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Isocratic liquid chromatographic assay for monitoring the degradation of luteinizing hormone releasing hormone by extracts from the gastrointestinal tract of possums

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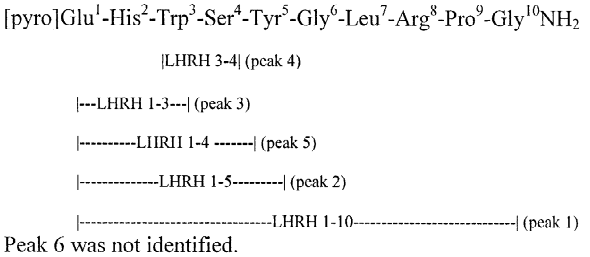
Abstract

A simple HPLC method to separate human luteinizing hormone releasing hormone (LHRH) from its metabolites using an isocratic elution is described. Intact LHRH and five metabolites were separated in 11.4 min. The calibration curve (peak area versus concentration) was linear over the concentration range 1.25–35 μg/ml (r^2=0.99) with the intercept not significantly different from zero (P>0.05). Intra-day and inter-day variability of the assay was less than 5% for repeat injections of 5, 14.5 and 29 μg/ml. The method was applied to evaluate the susceptibility of LHRH to enzymes present in the lumen and mucosal extracts of the gastrointestinal tract of possums. The major degradation products of LHRH were identified by HPLC separation, amino acid analysis and mass spectrometry as LHRH (1–5), LHRH (1–4), LHRH (1–3) and LHRH (3–4).

Keywords: Luteinizing hormone releasing hormone

1. Introduction

The decapeptide, human luteinizing hormone releasing hormone (LHRH, Fig. 1) regulates the secretion of both luteinizing hormone and follicle stimulating hormone from the anterior pituitary in a dose-dependent manner [1]. A single dose of LHRH stimulates release of pituitary gonadotrophins while



Peak 6 was not identified.

Fig. 1. Correlation of LHRH and its metabolites with HPLC peaks.

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multiple doses or continuous infusion can produce reversible pituitary desensitization [2]. Its ability to block release of gonadotrophins by pituitary down regulation is the basis for its use as a female contraceptive and hence its potential use in the population control of wild pests [3]. In the present study, LHRH was used as a model peptide to investigate the enzymatic barrier limiting oral delivery of peptide bioactives in the possum, a pest in New Zealand following their introduction from Australia in the early 1800s. In the short to medium-term, oral baits will be the most practical method of delivering such biocontrol agents to possum. However, it is well documented that orally delivered peptides are degraded by the large number of endo- and exo-peptidases present in the intestinal luminal contents and the mucosal membrane [4]. In order to develop systems to overcome these enzymatic barriers to the oral delivery of peptide-based biocontrol agents, it is essential to understand their metabolic fate following oral administration.

High-performance liquid chromatography (HPLC) is a readily available, cost-efficient technique to separate a parent compound from its metabolic products. To date, however, HPLC methods described in the literature that satisfactorily separate LHRH from its metabolites are based on gradient elution chromatography [5–13]. Since solvent compositions change continuously during gradient HPLC and detectors are sensitive to changing solvent compositions, there is a tendency for base-line shifting and increased variability with these assays. Additionally, the system requires time to restablize for each new sample [14]. The aim of this work was to develop a rapid isocratic HPLC assay which could separate LHRH from its metabolites. In this study, degradation of human LHRH by enzymes presents in the luminal and mucosal extracts of the possum gastrointestinal tract was determined in order to monitor the metabolic fate of the peptide in these biological matrices.

2. Experimental

2.1. Animals

Adult, male brushtail possums that had been live-captured in the Otago region of New Zealand (latitude 45°S) were used in this study. At the time of capture, each animal was screened for health status and only large (≥2.5 kg live weight), healthy animals were included. Prior to use, animals were housed (n=5–18/group) under conditions of natural day-length and temperature. Animals had free access to water and a mixed diet of fresh fruit, specially formulated cereal-based pellets and bread. This study had approval from the AgResarch Invermay Animal Ethics Committee under the Animal Protection (Codes of Ethical Conduct) Regulations 1989.

