Journal of the Korean Nuclear Society
Volume 7, Number 2, June 1975

≪Technical Report≫ Efficient Preparation of Radioiodine Labelled 3, 5, 3′-Triiodothyronine and Thyroxine for Medical Use

Jaerok Kim and Tae Ho Kim

Korea Atomic Energy Research Institute, Seoul, Korea

(Received December 31 1974)

Abstract

For isotopic exchange labelling of 3, 5, 3′-triiodothyronine (T_3) and thyroxine (T_4) with radioiodide in the presence of molecular iodine, T_3:I_2 or T_4:I_2 molar ratios, pH, and reaction time are. considered to be important factors.

A modified labelling and separation method is proposed in present paper, by which T_3-^{125}I and T_4-^{125}I can be obtained with the mean labelling yields of 45%, and 50%, respectively. The whole reaction products can be separated by adoption of thin-layer chromatography technique using silica gel plate and the solvent system composd of chloroform, methanol and ammonia.

요 약

3, 5, 3′-Triiodothyronine (T_3) 및 Thyroxine (T_4)의 요오드 동위원소 교환표지에 어서는 T_3:I_2 또는 T_4:I_2의 몰비, pH, 반응시간 등이 중요인자 임을 알수 있었다. T_3 및 T_4를 평균 45%, 50%로 각각 표지할 수 있는 개량된 표지반응 조건 및 전 반응생성물을 셀리카 겔 박판과 클로로포름, 메탄올, 암모니아 등을 전개용매로 사용하는 박층크로마토그래피로 신속 간편하게 분리, 정제하는 방법을 제시하였다.

1. Introduction

Many authors have described methods for preparing radioiodine labelled 3, 5, 3′-triiodothyronine (T_3) and thyroxine (T_4) in small scale [1-4]. However, the labelling conditions established so far are still so sensitive that the yields are generally low with poor reproducibilities.

The T_3 labelling conditions are more sensitive than those of T_4 bringing about sometimes more T_4-^{125}I than T_3-^{125}I even though T_3 is subjected to labelling.

These labelled thyroid hormones are quite unstable like others and much influenced by their specific radioactivity, radioactivity concentration, UV light, temperature, and impurities etc. Thereupon, it is considered that the isotope exchange labelling with radioiodide in the presence of molecular iodine without any oxidizing agent[1-3] is much better than the direct radioiodination method[4]. The isotopic exchange reactions occur as following via $^{125}I_3^-$ [2]

Table 1. Comparison of the Status of Labelling T_3 and T_4

Method	T_3 Scale and molar ratio T_3:I_2 (umole)	T_3 pH	T_3 Total vol. of reaction mix. (ml)	T_3 Reaction time (min.)	T_3-^{125}I (%)	Yields* T_4-^{125}I (%)	T_4 Scale and molar ratio T_4:I_2 (umole)	T_4 pH	T_4 Total vol. of reaction mix. (ml)	T_4 Reaction time (min.)	Yields*
I [1]	0.1 : 0.1	8 0.5M Na_2HPO_4	0.23	15	28 (—)	35 (—)	0.1 : 0.4	8 0.5M Na_2HPO_4	0.25	30	30 (—)
II [2]	1 : 0.5	7—8 sat'd Na_2HPO_4	6.0	0.16	25 (40)	10 (5)	1 : 0.5	7—8 sat'd Na_2HPO_4	6.0	0.16	30 (32)
III [3]	0.2 : 0.07	8 no buffer**	0.24	30	34 (33)	8 (14)	0.2 : 0.14	10 ** no bufer	0.24	30	35 (38)
Established in Present Work	0.1 : 0.03	7—8 no buffer**	0.12	10	45	7	0.1 : 0.07	7 no buffer**	0.12	1	50

* average yields of 3 runs. (The numbers in the parenthesis indicate the yields in ref.)
** the pH of the sodium radioiodide itself, and no buffer used.

