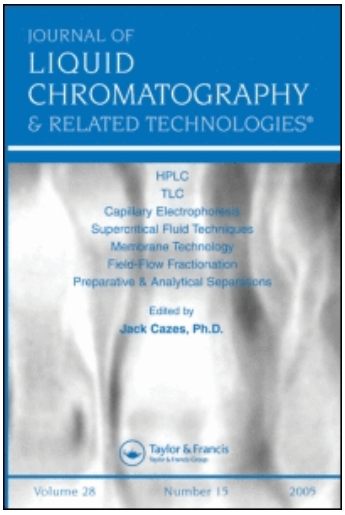

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RAPID HPLC ANALYSIS OF THYROID GLAND HORMONES TRI-IODOTHYRONINE (T_3) AND THYROXINE (T_4) IN HUMAN BIOLOGICAL FLUIDS AFTER SPE

V. F. Samanidou[a]; H. G. Gika[a]; I. N. Papadoyannis[a]
[a] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

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RAPID HPLC ANALYSIS OF THYROID GLAND HORMONES TRI-IODOTHYRONINE (T_3) AND THYROXINE (T_4) IN HUMAN BIOLOGICAL FLUIDS AFTER SPE

V. F. Samanidou, H. G. Gika, I. N. Papadoyannis

Laboratory of Analytical Chemistry
Department of Chemistry
Aristotle University of Thessaloniki
GR-54006 Thessaloniki, Greece

ABSTRACT

A rapid, accurate, and sensitive method has been developed for the quantitative determination of iodoamino acids, namely thyroxine (3,5,3',5'-tetra-iodothyronine, (T_4) and 3,5,3'-tri-iodothyronine (T_3). These compounds are essential indicators in the clinical diagnosis of thyroid gland diseases.

An Inertsil ODS-3, 150 x 4.0 mm, 5 μm analytical column was used with a mixture of CH_3OH-H_2O with 2% acetic acid, at a volume ratio 65:35, with a flow rate 1 mL/min. Detection was performed with a variable wavelength UV-visible detector at 240 nm, resulting in detection limits of 1 ng and 2 ng for T_3 and T_4, respectively, per 20 μL injection.

For the quantitative determination, anthraquinone was used as internal standard at a concentration of 1.0 ng/μL. A rectilinear relationship was observed up to 28 and 40 ng/μL for T_3 and T_4, respectively.

Analysis time was approximately 10 min (retention time of internal standard) while the two compounds are eluted within 5

min. The statistical evaluation of the method was examined, performing intra-day (n=8) and inter-day calibration (n=8) and was found to be satisfactory with high accuracy and precision results.

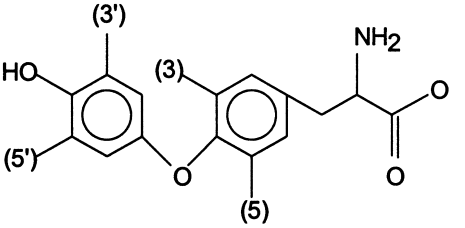
The method was applied to the analysis of the iodothyronines in biological fluids, blood serum and urine, after solid phase extraction for sample clean-up and analyte retention, using diol cartridges. Percentage recovery of iodothyronines in spiked samples ranged from 79.90 to 103.15 for T_3 and from 83.65 to 106.15 for T_4, over the range of 0.5-3 ng/μL.

No interferences were observed from endogenous compounds of human serum and urine.

INTRODUCTION

The identification, separation, and quantitation of iodoamino acids, iodothyronines, is essential for structural and functional studies of Tg thyroglobulin and related proteins in the thyroid gland, as well as in clinical diagnosis of thyroid disease. The synthesis of the important thyroid hormones T_3 (3,5,3'-tri-iodothyronine) and T_4 (3,5,3',5'-tetra-iodothyronine) takes place in the follicular cells and in the inner membrane between them and the colloid inner space. Effects of toxic compounds on the thyroid will be reflected by a change in the relative amount of the iodinated hormones and their precursors in thyroglobulin. The sodium salts are used as therapeutic agents.[1-2] A rapid method is, therefore, required to quantitate the iodoamino acids in biological fluids.

