REVISTA ESPAÑOLA DE FISIOLOGIA, 46 (3), 269-272, 1990

Immunological Characterization of L-Triiodothyronine and L-Thyroxine Labelled with Rhodamine B Isothiocyanate

A. Reyes*, J. L. Diéguez, M. Ruiz, A. Alonso and M. Morell

Departamento de Bioquímica y Biología Molecular
Facultad de Medicina
29080 Málaga (Spain)

(Received on November 7, 1989)

A. REYES, J. L. DIÉGUEZ, M. RUIZ, A. ALONSO and M. MORELL. *Immunological Characterization of L-Triiodothyronine and L-Thyroxine Labelled with Rhodamine B Isothiocyanate.* Rev. esp. Fisiol., 46 (3), 269-272, 1990.

In the present work the label of L-T_3 and L-T_4 with RBITC have been developed by means of a relatively easy procedure. A purification by TLC, previously optimized, offers the possibility of obtaining a good recovery of conjugates T_3 and T_4-RBITC. The immunoreactivity of conjugates was calculated by a formulas especially designed for this purpose. This formulas could be applied to any molecule labelled with fluorescent compounds.

Key words: L-Thyroxine, L-Triiodothyronine, Rhodamine B, Isothiocyanate, Immunoreactivity.

The methods currently used for thyroid hormone measurement rest on the three commonest variants of immunoassay: radioimmunoassay (RIA), fluoroimmunoassay (FIA) and enzymoimmunoassay (EIA) (1). Both FIA and EIA are at present challenging RIA (2). The FIA for thyroid hormones presents several advantages over RIA and EIA i.e. it is an assay which neither requires separation (3) nor addition of reagents for the determination of the end point of reaction. Different methods of labelling thyroid hormones, using isotopes, enzymes (1, 4) and fluorescent (5) and chemiluminescent substances, have been described. In all cases the aim was the development of simple methods for thyroid hormone measurement based on immunoassay. To date fluorescein (3, 6, 7) and tetramethyl-rhodamine (5, 7, 8) have been used for L-triiodothyronine (T_3) and Thyroxine (T_4) labelling. The versatility of fluorescence phenomena allows several approaches for the detection of the end point of a reaction while fluorescence polarization (6) overcomes one of the most difficult methodological problems of RIA i.e., the separation step.

In this work Rhodamine B isothiocyanate (RBITC) is used for T_3 and T_4 labelling and the usefulness of this label is

* To whom all correspondence should be addressed.

evaluated in comparison with other reported procedures.

Materials and Methods

All reagents were of analytical grade or equivalent, LT_3, LT_4, RBITC from Sigma and chromatographic reagents from Merck. Antiserum anti-LT_3, anti-LT_4 and magnet-coupled antiserum were supplied by Amersham. The following instruments were used: a Kontron gamma counter (model Gammamatic), spectrophotometer Shimadzu model UV-240 and spectrofluorimeter Perkin-Elmer, model 650-10S.

RBITC T_3 and T_4 labelling was carried out in an aqueous solution of pyridine (1:1, v:v) at 18 °C and a T_3/RBITC, T_4/RBITC molar ratios of 1. The reaction time was extended for 8 h with continuous stirring. T_3-RBITC and T_4-RBITC conjugates were identified by TLC using 5 × 10 cm plates of silica gel and a mixture of isopropanol-ammonia-water (200:10:20, v:v:v, pH 11.5) as the optimal development solution. The degree of labelling was quantified by direct photodensitometric reading (Zeiss, model PMQ-3) of the chromatographic spots.

Purification of the labelled hormones was carried out by preparative chromatography on 20 × 20 × 0.1 cm silica gel using the development solution described above. Labelled hormones were extracted from the gel by repeated resuspension in ethanol. A spectrofluorimetric study was carried out on all chromatographic fractions isolated as well as on conjugates.

In order to assess the immunoreactivity of the conjugates this parameter was previously evaluated by RIA on known concentrations of unlabelled hormone. Similarly the concentration of labelled hormones was determined by RIA using known amounts of these hormones and taking into account the change in molecular weight induced in them by the labelling process.