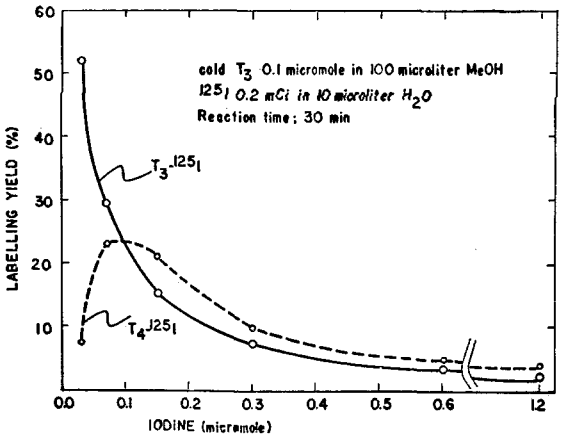

Fig. 1. Molar ratio vs. labelling yield % in the preparation of T_3-^{125}I

$$I_2 + {}^{125}I^- \rightleftharpoons {}^{125}I_2 + I^- \quad (1)$$

$$T\text{-}I + {}^{125}I_2 \text{ (or } {}^{125}I_2\text{-}I^-) \rightarrow T\text{—}{}^{125}I + {}^{125}I\text{—}I \quad (2)$$

(where T is T_3 or T_4)

In case of conversion of T_3 to labelled T_4, direct radioiodination on the 5′ position might be occured due to the presence of excess iodine molecules. Anghileri's results[3] of the studies on the exchange reaction varying the molar ratios of reactants and pH are not consistent; ie, even in case of similar

Table 2. Dependence of Labelling Yield on Radioactivity Concentration in Case of Labelling T_3

Radioactivity of ^{125}I (μCi) in 3 μl	T_3-^{125}I (%)	T_4-^{125}I (%)	$^{125}I^-$ (%)
1,000	57	0.7	43
200	56	0.7	44
20	50	3.0	47
1	42	5.5	52
0.5	34	6.7	59

Table 3. Dependence of Labelling Yield on Reaction Scale

Scale T_3 (μmole)	Scale I_2 (μmole)	Scale Total volume of reaction mix. (μl)	T_3 Labelling Yield (%)
0.2	0.07	240	25
0.1	0.035	120	36
0.05	0.017	60	35
0.025	0.008	30	7

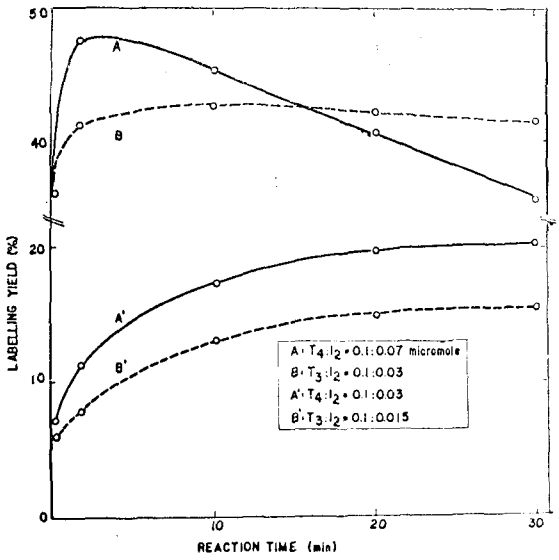

Fig. 2. Reaction time vs. labelling yields

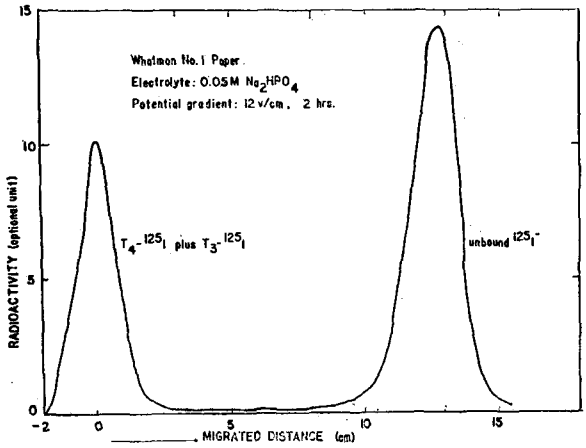

Fig. 3. Typical electrophorogram scan for the reaction mixture of T_3-^{125}I or T_4-^{125}I

reaction conditions, wide variation of yields was reported, and no detailed description was made for efficient and systematic preparation for labelled T_3 and T_4.

