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Note

Iodination of a long-acting analogue of luteinizing hormone releasing hormone: separation of reaction products by column liquid chromatography and measurement of biological activity

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There is considerable controversy concerning the effect of iodination on the biological activity of luteinizing hormone releasing hormone (LHRH). In particular, a report by Marshall and Odell [1] describing complete retention of biological activity after iodination contrasts sharply with the work of Terada et al. [2] showing that a well characterized monoiodinated derivative of LHRH retained only 3.9% of full activity. Clarification of the influence of iodination on LHRH activity may now be possible with the development of high-performance liquid chromatography (HPLC) systems for peptides [3—5] which should allow the preparation of iodinated derivatives of sufficient purity for reliable assessment of biological potency. Indeed, recent reports clearly recommend reversed-phase HPLC for the purification of iodinated peptides [6—10].

In the present study, iodinated derivatives of D-Ala^6-des-Gly^{10}-LHRH ethylamide (D-Ala-LHRH) have been separated using an elution system based on mixtures of acetonitrile and acidified 0.9% (w/v) sodium chloride (pH 2.0). This system originally shown by O'Hare and Nice [11] to provide resolution of peptides comparable with that obtained using phosphate-based systems, has the advantage that final solutions of peptides in 0.9% (w/v) sodium chloride, containing a minimum of non-physiological ions, may be added directly to bioassay systems.

EXPERIMENTAL

Materials

1,3,4,6-Tetrachloro-3α,6α-diphenyl glycoluril (iodogen) was obtained from Pierce and Warriner (Chester, U.K.) and carrier-free $Na^{125}I$ (15.7 mCi/μg iodine) from Amersham International (Amersham, U.K.). HPLC was performed using an octadecylsilyl silica (ODS) packing (Hypersil-ODS, 5 μm, particle size; HPLC, Queensferry, U.K.) and HPLC-grade solvents from Rathburn Chemicals (Walkerburn, U.K.). D-Ala-LHRH, 3-monoiodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT) and insolubilized protease (enzyme from *Streptomyces griseus* attached to carboxymethyl cellulose) were purchased from Sigma (London) (Poole, U.K.).

HPLC of D-Ala-LHRH iodinated with $Na^{127}I$ containing a trace of $Na^{125}I$

Iodogen (10 μl of a 0.1 mg/ml solution in chloroform) was added to a glass tube (10 × 50 mm) and dried down under nitrogen. A solution of D-Ala-LHRH (8 nmol in 100 μl of sodium phosphate buffer, 0.05 mol/l, pH 7.4) was added to the coated tube followed by iodide (8 nmol $Na^{127}I$ and 10^5 dpm $Na^{125}I$ in a total volume of 2 μl). The iodination reaction was allowed to continue for 10 min, with occasional agitation, then terminated with 900 μl of 0.9% (w/v) sodium chloride adjusted to pH 2.0 with concentrated hydrochloric acid. Immediately, the iodination mixture was applied to an ODS column (15 cm × 4.6 mm I.D.) for HPLC by a method involving elution with an acetonitrile gradient in 0.9% (w/v) sodium chloride, pH 2.0 [11]. Using a flow-rate of 1.5 ml/min, the following gradient of acetonitrile was used: 10—19% over 2 min, 19—34% over 20 min and 34—40% over 1 min. Times given are for programmed changes occurring in the relative pumping speeds of the two pumps which form the gradient. There is a delay of approximately 5 min before these pumping changes lead to consequent alterations in the acetonitrile concentration in the eluate. Eluted peptides were monitored by absorbance (280 nm) and collected fractions estimated for radioactivity using a gamma counter.

Digestion of iodinated peptides and identification of radioactive amino acids

Iodination and subsequent purification of iodinated peptides were carried out as described above. Peak fractions of each of the main radioactive components were collected and then incubated (37°C for 72 h) with insolubilized protease (10 mg containing about 1.5 U) in sodium phosphate buffer (0.05 mol/l, pH 7.4; total volume, 2.0 ml). After digestion, a 0.6-ml portion of each incubate was taken and non-radioactive standards of tyrosine, MIT and DIT were added. Following adjustment to pH 2.0, HPLC of each digest was carried out using an acetonitrile gradient (0—30% over 30 min) in 0.9% (w/v) sodium chloride adjusted to pH 2.0. The eluate was monitored for absorbance (280 nm) and collected fractions were estimated for radioactivity.