2.2. Reagents

Human luteinizing hormone releasing hormone (LHRH, purity 99%) was purchased from American Peptide Company (Sunnyvale, CA, USA). Sodium hydroxide, disodium hydrogen orthophosphate and acetonitrile (HPLC grade) were purchased from BDH Chemicals (Poole, Dorset, UK). Sodium dihydrogen orthophosphate and hydrochloric acid was purchased from Ajax Chemicals (Auburn, NSW, Australia). Trifluoroacetic acid (anhydrous protein sequencing grade) was purchased from Sigma (St. Louis, MO, USA). Euthal (sodium pentobarbitone 170 mg/ml and sodium phenytoin 25 mg/ml) was purchased from Delta Veterinary Laboratories (Hornsby NSW, Australia). Distilled, deionized water was used throughout and was obtained from a Millipore Milli-Q reagent water system.

2.3. Collection of luminal and mucosal extracts

Possums were fasted for 24 h and then euthanized by intra-cardiac injection of 4-ml Euthal™ while under halothane-induced anesthesia. Immediately following euthanasia, an incision was made along the abdominal centre line, the intestine removed and dissected into six regions: duodenum, jejunum, ileum, caecum, proximal colon and distal colon. The contents of each region were carefully squeezed into a centrifuge tube, and remaining contents were flushed into the centrifuge tube with a minimal volume of cold saline. The recovered intestinal contents were then diluted with cold 50 m*M* sodium phosphate buffer (pH 7.0) to give a final volume of 10 ml, homogenized for 2 min at 4 °C using a Ystral homogenizer and centrifuged at 3000 *g* for 15 min at 4 °C. Supernatants were collected and 200-μl

aliquots stored in 0.5-ml Eppendorf tubes at −80 °C until required.

The mucosal surface of each region of gastrointestinal tract was washed with cold 50 m*M* phosphate buffer (pH 7.4). The mucosa was then removed by scraping off the epithelial cell layer with a microscope slide. The mucosal homogenate was transferred into a centrifuge tube and processed as described above for luminal contents. All equipment and solutions used in sample preparation were maintained at 4 °C to minimize enzyme degradation.

The concentration of total protein in the luminal and mucosal samples, used as a measure of enzyme concentration was measured by the modified Lowry assay [15].

2.4. Degradation of LHRH by luminal and mucosal extracts

Luminal extracts (250–500 μg/ml protein, 20 μl) or mucosal extracts (2500 μg/ml protein, 20 μl) were diluted to 210 μl with either pH 7 or 7.4 phosphate buffer (50 m*M*), respectively, and incubated at 37 °C for 10 min. Proteolytic reactions were initiated by addition of 40 μl LHRH solution (1.5 mg/ml, pre-warmed to 37 °C for 10 min) to the diluted enzymatic solutions. The final concentrations in the reaction mixture were 0.24 mg/ml LHRH and either 20–40 μg/ml luminal or 200 μg/ml mucosal protein. The reaction mixtures were incubated at 37 °C for a further 180 min. At predetermined times (0, 10, 15, 20, 30, 40, 60, 90, 120, 180 min), 20-μl aliquots of the reaction mixture were dispensed into 0.5-ml Eppendorf tubes containing 20-μl hydrochloric acid (0.2 *M*) to induce protein precipitation and terminate the reaction. The precipitated mixtures were allowed to stand for 15 min at room temperature, then centrifuged at 20 000 *g* for 10 min. Aliquots of the supernatant (20 μl) were diluted (1:10) with HPLC water before HPLC analysis.

2.5. HPLC analysis

The Shimadzu HPLC system consisted of a solvent delivery system (Model LC-6A), a UV detector (Model SPD-6AV), an auto injector (Model SIL-9A) fitted with a 50-μl loop connected to a Hewlett-Packard HP (Model 3396A) integrator. Chromatography was performed on a Luna C_{18} (2) column (250×4.6 mm, 5 μm particle size; Phenomenex) fitted with a C_{18} guard column (10×3.0 mm). The mobile phase was 0.1% trifluoroacetic acid in 21% (v/v) acetonitrile in water adjusted to pH 1.9–2.5 with sodium hydroxide. Chromatographic separation was performed isocratically at a flow-rate of 1 ml/min at room temperature. The column eluent was analyzed at 215 nm.

