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Note

Separation of iodinated compounds of L-tyrosyl-L-tyrosine from iodothyronines by reversed-phase high-performance liquid chromatography

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The dipeptide 3,5,3′,5′-tetraiodo-L-tyrosyl-L-tyrosine (I_2Tyr-I_2Tyr) has been extracted from trypsic digests of bovine thyroglobulin and investigations conducted in our laboratory have shown that this sequence forms part of the primary structure of thyroglobulin[1]. "*In vitro*" experiments demonstrated that I_2Tyr-I_2Tyr led to synthesis of the iodothyronines by a mechanism involving a cyclic agent without breaking the peptide bond[2,3]. This suggested that tyrosyltyrosine sequences in thyroglobulin might be hormonosynthesis sites "*in vivo*". To check this hypothesis we studied the "*in vitro*" enzymatic iodination of synthetic peptides which include the tyrosyltyrosine sequence. The iodination led to a mixture of iodotyrosines, iodinated derivatives of tyrosyltyrosine and iodothyronines. Previously we described a procedure allowing the complete separation of these compounds by column chromatography on Bio-Gel P-2[4]. However, this technique is limited in application due to the 24 h needed for a single analysis.

Recent developments in chromatography have yielded highly efficient reversed-phase columns employing ion-pair partition. In 1978 Hearn *et al.*[5] described a procedure for the analysis of thyroidal iodoamino acids by hydrophilic ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC). This method permits the rapid separation of a mixture of iodinated compounds, by use of a chemically bonded C_{18} hydrophobic support as the stationary phase and water–organic solvent mixtures containing phosphoric acid or other ion-pairing reagents as the mobile phase. Burman *et al.*[6] measured serum thyronines by column chromatography on μBondapak C_{18} with a linear gradient of 25 to 90% acetonitrile in 0.025 *M* sodium acetate buffer, pH 4.

Neither method allowed a convenient separation in our particular case, but using a similar approach, we have developed a method for the rapid chromatographic analysis of the iodinated compounds of L-tyrosyl-L-tyrosine by reversed-phase partition HPLC.

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EXPERIMENTAL

Apparatus

An Altex (Chromatem, Touzart et Matignon, France) HPLC system equipped with two C380 solvent delivery pumps and a 420 Altex solvent programmer was coupled to a UV absorbance detector operated at a wavelength of 254 nm and/or to a Berthold LB 5026 radioactivity detector and to a double-channel chart recorder.

Reagents

All solvents were AnalaR grade. Methanol supplied by E. Merck (Darmstadt, G.F.R.) was further bidistilled. Potassium dihydrogen phosphate and orthophosphoric acid were supplied by Riedel de Haen (Hannover, G.F.R.). The iodoamino acids, monoiodotyrosine (ITyr), diiodotyrosine (I_2Tyr), triiodothyronine (T_3) and thyroxine (T_4), were obtained from Sigma.

Iodinated derivatives of L-tyrosyl-L-tyrosine (Tyr-Tyr) were synthesized in the laboratory by coupling with dicyclohexylcarbodiimide (DCC) the N-carboxybenzoxy (Cbzo) derivatives[7] of L-tyrosine (Tyr), 3-iodo-L-tyrosine (ITyr) and 3,5-diido-L-tyrosine (I_2Tyr) with their methyl ester analogues[8]. Eight compounds were obtained: ITyr-Tyr, Tyr-ITyr, I_2Tyr-Tyr, Tyr-I_2Tyr, ITyr-ITyr, ITyr-I_2Tyr, I_2Tyr-ITyr and I_2Tyr-I_2Tyr.

$^{125}IT_3$ and $^{125}IT_4$ were obtained from NEN; their specific activity was 100–150 μCi/μg. ^{125}ITyr and $^{125}I_2$Tyr were synthesized and labelled with ^{125}I by the Chloramine T method[9]; their specific activity was 100 μCi/μg. Iodinated derivatives of L-tyrosyl-L-tyrosine were labelled by isotopic exchange with unlabelled compounds; their specific activity was 10–20 μCi/μg.

