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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF ^{125}I-LABELED RAT GROWTH HORMONE-RELEASING HORMONE FOR RADIOIMMUNOASSAY

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SUMMARY

Using reversed-phase high-performance liquid chromatography, an effective purification was developed for radioiodinated rat growth hormone-releasing hormone (rGHRH) with isolation of monoiodo-rGHRH. The use of this purified radiolabel resulted in improvement in binding, half-maximum displacement and sensitivity in radioimmunoassay for rGHRH. The improved radioimmunoassay performance allowed the measurement of *in vitro* basal and stimulated the release of rGHRH from incubated hypothalamic preparations.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used to improve the purification of iodinated peptides for radioimmunoassay (RIA)[1]. In comparison with classical separation methods such as gel filtration and ion exchange, optimized RP-HPLC separation can achieve the resolution of monoiodinated peptides, which are separated from unlabeled peptides and other iodination products[2]. A further advantage of separation by RP-HPLC is that purification is faster.

Because of the need to improve the sensitivity of RIA for rat growth hormone-releasing hormone (rGHRH) for physiological experiments, an RP-HPLC purification for radiolabeled rGHRH was developed using shallow-stepped gradient elution. Other laboratories have reported RP-HPLC separation methods for radiolabeled rGHRH, but have not reported complete characterization or optimized separation[3,4]. In this paper, an effective RP-HPLC purification is described that improves resolution and is fully characterized with respect to rGHRH and oxidized rGHRH.

The regulation of growth hormone secretion by the hypothalamus is complex, depending on neural, hormonal and metabolic influences. The final pathway of regulation is regulated by stimulatory and inhibitory peptide-releasing hormones, GHRH and somatostatin[5,6]. A physiological approach to the examination of the regulatory control of hypothalamic releasing hormones, such as GHRH, is to study the *in vitro*

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release from hypothalamic preparations. Experience with perifusion and static incubation systems has established the validity of these techniques for physiological questions[7–9]. These studies can be technically difficult, as the content of hypothalamic releasing hormones is generally low (of the order of nanograms in the rat); for example, there is 2–3 ng of rGHRH in the rat hypothalamus[10]. The basal release of hypothalamic releasing hormones is usually in the range 0.02–0.3% of the hypothalamic content per incubation epoch[9], which would predict in a typical release experiment that picogram amounts per hypothalamus are released, close to the detection limit for many peptide RIAs. Preliminary experiments in this laboratory on the measurement of hypothalamic *in vitro* rGHRH release indicated that the amount released was near the sensitivity of 5 pg per tube for the rGHRH RIA established using gel filtration-purified [^{125}I]rGHRH[10]. With the development of an effective RP-HPLC purification of radiolabeld rGHRH as described here, the performance of the rGHRH RIA was substantially improved, permitting the measurement of *in vitro* rGHRH release.

EXPERIMENTAL

A Beckman HPLC system (Model 421A controller, Model 110B solvent-delivery modules and Model 163 variable-wavelength UV detector) with a Rheodyne Model 7010 injector employing a 500-μl sample loop was used for chromatographic separations. All separations were performed with a Vydac 218TP-54 250 × 4.6 mm I.D. reversed-phase C_{18} column with 5 μm particle size and wide pores of 300 Å (Separations Group, Hesperia, CA, U.S.A.). The components of the solvent system were acetonitrile and trifluoroacetic acid (TFA): solvent A, 0.1% TFA; solvent B, acetonitrile–water (80:20) containing 0.1% TFA. The flow-rate was 1 ml/min. Peptides were detected by measuring the UV absorbance at 210 nm and the absorbance was integrated with a Hewlett-Packard 3390A reporting integrator. Fractions were collected in glass tubes containing 5 μl of 10% bovine serum albumin (BSA) to minimize peptide absorption. For monitoring radioactivity, a portion of each fraction (10 μl) was counted in a Beckman Model 9000 gamma counter. For storage of radiolabeled peptides, fractions were centrifugally evaporated to dryness in a Speed-Vac (Savant Instruments, Hicksville, NY, U.S.A.) and reconstituted with 0.1 *M* acetic acid containing 0.1% BSA to $1 \cdot 10^6$ cpm in 40 μl and stored at −20°C.

