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Development of a validated HPLC method for the determination of iodotyrosines and iodothyronines in pharmaceuticals and biological samples using solid phase extraction

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Abstract

Identification, separation and quantitation of iodoaminoacids, is essential for the biological research and the clinical diagnosis of thyroid gland disease. Under this aspect a reversed-phase high-performance liquid chromatographic method was developed for the determination of thyroid gland hormones and some of their primary metabolites, 3,3′,5,5′-tetra-iodo-L-thyronine (L-thyroxine), 3,3′,5-tri-iodo-L-thyronine, 3,5-di-iodo-L-thyronine, L-thyronine, 3,5-di-iodo-L-tyrosine, 3-iodo-L-tyrosine and L-tyrosine. Analysis was performed on an Inertsil C_{18} column with photodiode-array detection, using a 25 min gradient scale program of a binary mobile phase consisted of 0.1% aqueous solution of trifluoroacetic acid at pH 3 as solvent A and acetonitrile as solvent B, at a flow rate of 1 mL/min. Quantitation was performed using were obtained using theophylline as internal standard. The method was applied to commercial pharmaceuticals and biological samples (serum, urine and tissue). Drug-free urine and serum samples were spiked with known concentrations of the analytes standards and pretreated by solid phase extraction to remove matrix interferences. C_{18} cartridges were used, yielding recoveries ranging from 87.1% to 107.6% for serum samples and from 92.1% to 98.7% for urine samples. With regard to total-T_4 concentrations in serum samples, results are cross-validated with RIA and found to agree well.

Keywords: Thyroid gland hormones; Iodotyrosines; Iodothyronines; Pharmaceuticals; HPLC; SPE biological samples; Serum; Urine; Tissue

1. Introduction

The thyroid gland and its hormonal products play an indispensable role affecting a variety of biochemical reactions. The mammalian thyroid gland biosynthesizes, stores and secrets the two so-called thyroid hormones; 3,3′,5,5′-tetra-iodo-L-thyronine (L-thyroxine-T_4) and 3,3′,5-tri-iodo-L-thyronine (T_3) with, four and three atoms of organically bound iodine, respectively. T_3 is of some five to eight times higher activity than T_4, whereas other analogs as reversed tri-iodothyronine (rT_3), di-iodothyronine (T_2) and the parent compound of the iodinated series of thyroid-active hormones, thyronine (T_0), have a low or undetectable thyromimetic action. Though T_2 and T_0 along with the iodinated analogues of tyrosine (tyr), diiodotyrosine (DIT), and monoiodotyrosine (MIT) have no thyroxine-like activity yet are of great significance too, since they are involved in the mechanism of T_4 formation and are products of its metabolism [1–4].

The biological and pharmacological importance of this class of compounds has been of great interest to many researches during the past years. A number of works is reported, focused on the elucidation of the actual biological activity of the thyroid gland hormones and on the study of synergistic or competitive interrelation with other endogenous compounds. Moreover, T_4 is the most often prescribed drug for hypothy-

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roidism replacement therapy and side effects, drug interaction, impurities, and effectiveness of the drug are always of the question [5–13].

High performance liquid chromatography (HPLC) has proved to be a suitable technique for the iodothyronines and iodotyrosines analogues separation and analysis and during the last decade several works have been reported concerning their determination in tissue and biological fluids, in thyroglobuline hydrolysates and pharmaceutical preparations. More specifically, quantitation of iodothyronine in body tissues and fluids of animals using HPLC and fluororimetric detection was achieved by gradient elution on a C_{18} column with an acetonitrile–water mixture and phosphate buffer. Derivatisation of the compounds by 5-dimethylaminonaphthalene-1-sulfonyl chloride is involved [14,15].

The assay of L-thyroxine in serum samples by electrochemical detection has been also described, where L-thyroxine was determined as *o*-phthalaldehyde-*n*-acetylcysteine derivative after elution with methanol–phosphoric acid–water (71:0.1:28.9, v/v) on a glass cartridge column C_{18} [16]. Thyroxine and trioiodothyronine were determined in serum and urine samples, after clean-up with SPE. HPLC analysis was performed on a C_{18} column with methanol–2% acetic acid (65:35, v/v) yielded detection limits of 1 and 2 ng per 20 μL injection [17].

Other reported methods refer to the determination of different iodo-thyronine and tyrosine analogues after enzymatic digest of thyroglobuline [18–20].

Concerning the pharmaceutical formulation analysis, sodium levothyroxine has been separated from excipients and determined in bulk drugs, tablets and injections by HPLC on a cyanoalkyl column using an acetonitrile–water–phosphoric acid mobile phase [21].

Liothyronine and levothyroxine were quantitatively determined by HPLC in samples of commercial thyroid tablets and bulk powders, in hydrolyzed samples using a bacterial protease, on a C_{18} column with a mixture of 28% acetonitrile and 72% of a 1:200 mixture of phosphoric acid in water, as mobile phase. Limits of 8.1–9.9 μg of liothyronine (T_3) and 32.3–43.7 μg levothyroxine (T_4) per 65 mg were achieved [22].

Identification and quantification of sodium-thyroxine and its degradation products of the thermally stressed amino acid comprising diiodothyronine, diiodotyrosine, iodotyrosine and tyrosine by HPLC, using electrochemical and MS detection was applied to elucidate the light and high temperature sensitivity of Na-T_4 [23]. Chromatography was performed on an RP-select B column with 0.5% formic acid in 40% acetonitrile (pH 3.1) as mobile phase and detection limits of 0.1 ng μL^{-1} were achieved. HPLC with fluorescence has been also used for the determination of liothyronine and levothyroxine sodium in pharmaceuticals preparations after fluorogenic derivatization [24]. The 9-anthroylnitrile derivatives were separated on a C_{18} column with acetonitrile–0.02 M sodium dodecylsulfate as eluent. The detection limits were 0.2 ng per injection.

Yet, a simple, direct without derivatization and reliable method would be of great interest attributing to the wide biological and pharmacological research field of these compounds.

