Detection of Thyroxine in Dietary Supplements Using an Enzyme-Linked Immunosorbent Assay

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(Received August 18, 2003; Accepted August 29, 2003)

Thyroxine (T4), one of the thyroid hormones, in adulterated dietary supplements was analyzed using two different methods; enzyme-linked immunosorbent assay (ELISA) and LC/MS. To release T4 from thyroglobulin, samples were first hydrolyzed with proteolytic enzyme, and then the supernatant was diluted and directly analyzed using a commercial free T4 ELISA kit for diagnostic discrimination. In contrast, T4 was extracted with ethyl acetate from the supernatant, and then ethyl acetate layer was evaporated. The residue was dissolved in the mobile phase and analyzed by LC/MS with electrospray ionization (ESI) interface under positive ion mode. These methods were applied to the analyses of 13 dietary supplements advertised as weight reducers. T4 was detected in four of the samples and the analytical results by ELISA agreed well with those obtained by LC/MS. The ELISA technology described here is available for the screening of T4 in adulterated supplements.

Key words —— thyroxine, thyroid hormone, dietary supplement, enzyme-linked immunosorbent assay, LC/MS

INTRODUCTION

The presence of therapeutic medicinal ingredients often added to dietary supplements as part of the intended use has been reported.[1–3] Serious adverse health consequences due to uncontrolled use of drug-based dietary supplements have become a problem in Japan. For periodic inspection, the establishment of a more effective procedure for screening medicinal ingredients in supplements is required.[4]

Thyroxine [T4, *O*-(4-hydroxy-3,5-diiodophenyl)-3,5-diiodo-L-tyrosine, $C_{15}H_{11}I_4NO_4$, mol.wt. 776.87] is one of the thyroid hormones prescribed for the symptoms of hypothyroidism. It is also possible that mild thyroid hormones excess over many years may increase the risk for a serious heart rhythm problem and fractures caused by excessive calcium loss from bones. In general, LC/MS is used for the assay of T4 in supplements with enzymatic hydrolysis and extraction procedure.[5] This assay method is time-consuming and requires proficient techniques.

Enzyme-linked immunosorbent assay (ELISA) has become an increasingly important alternative detection method for the determination of pesticides,[6] toxins[7] and forensic drugs[8] as a screening tool. ELISA for the detection of T4 in serum have been developed as a specific and rapid method, and a commercial test kit based on ELISA for clinical diagnosis of various thyroid disorders is available on the market. The present study examined an ELISA procedure for the detection of T4 in supplements made from Chinese herbal preparations advertised as weight reducers.

MATERIALS AND METHODS

Materials —— Five samples were offered by consumers with thyroid gland disease and liver trouble. Eight samples were collected from Aichi prefecture (Japan) by drug inspectors in 2002. All samples were claimed to be extracts of animal organs and traditional Chinese herbs, for use as weight reducers.

Reagents and Instrumentation —— Dried thyroid of Japanese Pharmacopoeia quality was purchased from Teikoku Hormone Mfg. (Tokyo, Japan) and 0.01 mol/l phosphate buffered saline (PBS, for tissue washing) and protease (for biochemistry) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Reducing buffer solution was prepared as a solution in 0.11 mol/l sodium chloride

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that was 0.04 mol/l tris(hydroxymethyl)aminomethane and 0.05 mol/l methimazole, adjusted with 6 mol/l hydrochloric acid to pH 8.4. Proteolytic enzyme was prepared as a solution containing 40 mg of protease in 5 ml of reducing buffer solution. Enzyme deactivating solution was prepared as a 1 in 100 mixture of phosphoric acid in acetonitrile. Acetate buffer solution was prepared as a solution in 0.2 mol/l sodium acetate that was adjusted with 0.2 mol/l acetic acid solution to pH 5.0.

Absorbances in microplate at 450 nm were read using a Sceti handy photometer model-6 (Tokyo, Japan). LC/MS with electrospray ionization (ESI) interface analyses were carried out using a Micromass Quattro II mass spectrometer (Manchester, U.K.) on the effluent from an Agilent HP1100 LC system (Palo Alto, CA, U.S.A.).

ELISA Analysis —— A commercial free T4 kit (DRG International, Inc., NJ, U.S.A.) was employed for the ELISA analyses performed in this study. The free T4 kit is a 96-well microtiter plate design and immunochemical analysis was conducted according to instructions included with the kit using the provided reagents. These reagents included eight strips (12-wells each) containing monoclonal T4 antibodies immobilized on the walls of the test wells, T4 horseradish peroxidase-labeled enzyme conjugate, tetramethylbenzidine substrate, stopping solution, and washing solution. PBS was used as the negative control. The microtiter plate kit procedure was according to the manufacturer's instructions (Fig. 1).

