# RADIOIMMUNOANALYTICAL DETERMINATION OF THE SPECIFIC ACTIVITY OF [$^{125}$I]L-THYROXINE

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The possibility to determine directly the specific activity of [$^{125}$I]L-thyroxine by the usual radioimmunoanalytical procedure using the data required for the determination of the calibration graph is demonstrated in this study. This approach, which is from the point of view of the mathematical formalism similar to the double substoichiometric isotopic dilution, can be used — due to its simplicity and sufficient precision in certain cases — for the determination of the specific activity of radioindicators used in the radioimmunoanalysis, particularly for the univalent (from the point of view of immunochemistry) haptenes. The method is limited by the index of heterogeneity of the used antiserum.

Specific activity is an important characteristics of the radioindicator used in the radioimmunoanalysis. The requirement of its precise knowledge is still more important for those radioindicators the specific activity of which does not attain the maximum possible specific activity ($a_s$)[1]. In this case the substance under determination appears in the reacting system not only in the form of the standard or of the sample in which the substance has to be determined but also in a non-negligible amount in the form of the radioindicator or as its carrier. The specific activity of substances with small molecules is practically determined using various methods: *e.g.*, in the radioiodination of digoxine[2] or steroids[3,4] bound by covalent bonds to thyramine the mono-iodo or diiodo derivatives of this so-called conjugate are formed. Both forms can be separated by chromatography from each other and also from the non-iodinated conjugate. The specific activity can be calculated[5] from the measured activity of individual derivatives and from the specific activity of sodium iodide labelled with $^{125}$I used for radioiodination. This method cannot be used for substances that contain iodine atoms in their molecules already in their initial state. Thyreoidal hormones are a typical case: after the radioiodination it is impossible to separate the radioactive and non-labelled hormones by chemical procedures. While the determination of the total activity of the radioindicator usually does not present any problems, many problems can be met in attempts to determine the mass of the radioindicator (usually of the order of pg).

In our previous work we compared the results of the determination of the specific activity of [$^{125}$I]L-thyroxine by three different methods[6]. The first one used the known mass of the hormone added to the mixture for labelling and the measured distribution of radioiodine in various chemical forms, in which it appeared after labelling[7]. This procedure gives inaccurate results in the case that not only monoiodo but also diiodo derivatives are present. In the second case the specific activity was determined by the so-called "self-displacement" method[8]. Contrary

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to other authors[9] it has been found that the value of the non-specific bond is not negligible if 2—3 or n-fold excess of [$^{125}$I]L-thyroxine is added[10]. The third method uses the difference between the energies of gamma radiation of $^{125}$I and $^{131}$I. In principle, [$^{131}$I]L-thyroxine is used as the radioindicator and [$^{125}$I]L-thyroxine is determined by radioimmunoanalysis[11] as the unknown substance. In spite of rather good results the necessity to prepare [$^{131}$I]L-thyroxine is a serious shortcoming of this method.

In this work we demonstrate the possibility to determine the specific activity of [$^{125}$I]L-thyroxine directly in course of the usual radioimmunoanalytical procedure using the data measured for the determination of the usual calibration graph.

## EXPERIMENTAL

Where particularly mentioned, the reagents were adjusted in the veronal buffer solution (0·03 mo . $l^{-1}$, pH 8·6) with the addition of 0·2% (w/w) of the bovine serum albumine (BSA, ÚSOL Prague). The veronal buffer was prepared using reagents (diethylbarbituric acid and sodium diethyl barbiturate) of the analytical grade (Lachema, Brno). The rabbit's antithyroxine antiserum (ÚEE SAV Bratislava) used in the experiments was diluted by the buffer solution in the ratio 1 : r (relatively to the concentrate) as required. The radioindicator [$^{125}$I]L-thyroxine was prepared by the chloramine method [12–14] and adjusted in the buffer. [$^{131}$I]L-thyroxine used for the radioimmunoanalytical determination of the amount of [$^{125}$I]L-thyroxine was prepared by an analogous method. Polyethylene glycol (PEG 1 500, CHZWP, Nováky) in the form of 40% (w/w) aqueous solution[15] was used as the reagent for the separation of thyroxine bound into the complex with the antibody from the free thyroxine. 1% (w/w) buffer solution of the human gamma globuline (NORGA, Imuna, Šar. Michaľany) was for the intensification of the visual effect of the precipitation of the mentioned complex and for the higher effectiveness of centrifugation. Two sets of independently prepared standard solutions of thyroxine of a known concentration (Table I) were used for the radioimmunoanalysis. The lyofilized standards in buffer solutions were reconstituted before use by the addition of 1 ml of distilled water.

The following apparatuses were used: thermostat TER 5/1 (Chirana, Stará Turá), cooled centrifuge K-24 (Janetzki, GDR), gamma counter NRG 603 (Tesla, Liberec) with the 43% effectiveness for the gamma radiation of $^{125}$I, micropipettes Brand 100 µl, Gilson 1 000 µl, and 5 ml polypropylene probes (Chirana, Nové Město na Moravě). The mathematical treatment of the results was carried out on the computer ADT 4300 (ZPA, Čelákovice).