Since the labelled T_3 and T_4 are to be used in vitro studies of thyroid function, it is essential that they should have a relatively high specific radioactivity to meet the common characteristics of a tracer. Consequently, in case of only small amount of radioactivity of radioiodine is available, the labelling scale should be minimized as far as possible. Thus, the establishment of the minimum micro scale as well as a prompt separation technique are important in radiochemical point of view.

In present paper, the authors describe the optimum conditions in microscale, labelling, and a simple, rapid separation method.

2. Experimental

1) Radioiodine labelling of T_3 and T_4

a) Method *I*[1] ;One tenth μ mole of pure cold T_3 was dissolved in 50 μl of 0.05 M sodium hydroxide solution. Ten μl (0.2mCi) of sodium iodide-^{125}I (carrier and reducing agent free, pH 8, was then added. To the mixture 50 μl of 0.5M disodium phosphate solution was added to adjust pH to 8, followed by 100 μl of a methanolic solution of elemental iodine containing 0.1 μ mole of iodine. The mixture was shaken well and set aside for 30 min at the end of which 20 μl of conc sodium sulfite solution was added to reduce iodine to iodide.

b) Method II[2] ; To 15 ml of water containing tracer^{125}I (0.2mCi) and 0.5 μ mole of I_2 were carefuly added 2 ml of t-butanol to form an insulating layer. One μ mole of cold T_3 in 2 ml of the same solvent was then carefuly admitted without disturbing the layer. The reaction was initiated by thorough agitation of the system while at the same time the pH was adjusted to 7-8 with 4 drops of saturated disodium phosphate solution. After 10 sec, the reaction was stopped by adding 1 drop of 0.5M sodium sulfite solution.

c) Method III[3] ; Ten μl (0.2mCi) of sodium iodide-^{125}I was placed in a 1 ml stoppered vial and 0.07 μ mole of I_2 in ethanol (2.5mg/ml) was added. After 5 min. 0.2 μ mole of T_3 in methanol (0.31mg/ml) was added. After 30

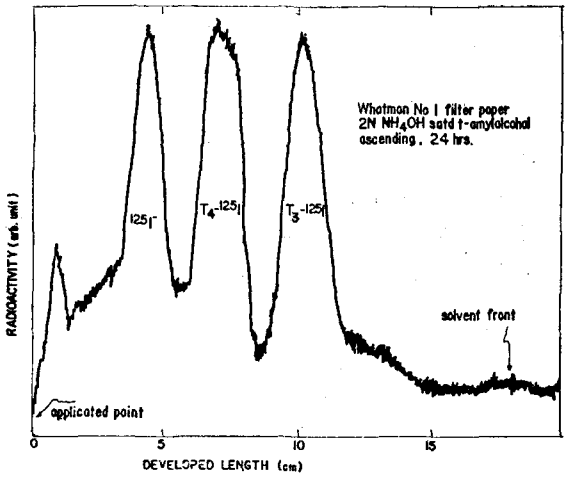

Fig. 4. Typical paper chromatogram scan for T_3-^{125}I reaction mixture

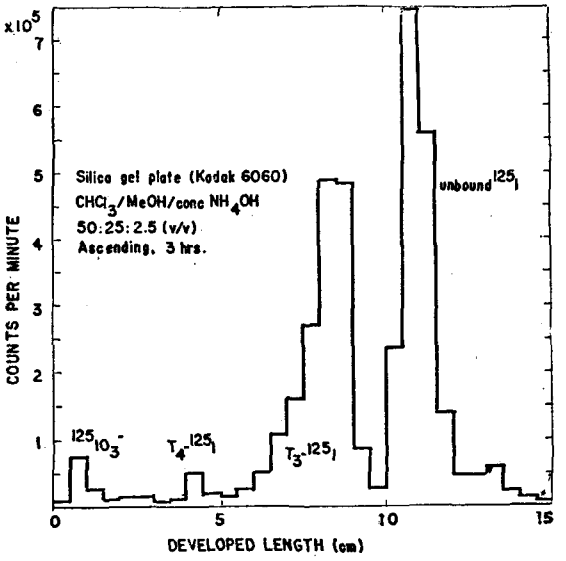

Fig. 5. Typical TLC gram scan for T_3-^{125}I reaction mixture

min. the reaction was stopped by adding 1 drop of 0.5 M sodium sulfite solution.