Herein a direct, convenient and sensitive approach of an HPLC method is proposed for the simultaneous determination of thyroid gland hormones and their major precursors to be applied in pharmaceutical formulations, serum, urine and thyroid tissue extracts.

2. Experimental

2.1. Chemical and materials

3,3′,5,5′-Tetra-iodo-L-thyronine L-thyroxine (L-T_4), 3,3′,5-triiodo-L-thyronine (L-T_3), 3,5-diiodo-L-thyronine (L-T_2), L-thyronine (L-T_0), 3,5-diiodo-L-tyrosine (L-DIT),3-iodo-L-tyrosine (L-MIT), L-tyrosine (L-Tyr) and theophylline reagents were obtained from Sigma–Aldrich (Steinheim, Germany). Their chemical structure is presented in Fig. 1. All other reagents and solvents used were of analytical grade. Solvents for HPLC were supplied from Merck (Darmstadt, Germany) and were of HPLC grade.

Representative pharmaceuticals used, Thyro-4, 0.1 mg (Faran, Greece), Dithyron, 50 μg (Uni-pharma, Greece), T4-100, T4-150, and T4-50 μg (Uni-Pharma, Greece) and Thyrohormone 0.2 mg (Ni-The, Greece) tablets are all commercially available.

Solid phase extraction cartridges used were Oasis HLB, supplied by Waters (Waters Corporation, Massachusetts, USA), Bond Elut C_8, C_{18}, PH, 2OH and Abselut NEXUS, by Varian (Harbor City, USA), Lichrolut RP-18 and Adsor-

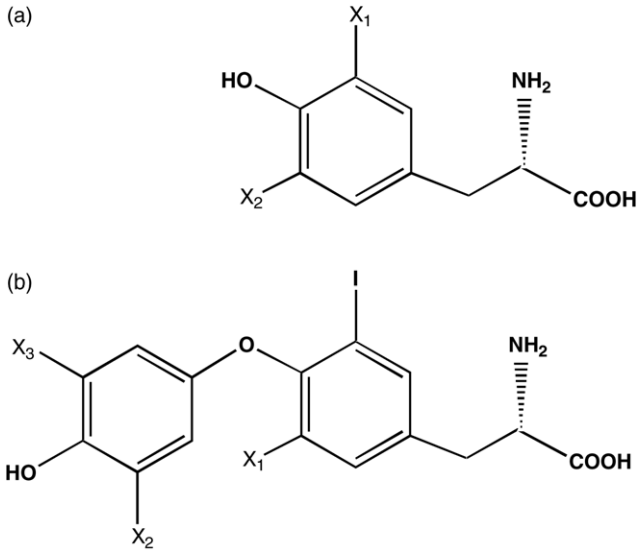

Fig. 1. Structures of (a) iodotyrosines (diiodotyrosine, DIT: $X_1 = I$, $X_2 = I$; monoiodotyrosine, MIT: $X_1 = I$, $X_2 = H$ and tyrosine, Tyr: $R_1 = H$, $R_2 = H$) and (b) iodothyronines (thyroxine, T_4: $X_1 = I$, $X_2 = I$, $X_3 = I$; triiodothyronine, T_3: $X_1 = I$, $X_2 = H$, $X_3 = I$; reversed-triiodothyronine, rT_3: $X_1 = H$, $X_2 = I$, $X_3 = I$ and diiodothyronine, T_2: $X_1 = I$, $X_2 = H$, $X_3 = H$).

bex RP-8 by Merck, C_8 by Alltech (Deerfield, IL, USA), and DSC-18 and Supelclean LC-18 by Supelco (Bellefonte, USA).

2.2. Instrumentation

All HPLC experiments were carried out on a Schimadzu (Kyoto, Japan) system consisted of an LC-9A pump, an SPD-M6A photodiode array detector, an SIL-9A automatic sample injector and a CTO-6A column oven module. Operation, data acquisition and analysis were performed using Class-M10A LC workstation software.

An Inertsil ODS (250 mm × 4 mm i.d., 5 μm particle diameter) analytical column purchased from MZ Analytical (Mainz, Germany) was used for chromatographic separation.

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Schleicher and Schuell (Dassel, Germany).

SPE assays were performed on a Vac-Elut vacuum manifold station purchased from Analytichem International (Varian, Harbor City, USA) with the aid of a 9-port Reacti-Vap evaporator (Pierce, Rockford, IL, USA).

A Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany), a Glass-col, Terre Haute 47802 small vortexer and an ultrasonic bath Transsonic 460/H (ELMA) were as well used in sample preparation procedure.

2.3. Chromatographic method development and validation

Optimization of the chromatographic system was performed in terms of elution program as well as constituents of mobile phase. Both isocratic and gradient elution was tested on the Inertsil C_{18}, 25 μm (250 mm × 4 mm) analytical column. Optimal separation of the seven analytes was achieved employing a multi-step 25 min gradient scale elution program.

The final chromatographic system was chosen after a thorough investigation of several mobile phases. Acetic acid was also tested as acidic modifier, but TFA was superior and acetonitrile was preferred to methanol as organic modifier.

The optimal mobile phase consisted of a mixture of solvent A: 0.1% aqueous solution of trifluoroacetic acid (TFA) and solvent B: acetonitrile, starting from B–A, 92:8 (v/v). The flow rate of the mobile phase was adjusted to 0.9 mL min^{-1} and the eluant was monitored at 240 nm detection wavelength under ambient temperature (25 ± 2 °C). Quantitation of analytes was performed at the wavelength of maximum absorbance for each analyte (λ_{max} for Tyr, T_2, T_3 and T_4 = 228 nm, λ_{max} for MIT = 215 nm, λ_{max} for DIT = 223 nm and λ_{max} for T_0 = 232 nm). An equilibration time of 10 min preceded each run.

