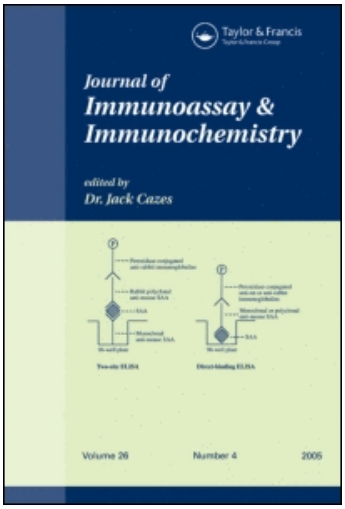

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New, Sensitive and Specific Elisa for the Detection of Neuropeptides in Culture Supernatants

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NEW, SENSITIVE AND SPECIFIC ELISA FOR THE DETECTION OF NEUROPEPTIDES IN CULTURE SUPERNATANTS

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ABSTRACT

Accurate and sensitive sandwich ELISA has been developed for the detection and identification of each of the three neuropeptides, namely, Vasoactive intestinal peptide, Somatostatin and Substance P. The neuropeptides conjugated with BSA and emulsified with Freund's adjuvant were used for immunisation of rabbits. Titres of polyclonal antibodies were checked by indirect immunofluorescence. The animals were bled when titres were high, sera separated, complement inactivated and IgG class of antibodies were purified using a protein G column. Purified IgG antibodies were used for coating the wells and for conjugation with HRPO and used for the detection of the synthetic neuropeptides in a standard solution or in the culture supernatant. The ELISA thus developed for the assay of each of the three neuropeptides had a sensitivity (0.01 ng - 12.8 ng / ml) equal to or better than that reported for these peptides by radioimmunoassay. The assay was highly specific and did not react with a panel of other neuropeptides

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tested. High level of sensitivity without compromising the specificity was achieved by using activated polyvinyl plates and using purified IgG from high titre rabbit anti-peptide sera. The non specific reaction was minimised by using 10,000 MW cut off amicon filtered supernatants.

(KEY WORDS : Neuropeptides, ELISA, Polyclonal antibodies)

INTRODUCTION

The term "neuropeptide" includes many peptides described originally in other seemingly unrelated contexts as "hormones" or "growth factors" (1,2,3). Most of the neuropeptides have been discovered serendipitously and it would not be surprising if the known peptides represent only about 10 percent or less of the total, whose number may exceed 200 (4). Peptides such as cholecystokinin and vasoactive intestinal peptide were first discovered in the gut and later recognised to be present in the brain also . Since their discovery about thirty years ago a lot of work has been done on most of the peptides elucidating their normal physiologic functions and pathophysiologic roles in different diseases (5,6). Alterations in the level of different neuropeptides have been reported in many diseases including some cancers (7,8,9,10). So far, the levels of neuropeptides have been assayed by radioimmunoassay which has a sensitivity in the nanogram to picogram range (11,12,13,14).In this study, we report highly sensitive ELISA for the assay of the neuropeptides Vasoactive intestinal peptide (VIP), somatostatin (SOM) and substance P (SP). The assays employ polyclonal antibodies for capture and detection of these neuropeptides. ELISA for all three peptides were highly specific with a sensitivity in the range of 10^{-9} to 10^{-10} M concentration. To our knowledge, this is the first report describing the detection and quantitation of neuropeptides using ELISA technique.

MATERIALS AND METHODS

Chemicals

Vasoactive intestinal peptide, Somatostatin, Substance P, gastrin, glucagon, secretin, cholecystokinin, Beta-Endorphin, Transforming growth factor-1, Insulin-like growth factor-1 and bombesin were obtained from Peninsula Laboratories Inc., USA. Bovine serum albumin, 1-ethyl-3- (3-diethylamino -propyl) carbodiimide hydrochloride, Horse radish peroxidase, Concanavalin A and ortho-phenylenediamine were purchased from Sigma, USA. Complete and incomplete Freund's adjuvant were obtained from DIFCO Laboratories, USA. Tween 20 was obtained from CDH, India. All other chemicals were purchased from Sigma, USA.

