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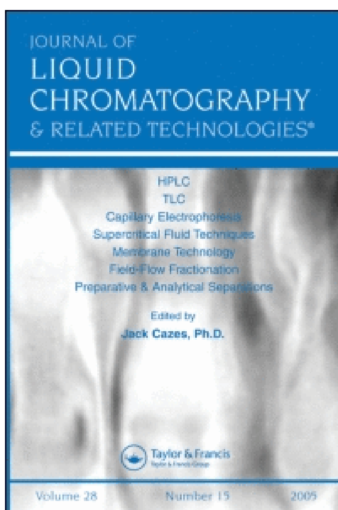

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IODINATION OF PEPTIDE HORMONES AND PURIFICATION OF IODINATED PEPTIDES BY HPLC

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ABSTRACT

Hexagastrin, Arg^8-vasopressin, oxytocin, Tyr^1-somatostatin, and ACTH 1-39 were iodinated in order to yield precursors for tritium labelling or radioiodinated tracers for radioimmunoassay, respectively. The heterogeneous mixture of iodination products was purified via reversed-phase high-performance liquid chromatography. Iodination of peptides resulted in a marked increase in retention time on the reversed-phase adsorbent. A simple and quick method was applied for purification of radio-iodinated peptides on a Sep-pak® C-18 cartridge for rapid sample preparation.

INTRODUCTION

The synthesis of iodinated peptide hormones has two aims:
1. Radioactive labelling for RIA and/or receptor binding studies.

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2. Synthesis of non-radioactive, halogenated precursor peptides for further work of tritiation /I → $^{3}_{1}H$ exchange by catalytic hydrogenolysis/.

1. Although numerous methods are known and have been applied for many years, there are some problems in the iodination of small peptides. One of the best-known methods is Hunter-Greenwood iodination /1/, using chloramin-T oxidizing agent for the iodid → iodine reaction in solution. During this procedure some amino acids /Met, Trp, His, Cys/ can also be oxidized partly or quantitatively by the excess of reagent in the solution. A short reaction time /5, 10 or 20 seconds/ is necessary to minimize side-reaction. Under the conditions applied /highly diluted reaction mixture, short reaction time/, only a part of the peptide reacts and it is necessary to separate the monoiodinated peptide from the oxidized by-products in order to increase the specific radioactivity. Numerous techniques have been employed for purification /high-voltage electrophoresis /2/, ion-exchange chromatography /3/, polyacrylamide gel electrophoresis /4/, thin-layer chromatography /5/ gel electrophoresis /6/ and isoelectric focusing /7//. Sediah /8/ used high-performance liquid chromatography for the purification of iodinated Leu-enkephalin, β-MSH, angiotensin, lysin-vasopressin and ACTH.

More recently, a new oxidizing reagent, "iodogene" /11/ has been introduced for the iodination of peptide hormones. It has some advantages over chloramin-T: the reagent has a very low solubility in water and present adhered as a thin film on the wall of the reaction vials, thus not being in a less direct contact with the peptide molecule in solution. The peptide is dissolved in water, radioactive iodide is added to the solution and the mixture is poured into the reaction

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vial containing iodogene on the wall. After some minutes the reaction mixture can be poured out of the vial and purified.
2. For introduction of non-radioactive iodine into the Tyr--residue of peptides, there are two possibilities:

a/ working with mono- or diiodotyrosine during peptide synthesis,

b/ iodination with unlabelled iodine by the methods mentioned for radiolabelling /1,11, 10/ or directly with elemental iodine /9/.

Our aim was to iodinate peptides /hexagastrin, Arg^8-vasopressin, oxytocin, somatostatin and ACTH/ in order to obtain radioactive-labelled hormones for RIA or to have iodinated peptide precursors for tritiation. HPLC proved to be the method of choice for the separation of mono- and diiodinated peptides from the starting material and the oxidized by-products.

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MATERIALS

Protected hexagastrin /Boc-Tyr-Ala-Trp-Met-Asp-Phe-NH_2/, mono- and diiodo-hexagastrin, Arg^8-vasopressin and oxytocin were synthetized in our laboratory. Tyr^1-somatostatin was purchased from Beckman /Geneve, Italy/, ACTH 1-39 was a generous gift from NIAMDD /Bethesda, USA/. ^{125}Iodine /as $Na^{125}I$/ was purchased from the Central Isotope Institute, Budapest. Vycor glass powder 140 mesh was bought from Vycor Glass Works, Corning N.Y. USA.

The high-performance liquid chromatograph consisted of a Waters 6000 A pump and a universal liquid chromatog raph injector /Waters U6K/, coupled to an LKB Uvicord III fixed wavelength /20 nm/ UV monitor with an 8 µl. flow-through cell. Methanol and acetonitril were used as supplied by E. Merck /Darmstadt,

Germany/. Water was glass-distilled and deionized. The freshly prepared buffers were passed through a 0.45 um Sartorins membrane filter. Elution was effected isocratically at room temperature.

