Instruction

EURIA-NPY

Neuropeptide Y radioimmunoassay

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For Research Use Only. Not for use in diagnostic procedures.

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Σ 100

INTRODUCTION

Neuropeptide Y (NPY) is a 36 aminoacid residues peptide which was initially isolated from pig brain (1,2). NPY was later extracted from human adrenal-medullary phaeochromocytoma tissue. The human NPY differs from the pig NPY only by the replacement of leucine at position 17 by methione (3). NPY shares considerable sequence homology with pancreatic polypeptide and peptide YY (4). NPY-like immunoreactivity has been found in the central nervous system and in peripheral noradrenergic neurons and intestinal neurons (5,6,7,8). Potent vasoconstrictor activity is exhibited by NPY (9). NPY also inhibits noradrenaline release via a presynaptic action (10) and has stimulatory effects on the contraction of the heart (11). Phaeochromocytoma tumours (3,12,13) contain NPY and elevated levels of NPY in plasma from phaeochromocytoma patients have been reported (14). Increased plasma concentrations of NPY have been found in patient with neuroblastoma (15). Increased plasma concentrations of NPY have also been found in pediatric B-cell precursor leukemia (16).

PRINCIPLE OF THE METHOD

The intended use of these reagents is for assay of NPY in human serum/plasma by direct assay without extraction. NPY in the serum/plasma samples is assayed by a competitive radioimmunoassay using an antiserum raised against synthetic NPY conjugated to bovine thyroglobulin. NPY in standards and samples compete with ^{125}I-labelled NPY in binding to the antibodies. ^{125}I-NPY binds in a reverse proportion to the concentration of NPY in standards and samples. Antibody-bound ^{125}I-NPY is separated from the unbound fraction by using double antibody coupled to solid phase. The radioactivity of the antibody-bound ^{125}I-NPY is measured. The antiserum used in this method crossreacts less than 2.0% with human Peptide YY.
The result shall not be used for clinical diagnosis or patient management.

PHYSIOLOGICAL CONSIDERATIONS

Increased serum/plasma concentrations of NPY have been found in subjects with neuroblastoma (15). Increased serum/plasma concentrations of NPY have also been found in pediatric B-cell precursor leukemia (16).

Elevated levels of NPY have been found in serum/plasma from subjects with phaeochromocytoma tumours (3,12,13). Assay of NPY in serum/plasma may serve as valuable tool in the identification of neuroblastoma and pediatric B-cell precursor leukemia and phaeochromocytoma.

Normal level of NPY in human plasma

Subjects	**Number**	**Range**	**Mean**
Healthy normals (age 20-60 years)	109	36-120 pmol/L	74 pmol/L (SD= ± 15)

Normal level obtained in healthy people in the age 20-60 years.

PRECAUTIONS

For research use only. Not for use in diagnostic procedures.

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory are familiar with current local regulations, concerning all aspects of radioactive materials of the type and quantity used in this test.

This kit contains components of human origin. They have been tested by immunoassay for hepatitis B surface antigen, antibodies to HCV and for antibodies to HIV-1 and HIV-2 and found to be negative. Nevertheless, all recommended precautions for the handling of blood derivatives, should be observed.

This kit contains ^{125}I (half-life: 60 days), emitting ionizing X (28 keV) and γ (35.5 keV) radiations. Steps should be taken to ensure the proper handling of the radioactive material, according to local and/or national regulations. Only authorized personnel should have access to the reagents.

The following precautions should be observed when handling radioactive materials:

- Radioactive material should be stored in specially designated areas, not normally accessible to unauthorized personnel.

- Handling of radioactive material should be conducted in authorized areas only.

- Care should be exercised to prevent ingestion and contact with the skin and clothing. Do not pipette radioactive solutions by mouth.

- Drinking, eating or smoking should be prohibited where radioactive material is being used.

- Hands should be protected by gloves and washed after using radioactive materials.

- Work should be carried out on a surface covered by disposable absorbing material.

- Spills of radioactive material should be removed immediately, and all contaminated materials disposed as radioactive waste. Contaminated surfaces should be cleaned with a detergent.

