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Method for the quantitation of iodothyronines in body tissues and fluids using high-performance liquid chromatography

Chester E. Hendrich*, Joseph Berdecia-Rodriguez, Vernon T. Wiedmeier and Susan P. Porterfield

Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA 39012-3000 (USA)

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ABSTRACT

The separation and quantitation of iodotyrosines and iodothyronines [3-monoiodo-L-tyrosine, 3,5-diiodo-L-tyrosine, 3,5-, 3,3′ and 3′,5′-diiodo-L-tyronines, 3,5,3′-triiodo-L-thyronine (T_3), reverse 3,3′,5′-triiodo-L-thyronine and 3,3′,5,5′-tetraiodo-L-thyronine (T_4)] from animal tissues (brain, liver and serum) by a new high-performance liquid chromatogrpahic (HPLC) method is described. Rats were infused with iso-osmotic sodium chloride containing 100 μM phloretin to block deiodination. The tissues were extracted using differential pH values to separate other amines from the amine containing iodothyroid hormones. Aliquots of tissue extracts (25–100 μl) were reacted overnight with 5-dimethylaminonaphthalene-1-sulfonyl chloride and their iodotyrosine and iodothyronine content determined by HPLC utilizing fluorimetric detection. Resolution of the individual compound peaks was achieved by gradient elution with a 3.0 mM H_3PO_4 buffer. Greater sensitivity has been achieved (< 1.0 pmol/g) utilizing fluorescence rather than ultraviolet absorbance for the quantitation of these iodinated compounds. The method is superior also to other methods in that recoveries, based on those of ^{125}I-labelled T_4 and T_3, were 89–97%.

INTRODUCTION

Several methods have been utilized for the characterization, separation and quantitation of iodotyrosines and iodothyronines. Under various conditions, purified standards of iodotyrosines and iodothyronines have been separated by high-performance liquid chromatography (HPLC) [1–4]. These methods have utilized underivatized compounds and absorbance at wavelengths of either 254 or 280 nm. Although these methods have many useful chemical and biomedical applications, they suffer from a lack of sensitivity and require from 10–20 μg of substrate for easy detection [1–4]. HPLC methods of separation of iodotyrosines and especially the iodothyronines 3,3′,5-triiodothyronine (T_3), reverse 3,3′,5′-triiodothyronine (rT_3) and 3,3′,5,5′-tetraiodothyronine (thyroxine, T_4) have not been utilized extensively for the quantitation of these important compounds in biological fluids and tissues. Rather, the indirect methods of quantitation such as isotopic equilibration followed by thin-layer chromatography [5–7], column chromatography [8] and rather laborious extractions followed by paper chromatography and radioimmunoassay (RIA) [9] have been utilized to estimate biologically active thyroid hormones and their metabolites in body fluids and tissues. Generally these procedures have permitted estimation of only T_3 and T_4 in tissues. These methods frequently suffer from lack of sensitivity and reduced and variable recoveries.

There are, of course, numerous needs for a sen-

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sitive and direct method for the determination of thyroid hormones and their metabolites in body fluids and tissues. It has been universally recognized that thyroid hormones are essential for normal brain development and mentation. In recent years, it has been demonstrated that relatively small but important quantities of active thyroid hormones cross the placenta in the human [10] as well as the laboratory rat [9,11–13] and that even these small amounts of T_3 and T_4 may be essential for normal fetal development and well-being.

The HPLC method presented here was developed for the purpose of measuring the biologically active thyroid hormones (T_3 and T_4) and some of their major metabolites in the brains and livers of fetal [13], neonatal and adult rats [14]. The method has been utilized also to measure T_3 and T_4 in the sera of these rats and a comparison with RIA values has been made. We present here a method for sample preparation, derivatization of the iodotyrosines and iodothyronines with 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns), separation by HPLC and quantitation using fluorescence spectrometry.

EXPERIMENTAL

The experimental procedures used herein were adapted from several sources (1–4] and were modified for the purpose of quantitation of thyroid hormones and their metabolites in body tissues. The concept of derivatization of iodotyrosines and iodothyronines with Dns and fluorimetric detection came from our previous experience of developing a highly sensitive and reliable method for the measurement of amino acids in body tissues and fluids [15]. This method was based on and adapted from previous thin-layer [16] and column [17] chromatography methods that have shown Dns to form highly fluorescent derivatives with amino acids [18–20].

