Rapid Analysis for Iodotyrosines and Iodothyronines in Thyroglobulin by Reversed-Phase Liquid Chromatography

Nicholas M. Alexander and Miyako Nishimoto

We describe a 10-min reversed-phase "high-pressure" liquid-chromatographic procedure for measuring tyrosine, monoiodotyrosine, diiodotyrosine, 3,5-diiodothyronine, 3,5,3'-triiodothyronine, 3,3',5'-triiodothyronine, and thyroxine. Resolution and quantitation of a mixture of these amino acids were excellent on LiChrosorb (Altex) RP-8 with isocratic elution (1.5 mL/min) with acetonitrile/water/glacial acetic acid (50/49/1 by vol). As little as 100 ng of each iodoamino acid could be detected and quantitated with a conventional 1-cm, flow-through spectrophotometric (254-nm) detector coupled to a 10-mV strip-chart recorder. Analyses for monoiodotyrosine, diiodotyrosine, 3,5,3'-triiodothyronine, and thyroxine in hog and beef thyroglobulin hydrolyzates (sequential digestion with pronase and aminopeptidase) agreed well with results by anion-exchange chromatography and by competitive radioassays. To prevent interference by tryptophan in the analysis for diiodotyrosine, we batch-separated the iodoamino acids by anion-exchange chromatography before the procedure. The procedure we describe seems generally useful for detection and quantitation of thyroid hormones, thyroid hormone metabolites, and iodoamino acids.

Additional Keyphrases: thyroid status • iodoaminoacids in thyroglobulin • anion-exchange chromatography • radioassay

Iodotyrosines (MIT and DIT) and iodothyronines (rT3, T3, T4) have been analyzed by numerous chromatographic, spectrophotometric, and competitive radioassay procedures. More recently, while our work was in progress, two groups (1, 2) described the separation of artificial mixtures of the iodoamino acids by ion-paired reversed-phase "high-performance" liquid chromatography (HPLC) on octadecyl-silica columns. Here we describe the separation and measurement of iodotyrosines and iodothyronines by HPLC by use of isocratic elution with acetonitrile/water/acetic acid (50/49/1 by vol) on a reversed-phase octyl-silica column support. We also describe the first application of this technique to analysis for iodoamino acids in thyroglobulin.

Materials

The L-isomers of all of the iodoamino acids we used were from Sigma Chemical Co., St. Louis, MO 63178, except for DL-rT3, which was generously donated by Nuclear-Medical Laboratories Inc., Dallas, TX 75247. Stock 1 g/L solutions of these amino acids were prepared in 10 mmol/L NaOH and diluted with distilled water to obtain working solutions for chromatography. Acetonitrile was "spectral" grade solvent (E. Merck).

Beef and hog thyroglobulin were isolated by salt fractionation, followed by chromatography on DEAE-cellulose (3); they contained 1.03 and 0.98% iodine, respectively (measured by Bio-Science Laboratories, Van Nuys, CA 91405).

The HPLC apparatus (Altex Scientific Inc., Berkeley, CA 94710) consisted of a Model 110 solvent metering pump, Model 905-42 syringe loading sample injector, Model 153 analytical ultraviolet (254-nm) detector, and Model 300 series linear recorder. The reversed-phase octyl-silica column (25 × 0.32 cm), LiChrosorb RP-8 (10-μm average particle diameter) was also a product of Altex.

Methods

Thyroglobulin hydrolysis. This was performed by sequential digestion with pronase (B grade; CalBiochem, La Jolla, CA 92037) and "aminopeptidase M" (Sigma, cat. no. A7761), under nitrogen (4, 5).

Anion-exchange chromatography and HPLC of iodoamino acids. The iodotyrosines and iodothyronines in the hydrolysates were separated (4, 5) on 8 × 8.9 mm columns of AG1 × 2, 200–400 mesh, acetate form (BioRad Laboratories, Richmond, CA 94804), after the columns were pretreated with 5 mL of 3 mol/L sodium acetate and 20 mL of distilled water to assure conversion of the anion-exchange resin to the acetate form. The hydrolysate from 4 mg of thyroglobulin was transferred onto the column and successively eluted with 5 mL of distilled water, and 6 mL each of 0.25, 10, and 500 mL/L solutions of acetic acid. MIT was in the first acetic acid fraction, DIT in the second, and T3 and T4 (eluted together) in the third. The water eluate, which contained nearly all of the other constituents (buffer salts, neutral and basic amino acids, enzymes, etc.) in the digestion mixture, was discarded. The iodoamino acid content in a 5- or 10-μL portion of each fraction was quantitated by the ceric–arsenite reaction (20 min, 37 °C) after 5 min of pretreatment with bromine (6), a procedure that detected 90% of the iodine in these amino acids. The reaction was monitored in a 1-cm flow-through cell at 420 nm with a Model 240 Gilford spectrophotometer rather than as reported (6) with a Klett–Summerson photometer. Although tyrosine and tryptophan were eluted with the 0.25 mL/L acetic acid

