Instruction

EURIA-α-MSH

α-Melanocyte Stimulating Hormone radioimmunoassay

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

REF **RB 303 RUO**

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PURPOSE OF RESEARCH PRODUCT

α-Melanocyte stimulating hormone (α-MSH) is a 13 amino acids peptide with a molecular weight of 1665. The N-terminal serin is acetylated and the C-terminal valine is amidated. The amino acid sequence of α-MSH is identical to ACTH 1-13 in man.
α-MSH is derived from pro-opiomelanocortin, a precursor protein which contains, within its structure, the sequences of other melanotropic peptides like γ-, ß-MSH and ACTH.
α-MSH stimulates melanosome dispersion within dermal melanocytes and melanin biosynthesis within epidermal melanocytes.
α-MSH is a potent modulator of fever and inflammation. Plasma α-MSH increases in human subjects with high fever caused by endotoxin administration. The average plasma α-MSH level has been found higher in subjects with AIDS than in control subjects.
The result shall not be used for clinical diagnosis or patient management.

PRINCIPLE OF THE METHOD

The intended use of these reagents is for the determination of α-melanocyte stimulating hormone (α-MSH) in human plasma or cerebrospinal fluid. α-MSH is analysed by the competitive radioimmunoassay using an antiserum to an α-MSH-albumin conjugate. α-MSH in standards and samples compete with ^{125}I-labelled α-MSH in binding to the antibodies. ^{125}I-α-MSH binds in a reverse proportion to the concentration of α-MSH in standards and samples. In order to increase the sensitivity of the assay a sequential assay with delayed addition of ^{125}I-α-MSH is performed. Antibody-bound ^{125}I-α-MSH is separated from the free fraction using the double antibody polyethylene glycol precipitation technique. The radioactivity of the precipitates is measured. The antiserum used in this kit is directed to the C-terminal part of the α-MSH molecule and shows no crossreactivity with adrenocorticotropic hormone.

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.

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-α-MSH (Reagent A)

Rabbit antiserum raised against α-MSH conjugated to bovine serum albumin. Lyophilized in 2.0 mL 0.5 M phosphate buffer, pH 7.4, 2.5% human serum albumin, 2.5% EDTA disodium salt, 0.5% sodium azide and 5000 KIU Trasylol® per mL. For 100 tubes. Reconstitute in 22 mL distilled water.

2. ^{125}I-α-MSH (Reagent B)

Contains 0.75 μCi or 28 KBq at reference date. Produced by iodination of synthetic α-MSH. HPLC-purified, monoiodinated. Specific activity: 1700-2100 μCi/nmol (62-77 Mbq/nmol). Lyophilized in 2.5 mL 0.5 M phosphate buffer, pH 7.4, 2.5% EDTA disodium salt, 0.5% sodium azide and 5000 KIU Trasylol® per mL. Contains 0.12 mL normal rabbit serum. Reconstitution in 25 mL distilled water.

3. Double antibody-PEG (Reagent C)

50 mL diluted goat anti-rabbit-IG antiserum. 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA disodium salt and 0.05% sodium azide. Contains 5.0% w/v polyethylene glycol 6000.

4. Diluent (Reagent D)

25 mL 0.05 M phosphate buffer, pH 7.4, with 0.25% human serum albumin, 0.25% EDTA disodium salt, 0.05% sodium azide and 500 KIU Trasylol® per mL. To be used for dilution of the α-MSH standard.

5. α-MSH standard, 300 pmol/L (Reagent E)

5.00 mL, 300 pmol/L (500 pg/mL) α-MSH standard. Lyophilized in 0.05 M phosphate buffer, pH 7.4, 0.25% human serum albumin, 0.25% EDTA disodium salt, 0.05% sodium azide and 500 KIU Trasylol® per mL. Reconstitution in 5.00 mL distilled water.

6. Controls (Reagent F-G)

Lyophilized controls. 1.00 mL of each control after reconstitution. The α-MSH concentrations of the controls are given on the labels of the vials. Contains 0.05% sodium azide.

EQUIPMENT AND REAGENTS REQUIRED BUT NOT PROVIDED

Distilled water.

11-13 x 55 mm disposable tubes, glass (for dilution of the standard).

11-13 x 55 mm disposable tubes, polystyrene (for the radioimmunoassay procedure).

Pipettes glass: 1.00 and 5.00 mL.

Measuring cylinder: 25 mL.

Pipettes with disposable tips: 100, 200 and 500 µl.

Vortex mixer.

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 lyophilized reagents should be destilled 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 dates are valid for the unreconstituted reagents. Reconstituted reagents are stable for 10 weeks, or until the expiry date is reached, stored correctly.

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

Reagent B: ^{125}I-α-MSH
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
Ready for use. Store at 2-8° C.