Method validation was performed in terms of precision and accuracy. The precision of the method was investigated with repeatability and intermediate precision assays. Repeatability was evaluated at three concentration levels (4.0, 8.0 and 15.0 ng μL^{-1}), by seven replicates over the linear range of the method within a day. Intermediate precision at three concentration levels by three replicates was studied for a time interval of seven days. Accuracy was estimated in intra- and inter-assays based on the standard solutions of the analytes in three concentration levels.

Method was also validated by three replicate injections in a period of four days, using blood serum spiked at four concentration levels (1.0, 3.0, 5.0 and 8.0 ng μL^{-1}).

2.4. Preparation of standards

Stock solutions of the compounds were prepared at a concentration of 100 ng μL^{-1} in a mixture of MeOH and 0.01 M NaOH (1:1, v/v). Working standards solutions of their mixtures were prepared from the stock solutions at a concentration range between 1 and 20 ng μL^{-1}. These were stored protected from light at 4 °C. Under these storage conditions were proved to be stable.

Quantitative analysis was based on the internal standard method. Calibration curves were established over the range of 0.1–10 ng μL^{-1} based on the peak area ratio using an aqueous solution of theophylline as internal standard at a concentration of 10 ng μL^{-1}. Triplicate injections of a 20 μL volume of each solution were performed at seven calibration points (0.1, 0.5, 1.0, 3.0, 5.0, 8.0 and 10.0 ng μL^{-1}).

2.5. Sample preparation

2.5.1. Pharmaceuticals

Ten tablets of each drug formulation were pulverized and an accurately weighted mass of powdered tablets corresponding to their mean weight was suspended in 100 mL of a mixture consisted of MeOH and 0.01 M NaOH (1:1, v/v). The suspension was sonicated at room temperature for 10 min and undissolved particles were removed by filtration. The filtrate with T_4 sodium salt (levothyroxine sodium) concentration corresponding to 1 μg mL^{-1} for Thyro-4, 0.5 μg mL^{-1} for dithyron, 1.5 μg mL^{-1} for T4 and 2 μg mL^{-1} for thyrohormone formulation, was then injected to column.

2.5.2. Biological samples

Sample clean up in the case of blood serum or urine was performed by a solid phase extraction (SPE).

The final extraction protocol was chosen after SPE optimization in terms of sorbent used, as well as washing and elution solvents.

Human blood serum samples were collected from healthy volunteers and stored at −20 °C, whereas an aliquot of a 24 h urine collection, kept in 4 °C was used for analysis.

Supelclean LC-18, 500 mg cartridges were conditioned with 2 mL MeOH followed by 2 mL water. Serum (50 μL) or urine samples (200 μL), were spiked with 200 μL of analytes standard solutions and then 100 μL of acetonitrile were added. The sample mixture was vortexed and centrifuged

for 10 min at 3500 rpm. The supernatant was removed and residue was washed twice with 30 μL portions of acetonitrile. Extracts were combined and added to the SPE cartridge. The unwanted retained compounds were washed off with 100 μL of water and then the extraction was carried out by two sequential elution steps with 1 mL of methanol and 1.5 mL of acetonitrile–0.1 M HCl, 4:1.8 (v/v) mixture. The eluent was evaporated to dryness under a gentle nitrogen stream at 40 °C and finally the residue was re-dissolved in 200 μL of internal standard solution. Aliquots of 20 μL were used for the chromatographic analysis.

Thyroid tissue specimens, derived from two patients of different cases and obtained by incisional biopsy from a pathologoanatomical laboratory, were kept desiccated in paraffin. Ten to twenty milligrams of dry tissue were pulverized and suspended in 2.5 mL of MeCN–0.1 M NaOH 2:1 (v:v) mixture and submitted to ultrasonication at 40 °C for 20 min. The solid precipitate was removed by centrifugation and the supernatant was evaporated to dryness at 40 °C under nitrogen. The dry residue was reconstituted to 200 μL internal standard solution and a 20 μL aliquot was injected.

3. Results and discussion

3.1. Method development and chromatography

Method development consisted of the control of a number of parameters, related to the efficiency of the chromatographic system. Variant reverse phase chromatographic supports, and a series of aqueous mobile phases containing buffer solutions at different pH values in combination with

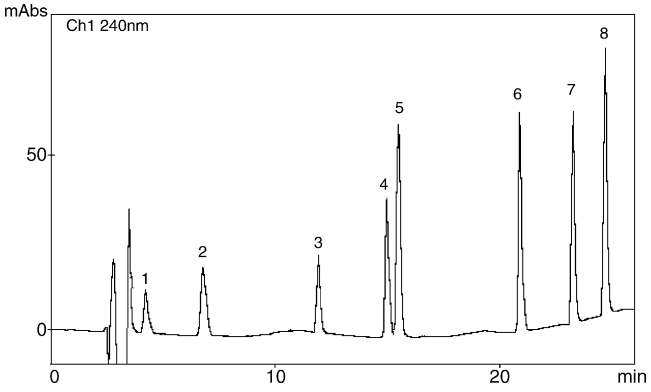

Fig. 2. Typical chromatogram of the iodothyronines and iodotyrosines separation using the chromatographic conditions described under Section 2.3. All analytes are at 10 ng μL^{-1}. Peaks: (1) Tyr (4.2 min), (2) theophylline (IS, 6.8 min), (3) MIT (11.9 min), (4) DIT (14.9 min), (5) T_0 (15.4 min), (6) T_2 (20.9 min), (7) T_3 (23.2 min) and (8) T_4 (24.7 min).

different organic modifiers as well as elution step programs were tested to adjust the optimum conditions. It was shown that a low-pH mobile phase, acetonitrile in combination with trifluoroacetic acetic acid produced the more efficient results regarding retention times, peak asymmetry (A_s), plate number (N), selectivity (α) and resolution (R_s). In Fig. 2 a chromatographic separation is illustrated for the seven analytes. The compounds are not strictly eluted in order of increasing hydrophobicity (log P values are: Tyr-2.28, thyronine 0.32, diiodothyronine 0.34, monoiodotyrosine 0.35, T31.29, diiodotyrosine 1.79, T41.65). It should also be considered that since at this pH value the analytes exist in their zwitterionic form with their amino group protonated and the carboxylic group ionized, ionic interactions with the