Rat GHRH (Peninsula Labs., Belmont, CA, U.S.A.) was iodinated with chloramine-T as described previously[10] using an optimized iodination technique[11]. The iodination reaction mixture consisted of 5 μg of rGHRH (1 nmol), 1 mCi of $Na^{125}I$ (0.46 nmol) (IMS.300, Amersham, Arlington Heights, IL, U.S.A.) and 5 μg of chloramine-T in 0.5 *M* phosphate buffer (pH 7.5) at room temperature. After reaction for 20 s, the reaction was stopped by the addition of 200 μl of 1% TFA containing 1% BSA. Incorporation was 75–85%; the iodide-to-peptide ratio of 0.46:1 favors the formation of monoiodo-rGHRH with substantial amounts of unlabeled rGHRH remaining at the completion of the reaction. For gel filtration purification the iodination mixture was separated on a 50 × 0.9 cm I.D. Sephadex G-50 Fine column (Pharmacia–LKB Biotechnology, Piscataway, NJ, U.S.A.), and the specific activity of the purified radiolabel calculated from the percentage incorporation was 150–170 μCi/μg.

Prior to RP-HPLC purification, peptides were separated from free iodide with octadecylsilylsilica disposable cartridges (Sep-Pak C_{18}; Waters Assoc., Milford, MA, U.S.A.) using methods described previously[10], with elution with 2 ml of acetonitrile–water (80:20) containing 0.1% TFA after a wash with 10 ml of 0.1% TFA. The eluate was centrifugally evaporated to 400 μl to remove acetonitrile preferentially, and then injected for chromatographic separation. Iodination with non-radioactive iodine was performed with $K^{127}I$ (0.46 nmol; equivalent to the amount of $Na^{125}I$) in a similar manner, and after stopping the reaction with 1% TFA containing 1% BSA, the reaction mixture was injected. Iodinations with Iodo-Gen® (1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril) and Iodo-Beads® (N-chlorobenzenesulfonamide-derivatized polystyrene beads) were performed[12–14] as recommended by the manufacturer (Pierce, Rockford, IL, U.S.A.). IodoGen was dissolved in chloroform and plated onto 12 × 75 mm glass tubes. Iodo-Gen and Iodo-Bead reactions were performed in 100 and 200 μl, respectively, of 100 m*M* phosphate buffer (pH 7.0) with 5 μg of rGHRH, 0.46 nmol of $K^{125}I$ and tracer $Na^{125}I$.

For time-course analyses, the reaction mixture was sampled at 2, 4, 6, 8, 10, 15 and 20 min. Incorporation was determined by removal of 5 μl, diluting in 0.5 ml of 1% BSA in 50 m*M* phosphate buffer (pH 7.0) and then precipitating peptide by adding 0.5 ml of cold 10% trichloroacetic acid. The precipitate and supernatant were counted, and the percentage incorporation was calculated from the ratio of the precipitate counts per minute and the sum of the precipitate and supernatant counts per minute.

To assess the oxidation state of methionine in peaks of RP-HPLC-purified [^{125}I]rGHRH, oxidation was performed with 1% hydrogen peroxide for 1 and 2 h at pH 2.5[15], followed by purification by RP-HPLC.

RIA of rGHRH was performed as described previously[10] with the specific antiserum 3138. Additional RIA were performed with antiserum 3140, which is specific but only half as sensitive as antiserum 3138. Radiolabeled rGHRH purified by gel filtration was used in comparison studies of binding and sensitivity. Prior to assay, [^{125}I]rGHRH was diluted to approximate volume in assay buffer and 5% (v/v) Dowex 1-X8–400 (Sigma, St. Louis, MO, U.S.A.) was added to remove free iodide. After low-speed centrifugation, the volume of the supernatant was adjusted so the number of counts added to each tube was 13 000 cpm. The assay buffer was 100 m*M* sodium phosphate (pH 7.5)–0.1% BSA (RIA grade, Sigma)–0.1% Triton X-100; all incubations were made at 4°C. The total volume of each tube was 500 μl; separation of bound from free ligand was effected by the second antibody technique.