Equipment

A high-performance liquid chromatograph (Beckman Instruments) consisting of a Model 421 microprocessor, two Model 110A pumps, a Model 210 sample injector with a 50-μl loop and a C-RIA recorder–integrator was used. Fluorescence was detected using a fluorimeter (Gilson Spectra/Glo) with a standard flow cell, 7-51X excitation filter and 3–72 M emission filter. Prepacked reversed-phase columns (Adsorbosphere HS C_{18} 20%, 5 μm, 250 mm × 4.6 mm I.D. and Spherisorb ODS-2 12%, 5 μm, 250 mm × 4.6 mm I.D.) (Alltech Assoc., Deerfield, IL, USA) were used for compound separation.

Chemicals and standards

The chemicals [water, acetonitrile, tetrahydrofuran (THF), triethylamine, acetone, NaOH, $NaHCO_3$, H_3PO_4] were all HPLC grade (Baker and Fisher). Dns (Pierce) was diluted in acetone to a concentration of 6.0 mg/ml.

Purified standards (Henning Berlin, Calbiochem, Sigma) were solubilized in a 0.02 *M* NaOH solution at a pH of 11.5. The standards were mixed together such that 25 μl of the final mixture contained 0.5 pmol of each of the standards.

Tissue extraction procedures

Rats were anesthetized with diethyl ether and, following a midline incision, blood was withdrawn from the inferior vena cava. The animals were then infused rapidly via the left ventricle with three times their estimated blood volume with a solution containing 0.9% NaCl and 100 μM phloretin [(3′),4′,4,6-(tetra)trihydroxyaurone] at a pH of 7.4 to clear the tissues of blood. Phloretin as well as other aurone flavonoids can displace thyroid hormones from their binding proteins and block deiodination, therefore, immediately inhibiting the peripheral metabolism of iodothyronines [21,22]. The tissues to be utilized, usually brain and liver, were removed rapidly and generally 1.0 g of tissue was used for analysis. The tissues were homogenized immediately in 6.0 ml of cold 80% ethanol, 0.02 *M* NaOH and 100 μM phloretin at a pH of 11.5. The samples were centrifuged and the supernatant was poured into a small beaker. This procedure was repeated two more times, and the supernatants were combined. The supernatants were dried in a vacuum oven at 40°C. The residues

were then resuspended in 4 ml of HPLC-grade water containing 100 μM phloretin at a pH of 6.0 in order to separate free amino acids from the iodo compounds. The samples were then centrifuged at 105 000 g for 30 min. The supernatants were discarded and the residues were solubilized in 0.02 M NaOH and 100 μM phloretin at a pH of 11.5 and frozen until analysis. Utilizing [^{125}I]T_4 and [^{125}I]T_3, added to the initial tissue samples, recovery rates were determined to be between 89 and 97%.

Preparation of Dns derivatives

Small (2.0-ml volume) vials were used for the derivatization procedure. A 100-μl volume of 0.5 M $NaHCO_3$ at a pH of 9.5 was added to the vials. This was followed with 25–100 μl of tissue or serum extract being added to the buffer. The amount of sample depended upon the tissue being analyzed, the age of the animal and prior treatment of the animal. Either 25 or 50 μl (0.5 or 1.0 pmol) of the mixed standards were added to each vial and finally 100 μl of Dns solution (6.0 mg/ml of acetone) were added to each vial. Several vials containing only standards (0.5 or 1.0 pmol) as well as a blank with buffer only were prepared for each run. The samples were vortex-mixed and placed in a refrigerator, and the reaction was allowed to proceed overnight. The following morning the volume of each vial was brought to 1.0 ml with HPLC-grade water. Volumes of 50 μl were injected onto the column. The utilization of internal standards makes for easy quantitation, prevents confusion when a peak is not completely resolved and is useful when a peak is absent.

Chromatography

Resolution of the peaks was achieved using gradient elution with the mobile phase in pump A containing a final concentration of 3.0 mM H_3PO_4 buffer with 0.2 mM triethylamine. This was in a solution of 670 ml of HPLC-grade water, 280 ml of acetonitrile and 50 ml of THF per liter at a pH of 3.25. Pump B solution consisted of 10% THF in acetonitrile. The mobile phase was begun at a flow-rate of 1.0 ml/min at 10% B, increased to 100% B over a period of 40 min and decreased back to 10% B over a period of 10 min. The column was then re-equilibrated with 90% A–10% B for a period of 10 min.