Table 1
Linearity and sensitivity data of iodotyrosines and iodothyronines determination in standards and biological fluids

	Analyte	Calibration range (ng μL^{-1})	Slope	Intercept (ng μL^{-1})	R^2	LOD (ng μL^{-1})	LOQ (ng μL^{-1})
Standardsolutions	T_4	0.1–10	0.154 ± 0.005	0.083 ± 0.028	0.998	0.02	0.1
	T_3	0.1–10	0.121 ± 0.004	0.081 ± 0.023	0.998	0.02	0.1
	T_2	0.1–10	0.128 ± 0.005	0.091 ± 0.029	0.997	0.02	0.1
	T_0	0.1–10	0.152 ± 0.008	0.181 ± 0.061	0.996	0.02	0.1
	DIT	0.5–10	0.079 ± 0.003	0.087 ± 0.021	0.997	0.05	0.5
	MIT	0.5–10	0.046 ± 0.002	0.044 ± 0.013	0.997	0.05	0.5
	Tyr	0.5–10	0.076 ± 0.007	0.289 ± 0.051	0.996	0.1	0.5
Serum	T_4	0.1–10	0.007 ± 0.003	0.006 ± 0.004	0.997	0.02	0.1
	T_3	0.1–10	0.009 ± 0.004	0.065 ± 0.012	0.999	0.02	0.1
	T_2	0.1–10	0.009 ± 0.002	0.116 ± 0.033	0.999	0.02	0.1
	T_0	0.1–10	0.014 ± 0.002	0.229 ± 0.050	0.998	0.02	0.1
	DIT	0.5–10	0.007 ± 0.004	0.088 ± 0.007	0.997	0.05	0.5
	MIT	0.5–10	0.004 ± 0.006	0.056 ± 0.014	0.998	0.05	0.5
	Tyr	0.5–10	0.003 ± 0.005	0.004 ± 0.045	0.996	0.1	0.5
Urine	T_4	0.1–10	0.016 ± 0.003	0.009 ± 0.004	0.997	0.02	0.1
	T_3	0.1–10	0.012 ± 0.005	0.011 ± 0.012	0.998	0.02	0.1
	T_2	0.1–10	0.008 ± 0.004	0.015 ± 0.033	0.997	0.02	0.1
	T_0	0.1–10	0.014 ± 0.002	0.024 ± 0.050	0.997	0.02	0.1
	DIT	0.5–10	0.009 ± 0.005	0.017 ± 0.007	0.998	0.05	0.5
	MIT	0.5–10	0.007 ± 0.006	0.025 ± 0.014	0.998	0.05	0.5
	Tyr	0.5–10	0.004 ± 0.005	0.008 ± 0.045	0.996	0.1	0.5

Table 2
Precision and accuracy assay data of iodotyrosines and iodothyronines determination

Analyte	Added amount							
	Day-to-day precision and accuracy study ($n = 7$)				Within-day precision and accuracy study ($n = 7$)			
	80 ng		160 ng		80 ng		160 ng	
	Found ± S.D. (ng)	RE (%)	Found ± S.D. (ng)	RE (%)	Found ± S.D. (ng)	RE (%)	Found ± S.D. (ng)	RE (%)
T_4	80.96 ± 6.76	1.20	171.34 ± 18.34	7.09	79.58 ± 1.23	−0.52	154.58 ± 4.83	−3.39
T_3	83.86 ± 6.40	4.82	171.91 ± 15.28	7.44	79.73 ± 1.56	−0.34	158.73 ± 5.23	−0.79
T_2	82.44 ± 4.23	3.05	171.24 ± 13.80	7.02	70.12 ± 1.03	−12.35	158.32 ± 5.28	−1.05
T_0	80.59 ± 6.81	0.74	178.11 ± 16.22	11.32	76.38 ± 1.52	−4.52	177.45 ± 5.65	10.91
DIT	79.36 ± 5.54	−0.80	169.54 ± 11.13	5.96	74.99 ± 1.60	−6.26	158.29 ± 5.41	−1.07
MIT	82.44 ± 6.93	3.05	176.14 ± 11.20	10.09	79.08 ± 2.01	−1.15	163.58 ± 6.39	2.24
Tyr	90.05 ± 4.52	13.12	145.59 ± 17.05	−9.01	87.14 ± 3.52	8.92	145.55 ± 5.21	−9.03

residual silanol groups of the silica surface are in the scenery too.

3.2. Method validation

Validation of the method comprising linearity, precision, and accuracy assessments was established according to the following:

Linearity was evaluated by regression analysis at seven concentration points. Calibration curves were established over the range of 0.1 −10 ng μL^{-1} for T_0, T_2, T_3 and T_4 and 0.5–10 for DIT, MIT and Tyr. Correlation coefficient of greater than 0.996 for each compound indicates that the system is linear over this range. The limit of detection considering a signal-to-noise ratio 3:1 was estimated to be 0.02–0.1 ng μL^{-1} whereas the limit of quantitation was determined to be 0.1–0.5 ng μL^{-1}. The analytical characteristics of the method are summarized in Table 1.

The precision of the method was investigated with repeatability and intermediate precision assays. Repeatability was evaluated at three concentration levels, by seven replicates over the linear range of the method within a day. Intermediate precision at three concentration levels by three replicates was studied for a time interval of seven days. The relative standard deviation values obtained for all analytes ranged between 1.3 and 8 (Table 2). Accuracy was estimated in intra- and inter-assays based on the standard solutions of the analytes in three concentration levels. Values expressed as relative error of the measurement were in the range of ±13%. Results from two concentration levels are summarised in Table 2.