2.6. Assay validation

Triplicate stock solutions of LHRH (1.5 mg/ml) were prepared in 10 m*M* hydrochloric acid containing 1 μg/ml inactive (boiled) luminal extract and diluted with mobile phase to give final concentrations of 35, 18, 9, 5, 2.5 and 1.25 μg/ml and analyzed by HPLC at pH 2.5. A standard curve was obtained by plotting the peak area against LHRH concentration. The slope, *y*-intercept and linearity of the curve were determined by linear regression analysis. Replicate analyses ($n=6$) of 5, 14.5 and 29 μg/ml standard solutions undertaken on four consecutive days were used to calculate the intra- and inter-day coefficients of variation and the accuracy of the assay at these concentrations. Standard curves were established on each day before sample analyses.

Reproducibility of LHRH and metabolite peak areas was evaluated by calculating the coefficient of variation for results of five replicate analyses of a single sample solution. The sample solution was prepared as discussed above.

2.7. Identification of LHRH metabolites

Fractions corresponding to discrete peaks were manually collected and evaporated to dryness under vacuum (Savant SpeedVac Plus SC 210A). The dried residue was dissolved in 100 μl water, 50 μl of this solution was lyophilized, hydrolyzed in vapor phase (0.1% phenol in 6 *M* hydrochloric acid under vacuum at 160 °C for 1 h) and derivatized with phenylisothiocyanate (PITC). The PITC-amino acids were analyzed on a narrow-bore binary RP-HPLC system according to the method of Hubbard [16].

The molecular mass of the fractions relating to the parent peak 1 and to peak 2 were measured by matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) as de-

scribed by Hubbard and McHugh [17]. The molecular masses of the fractions relating to peaks 3 and 5 were measured by electrospray mass spectrometry (ESMS). Mass spectra were collected on an API 300 triple quadrupole Mass Spectrometer (Perkin-Elmer Sciex Instruments) fitted with a nebulization assisted atmospheric pressure ionization source. All samples were infused into the spectrometer using a fused-silica capillary tube (I.D. 60 μm), at a rate of 5 μl/min using a syringe pump (Harvard Apparatus, model 2400-001). A coaxial spray of nitrogen at 0.9 l/min assisted nebulization of the liquid sample.

3. Results

3.1. HPLC assay and validation

LHRH and its metabolites were quantitatively analysed by HPLC using a mobile phase of 0.1% trifluoroacetic acid in 21% (w/w) acetonitrile in water over the range of pH 1.9–2.5. Typical chromatographs of LHRH with its degradation products following incubation with a luminal extract from jejunum eluted under these conditions are shown in Fig. 2A–D. Five metabolite peaks were identified which were completely resolved from the parent LHRH. Retention time and separation of the compounds were highly dependent on the organic content and pH of the mobile phase. For example, changing the acetonitrile content of the mobile phase from 20% (w/w) to 22.5% (w/w) reduced the retention time of LHRH from 18.4 to 7.66 min (figure not shown). Decreasing the pH of the mobile phase from 2.5 to 1.9 improved the separation of peaks 4 and 5 (figure not shown), which allowed easier collection of samples for amino acid analysis by MALDI-TOF-MS and ESMS. The peak area of parent LHRH decreased, whereas the peak areas of its metabolites increased, or increased initially then decreased during incubation of LHRH in jejunal luminal extracts (Fig. 3).