Reagent E: α-MSH standard, 300 pmol/L
Reconstitute with 5.00 mL distilled water. For preparation of working standards, see radioimmunoassay procedure.
Store at -18° C or lower if reused.

Reagent F-G: Controls
Reconstitute with 1.00 mL distilled water. Store at -18° C or lower if reused.

SPECIMEN COLLECTION

Blood is collected in tubes containing EDTA and Trasylol® (5000 KIU Trasylol in a 10 mL vacutainer). 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 -18° C or lower until assayed. Repeated freezing and thawing should be avoided.

ASSAY PROCEDURE

For an overview see page 11.
Accuracy in all pipetting steps is essential. The assay is performed with duplicates (standards, controls, samples, control tubes for non-specific binding and total activity).

A complete assay includes:

Standard (St-tubes): 7 concentrations: 0, 4.7, 9.4, 18.8, 37.5, 75 and 150 pmol/L.

Samples (S-tubes)

Controls (C-tubes): 2 different controls with known concentrations of α-MSH for quality control.

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

1. Reconstitute the reagents according to the instructions.

2. Prepare the α-MSH working standards by dilution of the α-MSH standard 300 pmol/L (Reagent E) with diluent (Reagent D) according to the following (use glass-tubes for standard preparation):
   a/ 1.00 mL standard 300 pmol/L + 1.00 mL diluent = 150 pmol/L.
   b/ 1.00 mL standard 150 pmol/L + 1.00 mL diluent = 75 pmol/L.
   c/ 1.00 mL standard 75 pmol/L + 1.00 mL diluent = 37.5 pmol/L.
   d/ 1.00 mL standard 37.5 pmol/L + 1.00 mL diluent = 18.8 pmol/L.
   e/ 1.00 mL standard 18.8 pmol/L+ 1.00 mL diluent = 9.4 pmol/L
   f/ 1.00 mL standard 9.4 pmol/L + 1.00 mL diluent = 4.7 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 (0-150 pmol/L), samples and controls in their respective tubes. Pipette 100 µL of the zero-standard in the NSB-tubes.

4. Add 200 µL anti-α-MSH (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-α-MSH (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 supernatants immediately after centrifugation.

13. Count the radioactivity of the precipitates in a gamma counter (counting time 2-4 minutes).

CALCULATIONS OF RESULTS

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 α-MSH standards. An example of a standard curve is given on page 12.

3. Interpolate the α-MSH concentrations of the samples and controls from the generated standard curve.

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

ASSAY CHARACTERISTICS

Sensitivity
The sensitivity calculated from a decrease in binding of 2 SD in the zerostandard is 3 pmol/L.

Precision
Intra assay variation

Level	Coefficient of variation
16.2 pmol/L	11.8%
33.6 pmol/L	4.7%
77.7 pmol/L	2.9%

Inter assay variation (total variation)

Level	Coefficient of variation
16.5 pmol/L	13.0%
37.8 pmol/L	8.4%
79.6 pmol/L	4.0%

Specificity
The following cross reactions have been found:

Polypeptide	Cross reaction
α-MSH	100.0 %
Des-acetyl-α-MSH	100 %
Des-amido-α-MSH	< 0.002%
ACTH 1-13	< 0.002%
ACTH 1-24	< 0.002%
ACTH 1-39	< 0.002%
Beta-MSH	< 0.002%
Gamma-MSH	< 0.002%

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. The found concentrations of the control sera
(Reagent F and G) are within the limits given on the labels of the vials.

2. Total counts
Counts obtained should approximate the expected CPM when adjusted for counter efficiency and radioactive decay. The content of ^{125}I-α-MSH in this kit will give 10 500 CPM (-5%, +20%) at the 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 50-70% at the reference date.