Precision was also studied using serum samples spiked at four concentration levels. Samples were extracted and triplicate measurements were conducted in a period of 4 days. Results from three concentrations are presented in Table 3. R.S.D. values were lower than 15%, while recovery ranged from 75% to 126%.

3.3. Pharmaceutical formulation analysis

Table 4 summarizes the results from the application of the method for the quantitative analysis of T_4 and T_3 in four pharmaceutical formulations. Recovery experiments in commercial tablets gave satisfactory recovery values between 88% and 99%.

3.4. Solid phase extraction

For the solid phase optimization step a number of parameters were studied. Various reversed-phase SPE materials, including silica-based and polymeric sorbents with different surface coverage, carbon load and endcapping, were tested for an efficient clean up of the sample and a quantitative recovery of the analytes. Among the several sorbents that were studied, i.e. Oasis, DSC-18, Lichrolut C_{18}, Varian C_{18}, Adsorbex C_8, Varian C_8, Varian PH, Varian 2OH and Supelclean

Table 3
Day-to-day accuracy and precision study of iodotyrosines and iodothyronines determination in blood serum samples

Analyte	Added (ng)	Found ± S.D.[a] (ng)	*R* (%)
Tyr	20	25.2 ± 3.5	126.2
	60	75.8 ± 2.0	126.2
	100	104.8 ± 5.3	104.0
MIT	20	20.2 ± 2	100.8
	60	45.2 ± 4.5	75.3
	100	93 ± 4.3	93.0
DIT	20	19.5 ± 2.1	97.6
	60	58.0 ± 4.6	96.7
	100	81.2 ± 7.1	81.2
T_0	20	23.1 ± 1.2	115.7
	60	56.8 ± 2.8	94.6
	100	79.7 ± 8	79.7
T_2	20	20.8 ± 2.7	104.0
	60	60.5 ± 1.3	100.8
	100	89.6 ± 3.7	89.6
T_3	20	21.5 ± 1.3	107.7
	60	55.3 ± 2.1	92.1
	100	117.6 ± 4.2	117.6
T_4	20	16.7 ± 2.5	83.5
	60	53.0 ± 3.3	88.3
	100	96.8 ± 4.6	96.8

[a] Mean values of three measurements in a period of 4 days.

Table 4
Results obtained from commercial pharmaceutical formulations analysis

Pharmaceutical samples	Declared amount (mg)		Measured mg ± S.D[a]		R.S.D.		Recovery (%)	
	T_3	T_4	T_3	T_4	T_3	T_4	T_3	T_4
Thyro-4	–	0.097	–	0.096 ± 0.52	–	8.5	–	98.9
Dithyron	0.012	0.048	0.011 ± 0.98	0.047 ± 0.91	5.1	4.4	91.6	97.9
T_4	–	0.145	0.021 ± 0.67	0.13 ± 0.73	9.2	5.8	–	89.6
Thyrohormone	–	0.194	0.008 ± 0.74	0.17 ± 0.55	6.7	8.3	–	87.6

[a] Mean of nine measurements.

LC-18, the latter as well as Varian C_8 provided the higher recovery rates as shown in Table 5.

The extraction steps were properly optimized by testing variant sample volumes and changing the nature and the volume of the eluent as well as the composition and the volume of the conditioning and wash solvent. Thus, conditioning step was tested with different organic solvents such as methanol, isopropanol and acetonitrile for "solvation" and aqueous buffers with variant pH for equilibrium of the sorbent with different volumes. However, best results were given

Table 5
Percentage recovery of iodothyronines and iodotyrosines using different sorbents and 2 mL of elution solvents

Compound	Oasis	DSC-18	Lichrolut C_{18}	Varian C_{18}	Adsorbex C_8	Varian C_8	Varian PH	Varian 2OH	Supelclean LC-18
Elution solvent: methanol									
Tyr	73.3	74.1	35.8	92.3	19.2	100.4	63.7	59.3	87.9
MIT	75.6	75.6	62.1	28.8	34.5	90.6	63.5	69.2	85.5
DIT	73.3	76.2	60.4	33.9	23.5	83.8	64.8	67.0	84.1
T_0	70.6	75.1	56.8	62.4	46.9	81.0	55.3	65.7	87.7
T_2	82.7	84.3	64.4	69.2	51.7	94.2	50.2	69.9	91.3
T_3	90.4	93.0	72.7	70.7	50.7	100.5	47.1	74.6	89.8
T_4	74.4	75.5	65.9	58.6	40.7	86.8	39.5	61.0	87.8
Elution solvent: methanol, 0.1% HCl									
Tyr	26.9	60.7	47.0	30.2	35.2	33.8	22.9	33.6	43.7
MIT	29.8	73.2	65.2	44.7	39.8	64.8	34.4	35.5	38.5
DIT	30.4	36.9	35.8	30.7	42.6	34.8	20.9	38.2	44.2
T_0	71.9	45.9	76.6	74.8	55.7	71.9	42.4	27.9	45.0
T_2	80.2	81.2	53.6	74.5	63.8	66.8	59.3	45.7	39.9
T_3	82.5	73.5	44.7	68.6	75.6	68.1	66.5	48.1	47.1
T_4	60.8	59.3	30.2	53.6	50.2	47.7	51.9	46.5	46.2
Elution solvent: acetonitrile									
Tyr	47.3	39.6	26.4	22.2	30.4	28.8	28.3	32.5	45.6
MIT	44.8	46.4	54.3	33.8	41.8	53.1	25.6	34.6	55.7
DIT	62.1	34.5	50.2	30.2	38.1	50.3	31.7	29.7	56.0
T_0	37.8	20.1	52.2	27.4	31.0	30.6	25.5	33.4	52.5
T_2	28.7	14.4	45.2	18.2	15.3	24.1	18.2	19.9	49.8
T_3	6.6	4.0	27.2	7.9	9.0	13.9	13.6	17.5	43.7
T_4	8.8	4.2	20.1	4.2	5.1	7.0	9.0	11.4	40.3
Elution solvent: 2-propanol									
Tyr	43.5	22.2	24.6	21.5	17.5	22.7	36.4	43.5	22.2
MIT	47.6	19.1	37.1	44.2	39.4	18.6	39.2	47.6	19.1
DIT	49.9	12.4	32.3	41.4	21.4	23.8	38.5	49.9	12.4
T_0	45.8	11.1	54.1	41.5	66.1	32.2	42.1	45.8	11.1
T_2	46.7	12.2	46.9	35.7	50.2	41.0	45.7	46.7	12.2
T_3	46.0	11.9	43.2	38.7	57.8	38.6	47.1	46.0	11.9
T_4	46.6	6.6	35.3	25.5	51.8	43.2	47.5	46.6	6.6
Elution solvent: tetrahydrofuran									
Tyr	40.9	16.9	15.8	14.9	21.2	30.9	32.7	40.9	16.9
MIT	42.8	31.4	17.1	14.6	15.8	55.9	38.4	42.8	31.4
DIT	45.9	38.4	15.2	11.2	18.5	53.9	35.4	45.9	38.4
T_0	37.8	42.9	12.6	21.5	16.9	49.3	28.6	37.8	42.9
T_2	45.4	20.8	16.2	27.2	22.4	56.3	31.0	45.4	20.8
T_3	45.2	23.6	15.4	23.4	19.5	58.6	33.4	45.2	23.6
T_4	39.5	14.4	7.0	16.7	24.6	41.8	29.2	39.5	14.4

