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Research Article

Preparation and a simple one-step purification of [His^{1}-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2

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Summary

A one-step purification of [His^{1}-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2, prepared using chloramine-T, by HPLC with isocratic elution is described. The labeled GHRH analog was suitable for GHRH receptor binding assays. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: mono-^{125}I-GHRH analog; isocratic; HPLC; binding assay

Introduction

Production and secretion of pituitary growth hormone (GH) is stimulated by hypothalamic GH-releasing hormone (GHRH). Receptors for this peptide (GHRH-Rs) occur on pituitary somatotropes. The GHRH-analog, [His^{1}, Nle^{27}]-hGHRH(1-32)-NH_2 has a high affinity for GHRH-Rs and has been radioiodinated for use as a radioligand to characterize GHRH-Rs.[1] It retains its high binding potency when radioiodinated due to the substitution of His^{1} for Tyr^{1} and to avoid iodination of this amino-terminal tyrosine in hGHRH and due to the

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substitution of Nle for Met in position 27 of the native peptide to avoid possible oxidation of this amino acid.[1,2] We show here a simple, alternative method to prepare the [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2 and demonstrate its utility in a binding assay for GHRH-Rs.

Materials and methods

Reagents and animals

[His^1,Nle^{27}]-hGHRH(1-32)-NH_2 was from American Peptide Co. (Sunnyvale, CA), acetonitrile (HPLC Grade) and HPLC grade water were from Fisher Scientific (Fair Lawn, NJ), carrier-free sodium ^{125}I (NEZ-033H) was from New England Nuclear (Boston, MA), monosodium phosphate, disodium phosphate, chloramine-T, sodium metabisulfite, potassium iodide, phosphoric acid, triethylamine, $MgCl_2$, Tris, EDTA, sucrose and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). The pituitary tissue used for the binding assays was obtained from 3-month-old Sprague–Dawley rats.

Radioiodination of [His^1,Nle^{27}]-hGHRH(1-32)-NH_2 by the chloramine-T method

The procedure for iodination of the GHRH analog was derived from the procedures of Hunter and Greenwood.[3] Two-hundred microliters of 500 mM sodium phosphate buffer (pH = 7.4), 25 µl synthetic [His^1,Nle^{27}]-hGHRH(1-32)-NH_2 (1.5 mg/ml), 20 µl Na ^{125}I (360 µCi/µl), 20 µl chloramine-T (4 mg/ml) were added into a culture tube successively. After mixing for 20 s at room temperature the labeling reaction was terminated by addition of 30 µl of $Na_2S_2O_5$ (5 mg/ml), with continued mixing for an additional 20 s. Ten microliters of KI (20 mg/ml) was then added as a carrier for the ^{125}I to minimize the amount of ^{125}I that is retained on the HPLC column. The mixture was loaded onto the chromatographic column. To increase the transfer of radiolabeled material from the reaction tube to the HPLC column, the culture tube was rinsed with 100 µl of mobile phase and the rinse was added to the injectate.

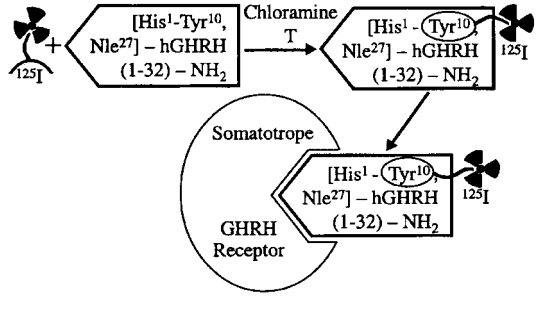
Reverse-phase HPLC

The HPLC comprised a single pump isocratic system connected to a flow-through UV detector capable of monitoring peptide bonds at

210 nm and to a flow-through radioiodine detector (Model 170 Radioisotope Detector, Beckman Instruments, Fullerton, CA.). The separation was carried out on a C_{18} column (Varian, Microsorb-MV, 0.46×25 cm, 100 Å). The mobile phase was 30% acetonitrile and 70% triethylamine phosphate (166 mM phosphate, pH = 2.35). The column was equilibrated with mobile phase and the labeled GHRH analog was eluted isocratically at a flow rate of 1.6 ml/min. The mono-^{125}I-labeled peptide was collected and BSA was added to this to yield a final concentration of 2 mg/ml.

Binding assay

GHRH receptors were assayed by a modification of the method described by Seifert *et al.*[1] Briefly, pituitaries from 3 to 4 rats were pooled for each determination, weighed together, and then placed in 2.2 ml of ice-cold assay buffer (50 mM Tris, 5 mM $MgCl_2$, 0.24 M sucrose, 2 mM EDTA, 2 mg/ml BSA, pH = 7.4). The pituitaries were homogenized mechanically (Tissuemizer, Tekmar, Cincinnati, OH) and a 100 µl aliquot was added to glass culture tubes each containing 50 µl of assay buffer and [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32) amide at



one of four different concentrations ranging from 50 to 500 pM. The pituitary homogenates were incubated at 22°C for 1 h. The mixture was then transferred to 1.5 ml microcentrifuge tubes (Low Retention Flattop Microtube, United Scientific Products, San Leandro, CA) and centrifuged for 2 min at approximately 13600 g. The supernatant was aspirated, the bottom of the microcentrifuge tube containing the tissue membrane pellet was chopped off, placed into a fresh culture tube, and the radioactivity was measured. To assess nonspecific binding, half of the tubes contained 2×10^{-7} M of the unlabeled GHRH analog to saturate the GHRH receptors with non-radioactive ligand. The binding

data were evaluated by regression analysis (PRISM, Graphpad Software, San Diego, CA). Protein in the tissue homogenate was determined by the method of Lowry *et al.*[4]

