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Short communication

# Determination of thyroid hormones in pharmaceutical preparations, after derivatization with 9-anthroylnitrile, by high-performance liquid chromatography with fluorescence detection

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**Abstract**

HPLC with fluorescence detection was used for the determination of low levels of liothyronine sodium and levothyroxine sodium in pharmaceutical preparations after fluorogenic derivatization. 9-Anthroylnitrile in dimethyl sulfoxide was used as a precolumn fluorogenic reagent. The 9-anthroylnitrile derivatives of liothyronine sodium and levothyroxine sodium were separated on a reversed-phase column with acetonitrile–0.02 *M* sodium dodecylsulfate (pH 3.5 with phosphoric acid) as the eluent. The calibration graphs were linear over a sample concentration range of 0.25–2.5 μg/ml. The detection limits for liothyronine sodium and levothyroxine sodium were 0.2 ng per injection. The proposed method was applied to the determination of thyroid hormones in pharmaceutical preparations. 

*Keywords:* Derivatization; LC; Thyroid hormones; 9-Anthroylnitrile

## 1. Introduction

Thyroid hormones are secreted from the thyroid which is in the pharyngeal front. The lack of production of these hormones causes hypothyroidism, leading to myxoedema with struma and debility. For the treatment of thyroidism, thyroid hormone preparations are used. Side effects attributed to overdosage of these formulations are hyperthyroidism, leading to arrhythmia and insomnia. The dosage levels are generally very low. For example, the smallest dosage form of liothyronine sodium ($T_3$-Na) tablet contains 5 μg/tablet and the smallest dosage form of levothyroxine sodium ($T_4$-Na) tablet contains 25 μg/tablet. Therefore, a sensitive analysis is needed to evaluate the quality of these formulations.

Many methods have been developed for the determination of thyroid hormones, including colorimetry [1], immunoassay [2,3], capillary electrophoresis [4] and HPLC [5–9]. Of these methods, HPLC with UV detection has been widely used as simplest method with highest selectivity in the determination of $T_3$-Na and $T_4$-Na in pharmaceutical preparations. However, the sensitivity of HPLC using UV detection is low for the determination of $T_3$-Na and $T_4$-Na in pharmaceutical preparations at minimum levels. In this case, improvements in the sensitivity of such a method are needed.

Some years ago, a fluorescence-labeling reagent

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that has an acyl cyanide group was developed for use in the HPLC analysis of hydroxy compounds [10–12]. This reagent is not only extremely fluorescent, but is also reactive with hydroxy groups under mild conditions. We found that $T_3$-Na and $T_4$-Na were derivatized quantitatively into fluorescent compounds by 9-anthroylnitrile (9-AN).

In this paper, we describe the precolumn reaction and fluorescence detection of $T_3$-Na and $T_4$-Na with 9-AN and their separation by reversed-phase HPLC. The proposed method was applied to the profile analysis of $T_3$-Na and $T_4$-Na in pharmaceutical preparations.

## 2. Experimental

### 2.1. Reagents and standards

$T_3$-Na and $T_4$-Na were obtained from Sigma (St. Louis, MO, USA). A stock standard solution was prepared by dissolving $T_3$-Na and $T_4$-Na in DMSO to give a concentration of 100 μg/ml. Working standard solutions were prepared by diluting the stock standard solution with DMSO. 9-AN (used as a reagent for fluorescence labeling) was purchased from Wako (Osaka, Japan). The fluorescent reagent was prepared using 4 mg of 9-AN per ml of DMSO. This solution was stable for several weeks in the refrigerator at 5 °C. HPLC grade acetonitrile, DMSO and magnesium powder were obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical reagent grade. Water was purified using a Milli-Q II water purification unit (Nihon Millipore, Tokyo, Japan). SPE cartridge columns (ion-exchange $NH_2$/NH bonded to silica gel, 40 μm diameter, 500 mg) were supplied by J.T. Baker (Phillipsburg, NJ, USA).

### 2.2. Derivatization

The derivatization procedure was as follows: 0.5 ml of 0.4% 9-AN and 30 mg magnesium powder were added to 2 ml of the working standard or sample solution (0.25–2.5 μg/ml), then the mixed solution was reacted at 80 °C for 80 min. After cooling to room temperature, the mixed solution was applied to a SPE cartridge column which had been preconditioned with 2 ml methanol and 2 ml DMSO. The cartridge was washed subsequently with 6 ml DMSO, 4 ml water and 4 ml 15% acetonitrile. The derivatives in the cartridge were eluted with 8 ml of 0.5 *M* sodium chloride solution in 50% acetonitrile. The eluted solution was made up to a volume of 10 ml with 0.5 *M* sodium chloride solution in 50% acetonitrile. An aliquot (20 μl) of this solution was injected into the HPLC system.

