

Determination of Liothyronine and Levothyroxine in Thyroid Preparations by Liquid Chromatography

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Abstract

Liothyronine and levothyroxine were quantitatively determined in samples of commercial thyroid tablets and bulk powders. Samples were first hydrolyzed using a bacterial protease and then analyzed by high-performance liquid chromatography. Various hydrolysis conditions were investigated. The liothyronine and levothyroxine contents of commercial tablets and bulk powders were found to be 8-11 mcg and 25-43 mcg, respectively, per 65 mg of thyroid. The stability of the iodothyronines in thyroid tablets was also investigated.

The U.S.P. method[1] for the high-performance liquid chromatographic (HPLC) determination of liothyronine (T3) and levothyroxine (T4) obtained from enzymatic digestion of desiccated thyroid and thyroid tablets is based on that of Rees-Jones and Larsen.[2] These authors reported that 65 mg (1 grain) of thyroid tablets contained 12 mcg of liothyronine (T3) and 64 mcg of levothyroxine (T4), determining the iodothyronine contents by radioimmunoassay (RIA) instead of HPLC. Other investigators have reported considerably lower recoveries of liothyronine (T3) and levothyroxine (T4) following enzymatic digestion of desiccated thyroid and thyroid tablets[3-8] Initial attempts to reproduce the results of Rees-Jones and Larsen[2] using the proposed HPLC method of analysis on a variety of thyroid tablets and bulk material gave liothyronine (T3) and levothyroxine (T4) recoveries of only 50-60% of those reported. Chromatographic difficulties were encountered using the cyano column specified, and the small incubation volume (0.55 mL) made it impossible to obtain adequate hydrolysis on most tablets in which the proportion of excipients to desiccated thyroid was large. Hence, studies were undertaken to determine the optimum conditions for the hydrolysis and chromatography of liothyronine (T3) and levothyroxine (T4) present in commercially available thyroid tablets. As a result of the studies which are reported here and studies performed collaboratively at other laboratories (Armour Pharmaceutical Co., Eli Lilly Pharmaceutical Co., and the National Center for Drug Analysis, Food and Drug Administration), the Pharmacopeial Convention made revisions in USP XXI via the Second Interim Revision Announcement.9 Limits of 8.1-9.9mg of liothyronine (T3) and 32.3-43.7 mg levothyroxine (T4) per 65 mg (11 grain) were adopted.

Experimental Section

Reagents and Materials-U.S.P. reference standards of levothyroxine and liothyronine were used undried. Moisture content was determined by the Karl Fischer titration method, and a correction was made. L-3,3',5'-triiodothyronine (T3, reverseT3, or iso-T3, Calbiochem-Behring Corp., San Diego, CA) was used as received. Bacterial protease derived from *Streptomyces griseus* was obtained from two sources (Pronase, catalog #53702, Calbiochem-Behring Corp.; Bacterial Protease, catalog #P5147, Sigma Chemical Co., St. Louis, MO). Reagents used were analytical reagent grade, and acetonitrile was HPLC grade. Iodine assays were done by potentiometric titration using silver nitrate and an iodide-sensing electrode.[10] Thyroid tablets were obtained from several manufacturers (Pharmaceutical Basics, Inc., Denver, CO; Armour Pharmaceutical Co., Scottsdale, AZ; Eli Lilly Co., Indianapolis, IN), and bulk thyroid was either full strength (0.8 iodine, American Laboratories, Inc., Omaha, NE) or cut with lactose to 0.2 iodine content (Pharmaceutical Basics, Inc., Denver, CO).

Apparatus-The high-performance liquid chromatograph (HPLC) (model 5500, Varian

Instruments, Palo Alto, CA) was equipped with a variable-wavelength detector (Varian model UV-200), column heater, auto sampler (Varian model 8000), and auto injector (model 7126, Rheodyne Corp., Cotati, CA) with a 200-L loop. Data was handled by a chromatographic data system equipped with a 144K memory, disk storage, and a printer plotter (Varian, Vista 402). A commercially available 4 mm ID x 30 cm octadecylsilane column (A-Bondapak-Cia, Waters Associates, Milford, MA) was used.

