— **Regular Article** —

Determination of Liothyronine and Levothyroxine in Dietary Supplements by HPLC Using a Pre-column Derivative

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A new method to identify liothyronine (T3, 3,3′,5-triiodo-L-thyronine) and levothyroxine (T4, L-thyroxine), which are thyroid hormones, in dietary supplements by high-performance liquid chromatography was developed using a pre-column derivative with 4-fluoro-7-nitrobenzofurazan (NBD-F), without using liquid chromatography (LC)/MS. T3 and T4 in a dietary supplement were extracted with 10% ammonia water. The extract was treated with a polyvinylpolypyrrolidone (PVPP) column. The T3 and T4 adsorbed on the PVPP were eluted with 60% acetonitrile. T3 and T4 present in the eluate from the PVPP column were then derivatized with NBD-F. The reaction of T3 and T4 with NBD-F was complete after 30 min at 60°C. These derivatives were measured by HPLC using an octadecylsilane (ODS) column with a fluorescent detector. 0.1% Phosphoric acid was added to 630 ml of acetonitrile to a final volume of 1000 ml, and the mixture was used as the mobile phase. The excitation wavelength on the fluorescent detector was 470 nm, and the emission wavelength was 530 nm. The stability of the peak area of T3-NBD and T4-NBD was maintained for 12 hr. The linearity of T3 and T4 as a coefficient of the correlation value was 0.9999, and the quantitation limit of T3 and T4 was 0.002 μg/ml. The results confirmed that T3 and T4 were added to eight dietary supplements. The recovery rate was in the range of 80.4–103.7% for T3 and 79.8–101.5% for T4, and the precisions, as measured by the standard deviation, were within 4.8% and 4.3%, respectively.

Key words —— 3,3′,5-triiodo-L-thyronine, thyroxine, 4-fluoro-7-nitrobenzofurazan, polyvinylpolypyrrolidone, HPLC

INTRODUCTION

Liothyronine (T3, 3,3′,5-triiodo-L-thyronine) and levothyroxine (T4, L-thyroxine) are thyroid hormones administered to patients with thyroid deficiencies such as myxedema and cretinism. The serious side-effects of thyroid hormones include shock, angina pectoris, and hepatic dysfunction, and thus, thyroid hormones must be administered with discretion. Some of the pharmacological effects include increased basal metabolic rate, oxygen consumption, and carbohydrate metabolism.[1] Recently, thyroid hormones have been detected in dietary supplements. The dietary effects of these hormones are mediated by their pharmacological effects. However, the health of people who used dietary supplements containing thyroid hormones was impaired. Between 2002 and 2010, there have been 28 cases where thyroid hormones have been found in dietary supplements according to administrative tests in Japan. In some cases, dried thyroid was found in the dietary supplements and results suggested that thyroid hormones originated from the dried thyroid. There is a possibility that dried thyroid and thyroid hormones are dispensed in dietary supplements because they have dietary effects. Thyroid hormones can be measured by HPLC with a UV detector.[2–6] However, it was very difficult to identify and determine peaks of T3 and T4 in dietary supplements using previously reported methods because of many interfering substances and the fact that UV detection has poor sensitivity and specificity. Therefore, liquid chromatography (LC)/MS was developed.[7,8] Although LC/MS is highly sensitive and specific, it is expensive, and the cost of maintaining the equipment is more expensive than that for a standard HPLC. Thus, smaller laboratories, like official institutions examining dietary sup-