Procedure

A 30 × 0.47 cm I.D. column was packed with 10-μm LiChrosorb RP-18 (Merck). The mobile phase used successively consisted of four buffers:

buffer 1: 5% to 80% methanol gradient in 0.1 *M* KH_2PO_4 containing 0.1% H_3PO_4; buffer 2: 30% methanol in 0.02 *M* KH_2PO_4 + 0.1% H_3PO_4; buffer 3: 50% methanol in 0.02 *M* KH_2PO_4 + 0.1% H_3PO_4; buffer 4: 20% to 40% methanol gradient in 0.02 *M* KH_2PO_4 + 0.1% H_3PO_4 for 8 min, 40% to 50% for 8 min and 50% to 70% for 8 min.

A flow-rate of 2 ml/min was maintained at a pressure of 800–1000 p.s.i. All separations were performed at ambient temperatures. The sample injections were made with Hamilton syringes (0.10 μl or 0.50 μl) by a Rheodyne injector with a 100-μl loop. Samples of the iodinated compounds were diluted in the first buffer system. The concentrations varied between 20 and 50 μg per 10 μl. The radioactivity of each compound was 0.05 μCi (maximum sensitivity of the detector 0.005 μCi). Simultaneously, the variations of optical density at 254 nm and the radioactivity in the eluate were measured.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a mixture containing ITyr, I_2Tyr, iodinated derivatives of Tyr-Tyr, T_3 and T_4, labelled with ^{125}I. In this preliminary experiment,

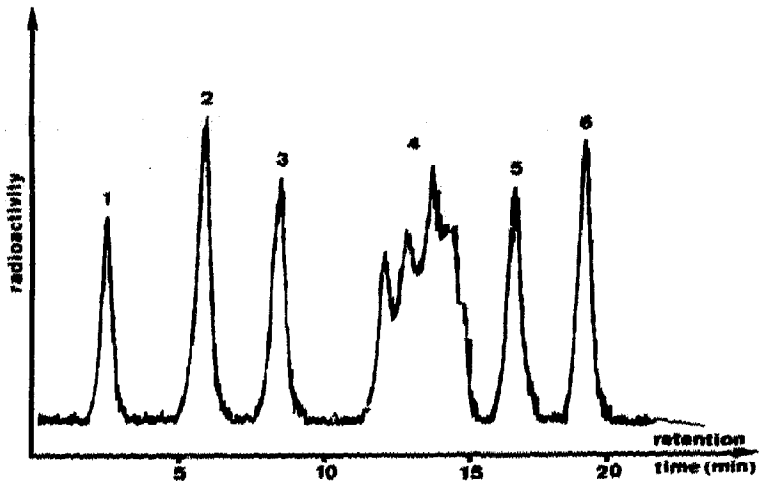

Fig. 1. Separation of a standard solution of iodide (1), ITyr (2), I_2Tyr (3), iodinated derivatives of Tyr-Tyr (4), T_3 (5) and T_4 (6). Mobile phase: 5% to 80% methanol gradient in 0.1 *M* KH_2PO_4 containing 0.1% H_3PO_4 for 20 min. Flow-rate: 2.0 ml/min on a column (30 × 0.47 cm I.D.) of LiChrosorb RP-18.

elution was performed as described by Hearn *et al.*[5] using a 20-min linear gradient of 5% to 80% methanol in 0.1 *M* KH_2PO_4, containing 0.1% H_3PO_4 as ion-pairing reagent. A good separation of ITyr, I_2Tyr, T_3 and T_4 could be achieved but this elution system did not resolve adequately the mixture of iodinated derivatives of Tyr-Tyr. With a concentration in methanol higher than 60% in 0.1 *M* KH_2PO_4, we noted the appearance of crystals which disturbed the elution. The crystals did not appear when the concentration of KH_2PO_4 was below 0.02 *M* whatever the concentration of methanol. Under these conditions, ITyr is eluted at 25% methanol, I_2Tyr at 40%, iodinated derivatives of Tyr-Tyr between 45 and 55% and T_3 and T_4 at 60 and 70% respectively.