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

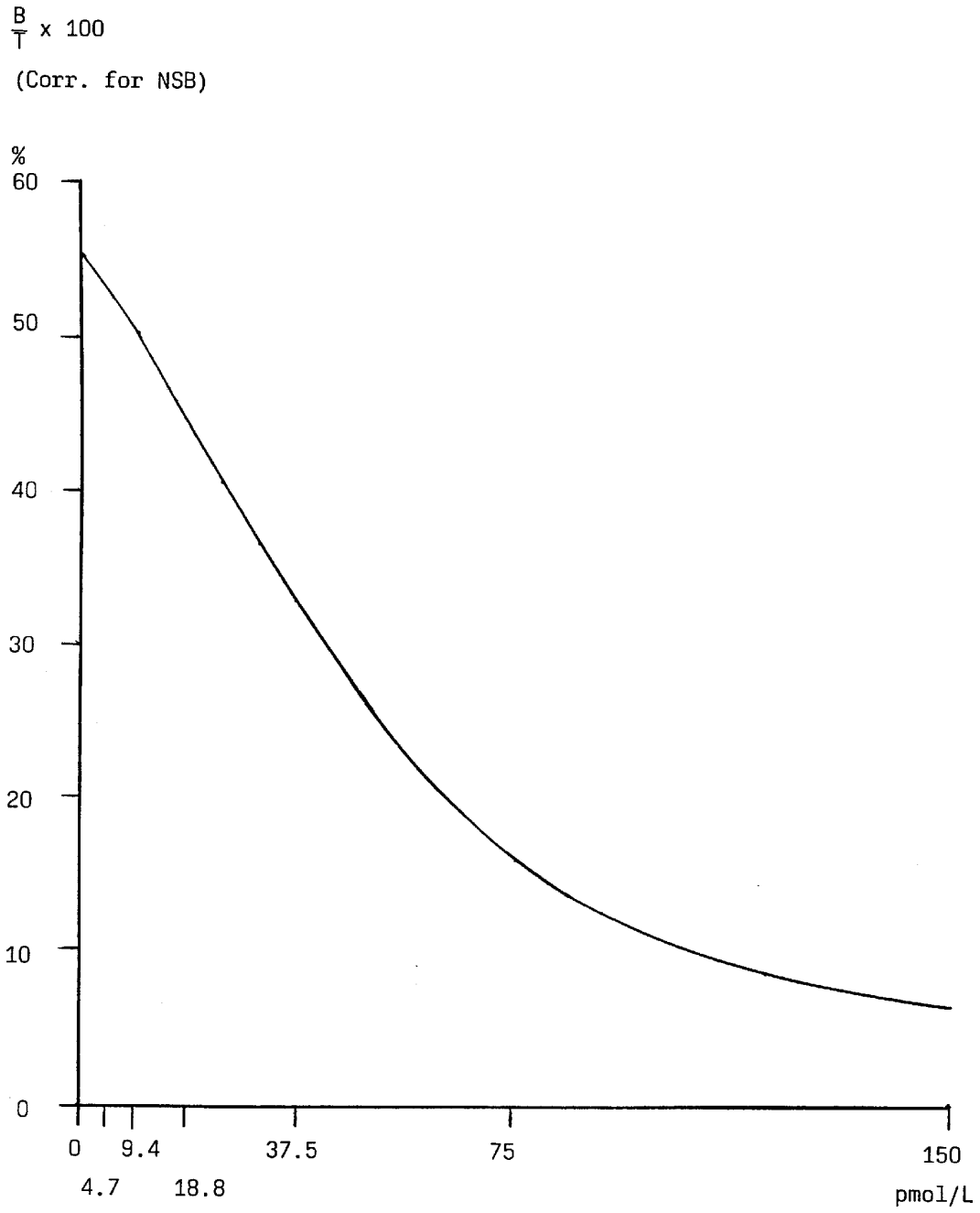
$\frac{NSB}{TOT} \times 100$ is less than 6%.

5. Slope 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-α-MSH (A)	Diluent D)		^{125}I-α-MSH (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 min. at 1700 x g at +4° C. Decant and count the radioactivity of the precipitates.
NSB	3- 4	100 µL	-	200 µL		200 µL		500 µL	
Stand 0	5- 6	100 µL	200 µL	-		200 µL		500 µL	
Stand 4.7	7- 8	100 µL	200 µL	-		200 µL		500 µL	
Stand 9.4	9-10	100 µL	200 µL	-		200 µL		500 µL	
Stand 18.8	11-12	100 µL	200 µL	-		200 µL		500 µL	
Stand 37.5	13-14	100 µL	200 µL	-		200 µL		500 µL	
Stand 75	15-16	100 µL	200 µL	-		200 µL		500 µL	
Stand 150	17-18	100 µL	200 µL	-		200 µL		500 µL	
Control (F)	19-20	100 µL	200 µL	-		200 µL		500 µL	
Control (G)	21-22	100 µL	200 µL	-		200 µL		500 µL	
Sample 1	23-24	100 µL	200 µL	-		200 µL		500 µL	
Sample 2 etc.	25-26	100 µL	200 µL	-		200 µL		500 µL	

EXAMPLE OF ALPHA-MSH STANDARD CURVE



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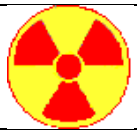
Explanation of symbols.

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

REAG A Ab	Anti-α-MSH.
REAG B Ag ^{125}I	^{125}I-α-MSH.
REAG C DAB	Double antibody-PEG
REAG D DIL	Diluent.
REAG E CAL 300	α-MSH standard, 300 pmol/L.
REAG F CONTROL	Control.
REAG G CONTROL	Control.

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