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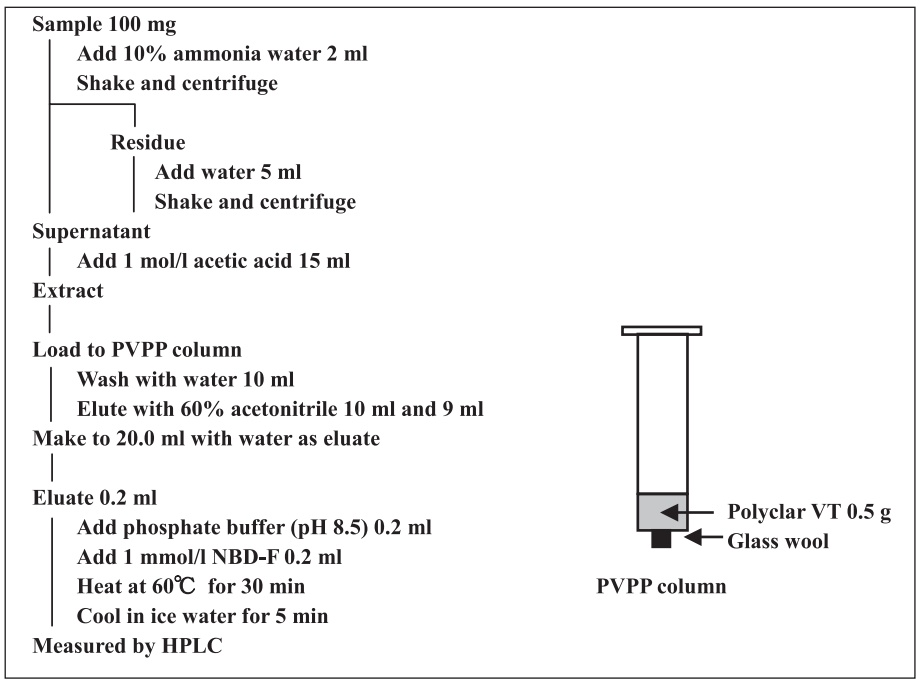

Fig. 1. Flow Chart of Recommended Procedure
This method requires three steps: T3 and T4 are extracted from dietary supplements, adsorbed onto the PVPP column, and derivatized with NBD-F.

plements, do not often have LC/MS machines. In the present study, in order to identify and determine the amount of T3 and T4 dispensed in dietary supplements without using LC/MS, we developed a new method that uses high-performance liquid chromatography with a pre-column derivative with 4-fluoro-7-nitrobenzofurazan (NBD-F). The development of a method to analyze T3 and T4 in dietary supplements leads to the selective analysis of T3 and T4 originating from dried thyroid.

MATERIALS AND METHODS

Materials —— L-Thyroxine sodium salt pentahydrate was used as a reference standard for T4 and 3,3′,5-triiodo-L-thyronine sodium salt served as a reference standard for T3. Polyclar VT was used as polyvinylpolypyrrolidone (PVPP). Their reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). NBD-F was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals used were HPLC or reagent grade. The NBD-F solution was dissolved in acetonitrile to make a 1 mmol/l solution. A reference standard was dissolved in 50% methanol to make 50 μg/ml standard stock solution. Each standard stock solution of T3 and T4 was mixed and diluted with water as standard solution, and spiked solution for recovery tests.

Recommended Procedure —— The flow chart of the method used is shown in Fig. 1. T3 and T4 were extracted from dietary supplements, adsorbed onto the PVPP column, and derivatized with NBD-F.

Extraction of T3 and T4 from Dietary Supplements —— First, 100 mg of sample and 2.0 ml of 10% ammonia water were added into a screw-capped culture tube. The tube was tightly capped, mixed for 10 min, centrifuged for 5 min at 2000 rpm, and the supernatant was separated. Next, 5 ml of water was added to the residue in the tube, and the solution was mixed and centrifuged. The supernatant was then added to a previous supernatant.

Treatment with PVPP Column —— First, 0.5 g of Polyclar VT was packed into a 10 ml disposable syringe, which served as a PVPP column. The PVPP column was previously saturated with 3 ml of water. Next, 15 ml of 1 mol/l acetic acid was added to the supernatant to produce a pH 5.1 loading solution, which was then loaded onto the PVPP column. The column was washed with 10 ml of water. The T3 and T4 adsorbing on the PVPP were eluted twice with 10 and 9 ml of 60% acetonitrile. The eluate was diluted to 20.0 ml with water.

Derivatization of T3 and T4 with NBD-F —— Here, 0.2 ml of the eluate from the PVPP col-

umn, 0.2 ml of 0.1 mol/l phosphate buffer containing 20 mmol/l EDTA (pH 8.5), and 0.2 ml of 1 mmol/l NBD-F were added into a 1.5 ml glass vial for HPLC. The vial was tightly capped, mixed, and heated at 60°C for 30 min. Then the vial was cooled for 5 min in ice water, and the solution was measured by HPLC.

Conditions of HPLC —— A L-column ODS [150 × 4.6 mm internal diameter (i.d.), 5 μm, Chemical Evaluation and Research Institute, Tokyo, Japan] was used in these experiments. The column temperature was maintained at 40°C. 0.1% Phosphoric acid was added to 630 ml of acetonitrile to make up 1000 ml and the mixture was as the mobile phase. The flow rate was 1.0 ml/min, and the injection volume was 30 μL. The HPLC system consisted of a Shimadzu LC solution version 1.02 system equipped with a Shimadzu RF-10AXL fluorescence detector (excitation wavelength: 470 nm, emission wavelength: 530 nm), Shimadzu (Kyoto, Japan).