Both peak area calibration graphs for $T_3$-Na and $T_4$-Na were established over the working standard concentration range of 0.25–2.5 μg/ml.

### 2.3. Chromatography

The HPLC apparatus consisted of a Jasco Model 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne injector Model 7125, equipped with a 20-μl loop (Rheodyne, Berkeley, CA, USA), a Jasco Model 960-CO column oven, a Jasco Model 1520-FP fluorescence detector set at excitation and emission wavelengths of 257 and 425 nm, respectively, and a Jasco Model AS-950 autosampler. The HPLC column used was a Cosmocil $5C_{18}$-AR-II (Nacalai Tesque), 150×4.6 mm, 5 μm. The mobile phase was acetonitrile–0.02 *M* sodium dodecylsulfate (SDS), adjusted to pH 3.5 with phosphoric acid (56:44, v/v). Before use, it was filtered through a membrane filter (0.45 μm; Millipore, Bedford, MA, USA) followed by degassing using sonication under vacuum. The eluent was pumped at a flow-rate of 1.0 ml/min and with a column oven temperature of 40 °C.

### 2.4. Sample preparation

Twenty tablets containing $T_3$-Na or $T_4$-Na were weighed and finely powdered. An accurately weighed powder equivalent to about 50 μg of $T_3$-Na or $T_4$-Na was dissolved in 40 ml of 70% methanol–water in a 50-ml flask and ultrasonicated at 45 °C for 30 min. The flask was shaken for 15 min and the solution was made up to 50 ml with 70% methanol–water, and then filtered through a 0.45-μm membrane filter. A 2-ml volume of the filtered solution was evaporated to dryness at 50 °C under reduced pressure. The dry residue was redissolved in 2 ml of

DMSO and was subjected to derivatization and analyzed by HPLC as described above.

## 3. Results and discussion

Three kinds of acyl cyanides (9-AN, 1-anthroylnitrile and pyrene-1-carbonyl cyanide) were tested as derivatization reagents for the determination of $T_3$-Na and $T_4$-Na. The extent of each reaction using these labeling reagents was measured by HPLC with fluorescence detection. The following relative fluorescence intensities (%) were obtained: 9-AN, 100; 1-anthroylnitrile, 90 and pyrene-1-carbonyl cyanide, 60. Hence, the best sensitivity was obtained using 9-AN. The postulated reaction is shown in Fig. 1.

The optimum conditions for the fluorescence derivatization of $T_3$-Na and $T_4$-Na with 9-AN were determined. First, five organic solvents were tested and the results compared. The following relative fluorescence intensities (%) were obtained: DMSO, 100; hexane, 80; ethyl acetate, 40; tetrahydrofuran, 30 and chloroform, 25. DMSO was selected because it gave the highest fluorescence intensity.

The effect of the concentration of 9-AN was studied in the range of 0.02–5 mg per 10 μg of $T_3$-Na or $T_4$-Na. Constant peak areas were obtained above 1.5 mg of 9-AN. A 2-mg amount of 9-AN was chosen for subsequent experiments. The 9-AN was stable for more than 1 month at ordinary temperatures when shielded from light.

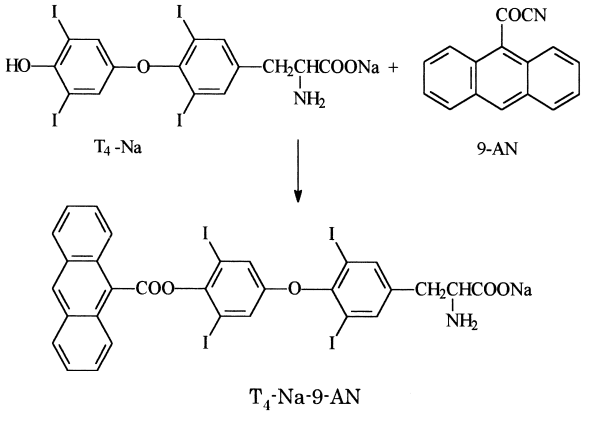


Fig. 1. Fluorescence derivatization of $T_4$-Na with 9-AN.

