β-Endorphin Ria kit

KIPERB301

DIAsource ImmunoAssays S.A. - Rue de l'Industrie, 8 - B-1400 Nivelles - Belgium

ß-ENDORPHIN-RIA

en

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For research use only

DIAsource ImmunoAssays S.A. - Rue de l'Industrie 8, B-1400 Nivelles, Belgium - Tel: +32 67 88 99 99 - Fax : +32 67 88 99 96

1 PRINCIPLE OF THE METHOD

ß-endorphin in sample (human plasma) is extracted using sep-pak C18 cartridges. The extracts are analysed by a competitive radioimmunoassay using antibodies against synthetic human ß-endorphin. ß-endorphin in standards and samples compete with ^{125}I-labelled ß-endorphin in binding to the antibodies. ^{125}I-ß-endorphin binds to the antibodies in a reverse proportion to the concentration of ß-endorphin in standards and samples. In order to increase the sensitivity of the assay a sequential incubation is performed. Antibody-bound ^{125}I-ß-endorphin is separated from the unbound fraction using the double antibody polyethylene glycol precipitation technique. The radioactivity of the precipitates is measured. The antiserum used is specific for the N-terminal region of the ß-endorphin with very low cross reaction with ß-lipotropin.

2 PRECAUTIONS

For research use only. This kit contains ^{125}I emitting ionizing X and gamma radiations.

As the regulations may vary from one country to another, it is essential that the person responsible for the laboratory is 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.

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 designed 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.

3 COMPOSITION OF THE REAGENT KIT

Contents of the Kit

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

1. | REAG | A | Ab |

Rabbit antiserum to synthetic, human β-endorphin (β-lipotropin 61-91). Lyophilized in 2.0 mL 0.5 M phosphate buffer, pH 7.4, 10% human serum albumin and 0.5% sodium azide.
For 100 tubes. Reconstitution in 22 mL distilled water. Colour: Yellow.

2. | REAG | B | Ag | ^{125}I |

56 KBq or 1.5 µCi. Produced by iodination of synthetic, human β-endorphin. HPLC-purified, monoiodinated. Specific activity: 62-77 Mbq/nmol (1700-2100 µCi/nmol).
Lyophilized in 2.5 mL 0.5 M phosphate buffer, pH 7.4, 10% human serum albumin, 0.5% sodium azide and 500 KIU Trasylol®/mL. Contains 0.12 mL normal rabbit serum. Reconstitution in 25 mL distilled water. Colour: Blue.

3. | REAG | C | DAb |

50 mL goat anti-rabbit-Ig antiserum. Contains 5% (w/v) polyethylene glycol 6000.

4. REAG | D | ASSAY | BUF

25 mL buffer for dilution of the β-endorphin standard and reconstitution of sample extracts. 0.05 M phosphate, pH 7.4, 0.25% human serum albumin, 0.05% sodium azide, 0.25% EDTA and 500 KIU Trasylol®/mL.

5. REAG | E | STD

5.00 mL, 500 pmol/L (1731 pg/mL), synthetic human β-endorphin. Lyophilized in 0.05 M phosphate, pH 7.4, 1.0% human serum albumin, 0.05% sodium azide and 500 KIU Trasylol®/mL. Reconstitution in 5.00 mL distilled water.
Conversion of pmol/L to pg/mL: pmol/L x 3.463 = pg/mL

6. REAG | F-G | Control

Lyophilized controls with two different levels of β-endorphin. 1.00 mL of each control after reconstitution. The concentrations of ß-endorphin are given on the labels of the vials. The controls should be assayed directly without extraction. Contains 0.05% sodium azide.

Equipment required but not provided

Distilled water
Aceton pro analysis
Acetic acid
Hydrochloric acid, 0.2 M
Sep-pak C18 cartridges
15 mL conical glass tubes
11-13 x 55 mm disposable test tubes (polystyrene)
Pipettes with disposable tips: 100, 200, 500 and 1000 µL
Glass pipettes: 1.00, 5.00 mL
Vortex mixer
Equipment for evaporation under vacuum
Centrifuge, refrigerated, giving minimum 1700 x g
Gamma counter

Reagent preparation and storage

Store all reagents at 2-8° C before reconstitution and use. The water used for reconstitution of the 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 label of the vials. For lyophilized reagents the expiry date is valid for the unreconstituted reagents. Reconstituted reagents are stable for 10 weeks or until the expiry date is reached.