Results and discussion

After radioiodination of [His^1,Nle^{27}]-hGHRH(1-32)-NH_2 using the chloramine-T method, the radioactive reaction mixture was separated isocratically by reverse-phase HPLC as described above. The initial double peak of radioactivity is ^{125}I that is not incorporated into the peptide. (Figure 1). Several small peaks of radioactivity also eluted from the column prior to the sharp, major radioactive peak containing putative [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2. To confirm the identity of this product, material from this peak was co-chromatographed with authentic [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2, (kindly provided by Dr Jean Rivier, Salk Institute, La Jolla, CA) using mobile phases with 29%, 31% and 32% acetonitrile. In all three conditions, the radioiodinated peptide and the

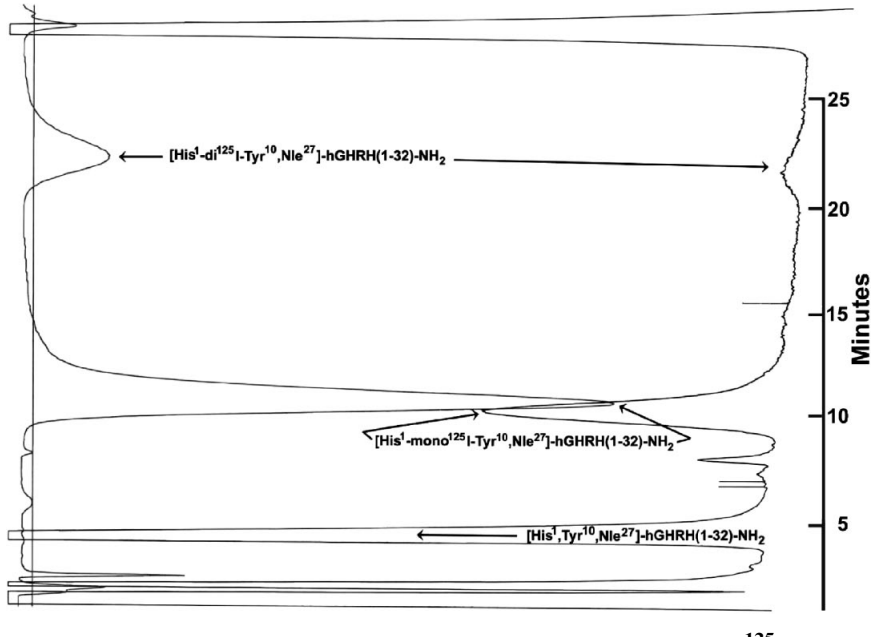


Figure 1. Chromatogram of radioiodination mix. Tracing on left is ^{125}I. Tracing on right is UV light absorbance at 210 nm

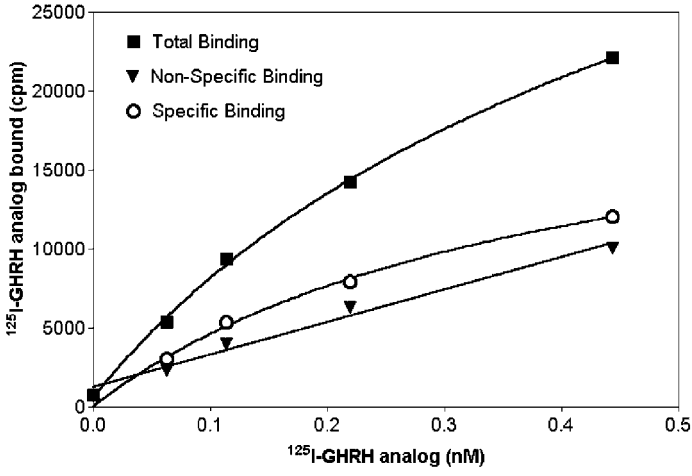


Figure 2. Saturation isotherm of [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2 binding to rat pituitary homogenate. Non-specific binding was assessed in the presence of 200 nM [His^1-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2. The K_d for this experiment was 0.39 nM, and the B_{max} was 22 550 cpm. The curve shown for total binding is the sum of the specific binding curve and linear regression line for non-specific binding

authentic peptide eluted from the column simultaneously. A smaller peak elutes later, probably representing the diiodinated analog. The [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2 yield of this reaction was approximately 35% of the ^{125}I added. The concentration of [His^1-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2 is in excess of 300 nM in the saved fraction. This allows considerable dilution of the radioligand for use in radioligand binding assays, precluding the likelihood that mobile phase components will adversely affect the binding of the radioligand to its receptors.

The binding of the GHRH analog to the rat pituitary was specific and saturable (Figure 2). The dissociation constant (K_d) = 308 $\pm$ 38.2 pM and the amount of specific binding (B_{max}) = 28.6 $\pm$ 1.95 fmol/mg protein in four pools of pituitaries.

Acknowledgements

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