The structure of the iodothyronines is:



Thyronine

T_3 (3,5,3'-tri-iodothyronine) T_4 (3,5,3',5'-tetra-iodothyronine)

Several HPLC separation methods for iodoamino acids have been reported. Radioimmunoassay methods have also been described to estimate biologi-

cally active thyroid hormones and their metabolites in body fluids and tissues. These methods have many useful chemical and biomedical applications, although they require long analysis time.

Most methods are based on radioactivity measurements of ^{125}I labeled T_4. Post column chemiluminescent detection of analytes labeled with acridinium esters has been reported.[1, 3-5]

It has been noted that L-enantiomers of thyroid hormones exhibit different biological activity. D-enantiomers show no basic metabolic rate enhancement but have a marked cholesterol level reducing effect. HPLC ligand exchange chromatography, using chemically bonded chiral stationary phase, has been used for the resolution of enantiomers of thyroid hormones.[6]

Derivatization methods for iodothyronine amino acids analysis have been proposed using various agents such as dansyl chloride or ethanolic pivalic anhydride containing 4-dimethylaminopyridine.

The latter has been suggested for HPLC or gas chromatographic determination of these compounds. With this procedure, T_3 and T_4 are converted to their corresponding N,O-dipivalyl ethyl ester derivatives, with a potential for losses during the derivatization procedure.[7,8]

Ion-pair RP-HPLC methods permit the separation of iodinated compounds by use of a chemically bonded C_{18} hydrophobic support as the stationary phase and water-organic solvent mixtures containing ion-pairing reagents as the mobile phase. Long retention times are noticed.[4,9]

The present paper provides a suitable simple, rapid and sensitive method for quantitative determination of thyroid hormones in extracts of biological fluids samples.

EXPERIMENTAL

Chemicals

Iodinated thyronine compounds, T_3 and T_4, were purchased from Aldrich (Aldrich-Chemie, GmbH, Steinheim, Germany). Acetic acid was “pro analysi” grade, from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Riedel-de-Haën (AG, Seelze, Germany). Bis deionised water was used throughout analysis.

HPLC Instrumentation

An SSI 222D pump (SSI, State College, PA, U.S.A.), was used to deliver the mobile phase to the analytical column, Inertsil ODS-3 150 x 4.0 mm, 5 μm purchased from MZ-Analysentechnik (Mainz, Germany).

Sample injection was performed via a Rheodyne 9125 injection valve (Rheodyne, Cotati California, U.S.A), with a 20 mL loop. Detection was achieved by SSI 500 UV-Vis detector working at a wavelength of 240 nm and a sensitivity setting of 0.002 AUFS.

A Hewlett-Packard (Avondale, PA, U.S.A.) HP 3396 Series II integrator was used for quantitative determination of eluted peaks.

A glass vacuum-filtration apparatus, obtained from Alltech Associates, was employed for the filtration of aqueous solutions, using 0.2μm membrane filters, obtained from Schleicher & Schuell (dassel, Germany). Degassing of solvents was achieved by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany) prior to use. A Glass-col, Terre Haute 47802 small vortexer and a Hermle centrifuge, model Z 230 (B. HermLe, Gosheim, Germany) were employed for the sample pre-treatment.

The SPE assay was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, a division of Varian (Harbor City, USA). All evaporations were performed, with a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

Chromatographic Conditions

Aqueous stock solutions of iodothyronines at a concentration of 100 ng/μL were prepared and stored refrigerated at 4 ^{0}C. These solutions were found to be stable throughout experimental analyses.

Working aqueous solutions were prepared from stocks at concentrations: 0.05, 0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 5.0, 8.0, 15.0, 20.0, and 30.0 ng/μL. Anthraquinone was used as internal standard at a concentration of 1.0 ng/μL.

The mobile phase consisted of CH_3OH-H_2O with 2% acetic acid, at a volume ratio 65:35, with a flow rate 1 mL/min. It was chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution. Inlet pressure observed with the eluent system, at a flow rate of 1 mL/min, was 3400 psi.