These three mothods were applied also to the T_4-^{125}I preparations under the conditions described in Table 1.

d) T_3:I_2 molar ratio control in T_3-^{125}I preparations; The procedure was just the same as described in method III except that the I_2 molarity was controlled to 0.03, 0.07, 0.15, 0.30, 0.60, 1.20, μmole, respectively, while the T_3 molarity of 0.1 μmole was kept constant (Fig. 1).

e) Radioactivity control; The procedure was just the same as described in method III except that the ^{125}I activity was controlled to 0.5, 1.0, 2.0, 200, 1,000, μCi respectively, in the same volume. (Table 2).

f) Reaction scale control; Experiments were conducted according to the procedure described in method III. The concentrations of the reactants were kept constant but the volumes of the reactants were proportionaly decreased to 1/2, 1/4, 1/8 etc. (Table 3)

g) Reaction time control; Experiments were conducted according to the procedure described in method III varying reaction time (Fig. 2).

2) Separation and Purification

a) Paper electrophoresis; It was conducted under potential gradient of 12 v/cm for 2 hrs using Whatman No. 1 filterpaper and the electrolyte of 0.05 M disodium phosphate solution[1]. After 2 hrs' run, the electrophoresis paper was sliced to 1 cm intervals, and the radioactivities were counted (Fig. 3).

b) Column chromatography;[5] After 20 g of Celite 545 powder (Jonn Manville Co.) was well moistened with 20 ml of 0.5 N sodium hydroxide in a mortar, it was put into a suction flask. One hundred ml of n-butanol which was saturated with equal volume of 0.5 N sodium hydroxide was poured into the flask. The contents was well mixed by vigorous rotation of magnetic stirrer bar under reduced pressure for 30 min. Then the kneaded Celite was packed in a column of 1 cm in diameter and 60 cm in length. The column was eluted slowly with about 5 ml of n-butanol which is saturated with equal

volume of 0.5 N sodium hydroxide solution, and subsequently with 2 to 3 ml of eluate A (n-butanol saturated with equal volume of 0.5 N NaOH: ethylene dichloride 190:20 in volume ratio) and followed with about 20 ml of eluate B (n-butanol saturated with equal volume of 0.5 N sodium hydroxide: ethylene dichloride 140:60 in volume ratio). Less than 1 ml of the T_3 or T_4 labelling mixture of known radioactivity was applied on the top of the column, eluted with eluate B in rate of 12 to 15 drops per min. for 4 hrs. Then it was eluted with eluate C (n-butanol saturated with equal volume of 0.5 N sodium hydroxide solution: ethylene dichloride 160: 40 in volume ratio) in the same rate for at least 12 hrs. The radioactivities of about 200 fractions were counted and the T_3 or T_4 fraction was collected in one vessel, and condensed with rotating evaporator (Fig. 3).

c) Paper chromatography[2]; Ordinary ascending radio paper chromatography was conducted using Whatman No. 1 filter paper and the solvent system of n-butanol saturated with equal volume of 2 N ammonium hydroxide. The radio chromatograms were scanned or sliced into definite size and counted their radioactivities (Fig. 4).

d) Thin layer chromatography; 20 cm×20 cm sized silica gel TLC plate (Kodak 6060) and the solvent system of $CHCl_3$: MeOH: conc NH_4OH 50:25:2.5 (v/v)[6] were used. In checking labelling yields or radiochemical purities of T_3 or T_4 the chromatostrips were sliced to definite size and counted their radioactivities.

The whole reaction mixture of about 150 μl volume was applied on one plate in case of product separations. After developing, the plate was verticaly cut into three strips in 1 cm width from left, right, and middle side of the plate. The strips were sliced into 5 mm intervals, and the consequent 5 mm×10 mm pieces were counted one by one in numerical order (Fig. 5). The T_3-^{125}I or T_4-^{125}I zone in the whole TLC plate detected by the way was marked, and the silica gel in the zone was scraped up. Then it was charged in a small column (9 mm in diameter and 5 cm in length) and eluted T_3-^{125}I (or T_4-^{125}I) with about 30 ml of methanol or ethanol which contains 1 drop of conc ammonium hydroxide solution. The solvent was evaporated to dryness by air blowing.