*“Pronase” is a mixture of proteinases, a principal component being microbial metalloproteinases (EC 3.4.24.4).
Table 1. Retention Times of Iodide and of Aromatic and Iodoamino Acids on LiChrosorb RP-8

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodide</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.4</td>
</tr>
<tr>
<td>3-Moniodotyrosine</td>
<td>2.7</td>
</tr>
<tr>
<td>3,5-Diodotyrosine</td>
<td>3.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>3.6</td>
</tr>
<tr>
<td>Thyronine</td>
<td>3.8</td>
</tr>
<tr>
<td>3,5-Diiodothyronine</td>
<td>4.2</td>
</tr>
<tr>
<td>3,5,3'-Triiodothyronine</td>
<td>6.2</td>
</tr>
<tr>
<td>3,3',5'-Triiodothyronine</td>
<td>8.5</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>9.6</td>
</tr>
</tbody>
</table>

*Conditions as in Figure 1.*

along with MIT from the resin, these aromatic amino acids did not interfere with the iodine assay. The overall analytical recovery of iodoamino acids after chromatography on AG-1 resin ranged from 94 to 100% when mixtures of MIT, DIT, T₃, and T₄ in the hydrolysis medium were analyzed.

The remainder of each acetic acid eluate was evaporated in a rotary evaporator and the residues were redissolved with 0.5 mL of 8.7 mol/L acetic acid. Five to 20 μL of each fraction was injected onto the chromatographic column.

Competitive radioassays for T₃ and T₄. Part of the thyroglobulin hydrolysate was diluted 200- (T₄) and 2000-fold (T₃) with a 5 g/L solution of human serum albumin, to assay for T₄ (7, 8) and T₃ (9). The proteolytic enzymes used for the digestion did not interfere in the competitive radioassays because they were excluded from the Sephadex G-25 columns. A relative molecular mass (Mr) of 670 000 for thyroglobulin was used to calculate the number of moles of iodoamino acid per mole of protein, and the thyroglobulin concentration was determined at 280 nm with use of an absorptivity (ε₂₈₀nm) of 10 (10).

Results

Figure 1 shows the separation of tyrosine, MIT, DIT, T₃, and T₄ within 10 min on isocratic elution with acetonitrile/water/glacial acetic acid (50/49/1 by vol). Other experiments (Table 1) showed that inorganic iodide, 3,5-diiodothyronine, and rT₃ were clearly separated from all of the compounds in Figure 1. Inorganic iodide emerged with the void volume at 1 min, while 3,5-diiodothyronine migrated (4.2 min) between DIT and T₃, and rT₃ appeared (8.5 min) between T₃ and T₄. Tryptophan and thyroxine were not entirely separated from DIT. The retention time for each compound was directly proportional to the flow rate, increasing about threefold when the solvent flow rate was decreased from 1.5 to 0.5 mL/min.

Figure 2 shows calibration curves for the quantitative detection of the common iodoamino acids and tyrosine. Plots for each of the amino acids were linear, whether peak heights or peak areas were plotted vs. concentration. The sensitivity of spectrophotometric detection is determined by the molar absorbances at 254 nm of each amino acid, and as little as 100 to 800 ng of each compound was detected with a CV of 4% with the recorder set at a full-scale deflection of 0.01 A. At maxi-
Table 2. Iodoamino Acid Analysis (moles/mole of thyroglobulin) of Beef and Hog Thyroglobulins Compared by HPLC, Anion-Exchange Chromatography, and Competitive Radioassay

<table>
<thead>
<tr>
<th></th>
<th>Beef thyroglobulin (54 atoms I/mol)</th>
<th>Hog thyroglobulin (52 atoms I/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>Anion-exchange</td>
</tr>
<tr>
<td>MIT</td>
<td>10.1±0.2</td>
<td>9.0±0.2</td>
</tr>
<tr>
<td>DIT</td>
<td>13.5±0.3</td>
<td>12.7±0.6</td>
</tr>
<tr>
<td>T₃</td>
<td>0.32±0.01</td>
<td>—</td>
</tr>
<tr>
<td>T₄</td>
<td>3.2±0.1</td>
<td>3.9±0.1 b</td>
</tr>
</tbody>
</table>

Sum c 51 53

a Calculated from the total iodine content.
b This fraction included T₃ and T₄, but was treated as T₄ in this calculation, because <10% of the iodine is in T₃.
c Total no. of iodine atoms per mole of thyroglobulin.

Values are the means (±difference from the mean) of duplicate assays.

maximum sensitivity (0.005 A full-scale deflection), quantitation of the peaks was less reliable because the baseline became more irregular, although this setting was useful for qualitative and semi-quantitative analyses.