Conditioning preceded by 2 mL of methanol and 2 mL of water.

Table 6
Percentage recovery results from serum and urine samples using different wash (W) and elution (E) solvents using Supelclean LC-18 cartridges, conditioned with 2 mL of MeOH, 2 mL of H_2O

Wash and elution solvents	Serum								Urine			
	Tyr	MIT	DIT	T_0	T_2	T_3	T_4	Tyr	MIT	DIT	T_0	T_2	T_3	T_4
W: 0.5 mL H_2O, E: 2 mL CH_3OH	32.2	36.2	36.4	34.1	24.4	25.2	20.4	34.5	33.7	39.8	41.4	35.2	32.6	33.3
W: 0.5 mL H_2O, E: 2 mL CH_3CN	32.1	28.5	34.3	34.9	40.3	36.2	31.1	37.4	35.6	34.8	45.6	37.4	39.5	40.4
W: 0.1 mL H_2O, E: 2 mL CH_3OH	67.8	61.4	66.4	68.3	56.2	59.1	55.7	66.1	68.3	70.2	65.7	62.4	59.8	62.1
W: 0.1 mL H_2O, E: 2 mL CH_3CN	54.3	51.4	53.6	50.7	51.6	54.0	53.3	56.4	55.0	60.7	58.9	52.6	52.1	57.3
W: 0.1 mL H_2O, E: 2 mL H_3OH, 0.1% HCl	65.5	71.4	69.7	73.2	66.4	68.1	67.7	69.5	50.4	55.7	56.7	45.0	47.4	46.3
W: 0.1 mL H_2O, E: 2 mL CH_3OH, 0.1% CH_3COOH	75.9	48.3	45.1	50.3	46.4	50.1	38.2	67.6	49.5	47.3	44.6	42.3	40.5	40.7
W: 0.1 mL H_2O, E: (a) 1 mL CH_3OH, (b) 1 mL CH_3CN, 0.1% HCl	71.9	51.7	49.6	57.5	68.1	73.9	53.4	74.3	68.6	69.7	70.5	72.4	75.5	73.7
W: 0.1 mL H_2O, E: (a) 1 mL CH_3OH, (b) 1.5 mL CH_3CN–0.1 M HCl (1:1, v/v)	96.0	73.5	70.8	78.5	73.0	72.9	70.4	89.9	80.3	85.2	90.0	87.6	82.3	81.6
W: 0.1 mL H_2O, E: (a) 1 mL CH_3OH, (b) 1.5 mL CH_3CN–0.1 M HCl (4:1.8, v/v)	98.4	87.8	88.1	94.5	91.3	89.9	90.5	99.4	88.5	89.7	95.3	92.6	87.0	89.5

when methanol and pure water were used at the conditioning step.

The results of a study focused on the elution step using three different eluents: methanol, 0.1% HCl in methanol and acetonitrile in combination with various sorbents: Oasis HLB, Bond Elut C_8, C_{18}, PH, 2OH and Abselut NEXUS, Lichrolut RP-18, Adsorbex RP-8, Alltech-C_8 and Supelclean LC-18 and DSC-18, after conditioning with methanol and water, showed better results when methanol was used with less polar sorbents like C_8; while acetonitrile gave the lower recovery values for all analytes. All SPE optimization results are presented in Table 5.

The repeatability and consistency of SPE procedure was furthermore studied. Thus, recovery was estimated in three concentration levels in serum sample treatment for three different series of cartridges and for four different days. The data reveal reproducible results for the SPE as well as for the overall analytical procedure with R.S.D. values less than 5.

Further SPE optimization was performed on spiked serum and urine samples using Supelclean cartridges and different washing and elution conditions. Results for serum samples and urine samples are presented in Table 6. Higher recovery rates were obtained when the sorbent was washed with 0.1 mL water, whereas an increased volume was leading to the removal of the analytes and low recoveries. Elution was performed in two steps using 1 mL CH_3OH and 1.5 mL CH_3CN −0.1 M HCl (4:1.8, v/v). The eluotropic strength of the elution solvents was adjusted with variations of the HCl portion in the organic phase. It was found that after the first step with methanol the more polar compounds were eluted in a percentage of 50–60%.

Testing different loading sample volumes proved that when using serum sample volume in the range from 50 to 400 μL, yielded recoveries were similar with low levels of contaminants. Regarding urine samples loading volume over 200 μL leaded to an inadequate clean-up and co-eluted impurities were not removed. Thus, 50 μL serum sample and 200 μL urine sample were used for further studies.