Chromatographic Conditions-The mobile phase was a mixture of 28 acetonitrile and 72 of a 1:200 mixture of phosphoric acid in water. The percentage of acetonitrile was increased to about 35 when a spherical 5- μ m Cig column packing was used. The flow rate was either 1.5 or 2.0 mL per minute, the column temperature was 34°C, and the detector was set at 225 nm.

Proteolytic Enzyme Solution-A pH 8.4 reducing buffer solution was prepared containing 0.11 M NaCl, 0.04 M Tris buffer, and 0.05 M methimazole. The pH was adjusted to 8.4 \pm 0.05 with 6 M HCl. On the day of use the proteolytic enzyme was dissolved in the reducing buffer to prepare a solution containing -150 protease units per milliliter (1 unit liberates a digestion product equivalent to 25 μ g of tyrosine per minute).

Stock Standard Solutions-Levothyroxine (95 mg) was dissolved in 100 mL of a 500:500:1 mixture of water:acetonitrile:ammonia hydroxide. Similarly, liothyronine (22.5 mg) was dissolved in 25.0 mL of the same mixture. A working combination stock solution was then prepared by combining 4.0 mL of the levothyroxine stock and 1.0 mL of the liothyronine stock and diluting to 10.0 mL with a 1:1 mixture of acetonitrile and water. This combination stock was stable for -2 months when stored at 4°C in the dark.

Working Standard-On the day of use, the combination stock was diluted 1:50 with the reducing buffer solution. Then, 2.0 mL of enzyme deactivating solution (1:100 phosphoric acid:acetonitrile) was added to 5.0 mL of the diluted standard. The final concentrations of liothyronine (T3) and levothyroxine (T4) were -1.3 mcg/mL and 5.4 mcg/mL, respectively.

Recovery Standard-The combination stock standard was diluted 1:50 with proteolytic enzyme solution instead of reducing buffer and treated in the same manner as the samples. **Sample Preparation**-An accurately weighed portion of powder equivalent to 65 mg (1 grain) of thyroid (proportionately less was used if the iodine content was greater than 0.2) was transferred to a screw-capped culture tube, 5.0 mL of proteolytic enzyme solution was added, and the contents were mixed well. The tubes were placed in an incubator maintained at 37 \pm 1°C, and the contents were agitated after 4-8 h and again after 20-24 h. At the end of the incubation period (28 h); 2.0 mL of enzyme deactivating solution was added, and the tubes were mixed well, and centrifuged at about 2000 rpm for 5-10 min. Some samples required filtration through a 0.45- μ m membrane filter in order to clarify the sample.

Results and Discussion

Chromatography-Table 1 shows a typical chromatogram obtained for a 65-mg thyroid tablet. The small peak at 14 min had the same retention time as L-,3',5'-triiodothyronine CIV, 1). The approximate amount of 1 was found to be about 1.0-1.6 μ g/65 mg of thyroid. The presence of 1 in thyroid has also been reported by other investigators,⁴⁻⁷ and its presence in samples precluded the use of 1 as a convenient internal standard.

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Liothyronine and Levothyroxine in Armour Thyroid

The current United States Pharmacopeia (U.S. Pharmacopeia) method[1] for the analysis of liothyronine (T3) and levothyroxine (T4) in desiccated thyroid requires a proteolytic enzymatic digestion followed by a high-performance liquid chromatographic assay. These procedures were validated through a collaborative study at four laboratories[2] in which one-grain thyroid tablets from three manufacturers were assayed in triplicate on five consecutive days. The final results of this study were submitted to the U.S. Pharmacopeia to assist them in establishing both the analytical procedures and the specifications for thyroid tablets, U.S.P.

In the February, 1986 issue of the Journal of Pharmaceutical Sciences, Richheimer and Jensen of Pharmaceutical Basics[3] published further data developed on thyroid products manufactured by Armour, Lilly, and Pharmaceutical Basics. The Pharmaceutical Basics data suggest that the Armour product failed to meet the current U.S. Pharmacopeia specifications for T3 (i.e., 8.1-9.9 µg/grain). Not included in the publication were the original data that were obtained from all the participating laboratories, including Pharmaceutical Basics, Inc. (PBI), and submitted to the U.S. Pharmacopeia. These results formed the basis for the current U.S. Pharmacopeia specifications for T3 and T4 in natural thyroid. The original results obtained for the Armour product (Lot 1516), as well as PBI published data, are shown in Table I.

