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USE OF GUANIDINE HYDROCHLORIDE IN THE PURIFICATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THYROXINYL- AND TRIIODOTHYRONYLPEPTIDES DERIVED FROM THYROGLOBULIN

CLAUDINE MARRIQ*, PIERRE-JEAN LEJEUNE, MARCEL ROLLAND and SERGE LISSITZKY
Laboratoires de Chimie Biologique et de Biochimie Médicale, Unité 38 INSERM, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 5 (France)
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SUMMARY

When peptides containing thyroid hormones are first solubilized in 6 *M* guanidine hydrochloride they can be perfectly separated by reversed-phase high-performance liquid chromatography on an octadecylsilica column using conventional elution conditions (trifluoroacetic acid–acetonitrile).

INTRODUCTION

Thyroglobulin (Tgb), the dimeric glycoprotein (2 × 330 kDa) specific to the thyroid gland, is the matrix on which thyroid hormones are synthesized. Only a limited number (about 7%) of tyrosine residues contained in the protein is involved in hormone synthesis, these residues probably being located in an appropriate spatiasl conformation of the polypeptide chain allowing, after iodination, the coupling of the resulting iodotyrosines (3-iodotyrosine or MIT and 3,5-diiodotyrosine or DIT) into hormones (3,5,3'-triiodothyronine or T_3 and 3,5,3',5'-tetraiodothyronine or thyroxine or T_4). The molecular mechanism of thyroid hormonosynthesis is still poorly understood and some complementary information on this subject could be obtained from the determination of amino acid sequences surrounding hormone residues in the polypeptide chain of Tgb.

However, the isolation and purification of peptide fragments containing T_4 or T_3 present numerous difficulties. The presence of hormone(s) in the peptide structure makes them very hydrophobic, rendering these peptides insoluble even in very acidic solvents. In alkaline media the fragments are relatively soluble, but are partially deiodinated even at room temperature. Moreover, hormonopeptides are strongly adsorbed on various chromatographic supports such as Sephadex G-25, Dowex 50, etc. Another difficulty is related to the iodination heterogeneity of Tgb[1,2] which is reflected in the derived iodinated peptides. For these reasons, attempts to purify the hormonopeptides (containing T_4 or T_3) on various chromatographic supports result in a mixture of derivatives of the same peptide having various levels of tyrosine

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iodination that is impossible to separate by conventional chromatographic methods.

In this paper, we demonstrate that when T_4- and T_3-containing peptides are dissolved in 6 *M* guanidine hydrochloride they can easily be purified by reversed-phase high-performance liquid chromatography (RP-HPLC), using an acidic mobile phase (trifluoroacetic acid–acetonitrile).

EXPERIMENTAL

Apparatus

All chromatographic experiments were carried out with a liquid chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisting of two M 6000 A pumps, a M 660 solvent programmer and a U 6 K universal injector. Separations were performed on a 7-μm LiChrosorb RP-18 column (250 × 4.6 mm I.D.) from Merck (Darmstadt, F.R.G.) equipped with a Lichroprep RP-8 (25–40 μm) pre-column. The column effluent was collected with a 201 Gilson collector and the peptides detected with a Waters GM 770 spectrophotometer. All separations were performed at ambient temperature.

Reagents

"HPLC water" was distilled from quartz, deionized in a Milli-Q system (Millipore, Bedford, MA, U.S.A.), then successively filtered onto a Merck Lichroprep RP-8 column (40–60 μm) and a Sep-Pak C_{18} cartridge (Waters). Acetonitrile was from Solvants-Documentation-Synthèse (Peypin, France) and trifluoroacetic acid (TFA, sequential grade) from Pierce (Rockford, IL, U.S.A.). Guanidine hydrochloride (Fluka, Buchs, Switzerland) was dissolved in HPLC water at a concentration of 6 *M* just before use and filtered onto a Sep-Pak C_{18} cartridge. All solvents were filtered using a Pyrex filter holder (Millipore) fitted with 0.45-μm membrane filters and degassed by sonication 10 min before use.