Assessment of biological activity of iodinated peptides

D-Ala-LHRH was iodinated as above using $Na^{127}I$ alone (i.e. with the omission of the $Na^{125}I$). HPLC was carried out and the three major components

(unreacted peptide and the two main iodinated products) were collected. The molar quantity of each was calculated by comparing the peak area (obtained by monitoring the eluate at 280 nm) with that peak obtained for a standard amount of D-Ala-LHRH. The following correction was made for the expected change in molar absorbance with the introduction of iodine into the peptide, assuming the label had been incorporated into the tyrosyl residue (see Results). The absorbance at 280 nm was compared for equimolar solutions of tyrosine, MIT and DIT dissolved in 0.9% (w/v) sodium chloride (pH 2.0). Using this comparison and assuming that the absorbance at 280 nm of the intact peptide is due solely to the residues of tyrosine and tryptophan, an adjustment factor was calculated. However, because the absorption due to tryptophan predominates at 280 nm, the correction necessary for iodinated peptides was relatively small (< 15%) and, therefore, made relatively little difference to the subsequent estimation of biological activity.

The biological activity of the iodinated derivatives was examined using cultures of enzymatically dispersed cells prepared from the pituitaries of 30-day-old male Wistar rats. The dispersion method was essentially that described by Vale et al. [12] modified to include filtration of the initial cell suspension through nylon gauze (mesh, 60 μm) and treatment of the filtrate with deoxyribonuclease [0.001% (w/v); BDH, Poole, U.K.]. The pituitary cells were maintained in culture (incubates contained about $2 \cdot 10^5$ cells in a total volume of 1 ml) for three days without hormonal additions. After washing with medium, cells were incubated for a further 24 h with the substance under test. Peptides resolved using HPLC could be added directly to the cell cultures following removal of the acetonitrile from the eluate with a jet of nitrogen. After the final incubation, the culture medium was aspirated and stored frozen (−20°C) prior to radioimmunoassay for rat luteinizing hormone (LH) using a protocol and reagents generously donated by the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD, U.S.A. (purified rat LH for iodination, NIAMDD-rat LH-I-5; antiserum to rat LH prepared in rabbits, NIAMDD-anti-rat LH-serum-S-5; rat reference preparation, NIAMDD-rat LH-RP-1).

Iodination with $Na^{125}I$ alone

Although the present paper does not include information on the properties of D-Ala-LHRH iodinated with $Na^{125}I$ alone, the HPLC procedure described may be used to prepare iodinated derivatives with high specific radioactivity. For this purpose, it is recommended that the HPLC column is loaded with iodinated material using one of the less expensive high-pressure pumps possibly in a fume cupboard. The HPLC column may then be transferred to the main HPLC system where a gradient of acetonitrile may be used to elute the iodinated peptides. Without passing the eluate through post-column detection systems, it is possible to collect a well defined peak of radioactivity corresponding exactly to monoiodotyrosyl-D-Ala-LHRH (see Results for identity of peaks shown in Fig. 1). In this way, the resolving power of gradient elution HPLC may be utilized without contaminating expensive chromatographic machinery with radioactive iodide.

RESULTS

Fig. 1 shows the separation by HPLC of reaction products following iodination of D-Ala-LHRH with $Na^{127}I$ containing a trace quantity of radioactive iodide. Peak A had a retention time consistent with its identification as unchanged D-Ala-LHRH. Two further components (peaks B and C), formed during the reaction, were clearly resolved both from each other and from unsubstituted peptide. Incorporation of radioactive iodide into peaks B and C would suggest they are both iodinated forms of D-Ala-LHRH. The increased retention time and greater ratio of radioactivity to absorbance for peak C compared to peak B, would be consistent with the preliminary identification of the two products, B and C, as being the mono- and diiodinated derivatives, respectively.

