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Journal of Chromatography B, 682 (1996) 219–224

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Rapid separation of gonadotropin-releasing hormone molecular forms by isocratic high-performance liquid chromatography on an ion-exchange column

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Received 5 September 1995; revised 6 February 1996; accepted 13 February 1996

Abstract

The purpose of the present work was to develop a chromatographic system for the separation of five molecular forms of the gonadotropin-releasing hormone (GnRH); mammalian GnRH (mGnRH) (LHRH), salmon GnRH (sGnRH), chicken I GnRH (cIGnRH), chicken II GnRH (cIIGnRH) and lamprey GnRH I (lGnRH-I). By using an ion-exchange HPLC column and isocratic elution, it was possible to separate properly the five peptides in approximately 20 min. The utility of the system in determining the GnRHs forms present in the brain of two species of vertebrates was examined.

Keywords: Gonadotropin-releasing hormone

1. Introduction

Gonadotropin-releasing hormone (GnRH) represents a family of decapeptides that control the release of gonadotropins from the pituitary in vertebrates [1]. The amino acid residues 1, 2, 4, 9 and 10 are identical in the nine forms described to date, while the residue in position 8 varies from species to species [2]. Most of the vertebrates have two or more molecular GnRH variants in the brain (within a single species) [3,4]. The known forms of these variants are mammalian GnRH (mGnRH) [5,6], chicken GnRH I (cIGnRH) [7,8], chicken GnRH II (cIIGnRH) [9], salmon GnRH (sGnRH) [10], catfish GnRH (cfGnRH) [11], seabream GnRH (sbGnRH) [12], dogfish GnRH (dfGnRH) [13], lamprey GnRH I (lGnRH-I) [14] and lamprey GnRH III (lGnRH-III) [15]. In mammalians and amphibians, a post-translationally modified form (hydroxyproline^9 mGnRH) was found also [16,17]. In addition, chromatographic and immunological evidence exists of other, as yet unidentified, molecular variants in different species of vertebrates [4,18,19]. We did not study the chromatographic properties of sbGnRH, dfGnRH and lGnRH-III in the present work because their standards were not available to us.

The procedures for characterisation of the GnRHs include HPLC separation and subsequent radioimmunoasay of the fractions for the different molecular forms using specific antisera.

Most of the HPLC systems described in the literature require solvent gradient systems in order to

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PII S0378-4347(96)00087-4

achieve satisfactory separation [20,21]. We report the development of an easy HPLC method for the separation of five of the six GnRH peptides studied on an ion-exchange column and isocratic elution, based on a mobile phase composed by acetonitrile (ACN), methanol (MeOH) and phosphate buffer (PB).

The applicability of this method was tested in tissues from species with molecular GnRHs form that have already been described, such as the carp (*Cyprinus carpio*) that contain sGnRH and cIIGnRH [22] and the chicken (*Gallus domesticus*) containing cIGnRH and cIIGnRH [7–9].

2. Experimental

2.1. Reagents

mGnRH, cI-GnRH, cII-GnRH, sGnRH and lGnRH-I were obtained from Peninsula Laboratories (Belmont, CA, USA) and cfGnRH was generously provided by Dr. Rüdiger Schültz (Research Group for Comparative Endocrinology, Department of Experimental Zoology, University of Utrecht, Utrecht, Netherlands). Antisera cII678 was generously donated by Dr. J.A. King (University of Cape Town, Cape Town, South Africa). HPLC-grade acetonitrile and methanol were purchased from Sintorgan (Buenos Aires, Argentina). Water for use in HPLC was prepared with a Milli-Q reagent water system (Millipore, Milford, MA, USA). The sodium [^{125}]iodide solution was purchased from New England Nuclear-Dupont (Boston, MA, USA), and carboxymethylcellulose was obtained from Sigma (St. Louis, MO, USA). All other reagents used were of analytical purity.

2.2. HPLC apparatus

The HPLC instrument, consisting of a Model 2150 pump with a 200-μl sample loop, a Model 2220 integrator and a Model 2151 UV variable spectrophotometer, was obtained from LKB (Bromma, Sweden) and the injector was a Rheodyne Model 7125 (Rheodyne, Berkley, CA, USA). Chromatography was performed on an Ultropac TSK SP5PW ion-exchange column (75×7.5 mm I.D., 10 μm particle size) obtained from Pharmacia (Uppsala, Sweden) that was protected with a TSK SP-5PW guard column (10×6 mm I.D.) from Pharmacia.

2.3. Preparation of standards

Standards of the peptides were disolved in 0.1 *M* acetic acid at 0.01 μg/μl.