Materials

Hormonopeptides were isolated and purified from porcine Tgb (pTgb) by conventional chromatographic methods using filtrations on DEAE-Sephadex and Biogels as previously described[3,4]. Table I shows the total or partial amino acid se-

TABLE I

TOTAL OR PARTIAL AMINO ACID SEQUENCES OF HORMONOPEPTIDES PURIFIED FROM PORCINE THYROGLOBULIN[3,4]

b_1Ad_3:	His-Asp-Asp-Asp-T_4-Ala-Thr-(Glx,Gly)-Leu-Tyr-Phe-Ser-Ser-Arg
b_1C:	Asp-Tyr-Phe-Ile-Leu-X-Pro-Val-(Asx_2-Pro-Leu)-Met MIT DIT T_4
b_3d_6	T_3 Leu-Ala-Ser-Lys-Ser- T_4

quences of these peptides. The identification and determination of the recovery of peptides purified by RP-HPLC were performed by amino acid analysis and microsequencing[5,6]. The estimation of hormones and iodoamino acids was carried out as described by Rolland *et al.*[7].

RESULTS AND DISCUSSION

Use of RP-HPLC for separation and purification of small and medium sized peptides is now well established and various elution methods have been described[8–12]. Nevertheless, none of them could be satisfactorily used for the purification of peptides containing T_4 and T_3, because of (1) the insolubility of these hormonopeptides in acidic or neutral media, even in high concentration of organic solvents (acetonitrile, propanol or methanol), and (2) the irreversible adsorption to reversed-phase supports. More recently, several authors have described various methods for the purification by RP-HPLC of large denatured polypeptides and hydrophobic proteins under experimental conditions allowing both a good solubilization of the protein material and satisfactory separations and recovery levels of the polypeptides studied[13–18]. Among these techniques, some investigators recommended the addition in the mobile phase of dissociating agents[16], non-ionic (*e.g.*, Brij or Triton X-100) surfactants[18] or even ternary elution systems[17]. Henderson *et al.*[19] used 6 *M* guanidine hydrochloride for solubilization of proteins from Rauscher murine leukaemia virus before RP-HPLC on an octadecylsilica column with a simple two-component linear gradient of 0.05% TFA in water, pH 2.1 (solvent A) and 0.05% TFA in acetonitrile (solvent B). Under these experimental conditions guanidine hydrochloride is eliminated just after the void volume of the column and the proteins kept in solution are satisfactorily separated by chromatography.

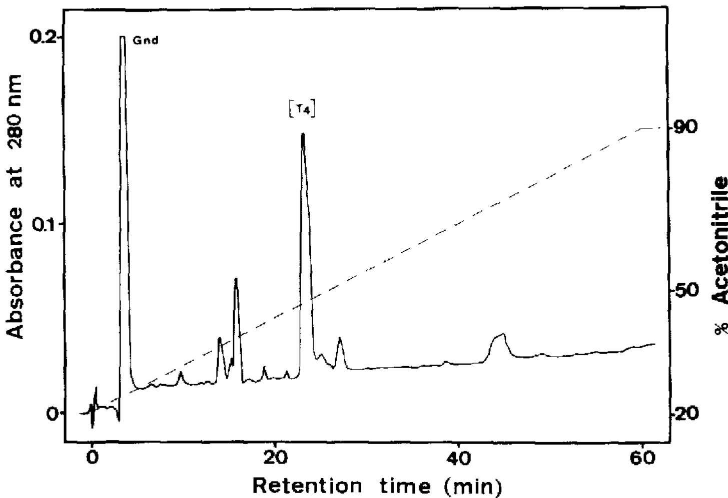

Fig. 1. Purification of hormonopeptide b_1Ad_3 by RP-HPLC. A 15-nmol amount of peptide dissolved in 60 μl 6 *M* guanidine hydrochloride was injected into a LiChrosorb RP-18 column. Elution conditions: solvent A, acetonitrile–water (20:80) containing 0.05% TFA; solvent B, acetonitrile containing 0.05% TFA; linear gradient from 0 to 90% solvent B for 60 min at a flow-rate of 1 ml/min. [T_4] corresponds to the T_4 peptide b_1Ad_3 and Gnd to guanidine hydrochloride.

We have used this method for the RP-HPLC separation of three hormonopeptides: b_1Ad_3 (Fig. 1), b_1C (Fig. 2) and b_3d_6 (Fig. 3) containing respectively 15, 13 and 6 amino acid residues with one hormone residue per mole peptide (Table I). These peptides were isolated from hydrolyzates (treatment with cyanogen bromide and/or enzymatic digestion) of pTgb by conventional techniques: ion-exchange chromatography (DEAE-Sephadex) and molecular filtration (Bio-Gel P-6)[3,4]. Due to the iodination heterogeneity of Tgb, b_1C and b_3d_6 were obtained as a mixture of different iodinated forms. For example, b_1C is represented by four forms of the same peptide differing only in the nature of the tyrosine residue in position 2 (Table I), the latter being a residue of tyrosine (uniodinated b_1C), of MIT (monoiodinated b_1C), of DIT (diiodinated b_1C) or of T_4 (hormonopeptide b_1C)[3]. b_1C was dissolved in 6 *M* guanidine hydrochloride, then chromatographed by RP-HPLC under the conditions described in Fig. 2, yielding an excellent separation of the four forms of b_1C.