Primary cultures

Rat brain and spleen cells have previously been shown to secrete vasoactive intestinal peptide, somatostatin and substance P (15,16,17) whereas CoLo 320, a subclone of HT-29 has receptors for VIP (18).

Rat brain : Brain from a 1-2 day old rat was removed and was chopped into small pieces and minced in a petri-plate containing 5 ml of RPMI 1640 (Rosewell Park Memorial Institute) with 10 % fetal calf serum (FCS). A single cell suspension of the brain cells was prepared by flushing the tissue mince through a 21-gauge needle. The resulting single cell suspension was incubated at 37°C for 48 hours, after which the cultures were fed with fresh

growth medium. The cultures attained confluence in 8-10 days. The supernatant was collected at different time points of culture and assayed for neuropeptides.

Spleen cells : Spleen cells were isolated by a method already standardised in the laboratory (19). Briefly, BALB/c mice, 6-8 weeks old were sacrificed by cervical dislocation and spleens were excised. The spleens were gently pressed between frosted glass slides and the resultant cells were collected and passed through a 21-gauge needle to make a single cell suspension in Dulbecco's Minimal Essential Medium (DMEM) containing 10 % FCS. The cells were cultured for 48 hours in presence of 5 ug/ml Concanavalin A. The supernatant was collected after 3 days and assayed for the presence of the three neuropeptides.

Cell line

CoLo 320 : CoLo 320 is a human colon adenocarcinoma cell line which is reported to have receptors for VIP. It was maintained in RPMI 1640 with 10% FCS and incubated at 37 °C with 5% CO_2 and 95 % O_2. The cultures were fed with fresh growth medium every fourth day and subcultured after every 10 days.

Preparation of Antigen

Three neuropeptides used in this study , namely, vasoactive intestinal peptide (MW 3323.74), somatostatin (MW 1636.72) and substance P

(MW 1347.6) were of high purity as indicated by a sharp, single peak on HPLC chromatogram. The three neuropeptides were conjugated to bovine serum albumin (BSA) using water soluble carbodiimide. Briefly, 50 μg of the neuropeptide was dissolved in water and the pH adjusted to 5.0 by addition of 1M HCl. To this was added 50 μg of BSA, allowing it to dissolve slowly without frothing. Freshly dissolved 500 μg of 1-ethyl-3-(3-diethylamino-propyl) carbodiimide hydrochloride in water was added to the peptide carrier solution (P-BSA) and incubated for 2 hours at room temperature with occasional stirring. The mixture was dialysed for 2 days at 4°C against several changes of 0.05 M PBS, pH 7.4 to remove the unconjugated reagent.

Immunization and Bleeding

Two healthy, adult rabbits of Newzeland White strain were selected for immunization with each P-BSA conjugate. The rabbits were primed intramuscularly (gluteal muscle) with 1.0 ml of the P-BSA conjugate emulsified with equal volume of the complete Freund's adjuvant. These animals were injected one month later subcutaneously in the abdomen with 1 ml of P-BSA emulsified with incomplete Freund's adjuvant. Second booster injection was given one month later. After the third booster, 10 ml of test bleed from each rabbit was collected under sterile conditions. Blood samples were allowed to clot overnight at 4°C. Following morning the clots were detached from the sera, separated and centrifuged at 2000 g for 10 minutes. Antibody titres were checked by indirect immunofluorescence assay. Briefly, spleen cells were incubated at 37°C for 1 hour with increasing dilutions of the

antisera (1:10, 1:20, 1:50, 1:100 & 1:200). The cells were washed by centrifuging at 2000 g and incubated under identical conditions with 1:200 dilution of goat anti-rabbit IgG-FITC conjugate. After washing, the cells were transferred to cover slips and mounted in a medium made of carbonate-bicarbonate buffer and glycerol in 1:1 ratio containing a crystal of paraphenyl diamine, and sealed in an inverted position on a glass slide with a clear nail polish solution. The cells were scanned under UV light on a Microphot FX microscope (Nikon) at 100 X magnification. Subsequently the animals were periodically reimmunized through the subcutaneous route. Test bleeds of 10 ml from each rabbit were collected 10 days after each booster injection and a final bleed of about 100 ml was collected when sufficiently high titres of the anti-neuropeptide antibodies were achieved. The separated sera were heat inactivated for 60 minutes at 60°C to destroy complement and esterase activity. Small aliquotes of sera were frozen at -20°C until used.

