Enzyme Immunoassay for Oxytocin

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Abstract

A competitive, double antibody enzyme immunoassay for oxytocin in a heterologous system was developed. Horseradish peroxidase was conjugated with oxytocin using N-succinimidyl 3-(2-pyridyldithio) propionate, and rabbit anti-oxytocin serum was produced by immunization of oxytocin-bovine serum albumin complex which was prepared by the carbodiimide method.

The sensitivity of the assay was 4 μ IU/tube, which corresponded to 10 μ IU per ml using 400 μ l of the sample which was extracted from the same volume of plasma by means of SEP-PAK C₁₈ cartridges.

The coefficients of variation for different levels of oxytocin ranged from 6.8–15.9% and 8.5–16.7%, for intra- and inter-assay. Recovery of oxytocin added to plasma after extraction was 99–117%.

No or little cross-reaction with arginine- and lysine-vasopressin was found.

Plasma oxytocin concentrations determined by the proposed enzyme immunoassay were well correlated with those determined by radioimmunoassay ($r=0.90$).

Oxytocin is a small peptide hormone that consists of nine amino acid residues, and is secreted from the posterior lobe of the pituitary.

In recent years, radioimmunoassay (RIA) has been used to measure oxytocin (Yamaji *et al.*, 1981). However, enzyme immunoassay (EIA) which can be done in routine

laboratory is preferred to RIA. This paper describes EIA for the measurement of oxytocin in plasma using oxytocin-horseradish peroxidase conjugate.

Materials and Methods

Buffers

0.1 M Sodium phosphate buffers, pH 6.0 and pH 7.0, were mainly used (buffer A and buffer B, respectively). The buffer used for the dilution of oxytocin and antiserum, was 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% bovine

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serum albumin (buffer C).

Chemicals

The following materials were purchased from commercial sources.

N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), Sephadex G-10, G-25, G-75, (Pharmacia Fine Chemicals AB, Uppsala, Sweden); BSA (Armour Pharmaceutical Co., Illinois); SEP-PAK C₁₈ (Water Associates Inc., Milford); 3-(4-hydroxyphenyl) propionic acid (HPPA), 4, 4-dithiodipyridine (4-PDS) (Dojindo Laboratories, Kumamoto, Japan).

Other chemicals used were obtained from Nacalai Tesque Inc., Kyoto, Japan.

Enzyme

Horseradish peroxidase (Grade I, 250 U/mg) was obtained from Boehringer Mannheim GmbH, Mannheim, FRG.

Hormones

Synthetic oxytocin (400 IU/mg) was obtained from Peptide Institute Protein Research Foundation, Osaka, Japan. Synthetic arginine-vasopressin (350 IU/mg) and lysine-vasopressin (200 IU/mg) were obtained from Sigma Chemicals Co., St Louis, Missouri.

Antiserum

Anti-oxytocin serum was obtained from rabbits immunized with oxytocin-BSA complex which was prepared with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (Yamaji *et al.*, 1981). This antiserum could bind 50% of ¹²⁵I-oxytocin at a dilution of 1:6000.

Goat anti-rabbit γ -globulin serum was obtained from Eiken Chemicals Co., Tokyo, Japan. This antiserum was dialysed against 0.05 M sodium phosphate buffer, pH 7.5, at 4°C overnight to remove NaN₃ that inactivated horseradish peroxidase.

Determination of pyridyl disulfide and thiol groups

Pyridyl disulfide groups of samples were determined by incubating with an excess amount of dithiothreitol and then measuring the absorbance of the 2-thiopyridone at 343 nm (E_{343} 8080 M⁻¹ cm⁻¹) produced (Carlsson *et al.*, 1978).

Thiol groups were determined with 4-PDS (Grassetti and Murray, 1967). Absorbance of the 4-thiopyridone produced was measured at

324 nm (E_{324} 19800 M⁻¹ cm⁻¹).

Preparation of SPDP-acylated oxytocin

Oxytocin (0.5 mg in 0.5 ml of buffer B) was mixed with 0.78 mg (5 fold molar excess) of SPDP in 60 μ l of ethanol, and the mixture was incubated at 25°C for 30 min. The reaction mixture was subjected to gel-filtration on a column (1 $\times$ 20 cm) of Sephadex G-10 using buffer A, and fractions containing oxytocin, which were monitored by absorbance measurement at 275 nm, were collected.

The number of pyridyl disulfide groups introduced to oxytocin was calculated to be 0.7 per oxytocin molecule by assuming that the recovery of oxytocin was 100%.

Preparation of SH-derivative of peroxidase

Peroxidase (0.5 mg) was reacted with 1.95 mg of SPDP (50 fold molar excess) to produce SPDP-acylated peroxidase as described above. Gel-filtration was performed on a Sephadex G-25 medium column using buffer A. Fractions containing peroxidase which were monitored by absorbance measurement at 403 nm, were collected. The number of pyridyl disulfide groups introduced to peroxidase was 2.3 per peroxidase molecule. (E_{403} and E_{343} of the enzyme were taken to be 91000 M⁻¹ cm⁻¹ and 23000 M⁻¹ cm⁻¹, respectively.)

