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Short communication

Identification and quantitation of iodotyrosines and iodothyronines in proteins using high-performance liquid chromatography by photodiode-array ultraviolet-visible detection

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Abstract

We describe a new method for the separation, identification and quantitation of iodotyrosines and iodothyronines [3-monoiodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT), L-thyronine (T_0), 3,5-diiodo-L-thyronine (T_2), 3,5,3′-triiodo-L-thyronine (T_3), reverse 3,3′,5′-triiodo-L-thyronine (rT_3) and 3,3′,5,5′-tetraiodo-L-thyronine (T_4)]. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Nucleosil C_8 column with photodiode-array UV–Vis detection. A clearly defined elution profile was obtained of each iodoamino acid (iodotyrosines and iodothyronines) using a linear gradient from 20 to 80% phase B (90% acetonitrile, 10% water, 0.1% TFA), phase A (water, 0.1% TFA, pH 2.0) eluted over 40 min. Iodoamino acid composition was determined, taking into account retention times and spectral characteristics. Thyroid protein samples were digested enzymatically and the complex mixture of IAA was then injected onto the RP–HPLC system. A photodiode-array detector with a dynamic range in the UV–Vis region was used in the HPLC system to monitor the absorbance at different wavelengths continuously, collecting data which were compared with standard samples. Each IAA was quantitated using linear calibration curves obtained at 280 nm. This method allowed identification and quantitation of iodoamino acids from diverse sources in the range 2–500 ng, avoiding the need to radiolabel samples. The technique was tested with in vitro iodinated and non-iodinated human thyroglobulin and the recoveries ranged from 84 to 91%.

Keywords: Iodotyrosine; Iodothyronine; Proteins; Iodoamino acids

1. Introduction

In the last few years, photodiode-array ultraviolet-visible (PDA-UV–Vis) detectors have been used as components of high-performance liquid chromatography (HPLC) systems. The advantage of these detectors is that they can continuously monitor all spectral wavelengths and collect data for future processing. An HPLC–PDA-UV–Vis system has therefore been used for detection and characterization of a large number of compounds [1–5].

The identification, separation and quantitation of iodoamino acids (iodotyrosines and iodothyronines) is essential for structural and functional studies of

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thyroglobulin (Tg) and related proteins in the thyroid gland, as well as in clinical diagnosis of thyroid disease. The synthesis of the important thyroid hormones, 3,5,3′-triiodo-L-thyronine (T_3) and 3,5,3′,5′-tetraiodo-L-thyronine (T_4), takes place in the follicular cells and in the inner membrane located between the follicular cells and the colloid inner space. The precursors 3-monoiodo-L-tyrosine (MIT) and 3,5-diiodo-L-tyrosine (DIT) are synthesized in a first reaction by the binding of iodine to the amino acid tyrosine in the Tg. In a second step, the iodinated tyrosines are coupled to form T_3, reverse 3,3′,5′-triiodo-L-thyronine (rT_3) and T_4 [6,7]. These reactions are catalysed by the enzyme thyroid peroxidase [8]. Effects of toxic compounds on the thyroid will be reflected by a change in the relative amount of the iodinated hormones and their precursors present in thyroglobulin. A method is thus required to quantitate the iodoamino acids (MIT, DIT, T_3, rT_3 and T_4) in Tg, as well as some other related analogues such as thyronine (T_0) and 3,5-diiodo-L-thyronine (T_2).

Several HPLC separation methods for iodoamino acids (IAA) have recently been described [9–14]. These techniques have many useful chemical and biomedical applications, although they show a lack of sensitivity and require approximately 1–20 μg of substrate for reliable detection [9–13]. Other indirect quantitation methods such as isotopic equilibrium followed by thin-layer chromatography [15,16] or radioimmunoassay (RIA) [17] have also been used to estimate biologically active thyroid hormones and their metabolites in body fluids and tissues. More recently, a method using fluorescence spectrometry has been described [14]. However, the technique requires iodoamino acid derivatization of IAA prior to detection, and the biological sample cannot be recovered for further analysis.

Here we report a new, sensitive and simple RP–HPLC method using PDA-UV–Vis detection (2–100 ng level) for identification and quantitation of IAA using several post-experimental data processing software routines. This method has the additional advantage of avoiding the use of radiolabeled samples.

The efficiency of the method was tested using in vitro ^{125}I-iodinated Tg and in vivo iodinated Tg purified from rat thyroid, yielding recoveries from 84 to 91% of the radioactivity incorporated into the protein.