Purification of Antibodies

Five ml of the serum was mixed with an equal volume of buffer made up of 0.05 M Tris hydrochloride and 0.25 M NaCl, pH 1.75. Antisera was eluted through a Protein G column with an 8 ml bed. The flow rate was maintained at 0.77 ml/minute. The eluate was immediately neutralized with 2.0 M Tris buffer to pH 7.4. A single peak containing mainly IgG fraction was obtained and hence all the fractions were pooled together. The eluate was dialysed 4 times at 4°C against 0.05 M phosphate buffered saline (PBS), pH 7.4 with 3-4 hourly changes of buffer. Protein content was estimated

spectrophotometrically at 280 nm using Lowry's method (20). The eluate was concentrated and stored at -70 °C.

Conjugation of Purified Antibody with HRPO

The purified antibody was conjugated to HRPO by using periodate method. Briefly, 5.0 mg of horse radish peroxidase was dissolved in 1.25 ml of water; to this 250 μl of 0.1 M Sodium metaperiodate solution was added and the mixture was gently agitated at room temperature for 20 minutes. The solution was dialysed for 18 hours against 0.001 M sodium acetate, pH 4.4 at 4°C. To the dialysate was added 25 μl of 0.2 M sodium carbonate buffer, pH 9.5 followed by immediate addition of 1 mg of the purified antibody. The mixture was stirred gently for two hours. To the mixture was added 125 μl of ascorbic acid (4 mg/ml) and conjugate was left at room temperature for 24-48 hours to stabilize. The mixture was dialyzed overnight at 4°C against 0.05 M PBS, pH 7.4 with 3-4 changes. Conjugate was filter sterilized by passing through a 0.22 μ filter and stored in 50% glycerol at -20°C.

Assay Protocol

Preparation of standard curve

Wells of a round-bottomed microtitre highly activated (MaxiSorp) plate (Nunc, Cat No. 449824) were first coated with 1 μg of the purified antibody in 100 μl of 0.05 M PBS, pH 7.4, with 0.05% Tween 20 (PBS-T), and incubated for 1 hr at 37°C. After incubation the wells were washed x2 times

with PBS-0.2 % Tween in an automatic Plate Washer (BDSL, UK). To each well 100 μl of 0.1 to 20 ng/ml of the neuropeptide in PBS-T was added and incubated for 1 hr at 37°C. The wells were washed x3 times with 0.05 M PBS - 0.2% Tween. This was followed by the addition of 100 μl of 1:2500 dilution of anti-neuropeptide antibody-HRPO conjugate in PBS-T to each well and incubation for 1 hour at 37°C. The wells were again washed x3 times as described previously. For color development, 25 μl of substrate (1 mg/ml OPD + 1 μl H_2O_2) in citrate-phosphate buffer, pH 5.5 was added to each well and incubated in dark for 5 minutes at 37°. The color development was terminated with addition of 10 μl of 5 N H_2SO_4. The absorbance in each well was determined at 490 nm on a multiscan microplate photometer (Biotech, USA).

Assay of neuropeptides levels in culture supernatant

For the detection and measurement of neuropeptide concentrations in culture supernatants of primary cultures and cell line, the assay procedure followed is identical to the one described for preparation of the standard curve, except instead of adding neuropeptide to antibody coated wells, 100 μl of primary cell culture supernatant is added. The supernatant of CoLo 320 cultures was filtered through a 10,000 MW cut off membrane (Amicon, Cat No. YM10) to concentrate the neuropeptides. (The loss of neuropeptides on filtration is theoretically negligible).