A modification to the previous technique was the addition of polyethylene glycol (PEG-8000) to the diluting buffer, following completion of the second antibody reaction. The final concentration of PEG after addition was 6.0%. This modification stabilized the second antibody pellet, permitting greater efficiency and consistency in aspirating the supernatant; there were no changes in percentage of non-specific binding or specific binding. Maximum sensitivity was achieved using non-equilibrium conditions. After an initial 36–48 h of incubation of first antibody and standard or sample, radiolabeled rGHRH was added for an overnight incubation of 16–18 h[16].

The working titer of antiserum was defined as the dilution of antiserum to achieve 25% binding after an overnight incubation of radiolabeled rGHRH. The

sensitivity of the RIA was equivalent to the number of picograms per tube of rGHRH resulting in 95% binding of radiolabel and the half-maximum displacement of the RIA (mid-point of the standard curve) was equivalent to the number of picograms per tube resulting in 50% binding of radiolabel. These values were determined from log–logit linear regression of the rGHRH RIA standard curve.

In vitro release of rGHRH was measured by incubation of hypothalamic preparations in siliconized 12 × 75 mm glass tubes in a shaking water-bath at 37°C. The entire hypothalamus was used as the hypothalamic preparation and was isolated as described previously[10] from male Sprague–Dawley rats weighing 100–150 g (Hilltop Lab. Animals, Scottdale, PA, U.S.A.). Krebs–Ringer hydrogencarbonate buffer (KRBB) gassed with oxygen–carbon dioxide (95:5) was used as the incubation medium at a volume of 300 μl with additions of 10 m*M* glucose, 0.05% BSA, 60 μM ascorbic acid and 100 μM bacitracin. For high-potassium medium (60 m*M* K^+), potassium chloride was substituted for sodium chloride in the KRBB. For calcium-free medium (0 m*M* Ca^{2+}), sodium chloride was substituted for calcium chloride (normal Ca^{2+} concentration 2.4 m*M*). The duration of incubations was 30 min and the preincubation period was 2 × 30 min incubations. After aspiration with a siliconized pipette, the incubation media were acidified with 10% TFA to a final concentration of 0.5% TFA. Media were extracted on octadecylsilylsilica cartridges (Bond Elut C_{18}; Analytichem, Harbor City, CA, U.S.A.), washed with 0.1% TFA and eluted with 2 ml of isopropanol–water (80:20) containing 0.1% TFA. After centrifugal evaporation to dryness and reconstitution with 0.01 *M* acetic acid, duplicate samples were measured by rGHRH RIA and the results were expressed as pg per hypothalamus per 30 min. Results are expressed as means ± standard error of the mean (S.E.M.). Statistical analyses were performed with the unpaired *t*-test with a significance level defined at $p < 0.05$.

RESULTS

Preliminary experiments using chloramine-T "cold" iodination of rGHRH with $K^{127}I$ demonstrated nine peaks with retention times of 15–21 min when monitored by UV absorbance using steep-gradient elution (similar to the gradient in ref. 4) (Fig. 1). With the large number of peaks in a short interval, it would have been difficult to collect fractions reliably for radiolabel purification. Attempts to reduce the number of peaks using solid-phase iodination reagents, such as Iodo-Gen or Iodo-Beads, which have been considered to be gentler iodination agents[12–14], were not successful. Incorporation of iodine with Iodo-Beads (one or two beads) was poor and erratic (38–60%), even with reaction times up to 20 min. On the other hand, incorporation with Iodo-Gen was consistent (75–85%). Experiments using 0.4–40 μg of Iodo-Gen and reaction times up to 20 min indicated that 4 μg and 15 min achieved maximum incorporation with the shortest exposure time and the minimum of oxidizing agent. When "cold" iodinations with Iodo-Gen were purified by RP-HPLC as above, no absorbance peaks at 15–21 min were detected. Experiments adding tracer amounts of $Na^{125}I$ to $K^{127}I$ revealed that greater than 90% of the iodinated peptide eluted at the solvent front, indicating severe damage to rGHRH by iodination with Iodo-Gen. Thereafter, all iodinations were performed with the established chloramine-T technique, and iodinations with Iodo-Beads and Iodo-Gen were discontinued.