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

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

Reagent C: double antibody-PEG
Ready for use. Mix thoroughly before use.
Store at 2-8° C.

Reagent D: Diluent
Redy for use.
Store at 2-8° C.

Reagent E: b-endorphin standard, 500 pmol/L
Reconstitute with 5.00 mL distilled water.
Store at -18° C or lower if reused.

Reagent F-G: Controls
Reconstitute with 1.0 mL distilled water.
Store at -18° C or lower if reused.

4 SPECIMEN

Specimen collection

Blood is collected in tubes containing EDTA and Trasylol® (5000 KIU Trasylol in a 10 mL vacutainer tube). The sample is cooled in an ice-bath immediately. Plasma is separated by centrifugation at +4° C. The plasma should be frozen within 1 hour and stored at -20° C or lower until assayed. Repeated freezing and thawing should be avoided.

5 ASSAY PROCEDURE

5.1 Procedure

The assay is performed in two steps:

I. Extraction of plasma samples
II. Radioimmunoassay of extracts

For an overview see section 8.

A complete assay includes:

Standard (St-tubes): 7 concentrations: 0, 15.6, 31.3, 62.5, 125, 250, 500 pmol/L

Controls (C-tubes): For recovery control (C_{REC}) and radioimmunoassay control (C_{RIA}).
The recovery of ß-endorphin in the extraction procedure is determinated by analysing a sample with exactly known concentration.

Samples (S-tubes)

Tubes for determination of the ***non-specific binding*** for standards and samples ***(NSB-tubes)***

Tubes for determination of the ***total radioactivity*** added ***(TOT-tubes).***

PERFORMANCE

I. Extraction of plasma samples

The described extraction procedure is based on the use of Sep-pak® C18 cartridges (available from Waters Assoc., Milford, M.A., USA). The procedure involves concentration of β-endorphin with a factor of 10.

1. Thaw the plasma immediately before starting the extraction. Store at 2-8° C before application on the sep-pak cartridge.
2. The sep-pak cartridge is activated by passing through it 2 mL of acetone.
3. Wash the sep-pak with 10 mL distilled water.
4. Apply 3.00 mL plasma sample on the sep-pak cartridge. The flow rate should not exceed 3 mL per minute.
5. Wash the cartridge twice with 2 mL 4% acetic acid in distilled water.
6. Elute the β-endorphin with 1.5 mL 0.2 M hydrochloric acid/acetone (25:75).
7. Dry the elute under vacuum.
8. Dissolve the extracted β-endorphin in 300 µL diluent (Reagent D).
   Vortex mix and allow the sample to stay for 30 minutes before assay with the radioimmunoassay procedure.

It is important that the recovery is controlled under the user's own experimental conditions.

RECOVERY CONTROLS

For the determination of the recovery in the extraction procedure prepare a control as follows:
To 3.00 mL blood donor plasma add exactly 75 µL of β-endorphin standard 500 pmol/L. The concentration will be 12.5 pmol/L. Extract the control according to method description. Extract also the same amount of plasma without added β-endorphin for correction for endogenous β-endorphin in the plasma.

II. Radioimmunoassay of extracts

1. Reconstitute the reagents according to the instructions.
2. Prepare the β-endorphin working standards by dilution of the 500 pmol/L standard (Reagent E) with the diluent (Reagent D) according to the following:
   a/ Reagent E after reconstitution = 500 pmol/L
   b/ 1.00 mL standard 500 pmol/L + 1.00 mL diluent = 250 pmol/L
   c/ 1.00 mL standard 250 pmol/L + 1.00 mL diluent = 125 pmol/L
   d/ 1.00 mL standard 125 pmol/L + 1.00 mL diluent = 62.5 pmol/L
   e/ 1.00 mL standard 62.5 pmol/L + 1.00 mL diluent = 31.3 pmol/L
   f/ 1.00 mL standard 31.3 pmol/L + 1.00 mL diluent = 15.6 pmol/L
   g/ Diluent = 0 pmol/L.
   Store the standard solutions at -18° C or lower if reused.
3. Pipette 100 µL of standards a-g (0-500 pmol/L), controls and reconstituted sample extracts in their respective tubes. Pipette 100 µL of the zero-standard in the NSB-tubes.
4. Add 200 µL anti-β-endorphin (Reagent A) to all tubes except the NSB- and TOT-tubes.