The reagents in this kit contain sodium azide. Contact with copper or lead drain pipes may result in the cumulative formation of highly explosive azide deposits. On disposal of the reagents in the sewerage, always flush with copious amounts of water, which prevents metallic azide formation. Plumbing suspected of being contaminated with these explosive deposits should be rinsed thoroughly with 10% sodium hydroxide solution.

COMPOSITION OF THE REAGENT KIT

The reagents provided in each kit are sufficient for 100 tubes.

1. Anti-NPY (Reagent A)
Rabbit antiserum raised against NPY conjugated to bovine thyroglobulin. Lyophilized in 2.0 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin, 2.5% EDTA, 1.0% Triton X-100, 0.5% sodium azide and 5000 KIU Trasylol® per mL. For 100 tubes. Reconstitution in 22 mL distilled water.
Colour: Yellow.

2. ^{125}I-NPY (Reagent B)
Contains 28 KBq or 0.75 μCi at reference date. Synthetic, human NPY is iodinated according to Bolton-Hunter. The monoiodinated form is purified by HPLC. Specific activity: 1700-2100 μCi/nmol (62-77 MBq/nmol). Lyophilized in 2.5 mL 0.5 M phosphate buffer, pH 7.4, with 2.5% human serum albumin, 2.5% EDTA, 1.0% Tween 80, 0.5% sodium azide and 5000 KIU Trasylol® per mL. Reconstitution in 25 mL distilled water.
Colour: Blue.

3. Double antibody solid phase (Reagent C)
Anti-rabbit-Ig coupled to cellulose particles in 0.01 M phosphate buffer pH 6.8 with 0.25% Human serum albumin, 0.045% NaCl, 0.05% NaN_3, 0.185% EDTA and 0.05% Tween 80.
11.0 mL suspension.

4. Standard diluent (Reagent D)
10.0 mL NPY-free human serum, lyophilized. Contains 500 KIU Trasylol® per mL. Reconstitution in 10.0 mL distilled water. For preparation of NPY working standards.

5. NPY standard 3000 pmol/L (Reagent E)
Lyophilized. 2.00 mL standard. Concentration: 3000 pmol/L after reconstitution. The standard is produced from synthetic, human NPY. Lyophilized in 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA, 0.1% Triton X-100, 0.05% sodium azide and 500 KIU Trasylol® per mL. For preparation of NPY working standards.

6. Assay buffer (Reagent F)
5.0 mL 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA, 0.1% Triton X-100, 0.05% sodium azide and 500 KIU Trasylol® per mL.
To be used instead of antiserum in non-specific binding test tubes.

7. Controls (Reagent G-H)
Lyophilized serum controls with low and high concentration of NPY. 2.00 mL of each control after reconstitution. The NPY concentrations are given on the labels of the vials.
Contains 0.05% sodium azide.

REAGENT PREPARATION AND STORAGE

Store all reagents at 2-8° C before reconstitution and use. The water used for reconstitution of lyophilized reagents should be distilled in an all-glass apparatus or be of corresponding purity. Dissolve the contents in a vial by gentle inversion and avoid foaming. The stability of the reagents is found on the labels of the vials. For lyophilized reagents the expiry dates are valid for the unreconstituted reagents. The reconstituted reagents are stable for 10 weeks (no longer than to the expiry date) if stored properly.

Reagent A: Anti-NPY
Reconstitute with 22 mL distilled water.
Store at 2-8° C.

Reagent B: ^{125}I-NPY
Reconstitute with 25 mL distilled water.
Store at -18° C or lower if reused.

Reagent C: Double antibody solid phase
Ready for use. Stir continuously during pipetting of this reagent.
Store at 2-8° C.

Reagent D: Standard diluent
Reconstitute with 10.0 mL distilled water.
Store at -18° C or lower if reused.

Reagent E: NPY standard, 3000 pmol/L
Reconstitute with 2.00 mL distilled water.
Store at -18° C or lower if reused.
For preparation of NPY-working standards, see radioimmunoassay procedure.

Reagent F: Assay buffer
Ready for use.
Store at 2-8° C.

Reagent G-H: Controls
Reconstitute with 2.00 mL distilled water. Store at -18° C or lower if reused.

EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

Disposable test tubes 11-13 x 55 mm, polystyrene
Pipettes with disposable tips, 100 and 200 µL
A repeating pipette, e.g. Eppendorf Multipipette, for volumes 100 and 200 µL will facilitate the dispensing of the reagents
Variable pipette 200-1000 µL
Vortex mixer
Centrifuge capable for min 1700 x g (refrigerated centrifuge is preferred)
Gammacounter
Distilled water

SPECIMEN COLLECTION

Vein blood is collected in tubes without additives. The sample is allowed to clot. The serum is separated by centrifugation at +4°C. The serum should be frozen at -20°C within 1 hour. The serum should be stored at -20°C or lower until assayed.

Plasma samples (EDTA or Heparin) can also be used but should not be diluted (dilution of plasma with the standard diluent may lead to clotting in the assay during incubation).

Repeated freezing and thawing should be avoided.

RADIOIMMUNOASSAY PROCEDURE

Reconstitute the reagents as specified. Reagents should be brought to room temperature prior to use. Accuracy in all pipetting steps is essential. All tests (standards, controls and samples) should be performed in duplicate.
A complete assay includes:
Standards (St-tubes): 7 different concentrations; 0, 9.4, 18.8, 37.5, 75, 150 and 300 pmol/L.
Controls (C-tubes): Controls with known concentrations of NPY.
Samples (P-tubes).
Tubes for determination of the ***non-specific binding (NSB-tubes)***.
Tubes for determination of the ***total radioactivity*** **added** ***(TOT-tubes)***.
For an overview se table 1 on page 11.

PERFORMANCE

1. Reconstitute the reagents according to the instructions.
2. Prepare the NPY-working standards by dilution of the NPY-standard 3000 pmol/L (Reagent E) with the standard diluent (Reagent D) according to the following:

   A/ 0.200 mL standard 3000 pmol/L + 1.800 mL diluent = 300 pmol/L
   B/ 1.00 mL standard 300 pmol/L + 1.00 mL diluent = 150 pmol/L
   C/ 1.00 mL standard 150 pmol/L + 1.00 mL diluent = 75 pmol/L
   D/ 1.00 mL standard 75 pmol/L + 1.00 mL diluent = 37.5 pmol/L
   E/ 1.00 mL standard 37.5 pmol/L + 1.00 mL diluent = 18.8 pmol/L
   F/ 1.00 mL standard 18.8 pmol/L + 1.00 mL diluent = 9.4 pmol/L
   G/ Standard diluent = 0 pmol/L.

   Store the standard solutions at -18° C or lower if reused.

3. Pipette 200 µL of the standards (0-300 pmol/L), samples and controls in their respective tubes. Pipette 200 µL of the zero-standard in the NSB-tubes.
4. Pipette 200 µL anti-NPY (Reagent A) to all tubes except the NSB- and TOT-tubes.
5. Add 200 µL assay buffer (Reagent F) to the NSB-tubes.
6. Vortex-mix and incubate for 20-24 hours at 2-8° C.
7. Pipette 200 µL ^{125}I-NPY (Reagent B) to all tubes. The TOT-tubes are sealed and kept aside.
8. Vortex-mix and incubate for 20-24 hours at 2-8° C.
9. Pipette 100 µL double antibody-solid phase (Reagent C) to all tubes except the TOT-tubes. (stir continuously during pipetting).
10. Vortex-mix carefully and incubate for 30-60 minutes at 2-8° C.
11. Centrifuge the tubes for 15 minutes at +4° C (minimum 1700 x g).
12. Decant the supernatants immediately after centrifugation.
13. Count the radioactivity of the precipitates in a gamma counter (counting time: 2-4 minutes).

CALCULATION OF RESULTS

1. Subtract the average count rate (CPM) of the non-specific binding tubes from the count rates (CPM) of the replicates of standards, controls and samples.

2. A standard curve is generated by plotting the precipitated CPM, bound fraction in CPM or % B/TOT against the concentrations of the NPY-standards. An example of a standard curve is given on page 12.

3. Interpolate the NPY concentrations of the samples and controls from the generated standard curve.

4. The standard curve and the calculations of the concentrations in samples and controls can also be done by a computer method.

DILUTION OF SAMPLES

Serum samples with NPY concentrations above 300 pmol/L should be diluted with standard diluent (reagent D) for determination of the real concentration of NPY.
The following procedure is recommended:
Dilution 1:2: Pipette 100 µL sample and 100 µL standard diluent in the assay tube (perform in duplicate).
Dilution 1:4: Pipette 50 µL sample and 150 µL standard diluent i the assay tube (perform in duplicate).