Recovery test of T3 and T4 from Dietary Supplements —— A spiked solution containing 1.0 μg/0.1 ml of T3 and T4 was prepared and 0.1 ml of this spiked solution was added to 100 mg of a dietary supplement that did not contain thyroid hormones. This dietary supplement spiked T3 and T4 was used as a sample. The sample was extracted, adsorbed onto the PVPP column, derivatized with NBD-F, and measured by HPLC. Each standard stock solution was mixed and diluted with water to make a 0.05 μg/ml solution, and then derivatized with NBD-F as a standard solution. The recovery rate was calculated using the peak areas of the standard solution and the sample solution by HPLC. A standard calibration curve was prepared by plotting peak area versus twelve different concentrations over the range of 0.002–0.2 μg/ml, and the linearity of the plot line was examined. The recovery rate, precision of recovery rate, and the specificity of this method were measured using these samples and the standard solution.

RESULTS AND DISCUSSION

Condition of PVPP Column Treatment

The effect of pH of the loading solution on the PVPP was examined. First, 1.0 ml of standard solution (1.0 μg/ml) and 2 ml of 10% ammonia water were mixed and 9–16 ml of 1 mol/l acetic acid was added. The solution was loaded on the PVPP column, derivatized with NBD-F, and measured by HPLC. The relationship between the recovery rate and the pH of the loading solution on the PVPP column is shown in Fig. 2. The recovery rate of T3 and T4 from the PVPP column was influenced by the pH of the loading solution. The recovery rate of T3 and T4 was stable in the pH range of 5.0–5.2. Thus, it was decided that 15 ml of 1 mol/l acetic acid should be added to the extract from a sample to increase the recovery rate. Next, the effect of the solvent used and its concentration was examined to choose the best eluting condition. Methanol, ethanol, isopropanol, acetonitrile, acetone, and tetrahydrofuran were examined as eluting solvents for the PVPP column. Acetonitrile yielded the maximum recovery rate of T3 and T4 from the PVPP column. The relationship between recovery rate and the concentration of acetonitrile is shown in Fig. 3. The recovery rate was stable when 50–70% acetonitrile was used. These results indicate that 60% acetonitrile should be used as the eluting solvent for the PVPP column. Eluting twice with 10 ml and 9 ml of 60% acetonitrile was necessary to achieve a high recovery rate.

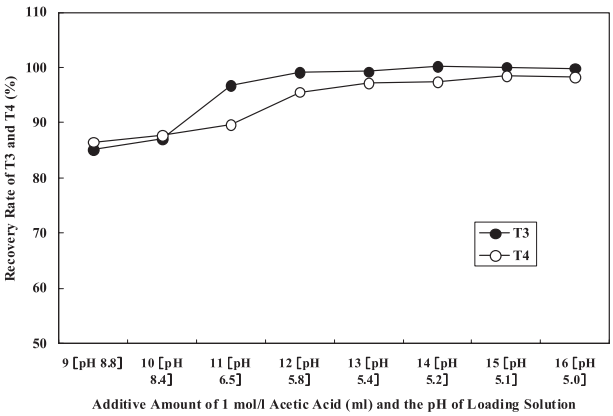

Fig. 2. Relationship between the Recovery Rates of T3 and T4 and the pH of the Loading Solution Used on the PVPP Column

Here, 1.0 ml of a standard solution of T3 and T4 (1.0 μg/ml), 2 ml of 10% ammonia water, and 9–16 ml of 1 mol/l acetic acid were added. The solution was loaded on the PVPP column using 60% acetonitrile as the eluting solvent, and the substances were derivatized with NBD-F and measured by HPLC. Each point was the average of three experiments.

Conditions for Derivatization of T3 and T4 with NBD-F

The optimum conditions were examined for the derivatization of T3 and T4 with NBD-F. First, the pH of a 0.1 mol/l phosphate buffer containing 20 mmol/l EDTA on the derivatization of T3 and T4 was examined in the pH 6.5–10.5 range. Further-

more, the effects of the temperature (50, 60, 70°C) and reaction time (5, 10, 20, 30 min) were examined. As a result, the optimum conditions for NBD-F derivatization occurred at pH 8.5, 60°C, and at a reaction time of 30 min, as derived on the basis of the peak area of the derivatized T3 and T4 (T3-NBD and T4-NBD). The peak areas of T3-NBD and T4-NBD were stable for a minimum of 12 hr.