Procedure. — T_3-RBITC and T_4-RBITC, 0.8 and 0.7 mg respectively, were diluted to obtain the concentrations shown in tables I and II. A solution containing 6.6 g of albumin/100 ml of 0.9 % saline was used as diluent. T_3 and T_4 concentrations were measured following the protocol suggested in the kit by Amersham.

The stability of the conjugate was evaluated by measuring the fluorescence intensity of 0.1 ml aliquots of pH 7.1 phosphate buffer solutions of T_3-RBITC (4.1×10^{-6} M) and T_4-RBITC (3.5×10^{-6} M) at weekly intervals during 65 days. Samples were kept at −20 °C during this period. The test was carried out at 18 °C and 552 nm and 584 nm emission and excitation wavelengths respectively.

Results and Discussion

Several parameters were studied to optimize T_3 and T_4 labelling with RBITC i.e. medium, molar ratio, temperature and time of incubation.

An aqueous solution of pyridine (1:1 v:v, pH 10.2) was finally used due to the poor solubility of both the dye and the hormone at high concentrations and to the good stability of the pH throughout the experiment. Labelling was most efficient at 18 °C and at an T_3:RBITC and T_4:RBITC molar ratios of 1:1. Although molar ratios higher than these increased the yield of conjugate, some nonspecific binding, due to impurities and isomeric forms of the dye, was detected by TLC. Incubation time was assayed from 1 h to 24 h. Although the highest amount of conjugate was obtained after 24 h incubation the experiments were performed with an incubation period of 8 h.

The identification and purification of the labelled hormone was carried out by TLC on cellulose and on silica. Cellulose was discarded due to the poor definition

of the spots. Among the media assayed for development a solution of isopropanol/ammonia/water (200:10:20 v:v:v, pH 11.5) allowed the identification of the maximum number of components (nine) on TLC and was used in all experiments. two of these components were easily identified as the labelled hormones by staining and Rf studies. Ethanol was selected for hormone extraction from the silica. Dry hormone extracts were obtained by desiccation.

The excitation and emission maxima of RBITC-T_3 are shifted 3 and 6 nm, respectively, towards relatively shorter wavelengths than those of RBITC. The shifts for RBITC-T_4 were of 5 an 7 nm respectively. These variations could be due to the thiocarbamylic covalent bond produced between RBITC and the hormone which changes the physico-chemical microenvironment surrounding the fluorochrome. Although these shifts were significant they were not large enough to allow the identification of both compounds in the same solution.

The fluorescent characteristics of the conjugate were studied at weekly intervals on frozen samples (−20 °C) for a period of six months. No changes were observed in the absorbance or emission maxima or in the intensity of fluorescence during this period.

In order to establish whether immunoreactivity of the hormones was last after labelling, a fraction of conjugate, extracted from TLC and desiccated, was assayed by RIA and the value obtained by RIA was compared with that of the extracts.

In order to calculate the immunoreactivity of the conjugates when dry extracts were obtained, the following formulae were developed:

$$P/MF \times M/P' = Z;\ M'/Z \times 100 = \%\ I$$

where P is weight of the labelled hormone; MF is the molar factor obtained by dividing the molar weight of the conjugate by the molar weight of the unlabelled hormone; P', is the weight of unlabelled hormone and M is the measure by RIA of P'; Z refers to the percentage of conjugate showing immune activity. M' is the conjugate determined by RIA. I % represents the loss of immunoreactivity expressed as a percentage.

The change of molecular weight of the hormones that the labelling involved was evaluated. Taking into account that the molecular weight in daltons of the components considered are: T_3-RBITC, 1180; T_3, 651; T_4-RBITC, 1305 and T_4 776 a correction factor was introduced to evaluate the quantity of immunologically active hormone. These factors were 1.81 for T_3-RBITC and 1.61 for T_4-RBITC.

Another factor related with the immunoreactivity studied was the measurements by RIA of known concentrations of unlabelled T_3 and T_4, in order to assess if the hormones used in the labelling process and those determined by RIA had any quantitative variation. It was observed that the concentrations of T_3 evaluated by RIA were 50 % of the amount evaluated by weighing. This figure was 90 % for T_4. These unexpected results are of special relevance when calculating the immunoreactivity of the conjugate.