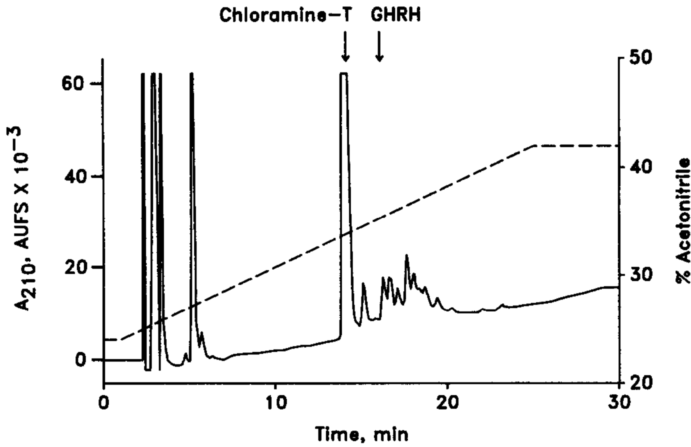

Fig. 1. RP-HPLC separation of [^{127}I]rGHRH (iodinated with chloramine-T) with steep gradient elution with UV detection at 210 nm. After equilibration at 24% acetonitrile, the gradient holds the starting concentration of 24% acetonitrile for 1 min and then increases it to 42% acetonitrile over 24 min. There are nine peaks of peptides eluting from 15 to 21 min. Oxidized rGHRH elutes within the chloramine-T peak.

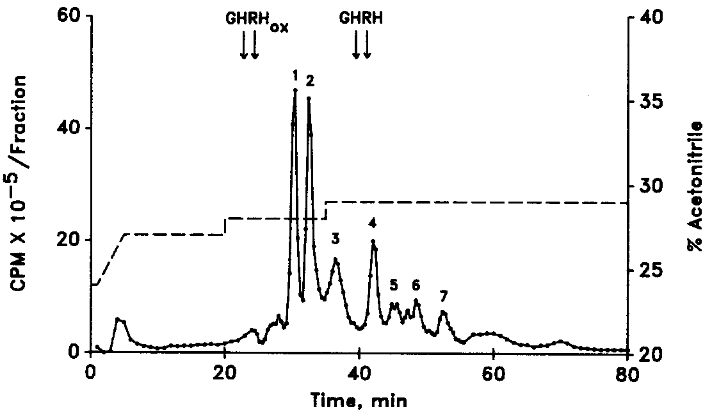

Fig. 2. RP-HPLC separation of [^{125}I]rGHRH iodinated with chloramine-T with shallow-stepped gradient elution. Major peaks of radioactivity, numbered consecutively, are chromatographically separated. After equilibration at 24% acetonitrile, the gradient holds the starting concentration of 24% acetonitrile for 1 min, increases it to 27% acetonitrile in 4 min, and then steps at 27% acetonitrile for 15 min, 28% acetonitrile for 15 min and 29% acetonitrile for 40 min. The positions of oxidized rGHRH (by chloramine-T) and synthetic (unoxidized) rGHRH are shown by the double arrows, as the peptide separates into two peaks by the elution gradient. The retention times of these peptides are clearly separated from peaks 1 and 2, which are used as the radiolabel in rGHRH RIA. Multi-iodinated rGHRH elutes at 60 and 63 min (see text). Free iodine elutes at the solvent front at 4 min and most has been removed by prior octadecylsilylsilica extraction. In this demonstration separation, 1-min fractions were collected, except during the period 24–56 min, when 0.4-min fractions were collected. In a typical separation, 80 0.4-min fractions are collected starting at 20 min.