RESULTS AND DISCUSSION

Calibration Data and Analysis Time

T_3 and T_4 are eluted within 5 min; however, total analysis time in the proposed method is approximately 10 min as anthraquinone is eluted later, as illustrated in Figure 1. The chromatogram was obtained under the experimental conditions developed in the present assay. Calibration of the method was performed by injection of standards, covering the entire working range. The sensitivity setting of the UV-VIS detector was adjusted at 0.002 AUFS. Each sample was injected six times.

Linear correlation between absolute injected amount or concentration and peak area ratio, with anthraquinone as internal standard, at a concentration of

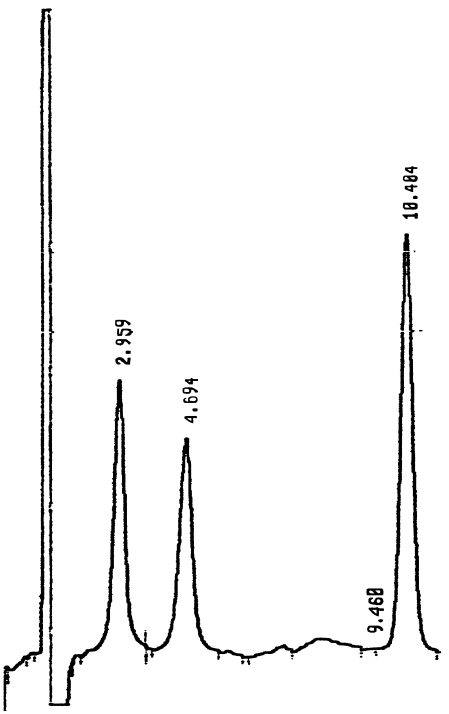


Figure 1. High performance liquid chromatogram of T_3 (2.959min) and T_4 (4.694 min) with anthraquinone (10.404 min.) as internal standard. Chromatographic conditions are described in the text.

Table 1

Calibration Data for T_3 and T_4 Determination with Anthraquinone as Internal Standard

Parameter	Value	T_3	T_4
Concentration range	ng/μL	0.15-28.0	0.2-40.0
Slope	AIU/ng	0.01518 ± 0.00031	0.01596 ± 0.00029
Intercept		-0.04541±0.04195	0,00559±0.04519
Correlation Coefficient		0.99918	0.99934
Limit of Detection	ng	1	2
Limit of Quantitation	ng	3	6
Upper limit	ng/μL	28	40

1.0 ng/μL was observed. The results of the statistical treatment of calibration data are summarised in Table 1.

Method Validation

Method validation, regarding within-day repeatability, was performed by eight replicate injections of standard solutions, at low, medium, and high concentration levels, where peak areas were measured with comparison to the peak area of the internal standard.

Statistical evaluation revealed relative standard deviations, at different values. Results are shown in Table 2.

A long-term stability study was conducted during routine operation of the system over a period of eight consecutive days. Day-to-day reproducibility results are illustrated in Table 2, using the mean values for five injections.

Solid Phase Extraction

Solid phase extraction protocol was selected among different solid phase cartridges. Diol cartridges presented higher recoveries, approximately 70%, in comparison to C_{18}, C_8, HLB cartridges, which provided only 40-50% recovery rates.

Table 2

Day-to-Day[a] and Within-Day Precision and Accuracy Study for T_3 and T_4 Determination

Day-to-Day

Added (ng)	T_3 Found ± SD (ng)	RSD (%)	T_4 Found ± SD (ng)	RSD (%)
20	19.98 ±1.29	6.46	21.11 ±1.50	7.10
40	39.81 ±1.69	4.24	39.71 ±1.40	3.53
80	79.63 ±4.40	5.52	78.78 ±2.54	3.22
100	98.50 ±4.31	4.38	100.10 ±4.24	4.24

Within-Day

Added (ng)	T_3 Found ± SD (ng)	RSD (%)	T_4 Found ± SD (ng)	RSD (%)
20	19.51 ±1.46	7.48	20.12 ±1.28	6.36
40	41.16 ±0,85	2.06	38.94 ±2.23	5.73
80	82.96 ±4.25	5.12	80.08 ±1.85	2.31
100	97.7 ±3.50	3.58	101.60 ±7.03	6.92

[a] Over a period of 8 consecutive days; n = 8.