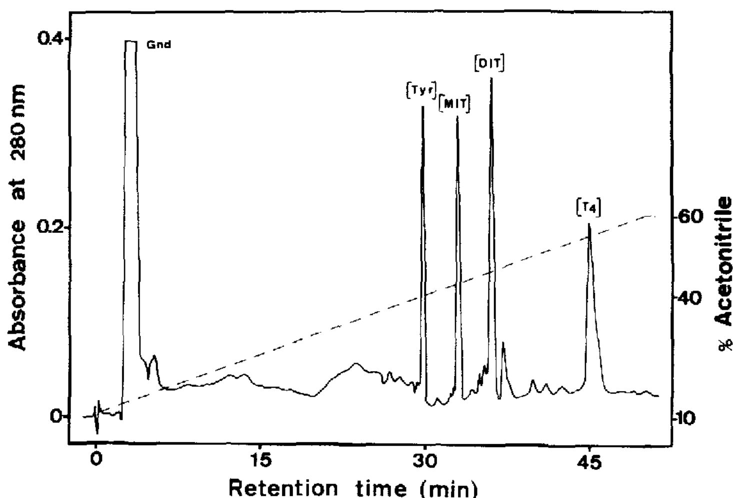

Fig. 2. Purification of hormonopeptide b_1C by RP-HPLC. A 40-nmol amount of peptide dissolved in 150 µl 6 *M* guanidine hydrochloride was injected into a LiChrosorb RP-18 column. Elution conditions: solvent A, acetonitrile–water (10:90) containing 0.05% TFA; solvent B, acetonitrile containing 0.05% TFA; linear gradient from 0 to 55% solvent B for 50 min at a flow-rate of 1 ml/min. The peptides of b_1C are indicated in square brackets.

Peptide b_3d_6 was obtained by filtration on Bio-Gel P-6 from a *Staphylococcus aureus* V8 protease hydrolyzate of a cyanogen bromide fragment of pTgb. Determination of the amino acid and iodoamino acid compositions and microsequencing suggested that b_3d_6 was a mixture of two forms of the same peptide, one containing T_3 and the other T_4[4]. RP-HPLC of this peptide dissolved in 6 *M* guanidine hydrochloride clearly illustrates the presence of the two hormonopeptide species (Fig. 3).

This method was also used to purify a T_4 peptide from human Tgb (hTgb). We have recently isolated the N-terminal part of hTgb[20]; this fragment of M_r 26 000 which contains 1 mole T_4 per mole of peptide represents a preferential hormonosynthetic site of hTgb. Indeed, at physiological hTgb iodination levels (0.2%) this peptide

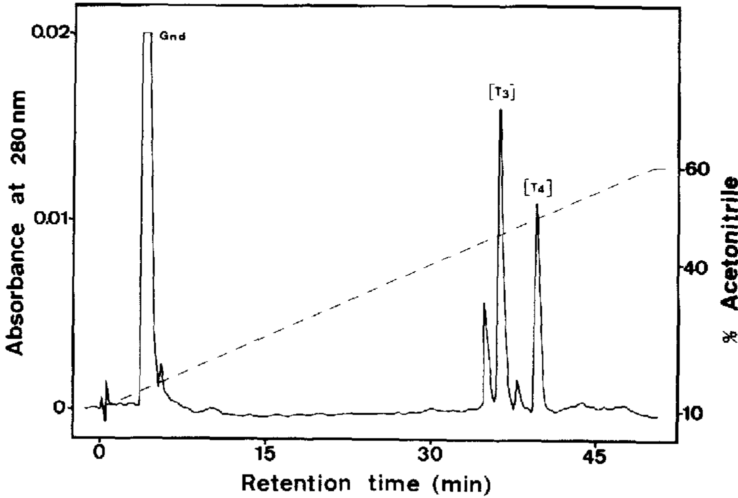

Fig. 3. Purification of hormonopeptide b_3d_6 by RP-HPLC. A 10-nmol amount of peptide dissolved in 40 μl 6 *M* guanidine hydrochloride was injected into a LiChrosorb RP-18 column. Elution conditions as in Fig. 2. The peptides of b_3d_6 are indicated as in Fig. 2.