Evaluation for specificity of the assay

The specificity of the ELISA was checked by using a panel of 10 other neuropeptides.The assay protocol described above was carried out to evaluate the specificity of the three anti-neuropeptide antibodies in the following manner. Anti-VIP antibody was coated and incubated with 1-100 ng/ml concentration of somatostatin, substance P, gastrin, glucagon, secretin, cholecystokinin, Beta-Endorphin, Transforming growth factor-1, Insulin-like growth factor-1, bombesin and VIP. HRPO-conjugated anti-VIP was used as detector antibody. The cross reactivity of anti-somatostatin and anti-substance P antibodies for a panel of above mentioned neuropeptides was checked in an identical manner.

RESULTS

Titration studies

Figure 1 (a,b & c) presents the standard titration curves for VIP, somatostatin and substance P obtained under the conditions described for the assay. The minimum amount detectable with these ELISAs were 0.4 ng, 0.8 ng & 0.01 ng for VIP, Somatostatin and Substance P respectively. These values are lower than the minimum detectable limit previously reported utilizing radioimmunoassay methods (11,12).The detection ranges of the assay were 0.4-12.8, 0.8-12.8 and 0.01-1.0 ng/ml for VIP, somatostatin and substance P respectively.

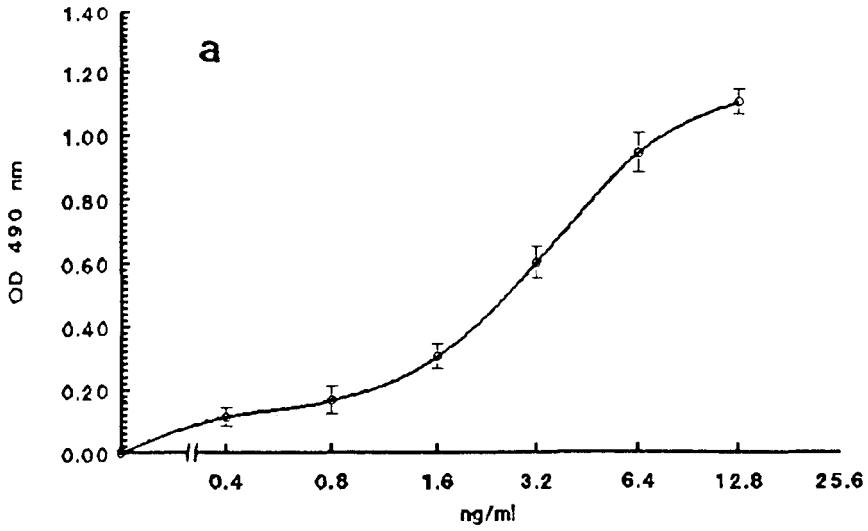
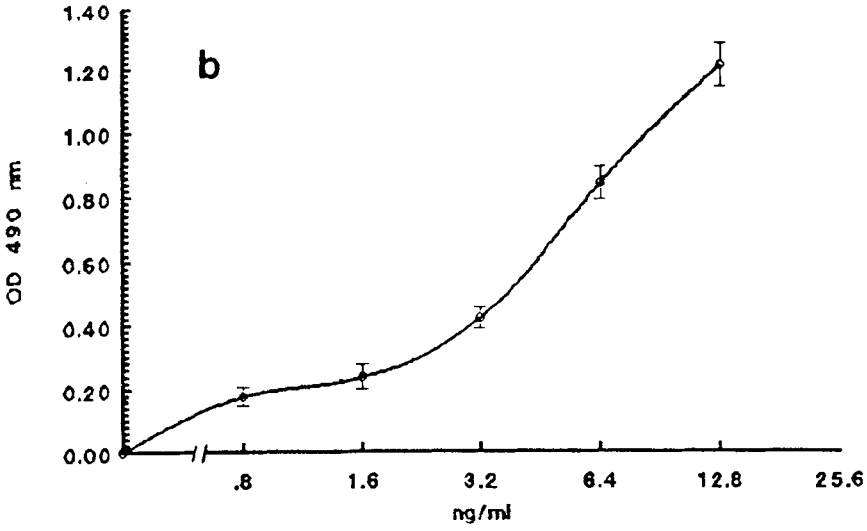


Figure 1. Standard curves of neuropeptides (a) VIP (b) Somatostatin & (c) Substance P. Each point is determined from triplicates in 11, 8 and 10 assays respectively. (The non specific background have been subtracted from the y-axis).