Weighed 200 mg powdered capsule type and tablet type samples, or weighed 500 mg powdered tea bag type samples were used in assay preparation procedure (Fig. 2). For ELISA, the supernatant was diluted one hundredfold with PBS. The screening spiking level of absorbance in this study was below the level of the standard solution obtained using 2.5 mg of dried thyroid with the same assay preparation procedure.

LC/MS Analysis —— An aliquot after proteolysis and the extraction procedure (Fig. 2) was used for confirmation by LC/MS. Mass spectra of T4 were investigated with ESI under positive ion mode. The operating parameters were as follows: electrospray voltage, 3 kV; cone voltage, 30 V; ion source temperature, 100°C; desolvation chamber temperature, 250°C. LC operating conditions for the MS system were carried out on a column of TSK gel ODS $80T_M$ (5 μm, 4.6 × 150 mm, Tosoh Co., Tokyo, Japan) with water/acetonitrile/trifluoroacetic acid (600 : 400 : 4.5, v/v) as the mobile phase, at a flow rate of 1.0 ml/min.

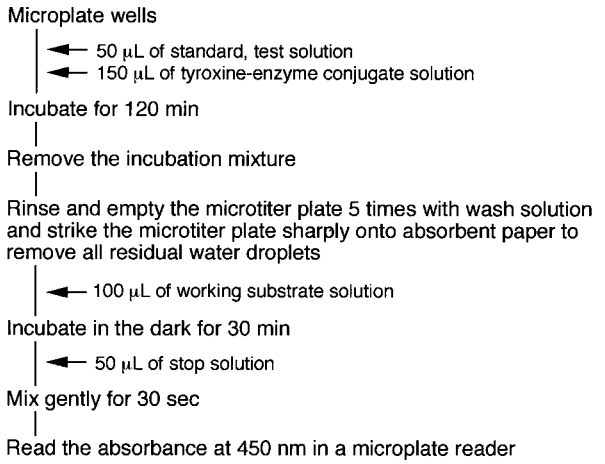


Fig. 1. Assay Procedure for Free Thyroxine by ELISA

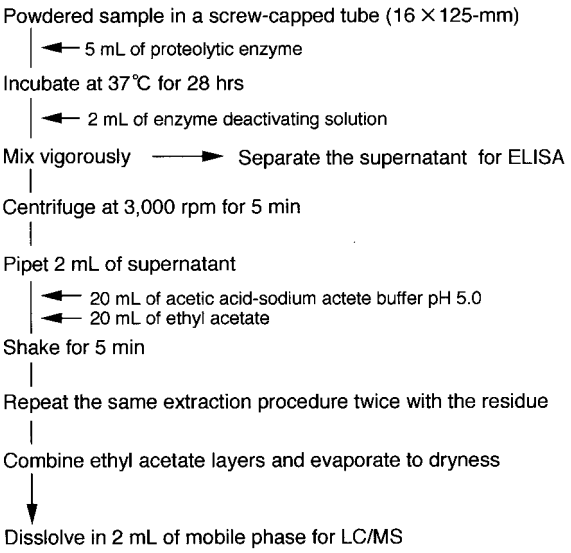
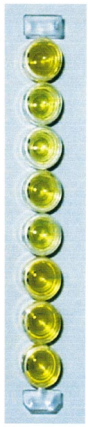


Fig. 2. Assay Preparation Procedure for ELISA and LC/MS

RESULTS AND DISCUSSION

Screening of T4 by ELISA

The synthesis of the important thyroid hormones, 3,5,3′-triiodothyronine [T3, *O*-(4-hydroxy-3-iodophenyl)-3,5-diiodo-L-tyrosine] and T4, take places in the thyroid gland. A great part of them is bounded with thyroglobulin, which is a macro-molecule synthesized exclusively in thyroid cells. The T4 concentration is higher than T3 in thyroid powder.[9] The supply of T3 or T4 into cells is governed by their unbound (free) concentrations in serum rather than



	Absorbance at 450 nm
1. Blank (PBS) :	2.50
2. Dried thyroid JP (5.0 μg/mL) :	1.10
3. Dried thyroid JP (6.2 μg/mL) :	0.95
4. Sample A :	1.05
5. Sample B :	0.91
6. Sample E :	2.50
7. Sample F :	2.50
8. Sample G :	2.50

Fig. 3. Typical ELISA for Free Thyroxine in Dietary Supplements

the protein-bound fractions. For the diagnosis of thyroid disease, the free hormone is generally accepted as an appropriate measure. In euthyroid subject, 0.01–0.02% of the total serum T4 is present as free T4.[10] A commercially available free T4 test kit for diagnostic discrimination has been applied to detect free T4 in dietary supplements. ELISA technology is often of slightly lower precision and accuracy caused by interaction with molecules with structures related to the target substance. Samples were hydrolyzed with proteolytic enzyme (Fig. 2) to yield a high free T4 concentration, thereby relatively reducing the endogeneous interfering component concentration. The supernatant was carefully decanted and diluted followed directly by an ELISA assay according to the manufacturer's instructions. The cut-off level of absorbance in this study was below the level (1.1 AU) of standard solution obtained using 2.5 mg of dried thyroid with the same assay preparation procedure (Fig. 3).