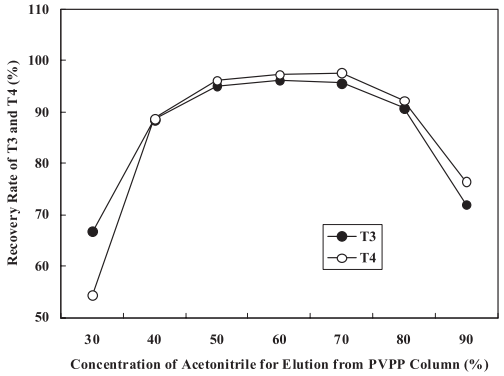

Fig. 3. Relationship between the Recovery Rates of T3 and T4 and the Concentration of Acetonitrile on the PVPP Column

In these experiments, 1.0 ml of a standard solution of T3 and T4 (1.0 μg/ml), 2 ml of 10% ammonia water, and 15 ml of 1 mol/l acetic acid were added. The solution was loaded onto a PVPP column using different concentrations of acetonitrile for elution, and the substances were derivatized with NBD-F and measured by HPLC. Each point was the average of three experiments.

Recovery Test of T3 and T4 from Dietary Supplements

Example HPLC chromatograms for a dietary supplement spiked with T3 and T4 are shown in Fig. 4. The chromatogram [A] is a chromatogram of dietary supplement No. 2 of Table 1, and had the greatest number of interfering peaks. T3-NBD and T4-NBD were eluted around 15 min and 21 min. No other peaks that interfered with both NBD-thyroid hormones were present on the chromatograms of all samples tested, so specificity was confirmed. The recovery rates of T3 and T4 from dietary supplements are shown in Table 1. The control sample demonstrates the recovery rate of a spiked solution without a dietary supplement. The recovery rate of the control sample was approximately 100%, and its standard deviation was very small. Both recov-