It can be clearly seen that with the exception of the second set of results provided by Pharmaceutical Basics, the Armour 1-grain (65 mg) thyroid product contains levels of liothyronine and levothyroxine that are well within the limits specified by the U.S. Pharmacopeia. It is interesting to note that the overall composite values for liothyronine and levothyroxine are quite similar to the mean values of the two sets of results from Pharmaceutical Basics. Additionally, the precision of the assay as demonstrated by Armour, Eli Lilly, and the FDA was significantly better than that obtained by Pharmaceutical Basics for their first analysis and for levothyroxine in the second set of data.

The above results should serve to correct any misrepresentations (implied or otherwise) reported previously (see below) regarding the liothyronine and levothyroxine content in Armour thyroid medications and the nature of the collaborative study for the U.S. Pharmacopeia. As determined by Armour Pharmaceutical Company and other participating laboratories, the liothyronine and levothyroxine content in Armour thyroid is well within the specifications set by the U.S. Pharmacopeia. The precision of the assay procedure as determined by Armour, Eli Lilly, and the FDA is considerably better than that reported by Pharmaceutical Basics.

Laboratory	Analysis	Liothyronine (T3)			Levothyroxine (T4)		
		Mean	Range	RSD	Mean	Range	RSD
Armour0		8.88			37.68		
Eli Lilly		8.58	8.58 - 9.04	1.81	37.30	36.62 - 38.32	1.39
FDA	14	8.60	8.33 - 8.76	2.99	39.47	35.6 - 38.2	3.26
PBI (1)	15	9.51	7.73 - 9.65	6.43	40.90	38.19 - 40.50	3.30
PBI (III)	13	7.96	8.57 - 11.21	7.9	36.66	34.16 - 45.48	9.9
Composite	13	8.71	7.27 - 8.56	5.3	38.30	33.53 - 44.27	8.1
PBI-Mean		8.74			38.78		

References and Notes

1. US Pharmacopeia, 21st rev.; U.S. Pharmacopeial Convention: Rockville, MD, 1985; pp 1893-1895
2. Armour Pharmaceutical Company, Eli Lilly Pharmaceutical Company, The National Center for Drug Analysis (FDA), and Pharmaceutical Basics, Inc.
3. Richheimer, S. L.; Jensen, C. B. J. Pharm. Sci. 1986, 75, 215-217.

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ALL OF THE FOREGOING EXTRACTS WERE TAKEN FROM THE MONOGRAPH ON ARMOUR THYROID USP AND WERE UNDERTAKEN AS A RESPONSE TO THE FOLLOWING REPORT

Response to "Liothyronine and Levothyroxine in Armour Thyroid"

We originally conducted the collaborative study to determine the levothyroxine (T4) and liothyronine (T3) content of thyroid tablets from three different manufacturers in late September 1984. Because of a deadline we had to send these results to R. Gamick at Armour Pharmaceutical Co. even though we were not happy with the results; that is, the T4 and T3 levels we found were 10-15% higher than we had gotten before and others had reported. As a result of this, we initiated a study to determine the cause of this anomaly and found that our standard stock solution had undergone extensive degradation (probably from being inappropriately stored at room temperature for several days). We then repeated the study using fresh standards and reported these new results directly to the U.S. Pharmacopeia on October 19, 1984, with instructions to disregard the previous data we had obtained. This second set of data is what is reported in our paper in the February 1986 issue of the Journal of Pharmaceutical Sciences. To the best of our knowledge this set of data was in the hands of the U.S. Pharmacopeia Committee on Revision when they decided upon the final specifications for thyroid tablets.

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a. USP XXI specification: 8.1 to 9.9 mcg/65mg (1 grain) b. USP specification: 32.3 to 43.7mcg/65mg (1 grain) c. Lot 1516; 1-grain, tablets. d. pharmaceutical basics, Inc e. reference 3