The SPDP-acylated peroxidase solution (45.4 nmol in 1.5 ml) was incubated with 50 μ l of dithiothreitol (5 μ mol in distilled water) at 25°C for 90 min. Then the reaction mixture was subjected to gel-filtration on a column (1 $\times$ 20 cm) of Sephadex G-25 medium using buffer A containing 5 mM EDTA. Fractions containing peroxidase were collected. The number of thiol groups introduced to peroxidase was calculated to be 2.3 per peroxidase molecule by assuming that E_{524} of peroxidase was 13700 M⁻¹ cm⁻¹.

Preparation of oxytocin-peroxidase conjugate

SPDP-acylated oxytocin (1.7 ml corresponding to 200 nmoles of oxytocin) was added to 1 ml of SH-derivative of peroxidase (corresponding to 13.5 nmoles of peroxidase). The mixture was incubated at 25°C for 20 hours and subjected to gel-filtration on a column (2 $\times$ 17.5 cm) of Sephadex G-75 using buffer A.

The eluate was collected as 1.0 ml fractions and 10 μ l of each fraction was used to monitor peroxidase activity. A single peak of peroxidase

activity was obtained. The three highest activity fractions were combined and stored at 4°C in the presence of 0.1% BSA and 0.002% merthiolate.

Standard oxytocin

Oxytocin was stored as a 100 mIU/ml stock solution in 0.1 M acetic acid at 4°C. Standard oxytocin (5, 10, 30, 100, 300, 1000 μ IU/ml) was prepared by diluting the stock solution with buffer C.

Extraction for oxytocin from blood plasma

The procedure used in extracting oxytocin from blood plasma employed SEP-PAK C_{18} cartridges according to the method of Schams (1983).

The cartridge was pre-wet with 2 ml of methanol and then with about 5 ml of distilled water. The mixture of 2 ml of blood plasma and 2 ml of 0.05 N HCl was centrifuged, and the supernatant was applied to the cartridge.

Most of the blood plasma proteins were eluted with 10 ml of 1.5% acetic acid, pH 4.8. Thereafter, oxytocin was eluted with 1.5 ml methanol and collected in glass tubes. The solvent was then evaporated to dryness under an air stream at 40°C, and the residue was dissolved in 2 ml of buffer C.

Procedure of EIA

To a polystyrene tube (10×70 mm), 400 μ l of standard oxytocin or extracted sample, 100 μ l of anti-oxytocin serum diluted 24000-fold with buffer C and 50 μ l of the oxytocin-peroxidase conjugate diluted 2000-fold with buffer A containing 0.1% BSA, were added. The reaction mixture was incubated at 4°C for 3 days. Then 200 μ l of goat anti-rabbit γ -globulin serum, diluted 30-fold with buffer C containing 1% normal rabbit serum, was added and allowed to react at 4°C overnight. At the end of the incubation, the solution was centrifuged at 3000 rpm for 30 min at 4°C and the precipitate was washed once with 2 ml of buffer B. The precipitate was suspended in 50 μ l of the same buffer.

Peroxidase activity in the suspension was determined fluorometrically (Zaitse and Ohkura, 1980). Fifty μ l of the suspension was mixed with 0.25 ml of 0.5% (w/v) HPPA in buffer B, and incubated at 30°C for 5 min. Fifty μ l of 0.03% hydrogen peroxide was then added. After incubating the mixture at 30°C for 90 min, the re-

action was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3.

Fluorescence intensity was measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm with a fluorophotometer (RF-510, Shimadzu Seisakusho, Ltd., Kyoto, Japan).

RIA Procedure

Plasma samples were extracted with SEP-PAK C_{18} cartridges described above and assayed for oxytocin by a competitive, double antibody RIA (Yamaji *et al.*, 1981). Oxytocin was labelled with ^{125} I by the chloramine T method and anti-oxytocin serum was the same as used for EIA.

Results

Standard curve

A typical standard curve for the assay of oxytocin is shown in Fig. 1.

The sensitivity was taken as the minimal amount of oxytocin which gave peroxidase activity bound significantly less than that bound in the absence of oxytocin (B_0). Peroxidase activity bound in the presence of 4 μ IU of oxytocin per tube was significantly lower than that in B_0 (*t*-test, $p < 0.05$, $n=6$). Since 400 μ l of the sample was used, the sensitivity was calculated as 10 μ IU/ml which corresponded to 25 pg/ml, and the measurable range of oxytocin was 10 to 1000 μ IU/ml of sample.

Specificity

Cross-reactivities with synthetic arginine- and lysine-vasopressin also are shown in Fig. 1. Little inhibition or none at all was observed with concentrations of up to 1000 μ IU/ml.

