is stable for longer than one year under these conditions. The

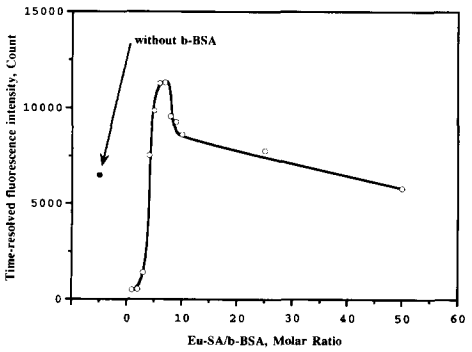

Fig. 1. Effect of europium (III) chelate labeled streptavidin and biotinylated BSA molar ratio on streptavidin-biotinylated BSA complex formation.

b-PACAP27 is considered more useful and stable than ^{125}I-labeled tracer with RIA.

3.1. Standard curve

We have attempted to use Eu-SA-*b*-BSA complex (Eu-ABC) for detection of *b*-PACAP27 on solid phase after immunoreaction. The conditions for Eu-ABC formation were optimized in series of experiments that included testing the effects of varying the molar ratios of Eu-SA and *b*-BSA. As shown Fig. 1, the optimal condition for assembly of Eu-ABC formation was 7:1 of a molar ratio for Eu-SA and *b*-BSA. In our proposed TR-FIA, the obtained fluorescence intensity with use the Eu-ABC as a detection system was 2-fold higher than use Eu-SA alone (without *b*-BSA) (Fig. 2). When

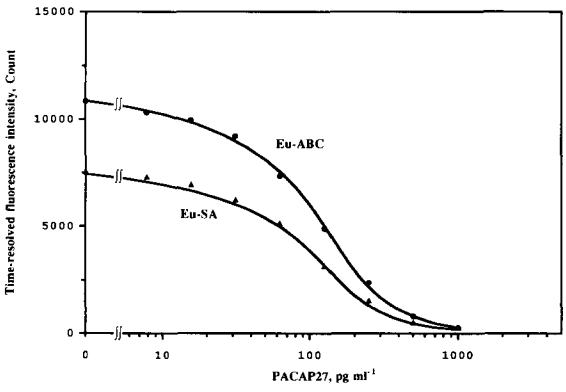

Fig. 2. Comparision of standard curves for PACAP27 using Eu-SA and Eu-ABC as a detection system.

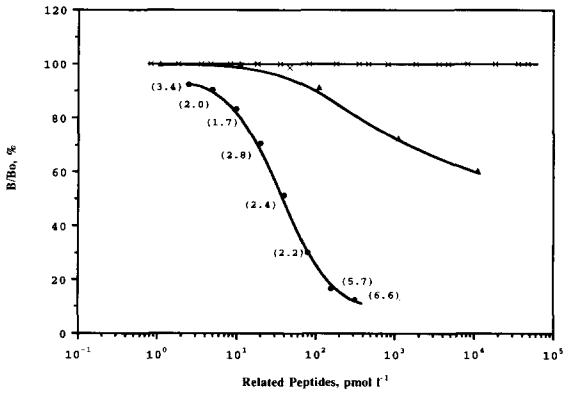

Fig. 3. Cross-reactivities of PACAP related peptides by the proposed TR-FIA ●: PACAP27, ▲: PACAP38, ×: VIP, secretin, adrenomedullin and PACAP fragments 16–38, 24–38, 28–38, 31–38. Values in parenthesis represents was percent of relative standard deviation ($n = 8$).

we used Eu-ABC as a detection system, the measurable range of PACAP27 was 7.81–1000 pg ml^{-1}. Although different anti-PACAP27 antiserum was used in the assay, a sensitivity obtained by the proposed TR-FIA was about 10-fold better than that obtained by RIA reported by Arimura [6]. The within-assay precision profiles for 8 replicates with each points of standard for PACAP27 were 1.7–6.6% (Fig. 3), respectively, and the between-assay precision profiles for 7 replicates value was 14.8% at value of the concentration yielding 50% inhibition (IC50).