Following digestion of the two iodopeptides (peaks B and C) with

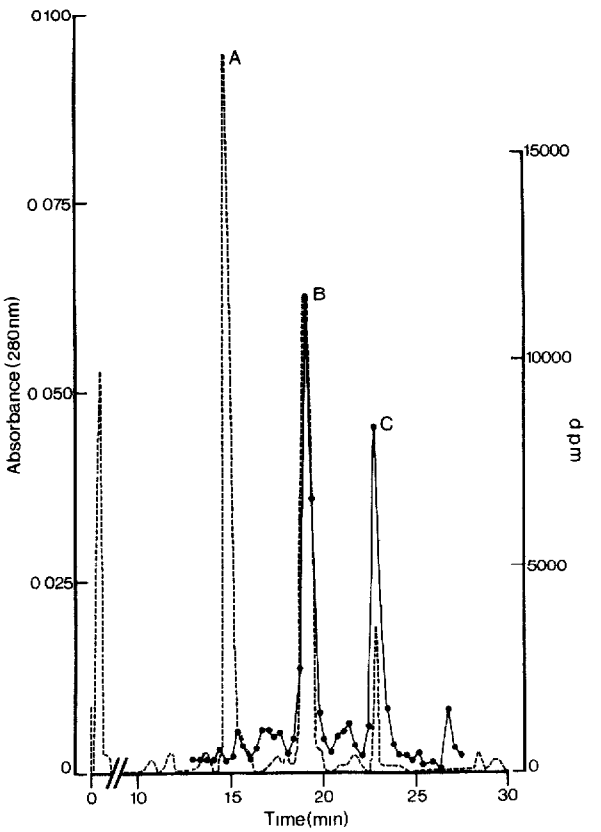

Fig. 1. Separation of iodination products of D-Ala^6-des-Gly^{10}-luteinizing hormone releasing hormone ethylamide (D-Ala-LHRH) by HPLC. The packing, flow-rate and gradient characteristics are as described in the text. Injection and start of the gradient are at time zero. The plot of absorbance measured at 280 nm (broken line) shows a peak shortly after injection which is an injection artefact. Peak A is the first major peptide fraction eluted and corresponds to unchanged D-Ala-LHRH. Fractions of the eluate were collected and radioactivity estimated. Radioactivity incorporated (solid line) into peaks B and C confirms their identification as iodinated forms of D-Ala-LHRH.

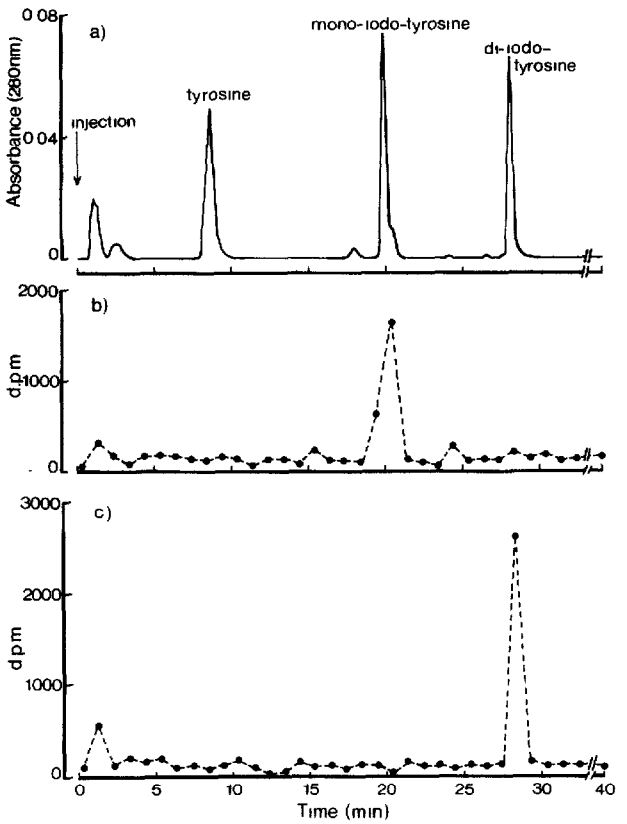

Fig. 2. HPLC of radioactive amino acids liberated by digestion of iodinated forms of D-Ala^6-des-Gly^{10}-luteinizing hormone releasing hormone (D-Ala-LHRH). Following iodination of D-Ala-LHRH with a mixture of $Na^{127}I$ and $Na^{125}I$, iodinated peptides (corresponding to peaks B and C shown in Fig. 1) were collected after chromatography and then digested using insolubilized protease. Each digest, adjusted to pH 2.0, was then subjected to HPLC using a gradient of acetonitrile (0—20% over 30 min) in 0.9% (w/v) sodium chloride (pH 2.0). Injection and start of the gradient are at time zero. Fractions of the eluate were collected and estimated for radioactivity. (a) HPLC of standards; (b and c) HPLC of digests of peaks B and C, respectively.

insolubilized protease, the liberated radioactive amino acids were subjected to HPLC using an acetonitrile gradient capable of resolving the unsubstituted mono- and diiodo forms of tyrosine (Fig. 2). The digest from peak B contained radioactive material with a retention time equivalent to that of mono-iodo-tyrosine whereas that from Peak B contained radioactivity which eluted at a position corresponding to the diiodo derivative. Peaks B and C were thus confirmed as being the mono- and diiodotyrosyl derivatives of D-Ala-LHRH.