2. Experimental

2.1. Chemicals

Acetonitrile was from Scharlau (Barcelona, Spain). Pronase E (4 U/mg solid), glucose oxidase (250 U/mg protein, EC 1.1.3.4), lactoperoxidase (81 U/mg protein, EC 1.11.1.7), aminopeptidase M (15–25 units/mg protein, EC 3.4.11.2), MIT, DIT, T_0, T_2, T_3, rT_3 and T_4 were from Sigma (St. Louis, MO, USA); trifluoracetic acid (TFA) was from Merck (Darmstadt, Germany). Ultra-pure water for HPLC, generated by a Milli-PO4 coupled to a Milli-Q water purification system (Millipore, Bedford, MA, USA), was used in the preparation of all buffers. ^{125}INa (17.4 Ci/mg) was from Dupont (Brussels, Belgium).

2.2. HPLC equipment

The chromatographic system consisted of a system controller, multisolvent system and photodiode-array detector. All sample injections were performed with a U6K universal injector (Waters Assoc., Milford, MA, USA).

RP–HPLC was performed with a Nucleosil C_8 column (250×4.6 mm I.D., 5 μm, 300 Å) protected by a guard Nucleosil C_8 pre-column (45×4.6 mm I.D., 10 μm, 300 Å) (Bio-Rad, Madrid, Spain). The column was operated at room temperature at a flow-rate of 0.5 ml/min, and was initially equilibrated with 80% phase A (H_2O, 0.1% TFA, pH 2.0), 20% phase B (90% acetonitrile, 10% water, 0.1% TFA) and eluted over 40 min from 20 to 80% phase B and then over 10 min from 80 to 100% phase B. The column was then re-equilibrated at initial conditions (80% phase A, 20% phase B) for a period of 10 min before a new run.

2.3. Proteins

Iodine-poor human Tg (660 kDa, 0.01% I<1 atom I/molecule) was purified and lyophilized as previously described [18]. Bovine serum albumin (BSA, 67 kDa) was from Sigma.

2.4. Enzymatic iodination

Iodine-poor Tg (1–2 nmol) and BSA (1–2 nmol) were enzymatically iodinated in vitro in 0.01 *M*

Tris–HCl (pH 8.0) at 37°C with 10^{-5} *M* iodide, labelled with radioiodine (^{125}INa), 1.5 μg/ml lactoperoxidase, 1.0 mg/ml glucose and 1.5 μg/ml glucose oxidase for 1 h. The number of iodine atoms bound per mole of protein was calculated as previously described [18]. The iodinated proteins were separated from excess iodide and reagents by gel permeation through disposable Econopac 10DG-P6 (Bio-Rad) columns in 0.01 *M* Tris–HCl, pH 8.0.

Aliquots of iodinated proteins were digested with pronase E, and IAA distribution determined using paper chromatography as previously described [19].

2.5. Proteolytic digestion

Proteins samples (1 nmol, in vitro iodinated or non-iodinated) were digested with pronase E (1:10, w/w) for 20 h at 37°C, followed by treatment with aminopeptidase M (1:10, w/w) for 6 h in 0.01 *M* Tris–HCl (pH 8.0) at 37°C [19,20].

2.6. Removal of undigested material

Undigested material was removed using gel permeation Econopac 10DG-P6 columns in distilled water. Fractions 12 to 18 (500 μl each), corresponding to IAA, were pooled, evaporated with a gyro-vap and redissolved in 300 μl Milli-Q water with 0.1% TFA. Fractions 5–11 were redigested and no IAA were found.

2.7. Data analysis

The data were obtained using a Waters 991 photodiode-array detector with a dynamic range in the UV–Vis region (190–800 nm) connected on-line to the above Waters HPLC system. A NEC PowerMate 386/25 personal computer and Powerline software were used to control the chromatographic system and collect data. All PDA spectra were run from 200–350 nm and analyzed using several post-run data evaluation programs. 'Spectrum index plot' and 'Spectrum analysis' were used to visualize and study the spectra (Fig. 1 Figs. 3 and 4). 'Library search' was used to identify IAA specific spectra, from 200–350 nm (Fig. 2A) and from 250–350 nm (Fig. 2B), obtained from a chromatogram peak. 'Peak purity', 'Integrator' and 'Calibration table'