ASSAY CHARACTERISTICS

Sensitivity
The lowest detectable concentration is 3 pmol/L. The figure corresponds to a decrease in binding of 2 x SD of the bound radioactivity in the zero-concentration standard.

Accuracy
A mean recovery of 82.4% (range 75-88%) was achieved in the assay of plasma samples with known amounts of NPY added. NPY was added in the range: 50-150 pmol/L.

Precision
Intra assay variation

Level	Coefficient of variation (%CV)	N
43.7 pmol/L	5.0	8
98.9 pmol/L	4.5	8

Inter assay variation (total variation)

Level	Coefficient of variation (%CV)	N
42.4 pmol/L	9.2	6
90.2 pmol/L	7.6	6

Specificity

The following cross-reactions with related peptides have been found:

Compound	**Cross-reaction**
Neuropeptide Y, human	100.0%
Peptide YY, human	<0.1%
Pancreatic Polypeptide, human	<0.1%
PYY 3-36	<0.1%
NPY 22-36	<0.1%
PP Bovine	<0.1%

Interference
Samples displaying cloudiness, hemolysis, hyperlipemia or containing fibrin may give inaccurate results.

QUALITY CONTROL

In order for the laboratory to completely monitor the consistent performance of the radioimmunoassay there are some important factors which must be checked.

1. Controls
The found concentrations of the controls (reagent G and H) should be within the limits given on the labels of the vials.

2. Total counts (TOT)
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-NPY in this kit will give 10 500 CPM (-5% to +20%) at the activity reference date (counter efficiency = 80%).

3. Maximum binding (Bo/TOT)
Calculate for each assay the % bound radioactivity in the zero-standard:

$$\frac{Bo}{TOT} \times 100$$

$\frac{Bo}{TOT} \times 100$ is generally 40-60% at the reference date and may have decreased a few % at the expiry date of the kit.

4. Non-specific binding (NSB/TOT)
Calculate for each assay the % non-specific binding $\frac{NSB}{TOT} \times 100$

$\frac{NSB}{TOT} \times 100$ is less than 8%.

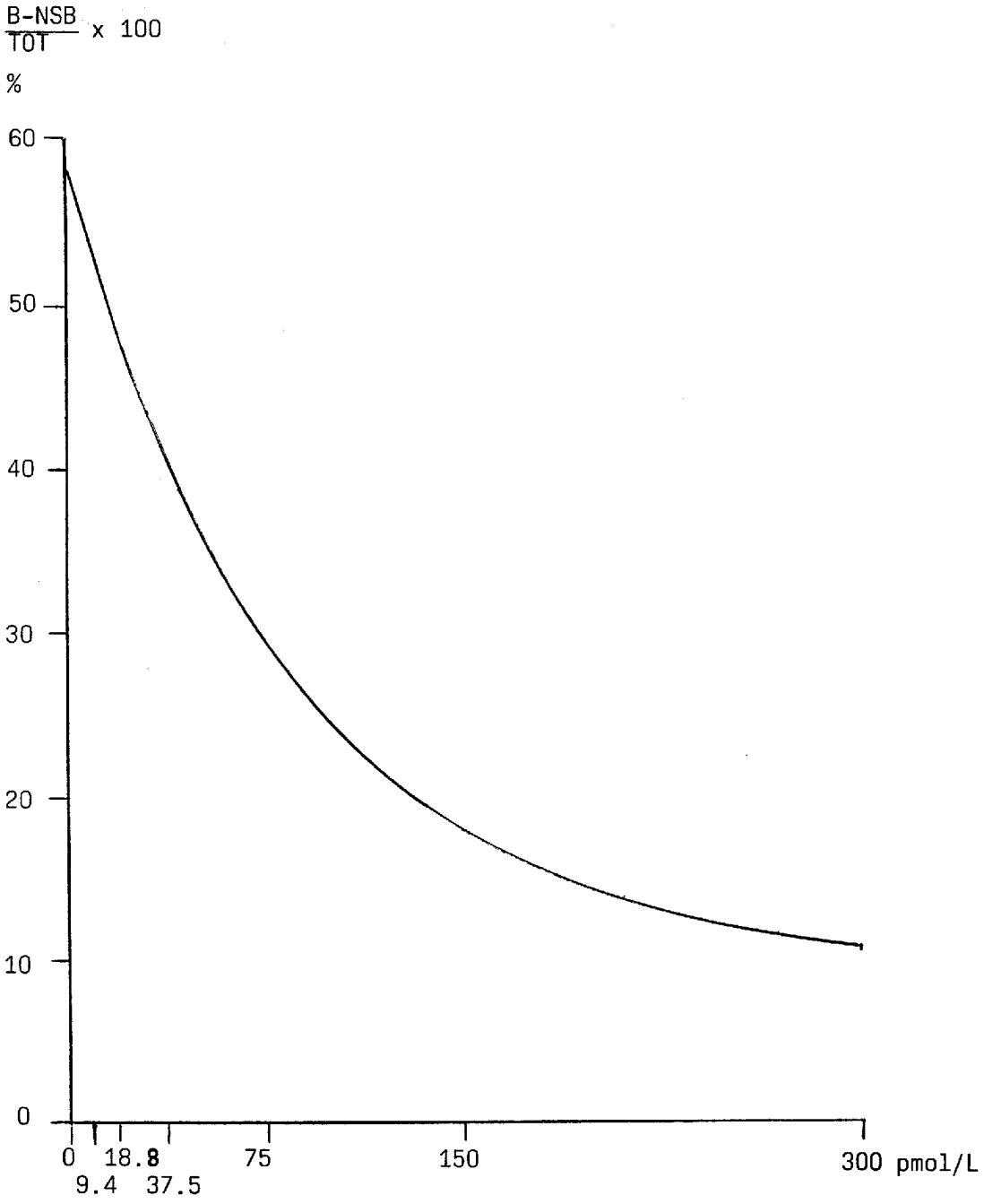
5. Shape of standard curve
For example, monitor the 80, 50 and 20% points of the standard line for run to run reproducibility.

OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-NPY (A)	Assay buffer (F)		^{125}I-NPY (B)		Double antibody solid phase (C)	
TOT	1-2	-	-	-	Vortex-mix and incubate for 20-24 hours at 2-8° C.	200 µL	Vortex-mix and incubate for 20-24 hours at 2-8° C.	-	Vortex-mix and incubate for 30-60 min. at 2-8° C. Centrifuge 15 min. at 1700 x g at +4° C. Decant and count the radioactivity of the precipitates.
NSB	3-4	200 µL	-	200		200 µL		100 µL	
Stand 0	5-6	200 µL	200 µL	-		200 µL		100 µL	
Stand 9.4	7-8	200 µL	200 µL	-		200 µL		100 µL	
Stand 18.8	9-10	200 µL	200 µL	-		200 µL		100 µL	
Stand 37.5	11-12	200 µL	200 µL	-		200 µL		100 µL	
Stand 75	13-14	200 µL	200 µL	-		200 µL		100 µL	
Stand 150	15-16	200 µL	200 µL	-		200 µL		100 µL	
Stand 300	17-18	200 µL	200 µL	-		200 µL		100 µL	
Control (G)	19-20	200 µL	200 µL	-		200 µL		100 µL	
Control (H)	21-22	200 µL	200 µL	-		200 µL		100 µL	
Sample 1	23-24	200 µL	200 µL	-		200 µL		100 µL	
Sample 2	25-26	200 µL	200 µL	-		200 µL		100 µL	

Table 1

EXAMPLE OF NPY STANDARD CURVE



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SYMBOLS USED ON LABELS

LOT	Batch code.
REF	Catalogue number.
	Use by date.
	Temperature limit.
	Date of manufacture.
	Contains radioactive substances.
	Biological risks.
	Consult instructions for use.
	Manufacturer.
 **100**	Contains sufficient for 100 tests.

REAG A Ab	Anti-NPY
REAG B Ag ^{125}I	^{125}I-NPY.
REAG C DASP	Double antibody solid phase.
REAG D DIL CAL	Standard diluent.
REAG E CAL 3000	NPY standard 3000 pmol/L.
REAG F BUF AS	Assay buffer.
REAG G CONTROL	Control, level 1 (low).
REAG H CONTROL	Control, level 2 (high).

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