2.4. Preparation of samples

Brain and pituitaries were dissected from adult specimens of carp and chickens. Fish were anesthetized in 0.05% tricaine methanesulfonate (Sigma), and tissues from the chickens were obtained at the commercial hatchery. The removed brains and pituitary glands were stored at −70°C until extraction.

Frozen pooled brains and pituitaries from 40 fish and 10 chickens, were homogenized in acetone–1 *M* HCl (100:3, v/v) at 4°C using an Ultraturrax homogeniser. The extraction mixture was stirred for 3 h at 4°C and filtered through a Whatman No. 1 filter. The insoluble material was re-extracted in acetone–0.01 *M* HCl (80:20, v/v) in 40% of the original volume, stirred for 5 min and refiltered. The combined filtrates were extracted five times with petroleum ether, in order to remove lipids, as previously described [14,23]. The final aqueous phases were then concentrated in a rotavapor apparatus to a final volume of 0.5–1 ml, stored at −20°C, resuspended in the mobile phase at a final volume of 2 ml and filtered through a 0.45-μm Millipore HPLV membrane before injection. The injection volume was 200 μl.

2.5. Mobile phase

The mobile phase was made by mixing 0.11 *M* phosphate buffer (PB) ($NaH_2PO_4 \cdot H_2O$, adjusted to pH 2.90 with phosphoric acid), ACN, and MeOH (70:15:15, v/v), then filtered through a 0.45-μm Millipore HPLV membrane and degassed.

2.6. Running conditions

Chromatographic separation was performed at room temperature with a flow-rate of 1.0 ml/min and

the column eluent was monitored at 280 nm. Collected fractions (1 ml) were lyophilised and resuspended in 300 μl of PBS–BSA (0.05 *M* phosphate buffer, 0.15 *M* NaCl, 0.01 *M* EDTA, 0.015 *M* sodium azide, pH 7.5, containing 0.5% bovine serum albumin) for subsequent RIA.

Each injection of the tissue extract was preceded by a blank run in which only mobile phase was injected, and the fractions were screened by RIA under the same conditions as the samples, to rule out any memory effects originating from the relatively high concentrations of previously injected synthetic peptides. A mixture of authentic standard peptides (0.2 μg per injection) was chromatographed after the tissue extract was run, for comparison. Finally, a pool of standards (2 μg each), which is a sufficient concentration for UV detection, was injected in order to allow an unequivocal assignment of retention times to individual peptides under the chosen running conditions.

2.7. Radioimmunoasay

mGnRH was labeled with ^{125}I according to the method of Yu et al. [23], with minor modifications. Briefly, 1 mCi of Na ^{125}I in 10 μl of 0.5 *M* PB, pH 7.5, and 5 μg of chloramine T in 10 μl of 0.05 *M* PB were added to 10 μg of each synthetic standard in 10 μl of 0.1 *M* acetic acid, and incubated at room temperature for 40 s. The reaction was stopped by addition of 200 μl of starting elution buffer (2 m*M* ammonium acetate buffer, pH 4.5). A carboxymethylcellulose (fibrous form, Sigma) cation-exchange column (6×0.6 cm I.D.) was used to separate the labeled peptide. The column was eluted with 15 ml of the starting elution buffer and then with 0.2 *M* ammonium acetate buffer (pH 4.5). Using this column, the radioactive free iodide was eluted first, followed by the labeled mGnRH peak. Fractions (3 ml) were collected into tubes supplemented with 0.3 ml of the starting elution buffer containing 5% (w/v) BSA, to minimize adsorption of the radioactive antigen onto the walls. Immunoreactivity in the lyophilised HPLC fractions was measured using the following protocol: each sample was resuspended in 300 μl of PBS–BSA, then 100 μl of antiserum and 100 μl of labeled hormone (approximately 12 000 cpm) were added to each tube. After incubation for 48 h at 4°C, 1.5 ml of cold (4°C) ethanol (96%) were added. The tubes were immediately vortex-mixed and centrifuged at 1500 *g* for 25 min. The supernatant was aspirated and the radioactivity in the precipitate was counted in a gamma counter.

The antiserum, cII678, was used at a final dilution of 1:40 000. It exhibits the following cross-reactivities: mGnRH (100%), cIGnRH (416%), cIIGnRH (81%), sGnRH (473%), lGnRH-I (3%) and cfGnRH (36%).

3. Results and discussion

The present study investigated the chromatographic properties of five members of the known vertebrate GnRHs. In order to find the optimum conditions to separate adequately the peptides, modifications in the ratio of the mobile phase components, its pH and ionic strength were performed.