Taking into account all these factors it is noticeable that for each 100 weight units

Table I. *Percentage of bound hormone (B) related to maximum binding (B_o) at different concentrations of T_3 and T_3-RBITC, and loss of immunoreactivity of T_3-RBITC.*

T_3 nmol/L	T_3 $B/B_o \times 100$	T_3-RBITC $B/B_o \times 100$	T_3-RBITC % loss immunoreactivity
50.00	35.32	79.39	68.13
25.00	62.26	88.44	69.36
12.50	85.48	95.91	71.83
6.25	92.40	97.34	73.44
3.12	96.20	99.66	69.87
	r = 0.994 $p \leq 0.001$	r = 0.994 $p \leq 0.001$	

Table II. *Percentage of bound hormone (B) related to maximum binding (B_o) at different concentrations of T_4 and T_4-RBITC, and loss of immunoreactivity of T_4-RBITC.*

T_4 nmol/L	T_4 $B/B_o \times 100$	T_4-RBITC $B/B_o \times 100$	T_4-RBITC % loss immunoreactivity
200	5.24	45.22	42.19
100	53.85	70.72	37.50
75	64.74	77.36	36.44
50	75.74	84.48	36.02
25	90.50	93.30	29.47
	$r = 0.999$ $p \leq 0.001$	$r = 0.998$ $p \leq 0.001$	

of T_3 it is necessary, first to divide by 1.81 as molar correction factor and second to deduct 50 % as deficit of immunoreactivity of the unlabelled hormone. Therefore, of each 100 weight units of conjugate only 24.5 are possibly immunologically active. Applying the same principles to T_4, this was found to be 50.3.

Considering these corrections and after measuring the labelled hormones (tables I and II) it was calculated that the loss of immunoreactivity of the T_3-RBITC was about 70 % and that of T_4-RBITC about 36 %.

It can be concluded from this study that the ease of labelling and purification of thyroid hormones with RBITC, the maintenance of the fluorescence properties of the conjugate and the high degree of immunoreactivity obtained with this technique can be developed as an alternative (2) to RIA and FIA (with other label) for the measurement of thyroid hormones. This would be favoured by the characteristic of the fluorochrome used. RBITC has a high quantic yield, a factor which significantly influences its sensitivity and whose maxima of absorption and emission are far from the possible interference normally experienced with biological fluid. The application of this technique offers the possibility of an improvement over the isotopic and fluorescent methods used at present.

Resumen

Se describe un método relativamente fácil de marcaje de L-T_3 y L-T_4 con RBITC. La purificación por TLC, previamente optimizada, ofrece la posibilidad de obtener una buena recuperación de conjugados de T_3 y T_4-RBITC. La inmunorreactividad de los conjugados se calcula utilizando una fórmula diseñada para este propósito, la cual podría ser aplicada a cualquier molécula marcada con compuestos fluorescentes.

Palabras clave: L-Tiroxina, L-Triiodotironina, Isotiocianato de rodamina B, Inmunorreactividad.

References

1. Proenca, M. T. and De Moura, L. S.: *Clin. Chem.*, 31, 767-768, 1985.
2. Kaplan, L. A., Chen, I. W., Gan, N., Fearn Ji Maxon, H., Volle, C. and Stein, E. A.: *Clin. Biochem.*, 14, 302-307, 1985.
3. Hassan, M., Landon, J. and Smith, D. S.: *J. Immunoassay*, 3, 1-15, 1982.
4. Eruk, A. A., Washington, R. J. and Laing, I.: *Ann. Clin. Biochem.*, 21, 434-443, 1984.
5. Symmons, R. G. and Vining, R. F.: *Clin. Chem.*, 31, 1342-1348, 1985.
6. Muratsugu, M. and Makino, M.: *J. Clin. Chem. Clin. Biochem.*, 20, 67-70, 1982.
7. Van der Werf, P. and Chang, C. H.: *J. Immunol. Methods*, 36, 339-347, 1980.
8. Liappis, N.: *Klin. Pediatr.*, 197, 302-307, 1985.