3. Results and Discussion

1) Labelling of T_3 and T_4

Our work was all performed at the micro level with analysis carried out by TLC described in experimental part. As table 1 shows, the method *I* gives more T_4-^{125}I than T_3-^{125}I even in case of labelling T_3. Such results may be due to the excess amount of I_2 in the reaction mixture. In method Ⅱ and Ⅲ, T_4-^{125}I is less formed than T_3-^{125}I in T_3 labelling since in these cases the molar ratios of T_3:I_2 are 1:0.5, and 1:0.3, respectively. The results of molar ratio control experiments indicate that the optimum molar ratio of T_3:I_2 for efficient T_3 labelling is 1:0.3, under which conditions less than 7% of T_4-^{125}I was formed (Fig. 1). In methods I and II, it could confirm that the T_3-^{125}I is slowly converted to T_4-^{125}I with increasing reaction time. Thus, T_3-^{125}I yield is maximum in 10 sec after cold T_3 solution is introduced, which is consistent with the data reported by Gleason[2]. Therefore, the results of the abnormally increased T_4-^{125}I product in T_3 labelling according to the procedure of method *I*, is partially attributable to the

relatively long reaction time.

As far as the reaction follows second order kinetics, the rate will be directly increased with increasing the molar concentrations of T_3 and I_2. As the molar concentrations of T_3 and I_2 are both 4.3×10^{-4} umole/1 in method *I*, and 1.6×10^{-4} and 8.0×10^{-5} umole/1 in method II, it is considered that the rate in method I will be faster than that in method II. Thus, the reaction time of 30 min is too long for T_3 labelling in method I.

In method II, the I_2 molarity in respect to that of T_3 is still too much, and consequently the exchange rate is too fast to control. By decreasing the I_2 molarity and by increasing the reaction time it is expected that the rate of T_3-^{125}I conversion to T_4-^{125}I can be decreased and consequently higher T_3 labelling yield can be obtained. As Fig. 2 shows, when T_3:I_2 molar ratio is 0.1:0.015 μmoles in 0.12 ml reaction volume (8.3×10^{-4} μmole/1:1.25×10^{-4} umole/1) and T_4:I_2 molar ratio is 0.1:0.03 μ moles in 0.12 ml reaction volume (8.3×10^{-4} umole/1:2.5×10^{-4} μ mole/1) the reaction rates are not so fast, and only negligible amount of T_4-^{125}I was formed. However, when T_3:I_2 and T_4:I_2 molar ratios are 0.1:0.03 and 0.1:0.07 μmoles in 0.12ml reaction volume the rates are considerably increased (Curve A, B, Fig. 2). Thus, it is considered that the Gleason's data on reaction time (10 sec) is due to the increased molar concentration of I_2 in respect to that of T_3. On the other hand, as Fig. 2 shows, the lower labelling yield of T_4 in method III is attributable to long reaction time. Upon it, we consider that the control of reaction time, and molar ratio of the reactants are essential factors as well as the pH of the reaction mixture and reaction scales. Reviewing the methods I to III, we have found that the T_4:I_2 molar ratio of 1:0.5—1:0.7 μ mole is optimum for T_4 labelling (Table 1). The Anghileri's data on pH control[3] suggest that the lower pH such as 4.1 gives better yield for T_4, and pH 7 to 8 for T_3. The scale control data shows that there is no effect of minimizing the scale on the yields as far as the reactants are well contacted with definite volume (Table 3). In view of simple separation by TLC and maintaining high specific activity of the products, micro scale preparations with the small amount of reactants in small reaction volume is preferable. As Table 2 shows the radioactivity concentration only slightly affects on labelling yields in minimized labelling scale. However, more than 1 mCi of ^{125}I is prefered.