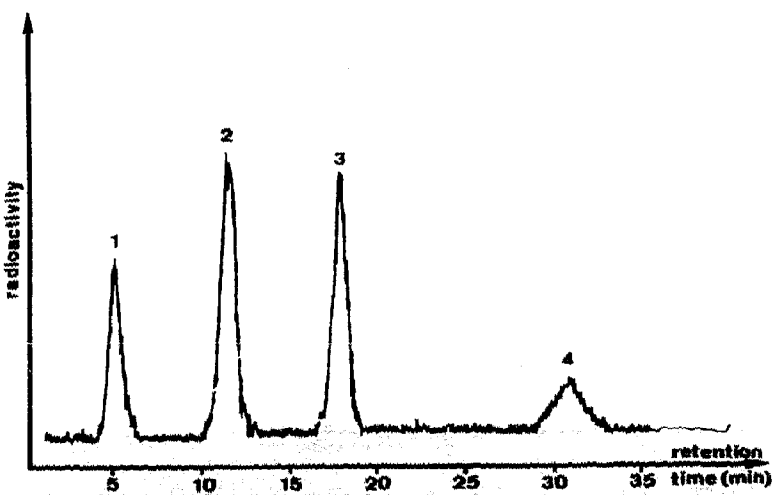

Fig. 2. Separation of iodide (1), ITyr (2), I_2Tyr (3) and I(Tyr-Tyr) (4) by isocratic elution with 30% methanol in 0.02 *M* KH_2PO_4 containing 0.1% H_3PO_4. Other conditions as in Fig. 1.

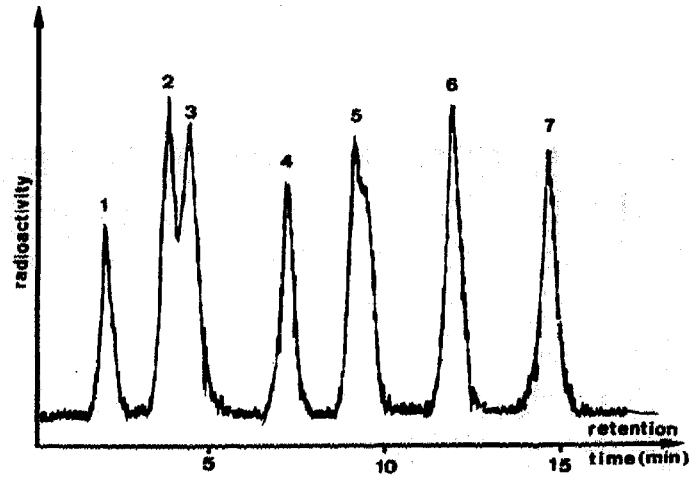

Fig. 3. Separation of iodide (1), ITyr (2), I_2Tyr (3), I(Tyr-Tyr) (4), I_2(Tyr-Tyr) (5), I_3(Tyr-Tyr) (6) and I_2Tyr-I_2Tyr (7) by isocratic elution with 50 % methanol in 0.02 *M* KH_2PO_4 containing 0.1 % H_2PO_4. Other conditions as in Fig. 1.

The elution of iodinated compounds with three different concentrations of methanol was then examined. Fig. 2 shows an isocratic elution with buffer 2. ITyr is well separated from I_2Tyr, but only monoiodinated derivatives are eluted and with poor peak shapes due to the long retention time of 30 min. This eluent does not allow the separation of the more highly iodinated derivatives of Tyr-Tyr.