By studying the retention times of the peptides, we have observed that variations in the ratio of the organic modifiers MeOH–ACN in the mobile phase affect retention times but have no influence on their elution order. With 20% MeOH, 10% ACN and 70% PB (0.125 *M*), pH 4.0, the complete chromatographic run lasted more than 30 min and it was not possible to separate sGnRH from lGnRH-I (cIGnRH, 3.75 min; sGnRH and lGnRH-I, 6.16 min; mGnRH, 13.89 min and cIIGnRH, 30.82 min).

The five peptides completely left the column in only 13 min when the mobile phase was composed of 20% ACN, 10% MeOH and 70% PB (0.125 *M*), pH 4.0. Most peaks were well resolved, but the pair sGnRH/lGnRH-I could not be sufficiently separated and left the column only 1 min after cIGnRH (cIGnRH, 3.09 min; sGnRH, 4.08 min; lGnRH-I, 4.55 min; mGnRH, 7.93 min and cIIGnRH, 12.97 min).

A better separation of the peptides was achieved by decreasing the molarity of PB (ionic strength) from 0.125 *M* to 0.11 *M*, pH 4.0, although separation of sGnRH and lGnRH-I was not totally satisfactory (cIGnRH, 3.05 min; sGnRH, 4.36 min; lGnRH-I, 5.51 min; mGnRH, 10.61 min and cIIGnRH, 19.39 min).

Under more acidic conditions (pH 2.9), it was

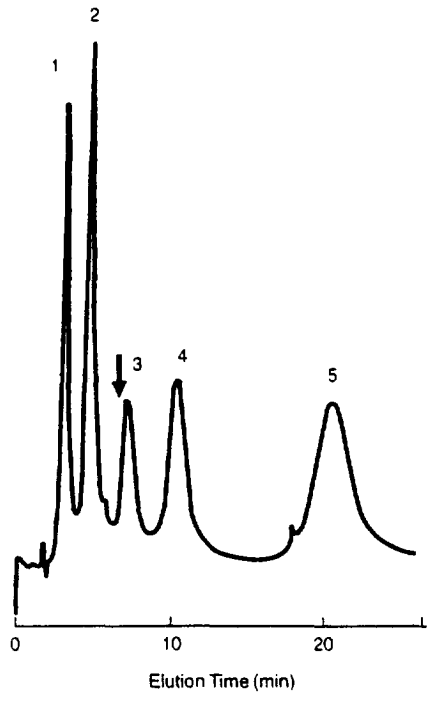

Fig. 1. Chromatogram of GnRH standards with UV detection. Eluent composition: 15% ACN, 15% MeOH, 0.11 *M* PB (70%, pH 2.9). Peaks: 1=cIGnRH (2.84 min); 2=sGnRH (4.38 min); arrow, cfGnRH (7.01 min); 3=lGnRH-I (7.12 min); 4=mGnRH (10.34 min); 5,=cIIGnRH (20.76 min). ACN, acetonitrile; MeOH, methanol; PB, phosphate buffer.

possible to achieve a "selective effect" only on lGnRH-I, which was retarded, while the variation in the pH did not affect the retention times of the other four peptides (cIGnRH, 3.05 min; sGnRH, 4.49 min; lGnRH-I, 7.24 min; mGnRH, 10.25 min; and cIIGnRH, 18.84 min).

Satisfactory resolution of sGnRH and cIGnRH was observed by replacement of 5% ACN by 5% MeOH. The ratio of 15% ACN, 15% MeOH, 70% (0.11 *M*) PB (pH 2.9), proved to be the optimum choice. The chromatogram of the five peptides is depicted in Fig. 1. The corresponding retention times were: cIGnRH, 2.84 min; sGnRH, 4.38 min; lGnRH-I, 7.12 min; mGnRH, 10.34 min; and cIIGnRH, 20.76 min. Using this eluent composition, we also studied cfGnRH, which had a retention time of 7.01 min, i.e. very close to lGnRH-I. The elution position of pure cfGnRH is indicated in Fig. 1 by an arrow.

In order to confirm the usefulness of this HPLC system, carp and chicken brain extracts were run. As shown in Fig. 2, screening of HPLC fractions obtained from carp brain by RIA using antiserum cII678 yielded two immunoreactive GnRH peaks. The first eluted in fractions 4–5 and the second in fractions 18–20, which indicated co-elution with authentic standards of sGnRH and cIIGnRH, respectively.