In order to improve the separation of the peaks of radiolabel so that reliable fraction collection could be performed, a shallow-stepped gradient elution was developed with steps of 27%, 28% and 29% acetonitrile, which resolved seven peaks of [^{125}I]rGHRH (Fig. 2). The peaks can be collected with 0.4-min fractions, and the peaks are defined by at least four fractions. Using this gradient, rGHRH and chloramine-T-oxidized rGHRH are clearly separated from the major iodination peaks. Although described as a single peak on RP-HPLC by the manufacturer, synthetic rGHRH under these chromatographic conditions separated into two peaks 1.5 min apart, with 76% eluting in the first peak. Iodination with a 10-fold increase in iodide (4.6 nmol of K^{127}I; ratio of iodide to peptide 4.6:1), which favors the formation of multi-iodinated rGHRH, resulted in two major peaks eluting at 60 and 63 min, suggesting that the peaks eluting earlier are less substituted rGHRH with the earliest peaks probably being monoiodo-rGHRH. Oxidation of peaks 1 and 2 with 1% hydrogen peroxide did not result in a change in retention time or amplitude for either peak 1 or 2, indicating that the methionine-27 in the [^{125}I]rGHRH of peaks 1 and 2 is already oxidized to methionine sulfoxide following iodination with chloramine-T[2].

The pattern of seven peaks remained consistent with respect to peak height and relative retention time. There is variation of the start of elution for the peaks of the radiolabel, as the retention time of peak 1 varied from 24 to 32 min in eleven iodinations. In practice, all the major peaks (peaks 1–4) can be collected using 80 fractions of 0.4 min starting at 20 min.

Binding experiments revealed that [^{125}I]rGHRH from peak 1 gave the best binding (highest titer) of all the peaks tested and was substantially better than gel filtration-purified [^{125}I]rGHRH (Table I). There were no differences in binding in fractions from the ascending or descending portion of peak 1, so all fractions of peak 1 were pooled. Using radiolabel from peak 1, the sensitivity of the RIA was improved 2.4-fold and at half-maximum displacement 3.5-fold (Table II). Additional modifications to the RIA by decreasing the assay volume to 225 μl and by decreasing the radiolabel to 5000 cpm improved the sensitivity 3.2-fold and the half-maximum displacement 4.7-fold overall. There was no difference in the kinetics of binding for gel filtration and RP-HPLC-purified [^{125}I]rGHRH, as the half-time to maximum bind-

TABLE I

BINDING OF GEL FILTRATION AND RP-HPLC-PURIFIED [^{125}I]rGHRH

Binding was determined by overnight incubation of antiserum and radiolabel using standard assay conditions. Working titers were determined from linear interpolation of a plot of log (reciprocal dilution) *vs.* percentage binding at various antibody dilutions.

Radiolabel	*Reciprocal titer* $\times 10^{-3}$
Gel filtration	35
Peak 1	82
Peak 2	43
Peak 3	42
Peak 4	34

TABLE II

COMPARISON OF BINDING, HALF-MAXIMUM DISPLACEMENT AND SENSITIVITY WITH GEL FILTRATION AND RP-HPLC-PURIFIED [^{125}I]rGHRH AND AFTER MODIFICATION OF RIA

Determinations were made using standard assay conditions or using modifications reducing the incubation volume and amount of radiolabel (see text). Half-maximum displacement and sensitivity were derived from log–logit regression of the rGHRH RIA standard curve.

Radiolabel	*Reciprocal titer* $\times 10^{-3}$	*Half-maximum (pg per tube)*	*Sensitivity (pg per tube)*
Gel filtration	45	68.9	5.8
RP-HPLC, peak 1	90	19.6	2.4
RP-HPLC, peak 1 + modifications	100	14.8	1.8

ing was 15–18 h with both. An important advantage observed with this separation is the longer useful life of the radiolabel. With gel filtration-purified peptides, the maximum useful life of the radiolabel in RIA has been generally 1–2 months (unpublished data and ref. 16). The radiolabel of peak 1 has been used for 4.5 months without a significant change in sensitivity with an acceptable decrease in binding (Table III). This is much improved from the maximum useful life of gel filtration-purified [^{125}I]rGHRH of 4–6 weeks when stored under the same conditions and similarly treated with Dowex prior to use in RIA. In contrast to the best RIA performance with [^{125}I]rGHRH from peak 1 for antiserum 3138, [^{125}I]rGHRH from peak 2 gave the best performance for antiserum 3140, suggesting that the best peak of radiolabel will depend on the recognition site(s) of polyclonal antiserum.