Fig. 3 shows an isocratic elution with buffer 3. Under these conditions, ITyr and I_2Tyr are poorly separated but a good separation of four peaks of the iodinated derivatives I(Tyr-Tyr), I_2(Tyr-Tyr), I_3(Tyr-Tyr) and I_2Tyr-I_2Tyr can be obtained. Iodothyronines are not eluted. From these chromatograms we can conclude that iodin-

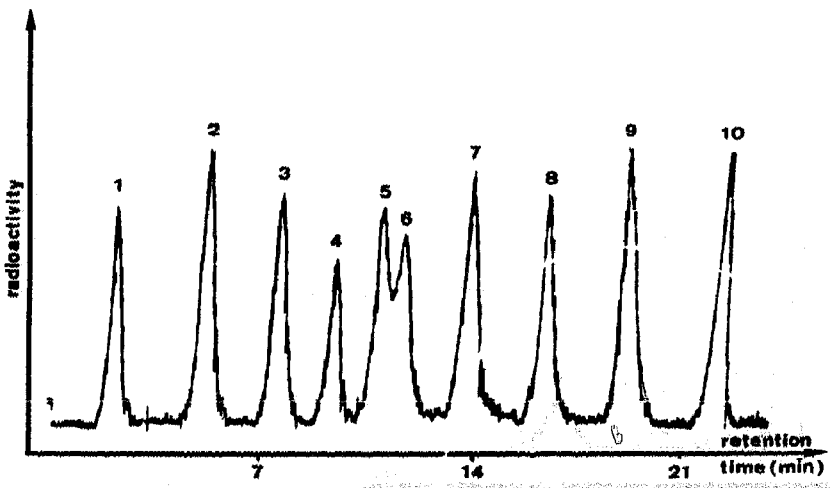

Fig. 4. Separation of iodide (1), ITyr (2), I_2Tyr (3), I(Tyr-Tyr) (4), ITyr-ITyr (5), I_2(Tyr-Tyr) (6), I_3(Tyr-Tyr) (7), I_2Tyr-I_2Tyr (8), T_3 (9) and T_4 (10). Gradient elution in 0.02 *M* KH_2PO_4 containing 0.1 % H_3PO_4: 20–40 % methanol for 8 min, 40–50 % for 8 min and 50–70 % for 8 min. Other conditions as in Fig. 1.

TABLE I

RETENTION TIMES OF IODOTYROSINES, IODOTYROSYLTYROSINES AND IODOTHYRONINES IN METHANOL MOBILE PHASES

Compound	*Retention time (min)*	*% Methanol in 0.1 M KH_2PO_4 + 0.1% H_3PO_4*
Iodide	2.4	25
ITyr	6.3	36
I_2Tyr	9.7	42
I(Tyr-Tyr)	12.1	45
ITyr-ITyr	14.5	48
I_2(Tyr-Tyr)	15.2	49
I_3(Tyr-Tyr)	16.6	52
I_2Tyr-I_2Tyr	18.6	56
T_3	20.5	62
T_4	23.2	68

ated derivatives of Tyr-Tyr have a polarity in between that of iodotyrosines and iodothyronines. In order to obtain an optimal separation of these compounds, we chose a 20–40% methanol gradient in 0.02 *M* KH_2PO_4 + 0.1% H_3PO_4 for 8 min, then 40–50% methanol for 8 min and 50–70% methanol for 8 min. A good separation (Fig. 4) was obtained of: I^-, ITyr, I_2Tyr, I(Tyr-Tyr), ITyr-ITyr, I_2Tyr-Tyr + Tyr-I_2Tyr, I_3(Tyr-Tyr), I_2Tyr-I_2Tyr, T_3 and T_4. The elution times and methanol concentrations are listed in Table I.

With this procedure, we are able to separate the iodinated derivatives of L-tyrosyl-L-tyrosine from iodotyrosines and iodothyronines in 24 min. This method should be suitable for quantifying the reaction products of "*in vitro*" iodination of synthetic peptides in order to elucidate the biosynthesis mechanism of iodothyronines applicable to thyroglobulin.

Application of this procedure associated with a derivatization method such as dansylation[6,10] will be of considerable value for the identification and the dosage of putative derivatives of L-tyrosyl-L-tyrosine in human blood.

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