RESULTS

A separation of purified standards of the iodotyrosines 3-monoiodo-L-tyrosine (MIT) and 3,5-diiodo-L-tyrosine (DIT), L-thyronine (T_0) and the iodothyronines 3,5-diido-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 (thyroxine, T_4) is depicted in Fig. 1. Unhydrolyzed Dns, Dns-OH and Dns-amide elute at the solvent front or close to it and well before any of either the iodotyrosines or iodothyronines elute. Multiple techniques were utilized to establish the identity of each of the peaks. Each of the tyrosine and thyronine peaks in Fig. 1 represents 0.5 pmol of standard at 20% of the maximal sensitivity of the fluorimeter.

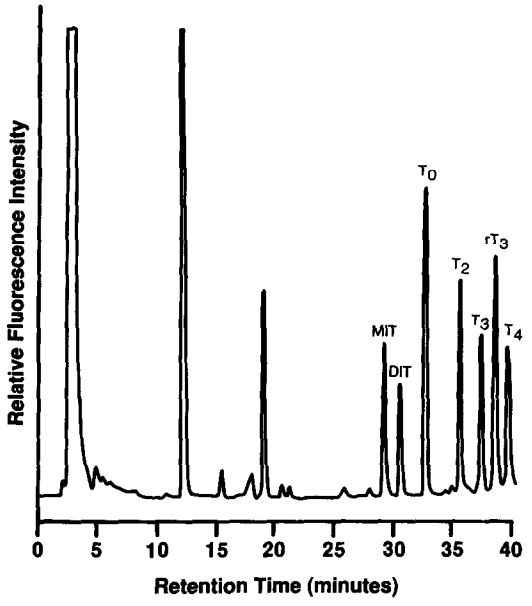

Fig. 1. Chromatogram demonstrating the relative fluorescence and the elution profile of a mixture of iodotyrosines, thyronine and iodothyronines. Each peak represents 0.5 pmol. Peaks: MIT = 3-monoiodo-L-tyrosine; DIT = 3,5-diiodo-L-tyrosine, T_0 = L-thyronine; T_2 = 3,5-diiodo-L-thyronine; T_3 = 3,5,3′-triiodo-L-thyronine; rT_3 = reverse 3,3′,5′-triiodo-L-thyronine; T_4 = 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine).

TABLE I

PERFORMANCE DATA WHICH DEMONSTRATE THE WITHIN-ASSAY AND ASSAY-TO-ASSAY PRECISION AS INDICATED BY COEFFICIENTS OF VARIATION (C.V.)

Concentration of each compound, 0.5 pmol injected onto the column. Mean = relative area ($\times 10^3$) computed by the integrating recorder. Abbreviations = MIT = 3-monoiodo-L-tyrosine; DIT = 3,5-diiodo-L-tyrosine; T = L-thyronine; 3,5-T_2 = 3,5-diiodo-L-thyronine; T_3 = 3,5,3′-triiodo-L-thyronine; rT_3 = reverse 3,3′,5′-triiodo-L-thyronine; T_4 = 3,3′,5,5′,-tetraiodo-L-thyronine.

MIT		DIT		T_0		3,5-T_2		T_3		rT_3		T_4	
Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
Within-assay (n = 6)													
109	6.3	96	4.7	185	5.0	155	7.0	127	6.0	172	7.9	104	9.8
118	5.8	86	5.2	211	2.6	161	4.1	129	5.6	180	5.8	91	3.5
128	4.4	87	13.9	196	5.3	165	6.6	127	5.1	188	4.2	100	8.9
113	5.5	93	6.8	192	4.1	172	5.6	118	5.6	171	4.0	110	4.5
120	4.9	95	6.1	208	3.5	154	7.5	120	6.3	180	3.4	107	5.4
116	4.0	83	7.0	215	4.4	175	3.8	114	5.3	186	5.1	108	4.9
Assay-to-assay (n = 6)													
117	5.5	90	6.0	209	5.9	164	5.3	123	4.9	180	3.9	103	6.8

Table I represents the within-assay and assay-to-assay precision of each of the standards as indicated by the coefficients of variation (C.V.). These data were obtained utilizing the same column, quartz halogen source lamp and the same batch of HPLC chemicals. These data are reproducible if careful adherence to constant derivatization and elution conditions are maintained. That is the pH of the derivatization media and the elution buffers must be carefully controlled on a daily basis.

