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Journal of Chromatography B, 814 (2005) 163–172
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

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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Received 9 June 2004; accepted 8 October 2004
Available online 11 November 2004

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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doi:10.1016/j.jchromb.2004.10.025

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-

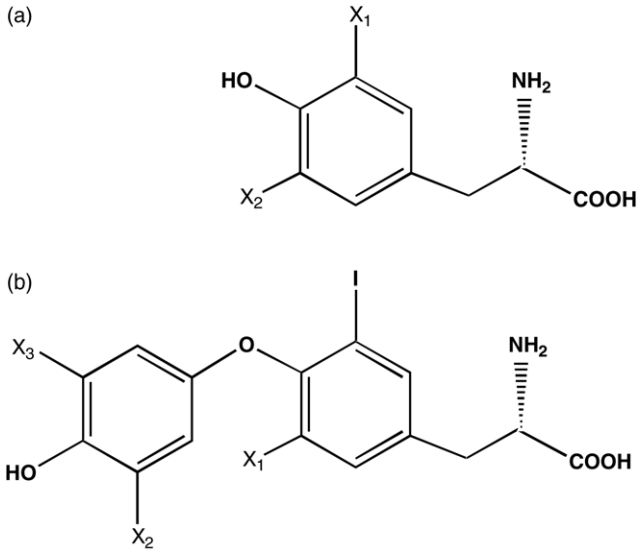

Fig. 1. Structures of (a) iodotyrosines (diiodotyrosine, DIT: $X_1 = I$, $X_2 = I$; monoiodotyrosine, MIT: $X_1 = I$, $X_2 = H$ and tyrosine, Tyr: $R_1 = H$, $R_2 = H$) and (b) iodothyronines (thyroxine, T_4: $X_1 = I$, $X_2 = I$, $X_3 = I$; triiodothyronine, T_3: $X_1 = I$, $X_2 = H$, $X_3 = I$; reversed-triiodothyronine,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