In release experiments using four hypothalami per tube in static incubation, basal rGHRH release was now detectable, and rGHRH release was stimulated 2.8-fold by high-potassium depolarization (Fig. 3). The calcium dependency of rGHRH release by high-potassium stimulation was demonstrated, consistent with the release of rGHRH from hypothalamic neurons. Without the improvement in assay sensitivity by HPLC purification, 12–20 hypothalami per tube (an impractical number) would have been necessary to achieve similar results, as the improvement in RIA performance is 3–5-fold with respect to sensitivity and half-maximum displacement.

TABLE III

STABILITY OF RP-HPLC-PURIFIED [^{125}I]GHRH

Values represent means ± S.E.M. of ten RIAs for each time period.

Time period	*Non-specific binding (%)*	*Binding (%)*	*Half-maximum (pg per tube)*	*Sensitivity (pg per tube)*
First 2 months	2.7 ± 0.1	24.0 ± 0.7	15.2 ± 1.0	1.8 ± 0.3
Second 2.5 months	3.0 ± 0.2	20.6 ± 0.5[a]	14.0 ± 0.5	1.8 ± 0.5

[a] $p < 0.005$ *vs.* First two months.

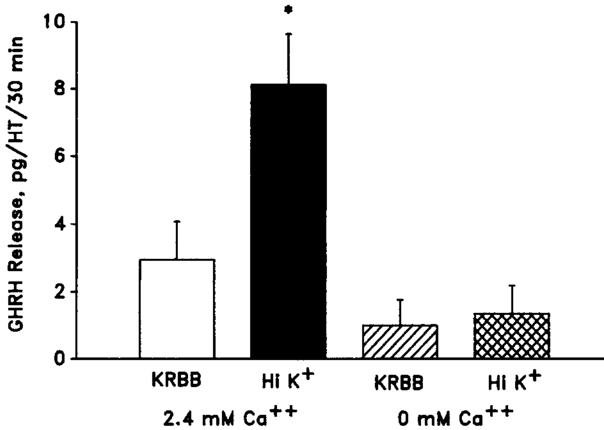

Fig. 3. Basal and stimulated *in vitro* release of rGHRH from hypothalamic preparations: calcium dependence of high-potassium stimulated release. Values are means ± S.E.M. (n = 4). Incubations were 30 min in duration, which followed 2 × 30-min preincubations. High-potassium stimulated release in the presence of normal Ca^{2+} is significantly different ($^*p < 0.05$ by unpaired t-test) from basal release, whereas there is no difference between basal or stimulated release in calcium-free media. The minimum detectable release is 1.0 pg per hypothalamus (HT) per 30 min.

DISCUSSION

RP-HPLC purification by shallow-stepped gradient elution achieved the resolution of radiolabeled rGHRH into seven discrete peaks, and selection of radiolabel from peak 1 resulted in an improved RIA performance with higher titers and improved sensitivity. The resulting improvement in sensitivity permitted the measurement of *in vitro* rGHRH release from hypothalamic preparations in static incubation.