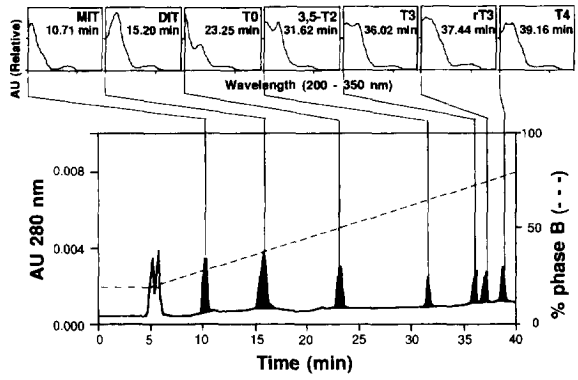

Fig. 1. RP-HPLC–PDA and spectral analysis of 10 ng standard iodoamino acids on a Nucleosil C_8 (250×4.6 mm I.D.) column. Samples were eluted at room temperature using a linear gradient from 20 to 80% phase B (90% acetonitrile, 10% water, 0.1% TFA) over 40 min. Phase A (water, 0.1% TFA, pH 2.0). Flow-rate 0.5 ml/min. The chromatogram was analyzed by monitoring the absorbance at 280 nm. Automatic spectra were acquired in the peak maximum from 200 to 350 nm wavelength to compare the different IAA.

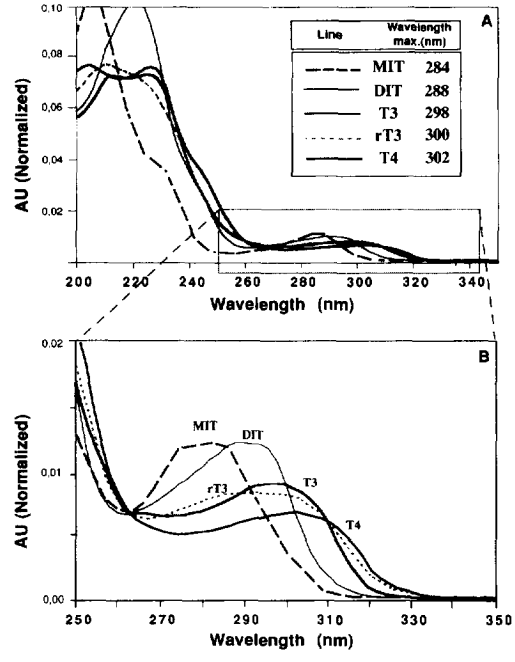

Fig. 2. Comparison of IAA spectra, from 200 to 350 nm, obtained using 'Spectrum Analysis' by software routines (A). IAA spectra in a more specific absorption range from 250 to 350 nm (B). Spectra were normalized at 263 nm to eliminate concentration differences and to obtain maximum correlation in the range of the selected absorbance.

were used to calculate areas and concentrations for each IAA.

The IAA contents of several biological samples were calculated based on standard linear calibration curves.

3. Results

In routine work with Tg and related proteins, we have found an absolute necessity to determine and quantitate the amount present in a variety of samples. Several reported methods have been used, but were found inappropriate for our needs. Some showed very low sensitivity and others required laborious preanalysis, usually resulting in samples loss.

To improve on the reported methods, we were urged to develop a simple technique based on RP-HPLC–PDA for easy, rapid detection and quantitation using software routines.

3.1. RP-HPLC and spectroscopic characterization of IAA

Fig. 1 shows a chromatographic separation of an IAA standard sample (MIT, DIT, T_0, T_2, T_3, rT_3 and T_4, 10 ng of each) at 280 nm, wavelength chosen for quantitation. Absorption data was acquired in a range of wavelengths from 200 to 350 nm.

Using a Nucleosil C_8 column with a Nucleosil C_8 pre-column and a linear gradient from 20 to 80% phase B (90% acetonitrile, 10% water, 0.1% TFA), phase A (water, 0.1% TFA, pH 2.0), we have identified each IAA by its retention time and characteristic spectrum.

Under these chromatographic conditions, all IAA display regular and well-defined peaks, and complete separation was achieved in a total time of 40 min. Iodotyrosines were eluted first, followed by iodothyronines according to their increasing iodo content and hydrophobicity.

A sensitive PDA was utilized for recording spectra of each peak maximum from 200 to 350 nm. Automatic detection and spectral analysis were performed by the 'Spectrum Index' program. Retention time of each standard IAA was reproducible and consistent with an array of sequential experiments carried out on the same or different days. Differences between retention times were always lower than 20 s, permitting a precise correlation between each IAA peak and its retention time.

Each IAA was identified by comparison of its retention time and spectrum with those obtained from the chromatography of a standard IAA. These data were registered and recorded for further analysis of biological samples.