We used our method to quantitate the iodoamino acids in beef and hog thyroglobulin, and compared the results to those for analysis for MIT and DIT by anion-exchange chromatography and for T₃ and T₄ by competitive radioassay procedures (Table 2). HPLC and anion-exchange chromatography yielded essentially identical results for MIT and DIT with either beef or hog thyroglobulin hydrolysates. The results for T₃ and T₄ by HPLC also agreed well with those for the competitive radioassays, although the T₃ analyses were somewhat higher, and the T₄ slightly lower, by HPLC. It is noteworthy that 97% of the total iodine in beef thyroglobulin (51 atoms per 54 total I atoms) and 102% in hog thyroglobulin were recovered in MIT, DIT, T₃, and T₄ by HPLC.

Figure 3 shows the HPLC patterns obtained with an hydrolysate of hog thyroglobulin (very similar patterns were obtained with beef thyroglobulin). As expected, the MIT fraction (chromatogram 2) showed large tyrosine and tryptophan peaks, in addition to the iodothyrosine peak. There was also an unidentified peak that migrated faster (1.5 min) than tyrosine; the small double peak at 1 min partly reflected the solvent front. The DIT fraction (chromatogram 3) primarily contained DIT, but several small unidentified peaks were apparent too. From chromatogram 4 it is clear that T₃ and T₄ were the most significant peaks in the iodothyronine fraction. The small peak at 8.5 min that preceded T₄ may have been r'T₃, and the little peak just before T₃ had a retention time of 4.2 min, similar to that of diiodothyronine. The small amount of deiodination (2%) that occurs during the digestion procedure may explain the appearance of these iodothyronines (10). Several other small peaks appeared between 1 and 4 min, and two large peaks were near the solvent front. Some of the unknown peaks may have been artifacts of the digestion procedure, or protease-resistant peptides and glycopeptides that contained tyrosyl and tryptophanyl residues. That all of the
iodoamino acids in thyroglobulin are released by the proteolytic digestion procedure is supported by previous data (10, 11) and by our results in Table 2, and therefore none of the unknown peaks were peptide-linked iodoamino acids.

Discussion

Adsorption of the aromatic and iodoamino acids onto hydrocarbonaceous-silica supports is primarily determined by the hydrophobic properties of these amino acids. Tyrosine, the most polar aromatic amino acid, was eluted before the iodotyrosines, tryptophan, thyronine, and iodothyronines. Clearly, enhanced hydrophobicity is related to iodine content, although the position of iodine atoms on the benzene rings is an important determinant as well, because T₃ was adsorbed less firmly than rT₃.

The chromatographic behavior of these amino acids was, as expected, significantly influenced by the composition of the eluting solvent. Retention times were decreased as the eluting solvent mixture was enriched with acetonitrile, but resolution of the iodotyrosines was incomplete with these less polar eluents. When the mobile phase contained either methanol or ethanol, rather than acetonitrile, the iodothyronines, but not the iodotyrosines, were effectively separated. Thus, our best separations were with acetonitrile/water/glacial acetic acid in the proportion of 50/49/1.

Other investigators have also recently described the separation, but not the quantitation, of iodoamino acids by HPLC on octadecyl-silica. Hearn et al. (1) used hydrophilic, ion-paired reversed-phase HPLC on μBondapak C₁₈ to resolve artificial mixtures of all of the iodoamino acids, similar to our results with RP8 LiChrosorb. Tyrosine was resolved from MIT, but the chromatographic behavior of tryptophan was not studied. Although these workers obtained about a 10-fold increase in sensitivity (1–10 pmol) by monitoring the eluate at 210 nm rather than at 254 nm, interference by ultraviolet-absorbing compounds other than the iodoamino acids probably restricts use of the 210-nm detector to purified mixtures of iodoamino acids.

By coupling HPLC on Nucleosil C₁₈ to a sensitive iodine detector (cerate–arsenite redox reaction), Nachtmann et al. (2) separated and detected sub-nanogram quantities of synthetic mixtures T₃ and T₄. This highly sensitive detector might possess sufficient specificity to eliminate potential interferences from non-iodinated, ultraviolet-absorbing compounds that might co-migrate with an iodoamino acid, such as tryptophan with DTT, but the analysis of iodoamino acids in complex biological mixtures with an HPLC–iodine detector system remains to be established.

Our results represent the first application of HPLC for iodoamino acid analyses in thyroglobulin, an iodoprotein that contains sufficiently large quantities of MIT, DIT, T₃, and T₄ for detection by ultraviolet spectrophotometry at 254 nm. We performed simple batch-elution chromatography of the thyroglobulin hydrolysate on a small anion-exchange resin column before HPLC in order to prevent tryptophan interference with DTT. Thyronine, another possible interfering compound, is not a constituent of thyroglobulin. Aside from the separation of tryptophan from DIT, the pre-HPLC separation of the iodoamino acids on an anion-exchange column removes potentially deleterious proteases and neutral and basic amino acids that might accumulate on the HPLC column.

Our confidence in the reliability of the HPLC method was reinforced by the excellent agreement with results we obtained by two other techniques, and by quantitative recovery of the iodine in MIT, DIT, T₃, and T₄. Moreover, our data are virtually identical to the iodoamino acid values reported for similar thyroglobulin preparations (10–13).

References