Accuracy

The recovery test was performed by adding 25, 50 and 100 μ IU of oxytocin to 1 ml of pooled plasma. The oxytocin concentrations of pooled plasma with oxytocin added were then determined by the pro-

Fig. 1. A typical standard curve for the enzyme immunoassay for oxytocin (○—○) and cross-reactivities with arginine-vasopressin (△—△) and lysine-vasopressin (▲—▲). Percent bound is calculated as follows;

$$\text{Percent bound} = \frac{(B - N)}{(B_0 - N)} \times 100$$

where B is peroxidase activities in the precipitates using various concentrations of standard oxytocin and B_0 is peroxidase activity in the absence of oxytocin. N is a blank value. Each point in a standard curve for oxytocin represents the mean value of 6 replicate determinations and vertical bars indicate standard deviations.

Table 1. Recovery of Oxytocin Added to Plasma

Sample No.	Oxytocin concentration (μIU/ml)*			Recovery (%)**		
	Pooled plasma	Added (a)	Observed (b)	A	B	N
1	nd	0	nd	—	—	5
2	nd	25	20.1 ± 3.2	80	—	5
3	nd	50	44.8 ± 6.9	90	99	5
4	nd	100	108.0 ± 7.3	108	117	5

* nd: Not detectable (Below 10 μIU/ml)

** A = $(b) \times 100 / (a)$ (Recovery is calculated against sample No. 1 assuming that oxytocin concentration of pooled plasma is 0 μIU/ml.)

B = $[(b) - 20] \times 100 / [(a) - 25]$ (Recovery is calculated against sample No. 2.)

posed method.

Since the oxytocin concentration of the pooled plasma was below sensitivity (<10 μIU/ml), the recovery was calculated by two methods (A and B) as shown in Table 1.

The recovery values ranged from 80 to

108% (A), and 99 and 117% (B), respectively.

Precision

Precision tests were performed by measuring oxytocin concentrations in oxytocin added samples at different levels.

As shown in Table 2, the coefficients of

Fig. 2. Correlation between oxytocin concentrations in extracts of plasma determined by enzyme immunoassay (Y) and radioimmunoassay (X).

Table 2. Intra-assay and Inter-assay Variations

Assay	Plasma	Oxytocin concentration* ($\mu\text{IU/ml}$)	Coefficient of Variation (%)
Intra-assay	1	20.1 ± 3.2	15.9
	2	44.8 ± 6.9	15.4
	3	108.0 ± 7.3	6.8
Inter-assay	4	21.6 ± 3.6	16.7
	5	83.3 ± 7.1	8.5

* mean $\pm$ SD for five determinations

variation were 6.8–15.9% (intra-assay) and 8.5–16.7% (inter-assays).

Comparison with RIA

The oxytocin concentrations in 16 plasma samples from pregnant women were determined by both EIA (Y) and RIA (X) (Fig.

2). The regression equation and correlation coefficient (r) were $Y = 1.06X + 19.0$, and 0.90, respectively.

Discussion

To develop a competitive EIA for oxytocin, we first tried to prepare oxytocin- β -D-galactosidase conjugate using maleimide compound by the same method as for other small peptide hormones such as arginine-vasopressin (Uno *et al.*, 1982), but the sensitivity and reproducibility were insufficient.

On the other hand, SPDP is known to be a heterobifunctional cross-linking agent with which intermolecular cross-linkage can

easily be introduced between proteins without the concomitant formation of intramolecular cross-linkages. We then attempted to improve the method by using horseradish peroxidase as a label and SPDP as a cross-linking agent.

Our proposed method for conjugating oxytocin with peroxidase consists of three steps. First, the 2-pyridyldisulfide group is introduced to oxytocin with SPDP. In the second step, the 2-pyridyldisulfide group is introduced to peroxidase with SPDP and then reduced to the thiol group with dithiothreitol. In the third step, the 2-pyridyldisulfide group of oxytocin is allowed to react with the thiol group of peroxidase. This method has the following characteristics.

Firstly, the average number of the 2-pyridyldisulfide group introduced to peroxidase is about 2.5 per molecule (Imagawa *et al.*, 1982). It is probable that the 2-pyridyldisulfide group is introduced into the individual peroxidase molecule, and then oxytocine/peroxidase ratio of conjugate is adequate.

Secondly, the 2-Pyridyldisulfide group is more stable than the maleimide group.

Thirdly, since the molecular weight of peroxidase is smaller than that of β -D-galactosidase, the background may be low.

The sensitivity of the proposed EIA for measuring the oxytocin concentration in plasma is 10 μ IU/ml, comparable with that of RIA (5 μ IU/ml). Arginine- and lysine-vasopressin, which were structurally similar to oxytocin, showed little inhibition or none at all of the binding of oxytocin-peroxidase conjugate to antibody. EIA and RIA methods showed a close correlation.

Therefore, this EIA could be a suitable method measuring oxytocin as an alternative to RIA.

Recently, Amico suggested that C-terminal nonamidated oxytocin was present

in primate plasma in states of estrogen dominance. Some antibodies recognised the nonamidated form as well as authentic oxytocin (Amico, 1988).

The cross-reactivity with nonamidated oxytocin of the antibody used in this study has not been studied. Further studies are required.

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