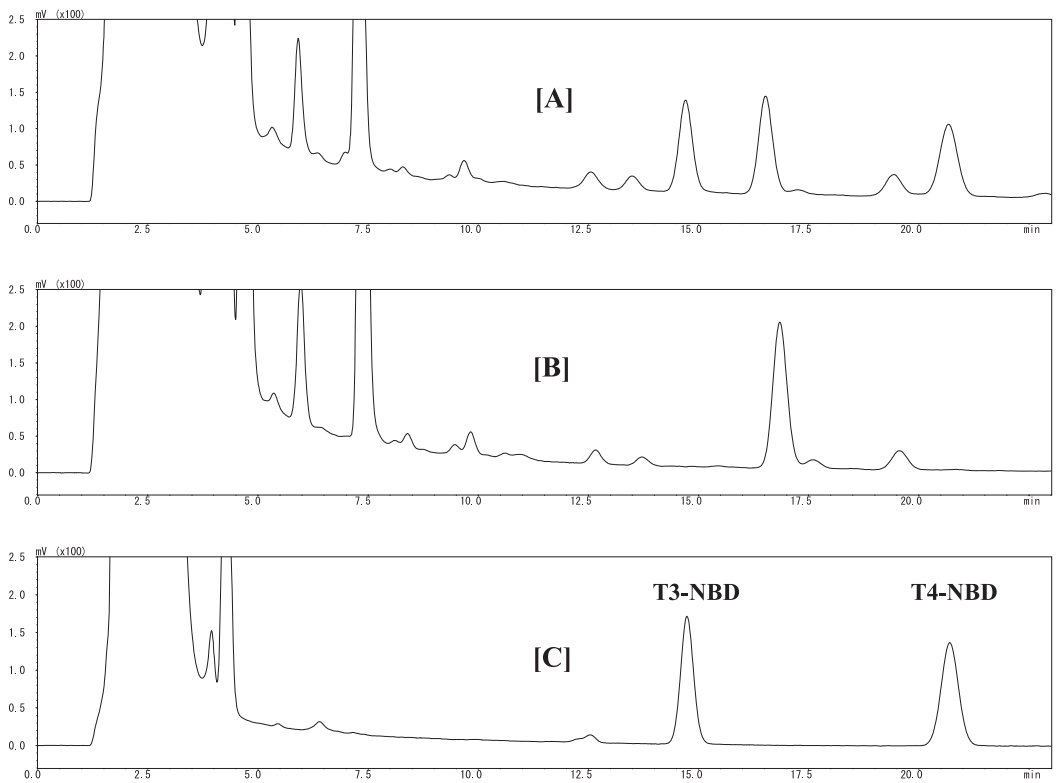

Fig. 4. HPLC Chromatogram of Dietary Supplements Spiked with T3 and T4

[A]: 100 mg of a dietary supplement spiked with 1.0 μg of T3 and T4 was measured by this method. [B]: 100 mg of a dietary supplement that did not contain T3 and T4 was also measured by this method. [A] and [B] are the samples of the dietary supplement No. 2 of Table 1. [C]: A standard solution containing T3 and T4 (0.05 μg/ml) was derivatized with NBD-F without PVPP column treatment. The retention times of T3-NBD and T4-NBD were 15 min and 21 min, respectively.

Table 1. Recovery Rates of T3 and T4 from Dietary Supplements

Dieray Supplement	Recovery rates (%)[a]	
	T3	T4
1	101.5 ± 4.8	100.4 ± 4.3
2	80.4 ± 1.4	79.8 ± 2.4
3	103.7 ± 3.3	101.5 ± 3.4
4	97.6 ± 0.2	98.7 ± 0.8
5	98.1 ± 3.3	96.0 ± 3.2
6	100.0 ± 2.2	100.9 ± 2.6
7	99.0 ± 4.1	98.9 ± 3.5
8	90.2 ± 2.9	80.7 ± 1.2
Control	100.3 ± 1.3	98.3 ± 2.1

a) Mean ± S.D. (n = 3). Dietary supplements spiking 1.0 μg of T3 and T4 were measured by this method.

ery rates for T3 and T4 were in the range of 79.8–103.7%, and their standard deviations were within 4.8%. The recovery rates of T3 and T4 from dietary supplements were also satisfactory. The linearity was good in the 0.002–0.2 μg/ml range, and the coefficients of the correlation values were 0.9999 in both cases. The regression equation of T3 was $Y = 3986438X - 70045$, while the regression equation of T4 was $Y = 4191474X - 137756$. The quantitation limit of both thyroid hormones was 0.002 μg/ml, and the reproducibility at the concentration of the quantitation limit was 2.84% for T3 and 7.57% for T4; this was verified by 6 experiments. The amount of T3 and T4 at the quantitation limit corresponds to 0.04 μg per 100 mg in a dietary supplement. The maintenance doses of T3 and T4 are 24–72 μg and 87–350 μg, respectively, per day. Therefore, this quantitation limit value was sufficiently low and verified that this method was highly sensitive.

Thyroid hormones in dietary supplements can be measured by LC/MS when they are prepared either by extraction with ethyl acetate or solid-phase extraction.[7,8] In this study, because PVPP adsorbs polyphenols, we hypothesized that T3 and T4, which are phenolic substances, might be adsorbed onto PVPP. In the field of chemical analysis, PVPP is used to adsorb and remove interfering substances including polyphenols from extract solutions. Here, the target substance of analysis is not adsorbed and passes through PVPP, allowing for a pure sample solution to be made after a pretreatment step that occurs prior to analysis.[9–13] However, the use of PVPP has not been previously reported as an analytical method that a target substance of analysis was adsorbed and eluted with PVPP. In this study, it was confirmed that T3 and T4 could be adsorbed onto PVPP and eluted using a 60% acetonitrile solution. PVPP was useful, allowing for identification and determination tests of T3 and T4 in dietary supplements. The peaks of T3 and T4 were buried in HPLC chromatograms because of many interfering substances when PVPP treatment was not used. Therefore, this method may be useful in the analyses of other phenolic substances.

NBD-F forms derivatives with primary and secondary amines that exhibits fluorescence at an emission wavelength of 530 nm and excitation wavelength of 470 nm.[14–17] T3 and T4 are primary amines, so we investigated the derivatization of T3 and T4 in terms of specificity and sensitivity. The peaks of T3-NBD and T4-NBD were identified on HPLC chromatograms with PVPP treatment and were not obscured by other substances. Given that the NBD-derivatives produce unique chromatograms, if thyroid hormones are dispensed in dietary supplements at concentrations that produce pharmaceutical effects, they can be identified and analyzed by this method. We are developing a new method to identify the thyroid hormones that originate from dried thyroids added to dietary supplements using this method.

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