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Identification and quantification of sea lamprey gonadotropin-releasing hormones by electrospray ionization tandem mass spectrometry

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ABSTRACT

Gonadotropin-releasing hormones (GnRH) are neuropeptide hormones that regulate reproduction in vertebrates. Twenty-five unique chordate GnRH isoforms have been identified, all with very similar molecular architecture. Identification and quantification of endogenous GnRH in biological samples is extremely challenging due to the high levels of sequence similarity among these GnRH peptides and complexity of the biological matrices laden with large numbers of other peptides and protein degradation fragments, and due to low levels of GnRH abundance. In this study, three lamprey GnRH (lGnRH-I, -II, and -III) were extracted from sea lamprey brain tissue and plasma samples by solid-phase extraction (SPE) and identified by a high resolution Q-TOF mass spectrometry (MS). A rapid quantitation method was developed and validated to determine the concentrations of these three lGnRHs by using a UPLC coupled tandem MS in positive ESI multiple reaction monitoring (MRM) mode. Luteinizing hormone-release hormone (LHRH, one of the mammalian GnRHs) was used as the internal standard. The developed quantitation method was fully validated for its recovery, matrix effect, linearity, repeatability, precision and accuracy, and storage stability. This method exhibited excellent linearity in a broad concentration range for all three lGnRHs ($R^2 > 0.99$) and limits of detection (LOD; as low as 0.007 ng/mL). Brain and plasma samples from a total of 280 sea lampreys were analyzed with the developed method to investigate the biological relevance of the lGnRH levels. The concentrations of these three lGnRHs were detected at levels of pictogram per microgram brain tissue and milliliter of plasma. The obtained analytical performance parameters and collected data from real biological samples have proven that this is a robust, sensitive, and fully validated LC–MS/MS method to simultaneously quantify three neuropeptide hormones in complex biological matrices.

1. Introduction

Gonadotropin-releasing hormones (GnRH) are neuropeptides found in the hypothalamus of vertebrates. GnRH stimulates the synthesis and secretion of gonadotropins and regulates reproductive activities through the hypothalamic-pituitary-gonadal (HPG) axis. To date, twenty-five unique chordate GnRH isoforms have been identified, all with a similar molecular architecture [1–3]. Chordate GnRHs are peptides consisting of ten amino acid residues with an *N*-terminal pyroglutamic acid and a *C*-terminal glycine. Many functions have been ascribed to these neuropeptides, including modulation of cell functions such as cell proliferation or apoptosis in different organs [4–8]. Different GnRH peptides are expressed in a variety of tissues, suggesting that they possess multiple functions and their syntheses are subjected to complex regulations [9]. Therefore, it is important to determine the concentrations of GnRHs in different tissues at different developmental stages to unravel their novel functions.

Sea lamprey (*Petromyzon marinus*) is an ideal model for this study. It is useful in basic neurobiological research, such as elucidating neural circuits of vertebrate brains,[10–13] as well as examining the adaptation and the evolution of vertebrate nervous systems.[14–16] Three lamprey (l) GnRHs, lGnRH-I, -II, and -III, have been identified in the sea lamprey.[17–19] These lGnRHs act as neurohormones that regulate the HPG axis in adult sea lampreys.[20–26] Further studies are necessary to elucidate the differential expression and function of these lGnRHs. It is hence pivotal to develop a simple detection method with high sensitivity

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and selectivity that simultaneously detects and quantifies various GnRHs to understand the physiological function and molecular process involving GnRH and GnRH-like peptides.

Analyses of endogenous GnRHs have been challenging due to their great diversity among chordates, similarity in primary structures and low concentrations within complex matrices. Somoza et al. reviewed the methodologies used to identify GnRH based on indirect approaches including radio-immunoassays (RIA) with specific antisera, *in situ* hybridization with homologous and heterologous probes, and RP-HPLC in combination with RIA [9]. RIA often suffers from poor specificity due to cross reaction with closely related peptides that are often present in the samples [27]. Analytical approaches that couple peptide separation (high-performance liquid-chromatography and capillary electrophoresis) and detection (absorbance, fluorescence, and electrochemistry) have been used for analyses of GnRH and GnRH-like peptides [28–36]. These methods are unable to meet the increasing requirements for routine analyses due to their poor sensitivity, selectivity and the necessity for derivatization steps. Mass spectrometry (MS) of different ionization techniques has been applied to identify GnRH primary structures [37–46]. An electrospray ionization-tandem mass spectrometry (ESI-MS/MS) approach was proposed for GnRH characterization to obtain sequence information using six model GnRH peptides [47]. Quantification of endogenous peptides (such as GnRH) with MS is more difficult than peptide characterization particularly in the aspect of purification, separation and sensitivity [48]. To date, MS techniques have been employed only to determine the molecular weight of purified GnRH or to isolate and identify known GnRH peptides [47,49].

The aim of this research was to establish a versatile, robust and sensitive method to identify and simultaneously quantify different GnRHs in biological sample matrixes. Three lGnRHs were sequenced and identified by a high resolution Q-TOF MS in brain tissue extracts and plasma. Their primary structures were confirmed by MS/MS spectra and their concentrations in 280 samples of lamprey brain and plasma were determined by a rapid UPLC–ESI-MS/MS method, developed and fully validated in this study. Significant biological relevancies of the lGnRH levels were reported based on the results of this study [54,55]. The applications of the developed method in the determination of GnRHs are important in neuroscience, reproductive biology, sea lamprey management and clinical studies. The developed method should be easily adaptable to the determination of other GnRH peptides and fulfill the routine measurement requirements.