Extraction efficiency in spiked serum and urine samples was estimated by applying optimum conditions samples at five concentration levels 1, 3, 5, 8 and 10 ng μL^{-1}. Corresponding regression equations were established for serum and urine for the seven analytes based on standard addition method and using theophylline as internal standard. Linearity range between 0.1 and 10 ng μL^{-1} is proved by correlation coefficients higher than 0.997. Results are summarized in Table 1.

Results obtained from the study concerning the solid phase extraction efficiency in terms of absolute recovery of the analytes in serum and urine samples are presented in Table 7. As it can be seen, satisfactory recovery rates were obtained, indicating no significant analytes loss derived from matrix effects in both samples.

In tissue samples the efficiency of analytes extraction was studied using variant extracting solvents or mixtures, equilibration and ultasonication terms and temperatures. Recover-

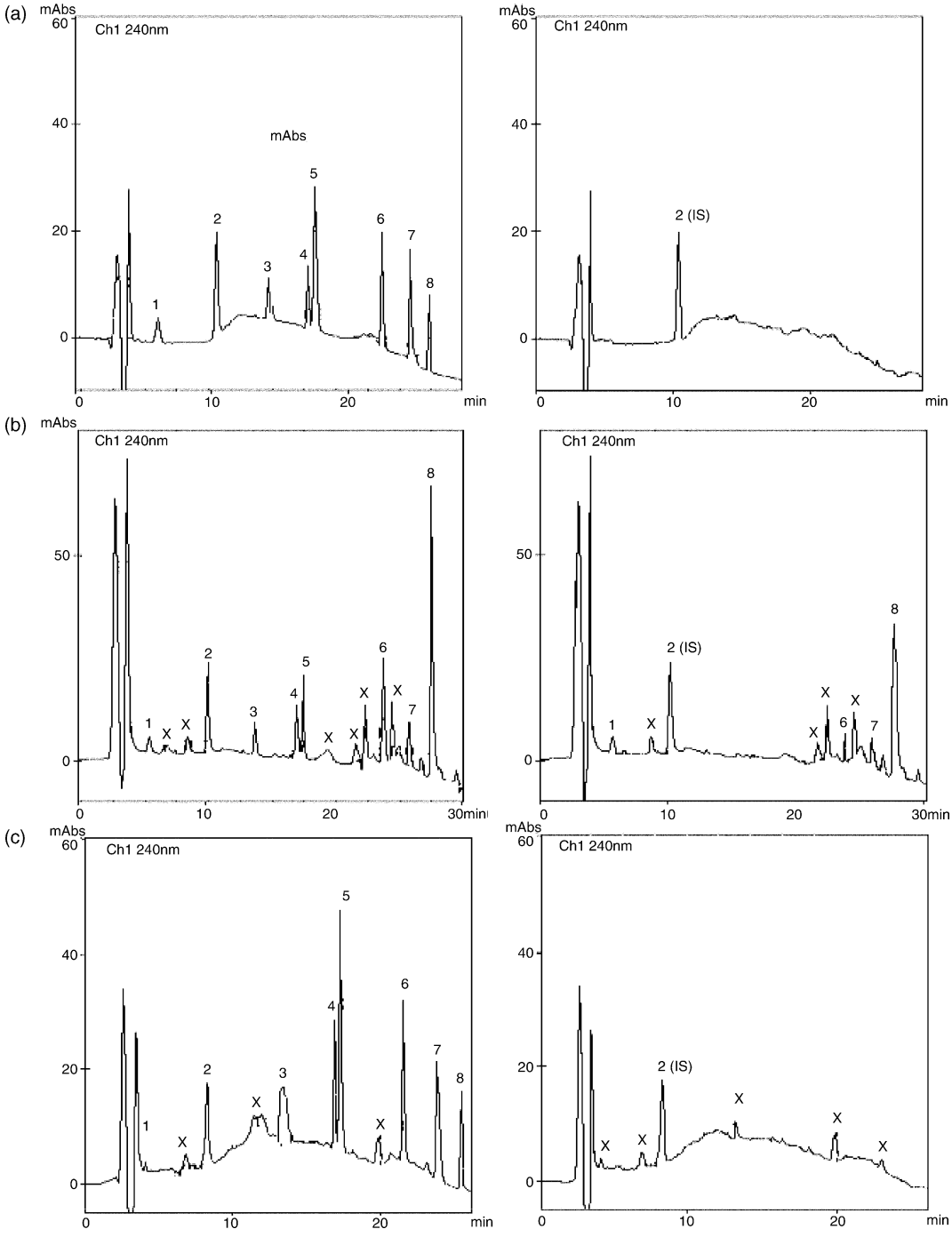

Fig. 3. Indicative chromatograms of spiked biological samples at 8 ng μL^{-1}: (a) serum; (b) urine and (c) tissue sample in left column, compared to their corresponding blanks in right column. Peaks: (1) Tyr, (2) theophylline (IS), (3) MIT, (4) DIT, (5) T_0, (6) T_2, (7) T_3 and (8) T_4. All unidentified compounds are marked by X.

ies were estimated based on spiked sample equilibrated with the matrix for 3 h in room temperature. However, quantitation was not feasible even after the optimum extraction procedure due to the co-eluted endogenous components of the tissue matrix that interfered the analysis as shown in Fig. 3 where representative chromatograms from biological samples both blank and spiked are illustrated.