contains about 50% of the total protein T_4. In the order to know the amino acid sequence surrounding the T_4 residue, the M_r 26 000 fragment was hydrolyzed with trypsin at 37°C for 4 h at an enzyme to substrate ratio of 1:25 (w/w). After acidification by acetic acid (pH 3), all the T_4 was recovered in the insoluble fraction or acid core. The latter was dissolved in 6 *M* guanidine hydrochloride and subjected to RP-HPLC (Fig. 4). Peaks T1 and T1a contain both T_4 (one mole per mole peptide). Amino acid sequencing[21] showed that the minor peak T1 contained a peptide with

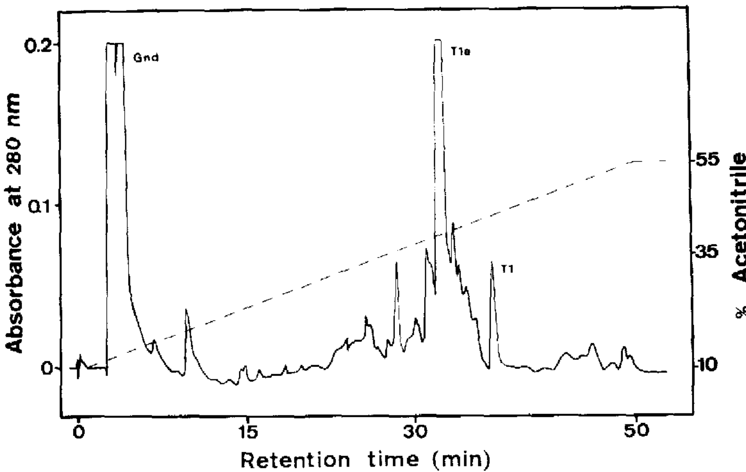

Fig. 4. Purification by RP-HPLC of the acid core of the tryptic digest of the M_r 26 000 peptide. Approximately 500 μg protein dissolved in 200 μl 6 *M* guanidine hydrochloride were injected into a LiChrosorb RP-18 column. Elution conditions as in Fig. 2, except linear gradient from 0 to 50% solvent B for 50 min at a flow-rate of 1 ml/min.

TABLE II

AMINO ACID SEQUENCES OF HORMONOPEPTIDES PURIFIED FROM HUMAN THYROGLOBULIN

The peptides correspond to T1 and T1a peaks separated by RP-HPLC (see Fig. 4).

T1:	Asn-Ile-Phe-Glu-T_4-Gln-Val-Asp-Ala-Gln-Pro-Leu-Arg-Pro-Cys-Glu-Leu-Gln-Arg
T1a:	Asn-Ile-Phe-Glu-T_4-Gln-Val-Asp-Ala-Gln-Pro-Leu-Arg-Pro-Cys

19 amino acid residues, whereas the major T1a peak was the result of a tryptic aspecific cleavage of the cysteinyl$_{15}$ bond of T1 (Table II). Thanks to RP-HPLC these two T_4 peptides differing only by four amino acid residues have been perfectly separated. Their identification allowed us to demonstrate that this part of the hTgb molecule which contains a preferential hormonosynthesis site has a particular susceptibility to proteolysis, which probably favours the release of the hormone *in vivo*[21].

Use of 6 M guanidine hydrochloride for solubilizing T_4 and T_3 peptides allowed their purification by RP-HPLC with a simple, fully volatile mobile phase of TFA–acetonitrile (permitting the dry unsalted peptides to be obtained by evaporation or freeze-drying) and at acidic pH values (so reducing the risk of deiodination). The manner in which guanidine hydrochloride interacts with hormonopeptides is presently unknown but it is possible that, in such a system, one or several molecule(s) of guanidine could form stable complexes with the peptides, favouring their solubilization in the mobile phase and reducing hydrophobic interactions between the peptides and the stationary phase. Indeed, all peptides studied had satisfactory recoveries in the range of 60–90%.

An extension of this procedure for the solubilization of peptides (other than T_4 and T_3 peptides) could resolve some difficulties encountered in RP-HPLC separation of various kinds of insoluble peptides.

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