2. Experimental

2.1. Liquid chromatography and mass spectrometry

High resolution MS was performed on a Xevo G2-S Q-TOF MS (Waters, Milford, MA, USA) in positive ESI mode. The sample separation was carried on a Waters ACQUITY H-Class UPLC with a BEH C18 column (2.1 mm × 100 mm, 1.7um particle size). The flow rate was 0.25 mL/min with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile (ACN) as mobile phase B. The capillary voltage, cone voltage, and rf lens energy setting were set at 3.00 kV, 35 V, and 100.0, respectively. The flow rates of cone gas and desolvation gas were 50 and 500 L/h, respectively. The source temperature and desolvation temperature were 90 and 200 °C.

The quantitation of lGnRH was performed on a Waters Xevo TQ-S mass spectrometry coupled to an H-Class UPLC system with a Waters BEH C18 column (1.0 mm × 50 mm, 1.7 μm particle size) and oven temperature at 35 °C. The injection volume was 10 μL, and the UPLC flow rate was 0.15 mL/min with a gradient shown as below (mobile phase A: 0.1% formic acid in water; B: 0.1% formic acid in ACN): initial, 88% A and 12% B; 0.5 min, 88% A; 4 min, 65% A; 7 min, 1% A; 8 min, 1% A; 8.01 min, 88% A; and 9 min, 88% A. Mass spectra were acquired using electrospray ionization in positive ion mode and MRM. The capillary voltage, cone voltage, and rf lens setting were set at 3.20 kV, 64 V, and 0.3, respectively. The flow rates of cone gas and desolvation gas were 20 and 400 L/h, respectively. The source temperature and desolvation temperature were 150 and 500 °C. Collision-induced dissociation employed argon as collision gas at a manifold pressure of 2×10^{-3} mbar, and collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. Data were acquired with MassLynx 4.1 and processed for calibration and quantification of the analytes with QuanLynx software.

3. Standard solutions

The lGnRH-I and -II were custom synthesized by GenScript USA Inc. (Piscataway, NJ, USA). lGnRH-III and LHRH were purchased from Bachem Americas, Inc. (Torrance, CA, USA). Each peptide was dissolved in 50% methanol/H_2O (v/v) to make individual stock solution at 1 mg/mL, and stored at −20 °C. Subsequent dilutions were made into 50% methanol/H_2O to minimize adsorption to tube and vial walls. Calibration standard spiking solutions were prepared in a range from 0.01 to 10 ng/mL by spiking appropriate stock solutions to brain tissue extracts. Internal standard (LHRH) solution (50 ng/mL) was prepared in 50% methanol/H_2O and 20 μL was added to each sample (1 ng total per sample). The quality control (QC) samples were prepared in homogenized brain tissue samples and plasma spiked with known amounts of analytes and internal standard at three concentration levels: 0.1 ng/mL (low, LQC), 1 ng/mL (middle, MQC), and 10 ng/mL (high, HQC).

3.1. Animal and sample collection

Two hundred and eighty sea lampreys were collected from the Laurential Great Lakes tributary streams by the U.S. Fish and Wildlife Service Marquette Biological Station (Marquette, MI, USA) and Canadian Department of Fisheries and Oceans Sea Lamprey Center (Sault Ste. Marie, Ontario, Canada). The body weight of sea lampreys ranged from 156 g to 405 g (average 261.3 g), and the body length ranged from 40.2 cm to 59.0 cm (average 51.1 cm). They were transported to the U.S. Geological Survey Hammond Bay Biological Station (Millersburg, MI, USA), where brain and blood were sampled. For each set of experiments, all test subjects were captured from the same stream on the same day to reduce variation in levels of maturity. Standard operating procedures for transporting, maintaining, handling, anesthetizing, and euthanizing sea lampreys were approved by the Institutional Committee on Animal Use and Care of Michigan State University. All animals were euthanized with 0.05% MS-222 (Sigma–Aldrich, St. Louis, MO, USA) before handling or tissue sampling. Plasma samples were obtained by centrifuging blood at 1000 × *g* for 20 min at 4 °C. Supernatants (plasma) were stored at −20 °C until use. Forebrain and hindbrain were separated, snap frozen in liquid nitrogen, and stored at −80 °C. The net weight of the collected forebrain and hindbrain tissue was in the range of 15.1–78.1 mg. The brain samples were weighed and homogenized with 400 μL of 1% formic acid in cold (−20 °C) acetonitrile with internal standard (LHRH, final concentration: 1 ng/mL) [50]. 1 mL 1% formic acid in cold (−20 °C) acetonitrile was added to the homogenate, incubated at −20 °C for 15 min, and centrifuged at 15,800 × *g* for 20 min at 4 °C. The supernatant was then transferred to a new tube, freeze-dried overnight, and reconstituted in 1 mL water solution with 3% acetic acid and 1% trifluoroacetic acid (TFA). 500 μL plasma samples were processed with the same procedure without the homogenization step.