Optimisation of the assay

Blocking of extra sites with BSA on the plate after coating with the antibody was not done, as for raising the polyclonal sera, peptide-BSA conjugate was used. The background and non-specific binding in the assay was minimised by adding different concentrations of Tween 20 in different steps of the assay. Tween concentration of 0.2 % in PBS was found to be best suitable for washing. The concentration of capture antibody was standardised to 1 μg/ml whereas 1:2500 dilution of the antibody-HRPO conjugate was found to be optimum. To prevent non-specific binding of the antibodies with



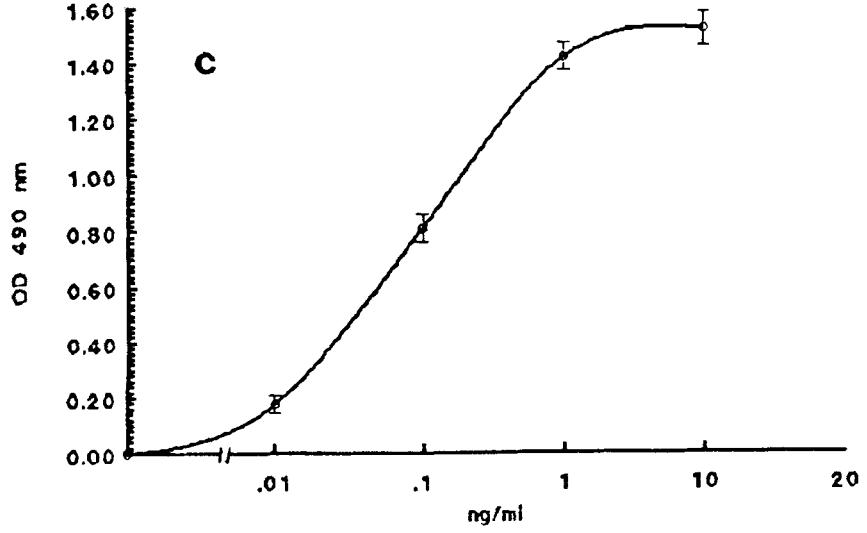


Figure 1. Continued

Table-1

Neuropeptide levels in culture supernatants

Culture supernatant	VIP	SOM	SP
Rat brain	1.8 ± 0.10	2.4 ± 0.15	0.025 ± 0.004
Spleen cells	0.2 ± 0.03	0.3 ± 0.02	0.008 ± 0.001
CoLo 320	2.1 ± 0.10	4.9 ± 0.24	1.0 ± 0.005

Concentrations of neuropeptides VIP, SOM & SP in ng/ml in culture supernatants of rat brain (day 10), spleen (day 1) and CoLo 320 cells (day 4).

high molecular weight proteins present in culture supernatants, the supernatant filtered through a 10,000 MW cut off membrane was used.

Neuropeptide concentrations in culture supernatants

The concentration of VIP, Somatostatin and Substance P were measured in the culture supernatants of rat brain cells, spleen cells and CoLo 320. Supernatants were collected from a 10 day, 1 day and 4 day old cultures of rat brain cells, spleen cells and human colon adenocarcinoma cells respectively. To each well of the previously described ELISA plate coated with neuropeptide specific capture antibody, 100 μl of the supernatant was added to measure the concentration of the neuropeptide. The results are presented in Table 1. The concentration of the three neuropeptides in the supernatants was in the range of 1-5 ng/ml.