Rat GHRH has four potential sites for iodination (tyrosines at positions 10 and 18 and histidines at positions 1 and 24), but the conditions (pH 7.5) employed during iodination strongly favor iodination of tyrosines. Further, with the molecular ratio of iodide to peptide of 0.46 in the iodination reaction, it is likely that only one tyrosine is monoiodinated for most [^{125}I]rGHRH. It is preferable to use monoiodinated radiolabel in RIA to limit potential steric hindrance of antigen–antibody binding by bulky multiple iodinated residues. Further evidence that the peaks 1 and 2 were monoiodo-rGHRH is that increasing the iodide to peptide ratio 10-fold to favor the formation of multi-iodinated rGHRH resulted in peaks with greatly increased retention times. Methionine residues in peptides, such as methionine at position 27 in rGHRH, are susceptible to oxidation by chloramine-T and characteristically result in elution at shorter retention times than unoxidized peptides by RP-HPLC[1,2], as observed in this study. On the other hand, iodinated peptides characteristically elute later than native peptides owing to the hydrophobic character of iodinated tyrosine residues[1,2]. Hence it is probable that peaks 1 and 2 are oxidized rGHRH with a single monoiodinated tyrosine residue at position 10 or 18, which elutes between rGHRH and oxidized rGHRH, permitting the complete separation of radiolabel from unlabeled rGHRH. The lack of change of retention time or amplitude for peaks 1 and 2 after treatment under mild oxidizing conditions with hydrogen peroxide, which oxidizes methionine

to methionine sulfoxide but not to methionine sulfone, supports the assertion that both peaks 1 and 2 contain methionine sulfoxide-27 and that peak 1 is not an oxidized form of peak 2. The two closely eluting peaks of synthetic rGHRH are not likely to be responsible for the separation of peaks 1 and 2, because of the differences in relative peak heights (synthetic rGHRH, 3.5 ± 1.1, $n = 3$; peaks 1 and 2, 1.2 ± 0.1, $n = 8$) and retention time separation (synthetic rGHRH, 1.5 ± 0.2 min; peaks 1 and 2, 2.3 ± 0.1 min). The later eluting peaks 3–7 may represent combinations of oxidized or unoxidized diiodo-rGHRH, either a single tyrosine residue iodinated with two ^{125}I atoms or two tyrosine residues each iodinated with a ^{125}I atom. In summary, the improved binding and sensitivity in rGHRH RIA probably results from the chromatographic isolation of methionine sulfoxide-27 monoiodo-rGHRH in peaks 1 and 2. However, the identities of peaks 3–7 are not established.

Although the solid-phase iodination reagents Iodo-Gen and Iodo-Beads have been considered gentler iodination agents[12–14], the results of these studies demonstrate that they are not practical for use with rGHRH using the methods described. An untested but attractive alternative for gentle iodination causing less peptide damage is use of the lactoperoxidase technique, although optimized chloramine-T iodination (as used in these studies) has been preferred[11]. With the effective purification by RP-HPLC of [^{125}I]rGHRH in these studies, it would be possible to evaluate peptide damage and the relative usefulness of these two iodination techniques.

Other methods for the purification of radiolabeled rGHRH by RP-HPLC have been published recently. In one method, using a Vydac 201TP reversed-phase C_{18} column and an acetonitrile–TFA solvent system, two peaks of [^{125}I]rGHRH were resolved[3]. The gradient of elution was steeper, which may account for the resolution of two peaks. Elution relative to unlabeled rGHRH was not described. The stability of the radiolabel was reported as 4–6 weeks. With the differences in binding of radiolabel from each of the peaks in the chromatographic purification in this work, resolution to multiple peaks may be important as only one of the peaks of radiolabel substantially improved the RIA performance for antiserum 3138. Vale *et al.*[4] used [norleucine-^{27}]-rGHRH as a non-oxidizable rGHRH analog for iodination. With the non-oxidizable residue substituted for the Met^{27} in rGHRH, there may be less change in the antigen–antibody interaction depending on the recognition site of the antiserum. The iodinated analog was purified on a Vydac C_{18} column using a 24 to 42% gradient (as in Fig. 1) with a flow-rate of 1.5 ml/min. The number of peaks and elution relative to unlabeled forms of rGHRH was not described. The stability of the iodinated analog was 2 months for RIA. In the purification described in this work, the separation of [^{125}I]rGHRH is fully characterized and the stability of the [^{125}I]rGHRH is improved, lasting 4.5 months.

The improvements in rGHRH RIA performance with the RP-HPLC methods described here were critical in enabling *in vitro* rGHRH release from hypothalamic preparations to be measured. The purification is well characterized, reproducible and represents an important application of RP-HPLC separation, which proved essential to the performance of a physiological experiment.

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