3.2. Cross-reactivities

We determined the specificity of the assay by measuring the cross-reactivities of the following related peptide hormones and PACAP fragments: PACAP38, PACAP16-38, 0ACA024-38, PACAP28-38, PACAP31-38, VIP, secretin and adrenomedullin (Fig. 3). Cross-reactivities of the antiserum were observed with PACAP38 (0.3%), which contains amino acid sequence of PACAP27 in their molecule. However, VIP and other peptides each had less than 0.1%

3.3. Recovery test and linearity of sample dilution

For determination of PACAP27, samples require pretreatment of non-specific interference with the tissue extract and plasma matrix in the

Table 1
Analytical recoveries of PACAP27 from rat testis by the proposed TR-FIA

Added (pg ml^{-1})	Determined (pg ml^{-1})	Found (pg ml^{-1})	Recovery (%)
0	17.9 ± 1.6		
31.25	41.0 ± 3.1	23.1	74.0
125	124.5 ± 1.4	106.6	85.3
500	342.5 ± 12.6	324.6	64.9
		mean ± S.D% (n = 12)	75.7 ± 10.2

proposed TR-FIA. Therefore, for the measurement of PACAP27, we attempted to eliminate the interference with use of reversed-phase solid column as a Sep-pak C18 cartridge. In the recovery test, we selected a rat testis as a target tissues. The rat testis extracts were spiked with different known quantities of PACAP27, pretreated and determined. As shown in Table 1, the mean recovery of PACAP27 was 74.7 ± 10.2% (n = 12).

Four samples of rat testis extracts after pretreatment with Sep-pak C18 cartridge were serially diluted with immunoreaction buffer (until 1:8) and measured. Good linearity was found with each sample at the relation of extent of dilution and assay result. The results were satisfactory.

3.4. Measurement of biological samples

We applied the proposed TR-FIA for PACAP27 to determination of biological samples from rat tissue. The PACAP27 concentrations in various tissues of SD strain rats of either sex, 300–400 g body weight were measured (Table 2). The concentrations of PACAP27 in rat tissues showed a distinction between the proposed method and RIA [6]. The distinction appeared to use another anti-PACAP27 antiserum in the assay and deferent conditions of rat which was perfused in our study. However, we found highest concentration of PACAP27 in hypothalamus, and other brain areas also were found higher concentration than other tissues. This result was similarly distribution of PACAP27 which was measured by using RIA [6]. Further, the plasma PACAP27 levels of normal rat (not fasted) could be measured by the proposed method and the mean level was 19.1 ± 10.4 pg ml^{-1} (n = 5).

4. Conclusions

In conclusion, we developed a sensitive TR-FIA for the determination of PACAP27. The proposed TR-FIA method makes use of a stable biotinylated PACAP27 as a labeled antigen and is free of the typical disadvantages of RIA systems. The sensitivities of the PACAP27 assay are 10-fold higher than that of RIA. When the proposed TR-FIA was applied to the determination of tissue extract of rat, brain areas were found to contain higher concentrations than other peripheral tissues.

Table 2
The PACAP27 levels in various rat tissues using the proposed TR-FIA

Rat tissue region	Mean ± S.D. (ng g^{-1} wet tissue)
Hypothalamus	5.51 ± 0.83
Cortex	1.83 ± 0.06
Hippocampus	1.47 ± 0.23
Anterior pituitary	2.58 ± 0.65
Posterior pituitary	3.35
Lung	0.19 ± 0.06
Atrium	0.18 ± 0.06
Liver	0.17 ± 0.06
Spleen	0.18 ± 0.08
Pancreas	0.22 ± 0.06
Stomach	0.44 ± 0.04
Duodenum	0.33 ± 0.06
Jejunum	0.32 ± 0.07
Ileum	0.36 ± 0.08
Colon	0.35 ± 0.06
Kidney	0.16 ± 0.05
Adrenal gland	0.44 ± 0.10
Testis	0.36 ± 0.03
Epididymis	0.09 ± 0.04
Ovary	0.26 ± 0.04
	(n = 6)

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