Therefore, we propose that the labelling method should be modified as the following conditions;

T_3: About 10 μl of Na^{125}I (more than 1 mCi, pH 7 to 8, carrier and reducing agent free for protein iodination) is placed in 1 ml stoppered glass vial, and 0.03 μ mole (3.5 μl) of I_2 in ethanol (250 mg I_2 in 100 ml ethanol) is added. After 5 min. 0.1 μmole of cold T_3 (100 μl) (12.7 mg T_3 in 20 ml ethanol) is added, and mixed well. After 10 min, 10 μl of 0.5 M sodium sulfite solution was added, and without any delay the entire reaction mixture is applied on TLC plate for separation of the product as described in experimental part.

T_4; About 10 μl of Na ^{125}I (more than 1 mCi, pH 7, carrier and reducing agent free for protein iodination) is added in 1 ml stoppered vial, and 0.7 μ mole (7 μl) of I_2 in ethanol (250 mg I_2 in 100 ml ethanol) is added. After 5 min 0.1 μ mole (100 μl) of cold T_4 in ethanol (16.8 mg in 20 ml ethanol) is added. After 1 min, 10 μl of 0.5 M sodium sulfite solution is added, and without any delay the

entire reaction mixture is applied on TLC plate for separations as described in experimental part, with good reproducibilities. (Table 1)

Under these reaction conditions, the mean labelling yields of T_3 and T_4 are 45%, and 50%, respectively, with good reproducibilities. (Table 1)

2) Separation and Purification of T_3 and T_4

In the paper electrophoresis, unbound $^{125}I^-$ was separated from the mixture in 2 hrs. The entity on the first peak in the original point was identified to be T_4-^{125}I, and the second to be unbound $^{125}I^-$ (Fig. 3). Therefore, for further purification of T_4-^{125}I or T_3-^{125}I, the radioactive zone in the electrophoresis paper must be eluted with solvent, condensed, and re-applied for chromatography. The results of the Celite column chromatography showed poor reproducibilities of separations probably due to the sensitivity in pre-conditionings in re-use the column. Further, this method needs quite long duration of more than 16 hrs for separation, and is quite complicated.

In paper chromatography, ammonia saturated t-amyl alcohol is well known solvent for separation of iodoaminoacids[2]. However, it takes more than 20 hrs for developing about 20 cm. The Rf values of T_3-^{125}I, T_4-^{125}I and unbound $^{125}I^-$ are 0.50, 0.27 and 0.20, respectively, which are coincide well with those of the literature[2]. However, as Fig. 4 shows the separation efficiencies are still poor, thus, developing more than 20 cm is required.

As shown in Fig. 5, TLC using silica gel plate and the solvent system of $CHCl_3$: MeOH: conc NH_4OH 50:25:2.5 (v/v) are effective. It takes only 2 hrs or slightly more for developing about 15 cm. Further, in this case the radioactive zone of T_3-^{125}I or T_4-^{125}I can be taken out of the TLC plate and eluted easily by using 30 ml of alkaline ethanol or methanol bringing about more than 70% of the product. The Rf values of T_3-^{125}I and T_4-^{125}I are 0.56, and 0.28, respectively, which are roughly coincide with those of the literature[6].

We propose TLC technique is the best for routine separation of T_3 or T_4 labelling mixture because it is simple and rapid.

4. Conclusions

T_3 or T_4 are radioiodine labelled in microscale of 1 mCi level in short time with mean yields of 45%, and 50%, respectively. For separation of the labelled T_3 or T_4 from the reaction mixtures or routine checking the radiochemical purities of the products, TLC using silica gel plate and the solvent system of $CHCl_3$: MeOH: conc NH_4OH 50:25:2.5 (v/v) is the most effective.

Acknowledgements

The authors wish to thank Dr. You Sun Kim for invariable encouragements and valuable advices throughout this work. They also thank Miss Young Hee Kim for her technical assistance in carrying out this work.

References

1) R. S. Mani. Private communications (1968)
2) G. I. Gleason, J. Biol. Chem., **213**, 837 (1955)
3) L. J. Anghileri, Int'l J. App. Rad. Isotopes, **14**, (6), 328 (1963)
4) J. C. Clayton et al, Biochem. J., **46**, 598 (1950)
5) Gross, J. and R. Pitt-Rivers, *ibid.*, **53**, 645 (1953) and its modifications
6) R. H. Osborn and T. H. Simpson, J. Chrom., **40**, 219 (1969).