Extraction protocol was optimized in terms of conditioning, washing and elution steps and optimum protocol was then applied to standard solutions of iodothyronines, as follows.

200 µL of standard solutions were applied to the diol SPE cartridges, which were conditioned by flushing with 3 mL MeOH and 3 mL H_2O prior to the addition of sample. After applying the sample, iodo-thyronines were eluted using 3 mL MeOH. After evaporation to dryness, under gentle nitrogen stream, in a 45 ^{0}C water bath, they were diluted to 200 µL internal standard anthraquinone solution. Aliquots of 20 µL volume were injected into the analytical column. Extraction efficiency was calculated by extracting standard solutions of iodothyronines, at five different levels, i.e., 0.5, 1.0, 2.0, 3.0, and 5.0 ng/µL. Regression equations obtained are as follows:

Y= (0.05150 ± 0.01146) + (0.02695 ± 0.00043) X, where X= ng of T_3, Correlation Coefficient R= 0.99987

Y= (0.55898 ± 0.01630) + (0.00657 ± 0.00026) X, where X= ng of T_4, Correlation Coefficient R= 0.99842

The reproducibility and accuracy of solid phase extraction of iodothyronines were investigated. Recovery results are shown in Table 3.

Application to Biological Fluids: Blood Serum-Urine

Human Blood Serum

Aliquots of 40 μL human blood serum (pooled sample) were treated with 50 μL of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was spiked with 200 μL of iodothyronine solutions, at concentration levels of 0.5, 1.0, 2.0, and 3.0 ng/μL.

Then, the sample was centrifuged at 3500 rpm for 15 min and the supernatant was evaporated, at 45°C, under nitrogen stream, to remove organic solvents. Subsequently, the sample was slowly applied to the solid-phase cartridge.

Table 3

Recovery of Iodothyronines from Standard Solutions After SPE on Diol Cartridges Using Internal Standard

Analyte	Added (ng)	Found (ng) ± SD	Recovery (%)
T_3	10	9.81 ± 0.65	98.14
	20	20.28 ± 1.13	101.40
	40	39.91 ± 3.12	99.78
	60	58.58 ± 2.55	97.63
	100	100.22 ± 4.61	100.22
T_4	10	9.56 ± 0.77	95.60
	20	19.23 ± 1.49	96.15
	40	39.12 ± 0.67	97.8
	60	62.86 ± 1.32	104.77
	100	98.78 ± 5.45	98.78

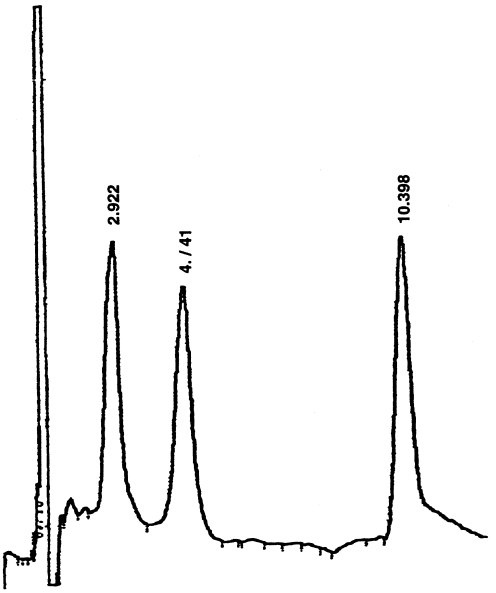


Figure 2. High performance liquid chromatogram of analysis of T_3 (2.922 min) and T_4 (4.741 min) in spiked human blood serum samples with anthraquinone (10.390 min) as internal standard. Chromatographic conditions are described in the text.

Then, the sample was treated according to the procedure described under solid-phase extraction paragraph. A high performance liquid chromatogram of iodothyronines, extracted from human blood serum, is shown in Figure 2. No interference from endogenous compounds from the sample matrix was noticed.