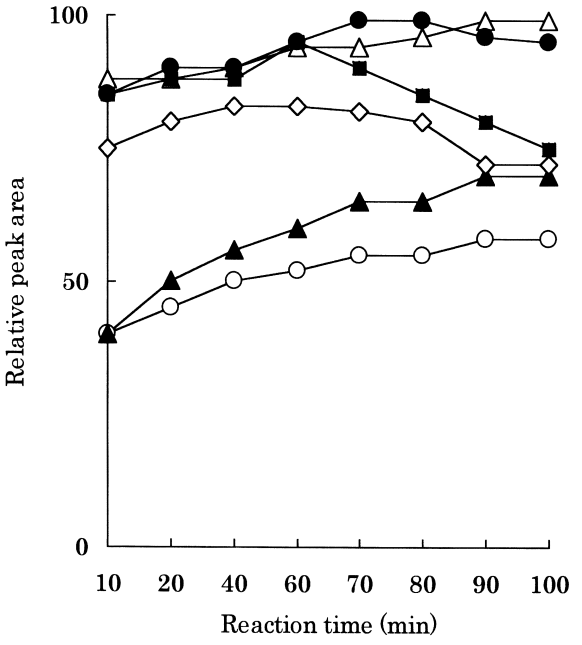


Fig. 2. Effect of reaction time and reaction temperature on the reaction yield of $T_3$-Na–9-AN. (○) at 40 °C, (▲) at 60 °C, (△) at 70 °C, (●) at 80 °C, (■) at 90 °C, (◇) at 100 °C.

Fig. 2 shows the effects of reaction temperature and reaction time on the production of $T_3$-Na derivatives with 9-AN ($T_3$-Na–9-AN). The effects of reaction temperature and reaction time on the production of $T_4$-Na derivatives with 9-AN ($T_4$-Na–9-AN) were the same as that of $T_3$-Na–9-AN. The derivatization reagent, 9-AN, tended to decompose at high temperatures and with long reaction times. The optimum reaction temperature and time were found to be 80 °C and 80 min.

Early in our studies, we found that the reactions with acyl cyanide groups were catalyzed by bases [13]. The effect of catalyst on the reaction was examined by using quinuclidine, magnesium powder, 4-dimethylaminopyridine, triethylamine, pyridine and triethylenediamine. Quinuclidine and magnesium powder gave the most intense fluorescence on reaction with 9-AN—however when quinuclidine was used, we observed the formation of impurities. Therefore, and because of the hazardousness, we decided to use the magnesium powder. The effect of the amount of magnesium powder on the reaction was studied in the range of 10–50 mg per 10 μg of

$T_3$-Na or $T_4$-Na. 30 mg of magnesium powder was sufficient for maximum fluorescence development.

It was necessary to purify the reaction mixture before injection into the HPLC column, because of a few interfering peaks around the retention times of $T_3$-Na–9-AN and $T_4$-Na–9-AN. For this purpose, SPE cartridge columns were tested: a $C_{18}$ column, a SiOH column and $NH_2$/NH column. The reaction mixtures applied to the $NH_2$/NH column, and washed subsequently with 6 ml DMSO, 4 ml water and 4 ml 15% acetonitrile and extraction with 8 ml of 0.5 *M* sodium chloride solution in 50% acetonitrile, gave the best results. Purification on a $C_{18}$ column and SiOH column by loading in DMSO of reaction solvent yielded lower recoveries.

The composition of the mobile phase had a strong influence on the separation of $T_3$-Na–9-AN and $T_4$-Na–9-AN. The optimum mobile phase composition was chosen for the separation of these derivatives from impurities of the derivatization. The influence of acetonitrile, SDS concentration and the pH of the mobile phase on the chromatographic characteristics of the derivatization products were examined systematically.

The capacity factor increased with decreasing concentration of acetonitrile and was not sensitive in the pH range 2.0–5.0. The relationship between the concentration of SDS and the capacity factor was examined by increasing the concentration from 0.005 to 0.1 *M*. The capacity factor rapidly increased with increasing concentrations of SDS. The optimum concentration of SDS in the mobile phase and the column temperature for separating the derivative peaks were 0.02 *M* and 40 °C, respectively. From the results, the appropriate solvent system chosen was acetonitrile–0.02 *M* SDS, adjusted to pH 3.5 with phosphoric acid (56:44, v/v).

When $T_3$-Na–9-AN and $T_4$-Na–9-AN were eluted in the flow-through cell of the detector, the flow of the mobile phase was stopped and both the excitation and emission spectra were optimized. The maximum excitation and emission wavelengths were found to be at 257 and 425 nm, respectively.

Typical chromatograms of $T_3$-Na–9-AN and $T_4$-Na–9-AN are shown in Fig. 3. The derivative peaks were clearly separated from impurities of the derivatization reaction.