Analysis of LHRH standard solutions demonstrated that the peak area of LHRH was a linear function of concentration over the concentration range 1.25–35 μg/ml (peak area=concentration×682.32±2.3–17.28±10.5, r^2=0.999, n=3). The intercept of the standard curve was not significantly different from zero ($P>0.05$). Intra-day and inter-day variability of the assay was less than 5% for the three concentrations tested (Table 1). In terms of the accuracy of the assay, the mean concentrations measured

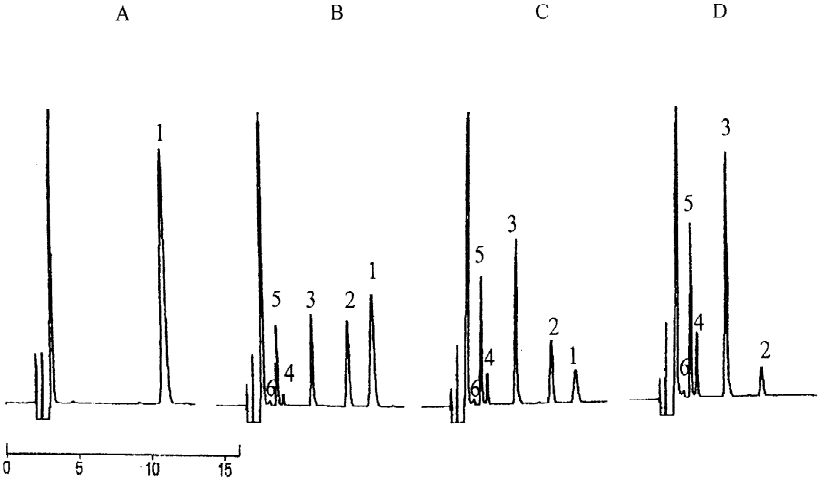

Fig. 2. Chromatogram of LHRH and its metabolites eluted by 0.1% trifluoroacetic acid in 21% (w/w) acetonitrile in water, pH 2.5 with UV detection at 215 nm. (A) Peak 1 LHRH 1–10 before incubation with luminal extracts, (B) after 6 min incubation with luminal extracts, (C) after 20 min and (D) after 30 min incubation with the luminal extracts. Peak 1 (LHRH 1–10), Peak 2 (LHRH 1–5), Peak 3 (LHRH 1–3), Peak 4 (LHRH 3–4), Peak 5 (LHRH 1–4) and peak 6 (Unknown).

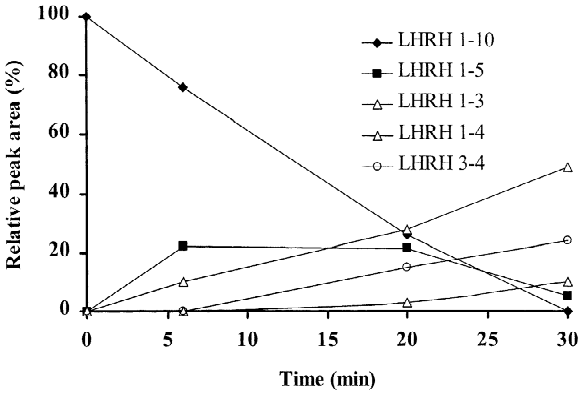

Fig. 3. LHRH peptidolysis and metabolite formation in jejunal luminal extracts, pH 7.0 when incubated at 37 °C for 20 min. Eluted by 0.1% trifluoroacetic acid in 21% (w/w) acetonitrile in water, pH 1.9, with UV detection at 215 nm. Each point is expressed as peak area relative to the initial peak area of LHRH.

Table 1
Intra- and inter-day variation and accuracy of HPLC assay

Spiked concentration of LHRH (μg/ml)	Calculated concentration of LHRH (μg/ml)	Variability (C.V) (%)	Accuracy (%)
Intra-day:			
5	4.73±0.20	2.01	95
14.5	13.71±0.18	0.95	95
29	27.99±0.30	0.66	97
Inter-day:			
5	4.74±0.20	4.23	95
14.5	13.71±0.34	3.21	95
29	27.95±0.43	1.57	96

agreed well with the spiked concentrations. In all cases, accuracy was greater than or equal to 95%. The lower limit of detection for LHRH (signal-to-noise ratio of greater than 3:1) was 0.46 μg/ml for a 50-μl injection volume. The reproducibilities of LHRH and metabolites after 20 min degradation by jejunal luminal extracts are shown in Table 2.