Regression equations obtained for blood serum samples, after SPE, are as follows:

$Y= (0.12510 \pm 0.02422) + (0.01032 \pm 0.00064)\ X$, where X= ng of T_3, Correlation Coefficient R= 0.99615

$Y= (-0.10416 \pm 0.00422) + (0.02188 \pm 0.00010)\ X$, where X= ng of T_4, Correlation Coefficient R= 0.99999

Urine

100 μL of pooled urine sample were extracted according to the procedure described under the SPE paragraph. Matrix interference was removed by wash-

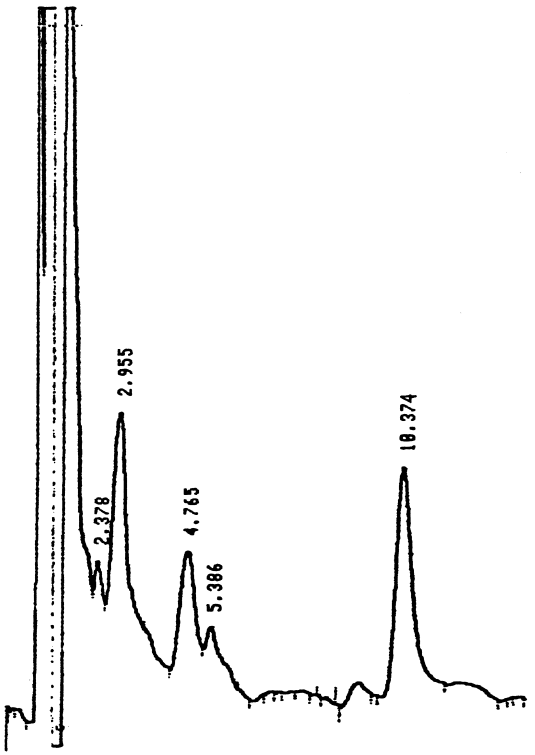


Figure 3. High performance liquid chromatogram of analysis of T_3 (2.955 min) and T_4 (4.765 min) in urine samples with anthraquinone (10.374 min) as internal standard. Chromatographic conditions are described in the text.

ing the diol cartridges with 3 mL H_2O. No interference from endogenous compounds from the sample matrix was observed, as shown in the urine sample chromatogram, in Figure 3. Extraction efficiency was calculated by extracting standard solutions of iodothyronines, at five different levels, i.e., 0.5, 1.0, 2.0, and 3.0 ng/μL.

The following regression equations were obtained for urine samples, after SPE:

$Y= (0.27025 \pm 0.00442) + (0.00678 \pm 0.00017) X$, where X= ng of T_3, Correlation Coefficient R=0.99970

Table 4

Recovery of T_3 and T_4 from Human Blood Serum and Urine After SPE

Added (ng)	T_3 Found ± SD (ng)	Recovery (%)	T_4 Found ± SD (ng)	Recovery (%)
		Blood Serum		
10	7.99 ± 0.32	79.90	9.95 ± 0.65	99.50
20	20.63 ± 1.12	103.15	21.23 ± 1.44	106.15
40	39.69 ± 1.19	99.22	40.13 ± 2.92	100.32
60	59.65 ± 2.26	99.42	59.92 ± 3.14	99.87
		Urine		
10	10.28 ± 0.16	102.80	10.45 ± 0.34	104.50
20	19.57 ± 1.46	97.85	16.73 ± 1.66	83.65
40	40.14 ± 2.23	100.35	38.13 ± 2.48	95.32
60	59.20 ± 3.11	98.67	58.90 ± 5.15	98.17

$Y = (0.20012 \pm 0.02258) + (0.00690 \pm 0.00038)\ X$, where X= ng of T_4, Correlation Coefficient R=0.99697

The precision and accuracy studies of SPE of iodothyronines from biological samples were conducted by spiking blood serum and urine samples with four known concentrations of the compounds and then comparing obtained results with those as calculated from the regression equations. Results of recovery studies for serum and for urine samples are given in Table 4. Each value represents the mean of six measurements carried out.

CONCLUSIONS

A simple, rapid, accurate and sensitive method for the simultaneous determination of tri- and tetra-iodinated thyronines has been developed in the present method. This method has been tested on samples of blood serum and urine; however, it can be applied to samples of diverse biological origins with the appropriate modifications in the sample pretreatment process. The method is useful in clinical assays, routine pharmacological analysis, and in pathways where the thyroid hormones play important roles.

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