METHODS AND RESULTS

1. Hexagastrin

In order to obtain precursor substances for tritiation and standard substances for HPLC purification, we synthesized hexagastrin, monoiodo- and diiodo-hexagastrin in a stepwise manner, using Boc-monoiodo tyrosine and BOC-diiodotyrosine for the syntheses /the details of the synthesis will be published elsewhere/. The three compounds were separated on a Partisil-10 ODS column /25 cm x 4.6 mm/, applying the following elution solvent: 0.1 M triethylammonium phosphate /pH = 3.20/ - acetonitrile 55:45 /v/v/ /flow rate 2 ml/min/

Compound	Retention time
BOC-hexagastrin	4.7 min
BOC-monoiodo hexagastrin	7.3 min
BOC-diiodo hexagastrin	12.7 min

Iodination of the hormone resulted in a marked increase in retention time on the reversed phase adsorbent, owing to the hydrophobicity of the iodo group.

BOC-diiodohexagastrin proved to be suitable as a precursor for tritiation. The radioiodination of hexagastrin was a difficult task: we found that a small amount of iodogene too dissolves in water and can oxidize the methionine residue. The iodination reaction is very slow /as a result of working in highly diluted solutions/, but a longer reaction time leads to oxidation

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of the peptide. We made a compromise: if the reaction time was 30-60 seconds, the yield of radiolabelled monoiodinated hexagastrin was ~ 10 % after HPLC purification on a Partisil column.

2. Vasopressin

Arg^8-vasopressin was synthesized with the solid-phase method on Merrifield polymer. It was found that the iodogene method was not suitable for iodination of vasopressin on a preparative scale: a large amount of unidentified oxidized vasopressin derivative was formed during the reaction. Therefore, iodination was performed with the method of Flouret /9/, applying elemental iodine in alcoholic solution and a reaction time of ~ 10 minutes. HPLC purification of the reaction mixture on a Partisil-10 ODS-2 column /15 cm x 4.6 mm/ yielded diiodo-Arg^8-vasopressin as main product /~ 40 %/. Only a small amount of vasopressin and monoiodo-vasopressin remained in the reaction mixture. The solvent system: 0.05 M ammonium acetate /pH = 6.5/ - methanol 6:4 /v/v/; flow rate 1.0 ml/min.

Compound	Retention time
free iodine	1.2 min
Arg^8-vasopressin	5.3 min
Monoiodo-Arg^8-vasopressin	19 min
Diiodo-Arg^8-vasopressin	36 min

Radioiodination of Arg^8-vasopressin with the Hunter-Greenwood method gave similar results, but owing to the short reaction time /35 sec/ and highly diluted solution /5 µg AVP in 50 µl solution/, monoiodo-Arg^8-vasopressin was the main product. Yields after HPLC purification with the above method: monoiodo--AVP ~ 40 %, diiodo-AVP ~ 8-9 %. /After radioiodination with $^{125}I_2$, the peptides were adsorbed on Vycor glass beads before HPLC purification./

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3. Oxytocin

Oxytocin was synthesized on solid phase and radioiodinated with $^{125}I_2$ /Hunter-Greenwood method, 5 ug oxytocin in 50 ul aqueous solution, reaction time: 35 sec/. The reaction mixture was separated on a Nucleosil 5 C-18 column /25 cm x 4.6 mm/. The solvent system: 0.01 M ammonium acetate /pH = 4.0/ - acetonitrile 4:1; solvent flow rate 2 ml/min.

Compound	Retention time
Oxytocin	4.8 min
Monoiodo-oxytocin	11.4 min
Diiodo-oxytocin	15.6 min

After purification the main product, monoiodooxytocin, had a very high specific radioactivity /1800 Ci/mmol/.

4. Somatostatin

Somatostatin does not contain any tyrosine-residue for iodination, and we therefore applied the Tyr^1-analogue of somatostatin. 2.5 ug peptide was radioiodinated with the Hunter-Greenwood method, the peptides were adsorbed on Vycor glass beads, and after desorption purified on a Partisil ODS-2 Column. The solvent system: 0.01 M ammonium acetate /pH = 4.00/ - acetonitrile 7:3, solvent flow rate 1.5 ml/min.

Compound	Retention time
Tyr^1-somatostatin	13.0 min
Monoiodo-Tyr^1-somatostatin	50.0 min
Diiodo-Tyr^2-somatostatin	91.0 min

The main product in this case too was the monoiodinated peptide, the amount of diiodinated compound being very small.

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5. A simple separation of iodinated peptides on Sep-Pak cartridge

Since the iodinated peptides have much longer retention times on reversed phase adsorbents than those of the original peptides, a very simple technique proved suitable for the separation of these compounds from the reaction mixture after iodination. This technique is also known from the literature /6/: the separation is performed on a Sep-Pak ® C 18 cartridge /Waters/ for rapid sample preparation. Solvent system: 1 % trifluoroacetic acid containing a methanol gradient from 5 to 90 %. The peptides containing tyrosine were iodinated as mentioned above /the reaction time for ACTH 1-39 was 20 sec/, pre-purified with adsorption on Vycor glass beads and purified on a Sep-Pak® C 18 cartridge equilibrated with 1 % trifluoroacetic acid. The results are as follows:

Compound	Methanol content of gradient
Monoiodo-Arg^8-vasopressin	45-50 %
Monoiodo-oxytocin	55 %
Monoiodo-Tyr^1-somatostatin	65 %
Monoiodo-ACTH 1-39	60-65 %

DISCUSSION

Four peptide hormones were iodinated and purified on reversed-phase high-performance liquid chromatography and on Sep-Pak® C-18 cartridge. Both methods are very simple, do not require any complicated instruments and give pure iodinated peptides suitable for RIA measurements.

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