Table-2

Specificity of the assays

	Anti-VIP	Anti-SOM	Anti-SP
VIP	5.80 ± 0.22	ND	ND
Somatostatin	ND	8.20 ± 0.56	ND
Substance P	ND	ND	0.11 ± 0.009
Gastrin	ND	ND	ND
Glucagon	ND	ND	ND
Secretin	ND	ND	ND
Cholecystokinin	ND	ND	ND
Beta-Endorphin	ND	ND	ND
Bombesin	ND	ND	ND
TGF-1	ND	ND	ND
IGF-1	ND	ND	ND

Concentration of VIP, SOM and SP in ng/ml detected by their respective antibodies. Note that the panel of other peptides are not detectable. (ND : Not detectable)

Specificity of the anti-neuropeptide antibodies

The reactivity of all the three anti-neuropeptide antisera was checked with a panel of neuropeptides. All the three antibodies did not detect peptides other than the one against which sera was raised even at 100 ng/ml concentration of the peptide. The results are presented in Table 2. These results reveal that the antisera to the neuropeptides VIP, somatostatin and substance P were highly specific and did not react with other neuropeptides.

Table-3

Inter-assay precision (n = 5)

	Mean concentration (ng/ml)	CV %
VIP	5.6 ± 0.09	1.7
SOM	5.9 ± 0.26	4.5
SP	1.3 ± 0.06	4.3

The inter assay precision was determined from 5 different experiments. The mean concentration ± SD (n = 5) along with CV % of all the 3 neuropeptides are presented. CV % = $\frac{\text{SD x 100}}{\text{Mean}}$

Intra- and Inter-assay precision

Intra-assay precision was checked in titration assays for VIP, somatostatin and substance P. The coefficient of variation of VIP (n=11) was below 6 %, and somatostatin (n=10) and substance P (n=7) was below 5 % over a concentration range of 0.4 - 12.8 ng/ml for VIP and somatostatin and 0.01 - 10 ng/ml for substance P.

The inter assay precision was calculated for all the assays performed. The calculations were based on the assays performed by taking standard solutions containing 5 ng/ml of the three neuropeptides. All determinations were made by taking mean value from 5 different experiments. The coefficient of variation of VIP was below 2 % while that of somatostatin and substance P was below 5 % . The results are presented Table 3.

DISCUSSION

It is becoming increasingly clear that neuropeptides have a very important role to play during the normal physiological functions and in the pathological states of the body. Initially the neuropeptides were discovered in the brain and were later found to be present in the other organs also. Amongst the panel of neuropeptides, VIP, somatostatin and substance P are known to be secreted by a variety of cells and the receptors for these peptides have been demonstrated on many cell types (21,22,23). The functions of these neuropeptides at cellular and molecular level are still not very well understood. What is becoming clear is that these neuropeptides also have a vital role to play in the pathology of many diseases including cancers (5,8). Such effects are induced by either low or high production of these peptides. The measurements of neuropeptides has not yet become a routine procedure as only radioimmunoassays have been described (24). It is very important to have technique(s) whereby alterations in the levels of neuropeptides can be detected and measured in biological fluid and culture medium easily and reproducibly. Although the sensitivity of the radioimmunoassay is high, there are several limitations of this assay system. Foremost among these is the use of high energy emitting radioisotopes. The iodinated peptide must then be used within the expiry of its half-life (59.6 days for ^{125}Iodine). The assay is also cumbersome since it involves handling of a large number of tubes. And finally, the assay duration varies between 1-6 days depending upon the sensitivity required.

In order to overcome the drawbacks of the radioimmunoassay and at the same time not sacrifice its high sensitivity and specificity, we developed a sandwich ELISA for detection and measurement of neuropeptides VIP, Somatostatin and Substance P in standard solution and culture supernatants. The assay developed has a potential to measure plasma concentrations of the neuropeptides and thus monitor the neuropeptide profile in the body. This assay will also be an important tool to measure neuropeptide levels and study their role in various diseases including cancers. This study demonstrates that it is possible to develop specific and highly sensitive ELISA for numerous other neuropeptides of physiological and pathophysiological significance.

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