Fig. 2 depicts a chromatogram of rat brain extract. Each peak contains an internal standard except for the first two peaks that are labelled T_2. The first of these peaks is 3,3′-diiodo-L-thyronine and the second peak is 3′,5′-diiodo-L-thyronine. They have been identified in the same manner as the other peaks. We do not routinely determine these two diiodothyronine metabolites because highly purified standards for these two compounds are quite expensive. The other compounds in this chromatogram contain an internal standard of 0.5 pmol. The brain extract obviously contains some contaminants but they all come off at the solvent front and do not elute near the iodotyrosine and iodothyronine peaks. Dansylamide is moved toward the iodotyrosines and iodothyronines but we have not experienced any difficulty of it eluting with one of the iodo compounds in brain, liver and serum extracts. The retention times are changed slightly in tissue extracts as compared to the elution times of purified standards.

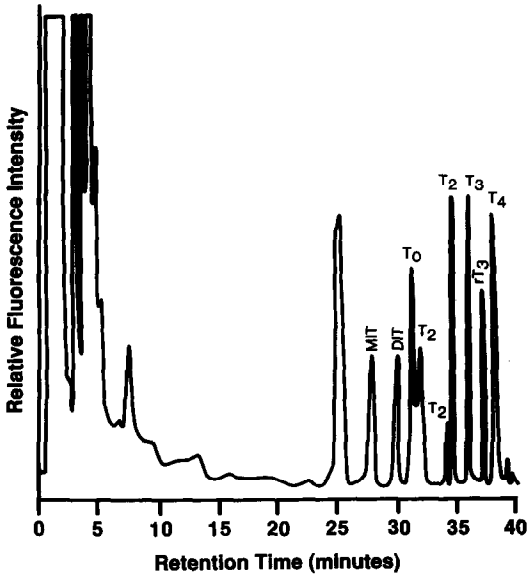

Fig. 2. Chromatogram of rat brain extract with Dns derivatization and with internal standards of 0.5 pmol. Compound abbreviations are given in Fig. 1 and also in the text.

We have chromatographed over 2000 brain, liver and serum extracts as well as several hundred purified standard mixtures, and the retention times have been constant. However, we utilize an internal standard for the major thyroid hormones T_4 and T_3, and the major metabolites. This prevents the possibility of confusing them with a minor metabolite. The use of the internal standard also makes for easy quantitation of the unknown. There is also an excellent dose response unless the amount of derivatized sample is too great and results in overloading of the column.

DISCUSSION

There are numerous needs for a sensitive method for the measurement of the active thyroid hormones and thyroid hormone metabolites. We attempted originally to utilize some of the existing separation and chromatography methods [9,23]. We utilized ^{125}I-labelled T_4 and T_3 but were not successful in obtaining acceptable recoveries (19–40%) and the day-to-day variability was likewise objectionable. Other investigators have been more successful with these methods of combined chromatography and RIA obtaining recoveries of approximately 50–75% [9,23]. Prior use of HPLC to quantitate thyroid hormones has been predominantly limited to the measurement of laboratory-prepared standards or the measurement of just T_4 and T_3 in rather extensively purified samples. These methods have utilized UV absorbance of underivatized samples which we found to be much less sensitive than fluorescence [1–4]. In fact, the level of hormone in tissues of mid to late gestation rat fetuses [13] is below the level of detection using UV absorption methods. Because of our prior experience in developing a sensitive method for measuring amino acids in body tissues and fluids [15], we chose this route of Dns derivatization of the amino-containing iodinated tyrosines and thyronines and therefore fluorescence.

At the same time that we were developing this method, another group of investigators have developed and compared several methods for the HPLC determination of iodinated tyrosines and thyronines and have applied this technology to the measurement of T_3, T_4 and their precursors in rat thyroid hydrolysates [24,25]. They have increased the sensitivity of detection by derivatization with either fluorenylmethyl chloroformate (FMOC-Cl) or dabsyl chloride rather than dansyl chloride. Although fluorimetric detection of FMOC derivatives was unacceptable due to high peaks which appeared in blank samples, UV detection resulted in acceptable chromatograms. However, the determination of dabsyl derivatives of iodinated tyrosines and thyronines utilizing UV and electrochemical detection resulted in excellent chromatograms [24]. The limits of detection of these methods are similar to those which we present utilizing Dns derivatives and fluorimetric detection.

The method presented here and those recently presented [24] greatly expand the thyroidologists potential for determining cellular actions of thyroid hormones, the biosynthesis of thyroid hormones from iodinated precursors and the cellular metabolism of thyroid hormones under physiological and pathological conditions. This is primarily due to the fact that small quantities of these iodocompounds can now be measured in biological samples utilizing these HPLC methods.

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