5. Add 200 µL diluent (Reagent D) to the NSB-tubes.

6. Vortex-mix and incubate for 20-24 hours at 2-8° C.

7. Add 200 µL ^{125}I-β-endorphin (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. Add 500 µL double antibody-PEG (Reagent C) to all tubes except the TOT-tubes (mix this reagent before pipetting).

10. Vortex-mix and incubate for 30-60 minutes at 2-8° C.

11. Centrifuge the tubes for 15 minutes at +4° C (1700 x g).

12. Decant the supernatant immediately after centrifugation.

13. Count the radioactivity of the precipitates in a gamma counter (counting time 2minutes).

5.2 Calculations

1. Subtract the average count rate (CPM) of the non-specific binding from the count rate (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 β-endorphin standards.

3. To obtain the β-endorphin concentrations in the samples and controls read the corresponding concentrations to their precipitated CPM or % B/TOT from the generated standard curve.

4. The received concentrations are corrected for the concentration factor in the extraction procedure. According to the method described the β-endorphin is concentrated 10 times. Divide with 10. Finally the found concentration is corrected for the recovery of the recovery controls.

5. The standard curve and the calculation of the concentrations in samples can also be done by a computer method. A spline method may be used.

6 ASSAY CHARACTERISTICS

6.1 Sensitivity
The sensitivity calculated from a decrease in binding of 2 SD in the zero standard is 3 pmol/L or 10 pg/mL. The sensitivity calculation is based upon a concentration with a factor of ten in the extraction procedure.

6.2 Recovery
The mean recovery in the extraction procedure is 80-90%.

6.3 Precision
Intra assay variation: 7.1%.

Inter assay variation (total variation): 7.2%.

6.4 Specificity
The following cross reactions have been found:

Polypeptide, human	Cross reaction
β-endorphin	100.0 %
β-lipotropin	<1 %
α-endorphin	69 %
Met-enkephalin	9 %
Leu-enkephalin	<0.04%
β-lipotropin 61-87	43 %
β-lipotropin 61-69	67 %
β-lipotropin 66-91	2 %
β-lipotropin 88-91	<0.04%

b-ENDORPHIN CONCENTRATION IN HUMAN PLASMA

Plasma from 27 healthy blood donors was assayed with these reagents. In 26 samples the concentrations were <3 pmol/L (10 pg/mL). In 1 sample the concentration was 5 pmol/L (17 pg/mL). See also reference number 1.

7 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 F and G) should be within the limits given on the labels of the vials.

2. Recovery control
The recovery should be controlled in each assay. It is important that the recovery is controlled under the user's own experimental conditions.

3. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-β-endorphin in this kit will give a total counts in the assay (TOT) of 21.000 CPM (-5%, +20%) at the activity reference date (counter efficiency = 80%).

4. 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 30-50% at the activity reference date