3.5. Cross-validation of results obtained by the developed HPLC method and those by RIA, with regards to total-T_4 serum concentrations

Ten real serum samples deriving from different patients were comparatively analysed for total-T_4 concentration, by RIA in another laboratory, and by the proposed HPLC

Table 7
Repeatability and accuracy results in spiked serum and urine samples

Analyte	Serum samples				Urine samples		
	Added (ng)	Measured ± S.D.[a] (ng)	R.S.D.	Recovery (%)	Measured ± S.D.[a] (ng)	R.S.D.	Recovery (%)
Tyr	20	28.4 ± 0.8	2.9	142.0	24.2 ± 1.1	4.7	121.0
	60	45.0 ± 1.8	4	75.1	54.2 ± 1.7	3.2	90.1
	100	100.1 ± 3.9	3.9	100.2	96.5 ± 5.9	6.1	96.5
	200	225.4 ± 7.0	3.1	112.7	174.3 ± 18.1	10.4	87.2
MIT	20	24.9 ± 1.1	4.5	124.3	21.4 ± 1.8	8.6	107.1
	60	61.4 ± 3.7	6.1	102.3	58.1 ± 1.6	2.7	96.9
	100	85.2 ± 2.3	2.7	85.2	85.7 ± 2.9	3.4	85.7
	200	188.6 ± 6.6	3.5	94.3	182.2 ± 7.7	4.2	91.1
DIT	20	24.6 ± 1.5	6.1	122.9	20.8 ± 0.2	1.2	103.8
	60	62.5 ± 2.6	4.2	104.2	55.4 ± 4.0	7.2	92.3
	100	87.6 ± 1.1	1.2	87.6	88.4 ± 3.9	4.4	88.4
	200	175.6 ± 21.2	12.1	87.8	176.2 ± 9.3	5.3	88.1
T_0	20	21.2 ± 1.1	5	106.1	22.2 ± 1.4	6.1	110.7
	60	57.3 ± 1.7	2.9	95.6	57.2 ± 1.5	2.7	90.4
	100	85.7 ± 1.2	1.4	85.6	84.8 ± 8.1	9.5	84.8
	200	190.4 ± 20.8	10.9	95.2	175.1 ± 8.9	5.1	87.5
T_2	20	21.9 ± 1.6	7.2	109.3	18.9 ± 0.9	4.7	94.5
	60	55.7 ± 5.1	9.1	92.9	58.1 ± 4.2	7.2	96.9
	100	94.8 ± 4.2	4.4	94.7	97.3 ± 3.5	3.6	97.3
	200	163.6 ± 9.0	5.5	81.8	185.1 ± 2.8	1.5	92.5
T_3	20	18.7 ± 1.8	9.6	93.6	18.5 ± 1.2	6.5	92.5
	60	50.6 ± 2.1	4.1	84.3	57.2 ± 3.0	5.3	95.2
	100	87.8 ± 2.0	2.3	87.8	87.2 ± 3.6	4.1	87.2
	200	213.7 ± 13.7	6.4	106.8	187.4 ± 17.1	9.1	93.7
T_4	20	17.8 ± 1.1	6.2	89.1	19.0 ± 1.4	7.4	95.1
	60	58.5 ± 2.5	4.3	97.4	51.9 ± 1.3	2.5	86.5
	100	77 ± 5.5	7.1	77.9	102.1 ± 6.6	6.5	102.1
	200	168.4 ± 15.7	9.3	84.2	179.2 ± 18.1	10.1	89.6

[a] Mean values of four measurements.

method. When total-T_4 concentration of the samples was below method's LOQ, preconcentration was achieved by SPE using three to four times higher sample volumes. Acquired results of the two methods for the same samples were statistically evaluated and compared using Student's *T*-test. Results of no statistically difference were obtained by the two meth-

Table 8
Cross-validation results of total-T_4 determination in serum samples by the developed HPLC method and RIA

Sample	Micrograms of T_4 in 100 mL of serum sample	
	RIA	HPLC
1	9.0	7.9
2	9.0	10.0
3	15.0	14.2
4	10.0	9.8
5	1.0	3.4
6	7.0	8.6
7	4.5	5.7
8	5.5	5.3
9	6.0	4.3
10	5.0	5.5

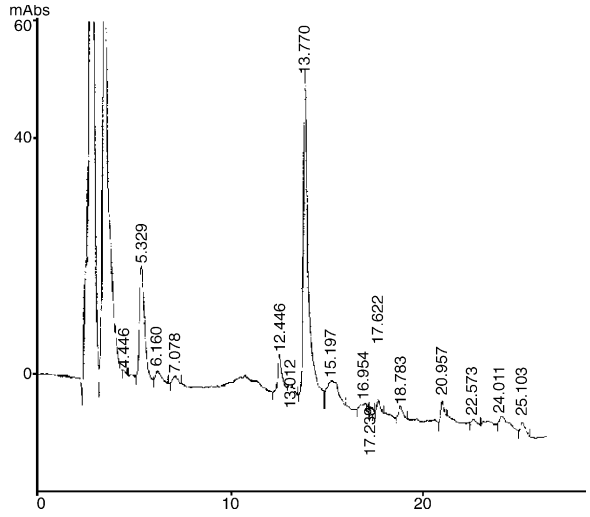

Fig. 4. Chromatogram of a real serum sample (No. 10 from Table 8) cross-analysed by the developed method and by RIA. Peaks: T_3 = 24.0 min and T_4 = 25.1 min.

ods as considering confidence interval of 95%, experimental t-value was lower than critical, $t = -0.659 < t_{critical} = 1.833$, with $P(T \leq t)$ of 0.263. These results are presented in Table 8 and they fully support the reliability of the method. In Fig. 4 a chromatogram of a real sample containing 0.08 ng μL^{-1} of T_4 analysed by both methods is given. T_3 and T_4 are eluted at 24.0 and 25.1 min, respectively. Identification was performed by UV spectrum comparison by means of the diode array detector.

4. Conclusions

Herein an HPLC method is developed for the determination of the thyroid hormones and their major analogues in commercial pharmaceuticals and biological fluids. The method can be applied with precision and accuracy in urine and serum samples employing a convenient and easy to-use solid phase extraction step. Preliminary studies for the application of the method in human thyroid gland tissue have shown not sufficient specification in this matrix and thus further studies need to be done. However, the advantageous simplicity over existing methods, the ability of simultaneous determination of seven analytes, efficient clean up, short time of analysis, high recoveries and no need of samples radio labeling suggest its proffer to biological and pharmacological research, rendering it valuable for diagnostic purposes.

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