Identification of T4 with ESI-LC/MS

LC/MS using the ESI interface with the positive ion mode was used to confirm screening results obtained by ELISA. The LC mobile phase was acidified with TFA to improve the sensitivity. The retention times of T4 in this LC/MS analysis were 11.9 min, and the peak detected in supplements was identified. A peak in the dried thyroid powder standard, a protonated molecule for T4 was at *m/z* 777.6 and fragment ion at *m/z* 731.6. A peak in the supplement, a protonated molecule at *m/z* 777.5 and fragment ion at *m/z* 731.5 were also observed. Fig. 4 shows the mass spectra generated at positive ion mode. As a result, a peak in the supplement was identified as T4 on mass and LC/MS spectra. The instrumental detection limits based on a signal-to-noise ratio of about 10 for standard solution of T4 was 0.5 μg/ml. Thus, it is therefore suitable to analyze T4 in adulterated dietary supplements using a single oral dose of marketed thyroid powder.

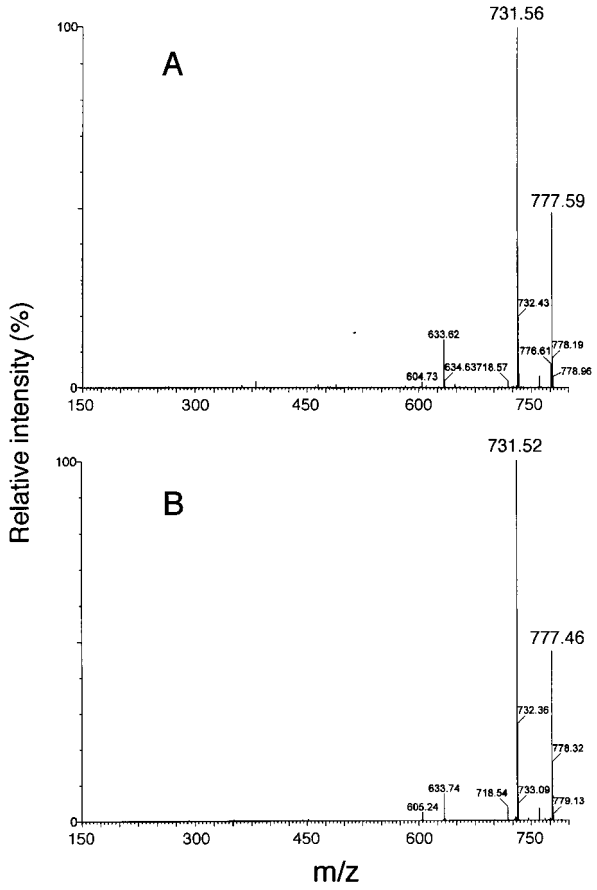


Fig. 4. Mass Spectra of Thyroxine by LC/MS at Positive Ion Mode

A: dried thyroid, B: dietary supplement containing thyroid hormone.

Sample Analysis

These methods were applied to the analyses of 13 dietary supplements advertised as weight reducers. The ELISA analysis revealed the presence of T4 in four samples, which were detected by LC/MS (Table 1). ELISA technology has advantages in rapid round times, ease for sample pretreatment and minimal need for specialized equipment. Although positive bias can be beneficial to a screening method as it reduces the possibility of generating false negatives, further studies are required to identify the factors responsible for its observed bias by free T4 ELISA with easier extraction.

This immunological test is the first reported case

Table 1. Analytical Results for Thyroxine in Commercial Dietary Supplements

Dietary supplement	ELISA	LC/MS
A. Capsule type	+	+
B. Capsule type	+	+
C. Capsule type	+	+
D. Capsule type	+	+
E. Capsule type	—	nd
F. Capsule type	—	nd
G. Tablet type	—	nd
H. Tablet type	—	nd
I. Tablet type	—	nd
J. Tablet type	—	nd
K. Tablet type	—	nd
L. Tea bag type	—	nd
M. Tea bag type	—	nd

+: positive, —: negative, nd: not detected.

of the detection of T4 as adulterants in dietary supplements. The ELISA presented here permits specific detection of free T4 in supplements using standard laboratory equipment and can be used for rapid detection. The procedure is considered to be available for the screening of T4 in adulterated supplements.

Acknowledgements This study was partly supported by a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan.

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