3.2. Identification of LHRH metabolites

Amino acid analysis and mass spectrometry of the metabolites of LHRH indicated that peak 1 (retention time of 11.4 min) was LHRH (1–10) having a mass:charge ratio of 1182.5, peak 2 (9.93 min) was LHRH (1–5) having a mass:charge ratio of 704, peak 3 (7.24 min) was LHRH (1–3) having a mass:charge ratio of 453.2, peak 5 (4.45 min) was LHRH (1–4) having a mass:charge radio of 540, peak 4 (5.05 min) was identified by amino acid analyses as LHRH (3–4) (Table 3 and Fig. 1). Due to the small amount of peak 6 produced (4.0 min), it was not possible to identify this compound either by amino acid analysis or mass spectrometry.

4. Discussion

The isocratic HPLC assay described allows for the rapid and simple quantitation and separation of human LHRH from its metabolites, previously only reported by gradient elution HPLC [5–13]. The application of the assay to monitor the proteolytic degradation of LHRH by enzymes present in the intestinal lumen and mucosal tissue of the possum is also demonstrated. Based on amino acid analysis and mass spectrometry of the metabolic fractions, the first metabolic product formed following incubation with luminal extracts from jejunum was LHRH (1–5) (peak 2, Fig. 2B). This was followed by formation of LHRH (1–3) and LHRH (1–4) (peaks 3 and 5, respectively, Fig. 2B). With time, metabolites LHRH (1–3), LHRH (1–4), LHRH (3–4) (peak 4) and an unknown metabolite eluting at 4.0 min (peak 6) increased in peak area, whereas metabolite LHRH

Table 2
Reproducibility of LHRH and metabolite peak areas after 20 min degradation by jejunal luminal extracts from brushtail possum

Peak area	LHRH (1–10)	LHRH (1–5)	LHRH (1–3)	LHRH (1–4)	LHRH (3–4)	Unknown
C.V. (%)	4.7	2.3	1.8	2.0	4.6	ND

ND, not detectable at 20 min incubation; $n=5$ (replicate analyses).

Table 3
Degradation products of LHRH obtained by digestion with luminal extracts from the jejunum of brushtail possums

Peak[a]	Amino acids[b]	m/z[c]	m[d]	ms[2e]	Assignment
1	Glu, His, Trp, Ser, Tyr, Gly, Leu, Arg, Pro	1182.5	1181.5	NA	[pyro]Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–GlyNH_2
2	Glu, His, Trp, Ser, Tyr	704	703	NA	pyroGlu–His–Trp–Ser–Try
3	Glu, His, Trp	453.2	452.2	110.1, 187.8, 220.9, 248.9	pyroGlu–His–Trp
4	Ser, Trp	NA	NA	NA	Trp–Ser
5	Glu, His, Trp, Ser	540	539	159, 221.2, 248.8, 275, 435	pyroGlu–His–Trp–Ser
6	Unknown	NA	NA	NA	

NA, not available.
[a] Peak numbers refer to Fig. 2.
[b] Amino acid composition by amino acid analysis.
[c] m/z values for the ion [M+H].
[d] Monoisotopic masses.
[e] ms^2 product ions refer to its precursor ion.

(1–5) decreased in peak area (Fig. 2C,D). The main metabolic products from mucosal enzyme digestion were LHRH (1–3), LHRH(1–4), LHRH (3–4) and an unknown. In contrast to luminal enzyme degradation, LHRH (1–5) was absent in samples from mucosal homogenate degradation (data not shown). Although these analyses were restricted to degradation of LHRH by gastrointestinal enzymes of the Brushtail possum, we have established that the assay is also applicable for the separation of salmon LHRH from its metabolites (data not shown).

Degradation of LHRH by luminal extracts and mucosal homogenates followed apparent zero-order kinetics for at least (30 min) and the rate constants were proportional to protein concentration of the extracts (up to 50 μg/ml). The greatest peptidolytic activity was found in luminal extracts from the jejunum and ileum. A detailed report of the metabolism of LHRH by extracts from the possum gastrointestinal tract will be reported elsewhere [18–20].

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