3.2. Comparative spectral analysis of IAA

Fig. 2 represents the absorption spectra of IAA. Spectra were obtained from the peak maximum of each compound after RP–HPLC using a sensitive PDA. The spectra (obtained utilizing the same experimental conditions as in Fig. 1 were normalized to eliminate concentration differences and for improved comparison, using the 'Spectrum Index' and 'Spectrum Analysis' subprograms. They were recorded at 1.3 nm, from 200 to 350 nm for complete spectra and from 250 to 350 nm for specific absorption spectra. It should be considered, that all iodothyronines displayed spectra with a common shape, differing only in the maximum position. The higher values of λ_{max} corresponded with the higher iodo content in the specific chromophore.

3.3. Verification of IAA quantitation accuracy

A quantitative method requires linear correlation between sample concentration and the parameter chosen to quantitate it. Ultraviolet detection provides chromatograms in which, at certain wavelength, each peak area could be consider proportional to the eluted material. We selected 280 nm for detection of IAA, as it is the best compromise between the maximum absorption of a specific spectrum (between 250–350 nm) of iodotyrosines and iodothyronines.

From 2 to 500 ng of each standard IAA were injected into the RP–HPLC system as shown in Fig. 1. The area was calculated at 280 nm. Linear calibration curves and coefficients of variation for IAA were obtained (Table 1). Each linear calibration curve is composed of ten points, and each point represents the average of five determinations. These data were obtained utilizing the same column and HPLC chemicals, the data are reproducible if elution

Table 1
Linear calibration curves over the range of 2 to 500 ng IAA obtained at 280 nm with its respective coefficient of variation (C.V.)

Linear calibration curves	r^2	C.V. (%)
[peak area]=0.00001474·[MIT] −0.00001927	0.99756821	2.1
[peak area]=0.00000830·[DIT] −0.00000763	0.99893769	3.2
[peak area]=0.00002178·[T_0] −0.00004615	0.99805076	2.8
[peak area]=0.00001389·[3,5-T_2] −0.00000674	0.99685951	2.4
[peak area]=0.00000974·[T_3] −0.00000389	0.99501105	3.3
[peak area]=0.00000935·[rT_3] +0.00002234	0.98701431	2.0
[peak area]=0.00001313·[T_4] −0.00005639	0.99597210	2.4

Each data point represents the average of five determinations and ten points were obtained for each linear calibration curve.

conditions are maintained. To do so, medium and elution buffer pH must be carefully controlled on a daily basis.

3.4. Distribution of IAA in Tg samples

Fig. 3 Fig. 4 show the distribution of iodoamino acids at 280 nm absorbance, of 1 nmol of iodine-poor Tg (Fig. 3) and 1 nmol of in vitro iodinated Tg (Fig. 4) after digestion. In Fig. 4, the distribution of ^{125}I-cpm in labelled control Tg (dashed line) is also shown. Each peak was automatically analyzed by its retention time and spectrum. 'Spectrum Analysis' and 'Library Search' identify specific IAA spectra. Quantitation was then performed by 'Integrator' using linear calibration curves at 280 nm.

Table 2 summarizes the results of Figs. 3 and 4. The sample in column 1, retention time in column 2, spectra identification with 'Library Search' in column 3, percent 'peak purity' in column 4, area (relative units: AU 280 nm×retention time) via software in column 5 and concentration (ng) in column 6. For each peak, the 'peak purity' value was obtained by comparing spectra from the right to the left valleys. The percent value corresponds to the number of spectra showing exactly the same peak maximum spectrum. The area boundaries were established from the left to the right valleys for each peak.

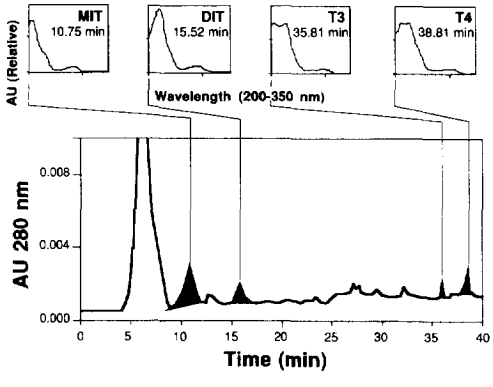

Fig. 3. Absorbance distribution at 280 nm of iodoamino acids obtained from iodine-poor Tg digested with pronase and aminopeptidase M. Undigested material (iodoamino acids not present) was removed. Samples were evaporated in a gyro-vap and redissolved in 300 μl phase A prior to injection. HPLC conditions as in Fig. 1.