Fig. 3 shows the results from the HPLC of the

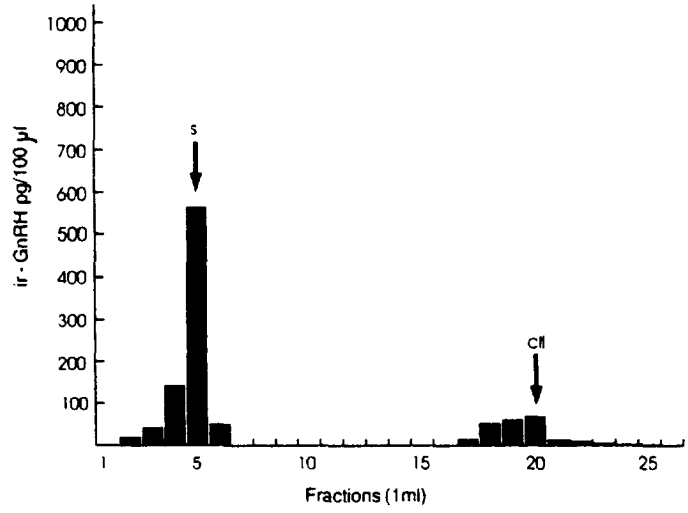

Fig. 2. HPLC of carp brain extract assayed with cII678 antiserum. Arrows show the elution position of standards of sGnRH (left arrow) and cIIGnRH (right arrow). Eluent composition as in Fig. 1.

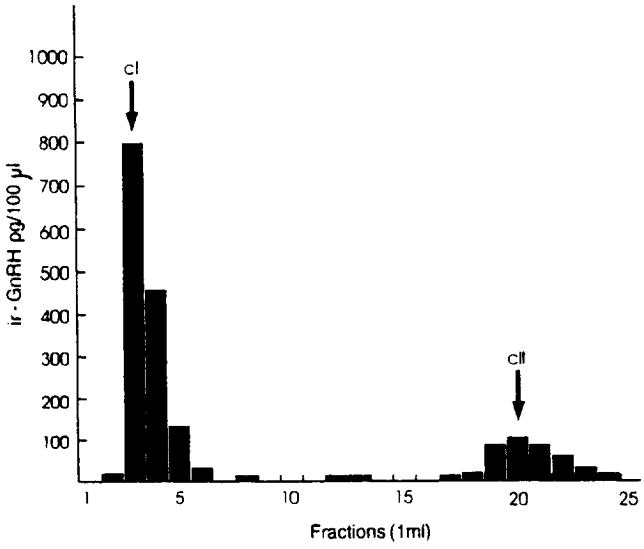

Fig. 3. HPLC of chicken brain extract assayed with cII678 antiserum. Arrows show the elution position of standards of cIGnRH (left arrow) and cIIGnRH (right arrow). Eluent composition as in Fig. 1.

chicken brain extract. After "off-line" HPLC–RIA coupling, as previously used for the carp brain extract, two main immunoreactive peaks were detected, the first eluting in fractions 3–4 and the second in fractions 19–21, thus co-eluting with authentic standards of cIGnRH and cIIGnRH, respectively.

The described isocratic chromatographic system offers several characteristics distinguishable from many others used to date. Firstly, it can separate mGnRH from lGnRH-I. Up to now, this could only be achieved by gradient HPLC. Secondly, the elution order of peptides of the GnRH family is different from that of other chromatographic systems. This peculiarity makes the method applicable for identification of peptides of the whole GnRH family. Thirdly, it is possible to obtain a satisfactory separation of the five GnRH variations in about 20 min under isocratic conditions. These two characteristics serves to decrease either the cost or the complexity of the required equipment.

Although cfGnRH and lGnRH-I eluted very close to each other, as depicted in Fig. 1, we believe that this small incovenience does not lower the efficiency of our recently developed HPLC system, because all the vertebrate species so far studied do not possess both of these two molecular forms together. Moreover, immunological detection systems exist that are capable of differentiating between them [11]. This isocratic HPLC system provides high resolution for five of the six tested vertebrate GnRHs. Nevertheless, further studies are required to complete the list of the nine described vertebrate GnRHs. Additionally, we are interested in the investigation of the chromatographic properties of the numerous GnRH analogues with this new HPLC system.

Acknowledgments

This research was supported by a grant from the National Research Council of Argentine (CONICET). The authors wish to thank Dr. A. Luchetti for providing the chickens and we are indebted to Dr. V.P. LoRiggio from Biocosmetica Exel Argentina S.R.L. We also thank Dr. J.A. King for the gift of the antiserum cII678 and Dr. Rüdiger Schültz for the standard of cfGnRH.

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