The calibration curves of both the $T_3$-Na and

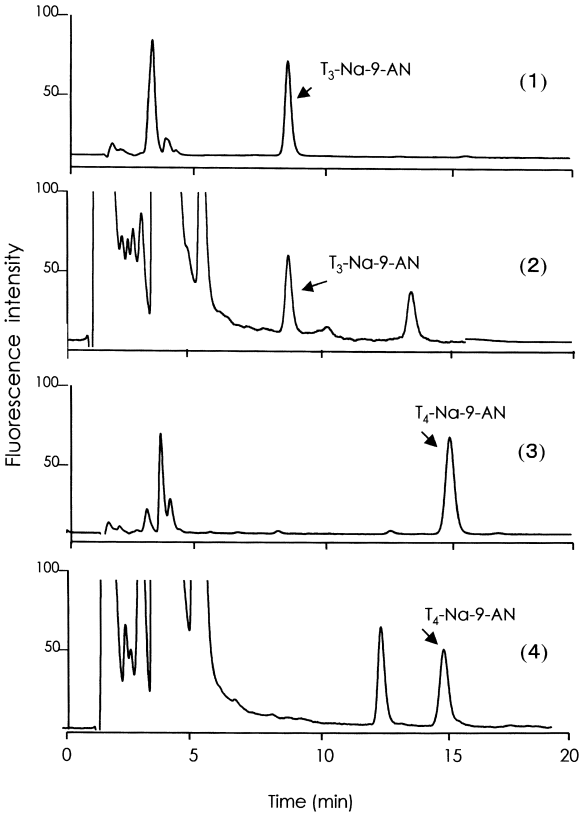


Fig. 3. Chromatograms of a derivatized standard solution (0.2 μg/ml) and a derivatized commercial tablet sample. (1) Derivatives standard solution of $T_3$-Na; (2) derivatives sample solution of (A) ($T_3$-Na tablet); (3) derivatives standard solution of $T_4$-Na; (4) derivatives sample solution of (C) ($T_4$-Na tablet).

$T_4$-Na were linear over the concentration range of 0.25–2.5 μg/ml. The parameters obtained by selected chromatographic conditions for $T_3$-Na and $T_4$-Na derivatives calibrations correspond to: $y = 96.294x - 1.668$ ($r = 0.998$), $y = 129.58x - 1.965$ ($r = 1.000$), respectively. Where $y$ = peak area, $x$ = $T_3$-Na and $T_4$-Na concentrations (μg/ml) and $r$ = correlation coefficient.

The repeatability was also satisfactory; the relative standard deviations (RSD) for 2.0 μg/ml of $T_3$-Na and $T_4$-Na were 2.5% and 2.3% ($n = 5$), respectively.

The detection limit for both $T_3$-Na and $T_4$-Na, under the selected conditions, was about 0.2 ng per injection ($S/N = 3$) which corresponds to a concentration of 0.01 μg/ml in the injected solutions. The limits of detection in most HPLC–UV methods

are 4–50 μg/ml in the injected solution, and these amount of injection are about 50 ng [6–8]. Therefore the low levels of $T_3$-Na and $T_4$-Na in pharmaceutical preparation cannot be determined.

The accuracy of the procedure was determined by spiking a placebo formulation with a known concentration of standard. Recoveries of $T_3$-Na and $T_4$-Na averaged 94.0% ($n=5$) and 97.0% ($n=5$) with RSD values of 4.0 and 3.0% for standard levels of 5 μg/tablet. respectively.

The proposed procedure was applied to the determination of $T_3$-Na or $T_4$-Na in pharmaceutical preparations. Table 1 reports the results obtained from the analyses. Fig. 3 (2,4) shows chromatograms of the $T_3$-Na–9-AN and $T_4$-Na–9-AN from commercial tablet samples. Neither the derivatization nor the chromatographic separation were influenced by pharmaceutical excipients. These results indicate that, in each instance, the values are within acceptable limits. Minimum recovery was 99.8%. The RSD is within 3.0% ($n=5$). The chromatograms of all other samples also showed a sharp peak for $T_3$-Na–9-AN or $T_4$-Na–9-AN with no interference from other substances.

Table 1
Determination of T3-Na and T4-Na in pharmaceutical preparations

| Preparations | Declared amount | Found (%)[a] | RSD ($n=5$) (%) |
|---|---|---|---|
| A (T3-Na tablet) | 5 μg/tablet | 101.9 | 3.0 |
| B (T3-Na tablet) | 25 μg/tablet | 102.2 | 2.2 |
| C (T4-Na tablet) | 50 μg/tablet | 99.8 | 2.9 |
| D (T4-Na powder) | 0.1 mg/g | 100.3 | 2.7 |

[a] Each value is the mean of five measurements.

## 4. Conclusion

HPLC using 9-AN as a derivatization reagent is very appropriate for the determination of $T_3$-Na and $T_4$-Na and offers a significant improvement in sensitivity over previously reported HPLC methods with UV absorbance detection. We believe that the availability of this new method, with its increased sensitivity and selectivity, will be very useful for the determination of thyroid hormones in pharmaceutical preparations.

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