4. Discussion

The important role of thyroid hormones in a variety of metabolic processes has led to a continu-

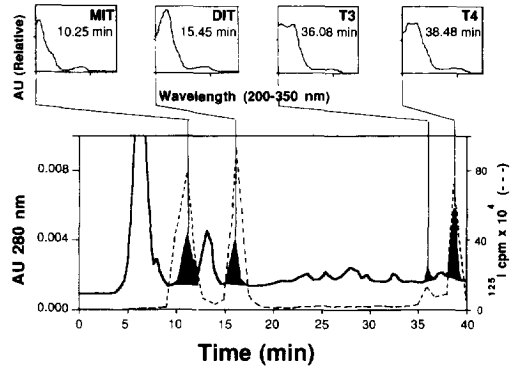

Fig. 4. Absorbance at 280 nm (——) and [^{125}I]-cpm (- - -) distribution of IAA obtained from in vitro iodinated Tg digested with pronase E and aminopeptidase M. Material not digested (no iodoamino acids present) was removed. Samples were evaporated in a gyro-vap and redissolved in 300 μl phase A prior to injection. HPLC conditions as in Fig. 1. Fractions of 1 min were collected and the radioactivity was measured.

Table 2
Results obtained after analysis of the data using several different post-run data programs

Protein	Retention time (min)	Spectrum library search	Percentage 'peak purity'	Area (AU·min)	Amount (ng/nmol Tg)
Tg	10.75	MIT	94.5	0.000232	16
	15.52	DIT	97.0	0.000043	6
	35.81	T_3	91.6	0.000031	4
	38.81	T_4	88.2	0.000113	11
^{125}I-labelled Tg	10.25	MIT	90.3	0.002317	157
	15.45	DIT	94.7	0.001830	221
	36.08	T_3	87.5	0.000063	7
	38.48	T_4	94.4	0.001556	97

The area (AU 280 nm×R.T.) and peak purity represent the average of duplicate determinations. The amounts were calculated with calibration curves over the range of 2 to 500 ng IAA. For more details see text in Section 3.4.

ous search for accurate methods to identify and quantitate them. The methodology has evolved together with the newly-available techniques, becoming progressively easier, faster and more reliable. HPLC has been more frequently used and gives better results than any other method due to its high efficiency and reproducibility.

The most sensitive methods require a previous treatment such as derivatization and transformation of the samples. This is time-consuming and makes impossible the recovery of active IAA for future studies in other clinical assays, or the preparation of pure IAA for studies of thyroid hormones in different metabolic pathways [15–17]. The methods that do not modify the sample during the analysis are insufficiently sensitive, or have low reproducibility for precise comparison of materials from different biological sources [9–14]. Any of the methods discussed above are adequate for the studies carried out in our laboratory.

The existence of PDA detectors, with high sensitivity, able to register spectra from the sample in real time and coupled to a traditional HPLC system, is an interesting option compared to previously-described methods. It is very important for samples with specific chromophores in the UV–Vis area. This is the case with IAA, which has a chromophore with specific absorption (approximately between 250–350 nm) in the UV area, due to the content in phenyl rings and the number of iodine atoms.

In our laboratory, due to routine work in thyroid physiology and more specifically on Tg and associated processes, we require a reliable, specific IAA detection method. For this reason, we have worked to obtain an easy, rapid and highly sensitive method for an HPLC–PDA-UV–Vis system. Using this method we have analyzed many different samples, obtaining high levels of reproducibility and efficiency. Another important aspect is the elimination of the use of radioactive products. The final yield obtained is from 84 to 91%, similar or better than that obtained with the current IAA analysis methods.

We have developed the present method to determine and quantitate IAA from proteins. However, it is applicable to samples of diverse biological origins, with small variations in the process prior to sample injection. In this way, it is possible to determine IAA in serum and urine from different physiological and pathological sources, as well as in clinical assays, routine pharmaceutical analysis and in pathways in which the thyroid hormones play important roles.

5. List of abbreviations

PDA	photodiode-array
TFA	trifluoroacetic acid
Tg	thyroglobulin
BSA	bovine serum albumin
MIT	3-monoiodo-L-tyrosine
DIT	3,5-diiodo-L-tyrosine
T_0	L-thyronine
T_2	3,5-diiodo-L-thyronine
T_3	3,5,3′-triiodo-L-thyronine
rT_3	3,3′,5′-triiodo-L-thyronine

T_4	3,3′,5,5′-tetraiodo-L-thyronine or thyroxine
IAA	iodoamino acids

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