Fig. 3 shows the effect of non-radioactive preparations of components A, B and C (recovered after chromatography) on the release of LH from cultures of rat pituitary cells. Peak A (unreacted peptide) stimulated LH release with a median effective dose (ED_{50}) of about 50 pmol/l which is similar to that obtained for standard D-Ala-LHRH in our laboratory. A higher concentration (about 1500 pmol/l) of peak B (monoiodinated peptide) was needed to give the same half-maximal response. At a level of

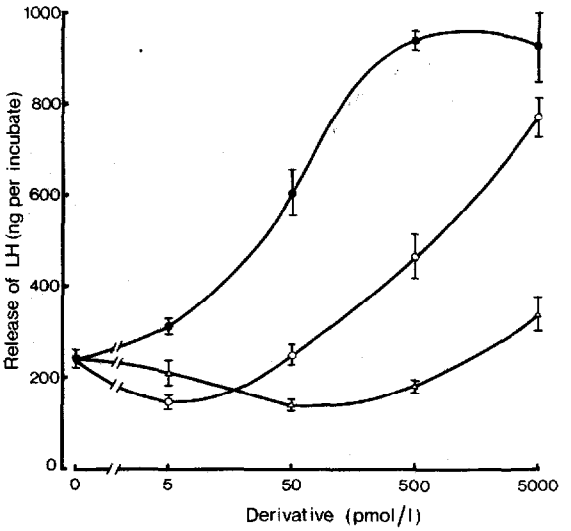

Fig. 3. Effect of D-Ala^6-des-Gly^{10}-luteinizing hormone releasing hormone ethylamide (D-Ala-LHRH) and its two iodinated derivatives on LH release from cultures of rat pituitary cells. Incubates containing $2 \cdot 10^5$ cells were pre-incubated for four days without hormonal additions. LH release in response to each peptide under test was then measured over a 24-h period. Values shown are means (± S.E.M.) of estimates on quadruplicate incubates. (•) Unsubstituted peptide (peak A of Fig. 1); (○) monoiodinated D-Ala-LHRH (peak B of Fig. 1); (△) diiodinated D-Ala-LHRH (peak C of Fig. 1).

peptide (500 pmol/l) just sufficient to elicit the maximum response to unreacted hormone, the relative effectiveness of the monoiodinated derivative was about 30%. The relatively inactive diiodinated form of D-Ala-LHRH (peak C) showed some residual biological activity at 5000 pmol/l.

DISCUSSION

The present study confirms that reversed-phase HPLC can be used for the purification of iodinated forms of LHRH providing a clear separation from unsubstituted peptide. The observed increase in retention time as iodine atoms are introduced into tyrosine (retention times: DIT > MIT > tyrosine) is in agreement with previously published data reviewed by Hearn [3]. Similarly, introduction of iodine into the intact peptide leads to progressive retention on the ODS packing (retention times: diiodo-D-Ala-LHRH > monoiodo-D-Ala-LHRH > D-Ala-LHRH). This has been described for a number of peptide hormones [6, 7, 10]. By combining the use of iodogen, recommended for a range of peptides by Salacinski et al. [7], and non-radioactive iodide, iodination products can easily be examined by direct application of the iodination mixture (after dilution and adjustment to pH 2.0) to the HPLC system. In this way, the exposure of the ODS packing to oxidizing agents or enzymes used in the iodination procedure is avoided.

The extent to which iodinated forms of LHRH retain biological activity has remained unclear over recent years because of a number of methodological problems. The first of these concerns the chromatographic purity of the iodinated species under investigation. Thus, Terada et al. [2] raise the possibili-

ty that an earlier study claiming full retention of biological activity [1] may have utilized a purification procedure (ion-exchange chromatography) which was unable completely to remove unsubstituted peptide from iodinated LHRH. Using a well characterized 3-iodo-Tyr^5-LHRH preparation free of native hormone, Terada et al. [2] reported a residual biological activity of only 3.9%. The second problem derives from methods used to quantify preparations of LHRH prior to bioassay. Marshall and Odell [1] for example, used an immunoassay technique whereby changes in bound:free ratio of radioactivity upon addition of LHRH were compared to parallel changes in this ratio with addition of [^{125}I]LHRH. While theoretically correct (assuming identical characteristics of binding to antibody of iodinated and non-iodinated species) such immunological methods are certainly less direct than equivalent spectrophotometric methods when available. A third source of disagreement between investigators may arise from differences in bioassay procedure as well as the range of concentrations tested. Thus, studies carried out by Piyachaturawata et al. [13] and Terada et al. [2] provide mean potencies (about 30 and 3.9%, respectively for [^{127}I]LHRH) on estimates at two dose levels of peptide. The present study has certainly revealed that the relative effectiveness of LHRH and its iodinated derivatives is very much effected by the concentration of peptide used.

The present investigation has attempted to overcome these problems. Highly purified mono- and diiodo-D-Ala-LHRH have been prepared free of contaminating unsubstituted peptide. After spectrophotometric quantification, biological activity has been assayed using a range of concentrations of each derivative. The finding that there was a considerable change in ED_{50} value (about 30-fold) with the introduction of one atom followed by a further reduction in activity with the second, demonstrate that each halogen atom has a profound effect on the interaction of LHRH with its target cells.

ACKNOWLEDGEMENT

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