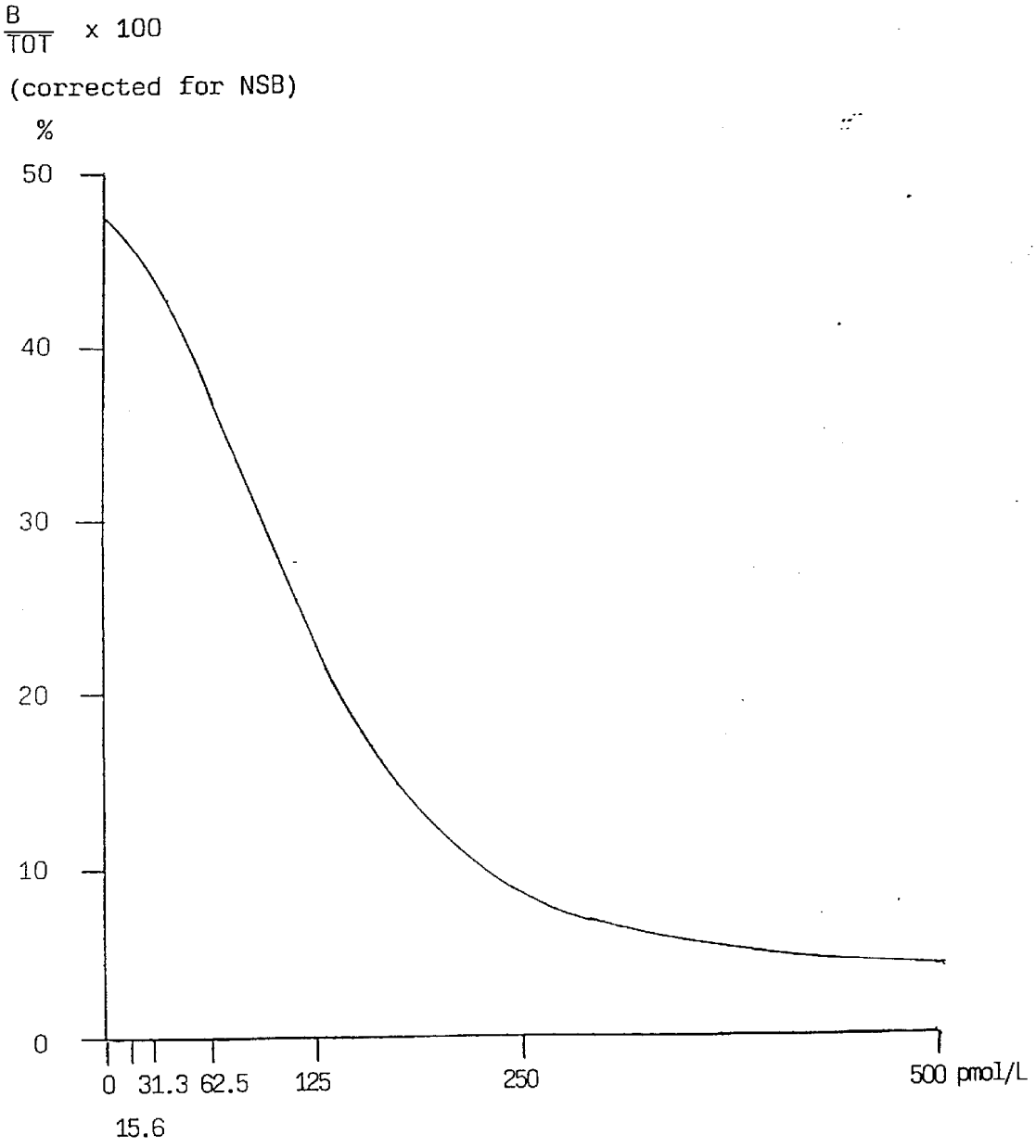
$\frac{Bo}{TOT} \times 100$ may have decreased a few % at the expiry date of the kit.

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

$\frac{Bo}{TOT} \times 100$ is less than 7% if decanting is made properly

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

EXAMPLE OF BETA-ENDORHPIN STANDARD CURVE



8 OUTLINE OF THE RIA PROCEDURE

Type of tubes	Tube no	Standard sample or control	Anti-ß-endorphin (A)	Diluent (D)		^{125}I-ß-endorphin (B)		Double antibody PEG (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 15-20 min. at 1700 x g at +4° C. Decant and count the radioactivity of the precipitates
NSB, St 0	3- 4	100 µL	-	200 µL		200 µL		500 µL	
Stand 0	5- 6	100 µL	200 µL	-		200 µL		500 µL	
Stand 15.6	7- 8	100 µL	200 µL	-		200 µL		500 µL	
Stand 31.3	9-10	100 µL	200 µL	-		200 µL		500 µL	
Stand 62.5	11-12	100 µL	200 µL	-		200 µL		500 µL	
Stand 125	13-14	100 µL	200 µL	-		200 µL		500 µL	
Stand 250	15-16	100 µL	200 µL	-		200 µL		500 µL	
Stand 500	17-18	100 µL	200 µL	-		200 µL		500 µL	
$Control_{RIA}$ (F)	19-20	100 µL	200 µL	-		200 µL		500 µL	
$Control_{RIA}$ (G)	21-22	100 µL	200 µL	-		200 µL		500 µL	
$Control_{REC}$ (a)	23-24	100 µL	200 µL	-		200 µL		500 µL	
$Control_{REC}$ (b)	25-26	100 µL	200 µL	-		200 µL		500 µL	
Sample1	27-28	100 µL	200 µL	-		200 µL		500 µL	
Sample 2 etc.	29-30	100 µL	200 µL	-		200 µL		500 µL	

9 REFERENCES

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Revision date : 2010-04-13