TABLE I

Molar concentrations ($[Ag]_x$ in nmol/l) of the standard thyroxine solutions used

| Set No | Standard | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 1·25 | 2·514 | 3·28 | 4·386 | 6·56 | 13·87 |
| 2 | 1·15 | 2·22 | 4·19 | 5·33 | 7·36 | 11·06 |

The specific activity of [$^{125}$I]L-thyroxine was determined also by reference methods[6]: by the computation from the known amount of thyroxine before labelling and from the measured distribution of various radiochemical forms of $^{125}$I, and also from the radioimmunoanalysis in which [$^{125}$I]L-thyroxine was used as the sample of unknown amount and [$^{131}$I]L-thyroxine as the radioindicator. For the direct determination of the specific activity of the radioindicator from the usual radioimmunoanalytical data the radioimmunoanalysis was carried out in the following way: 100 μl of solutions — mixed in the sequence: the respective x-th standard solution of thyroxine, gamma globuline, antiserum, and finally the radioindicator — were pipetted into the polypropylene probes and the reaction mixture was kept in the thermostat at 310 K for 1·5 h. Then the reaction in each probe was stopped by the addition of 500 μl of PEG solution. The mixture was vigorously shaken and the formed precipitate was separated by centrigufation at 277 K (2 000*g*, for 10 minutes). The supernatant was carefully removed by the water pump and the precipitate activity was measured on the gamma counter for 100 s. The value of the non-specific bond was subtracted from the measured value of activity. The obtained value of the specifically bound activity ($B_x$) was used in the subsequent calculations. The nonspecific bond[16] was determined by the measurement of the precipitate activity after an analogous treatment of the reaction mixture consisting of 100 μl of the radioindicator, 100 μl of gamma globuline, and 200 μl of the buffer solution. The total activity of the radioindicator $T_r$ was obtained by the simple measurement of the total activity of 100 μl of the radioindicator on the gamma counter for 100 s and subtracting the measured non-specific bond $Z$ from this value.

## THEORETICAL

Eqs (*1*) and (*2*) can be written for the molar activity of thyroxine in the separated fractions of the thyroxine complex with the antibody

$$a_x = B_x/[B]_x\,, \tag{1}$$

$$a_{x-1} = B_{x-1}/[B]_{x-1}\,, \tag{2}$$

where $[B_x]$ is the molar concentration of thyroxine bound into the complex, the computation of which is given in paper[17]. For the concentration of the radioindicator [*Ag], *i.e.* for the concentration of thyroxine added to the reaction mixture in the form of the radioindicator, the Eqs (*3*) and (*4*), respectively, derived from the simple condition of proportionality, can be written

$$[{}^*\mathrm{Ag}] = \frac{[\mathrm{Ag}]_x}{\left(\frac{a_s \cdot M_{\mathrm{Ag}}}{a_x} - 1\right)}\,, \tag{3}$$

$$[{}^*\mathrm{Ag}] = \frac{[\mathrm{Ag}]_{x-1}}{\left(\frac{a_s \cdot M_{\mathrm{Ag}}}{a_x} - 1\right)}\,, \tag{4}$$

where $a_s$ is the specific activity of the used radioindicator for which Eq. (*5*) can also be written

$$a_s = T_r/[{}^*\mathrm{Ag}] \,.\, M_{\mathrm{Ag}}\,, \tag{5}$$

$M_{\mathrm{Ag}}$ in these equations means the molecular weight of the antigen under determination. Solving

Eqs (*3*) and (*4*) the relation (*6*) can be obtained,

$$[^*\mathrm{Ag}] = \frac{[\mathrm{Ag}]_x \cdot a_x - [\mathrm{Ag}]_{x-1} \cdot a_{x-1}}{a_{x-1} - a_x} . \qquad (6)$$

Therefore, for the evaluation of the radioindicator concentration it is necessary to know — along with the experimentally easily obtainable values of $B_x$ — also the values of $[B_x]$. The evaluation of these values is more complicated and the specific activity of the radioindicator must be known for their correct determination. However, in one case these values need not be known exactly, namely when at least in two points (of all points on the calibration graph) the relation (*7*) is valid,

$$[B]_x = [B]_{x-1} . \qquad (7)$$

Using this relation in Eqs (*1*) and (*2*) we obtain for this special case (*i.e.*, for the case when Eq. (*7*) is valid) the relation (*8*),

$$[^*\mathrm{Ag}] = \frac{[\mathrm{Ag}]_x \cdot B_x - [\mathrm{Ag}]_{x-1} \cdot B_{x-1}}{B_{x-1} - B_x} \qquad (8)$$

which has the same form as the relation used in the double substoichiometric isotopic dilution[18].

Eq. (*8*) can be generalized for the case that the condition (*7*) is valid for $n$ points of the calibration graph. The number $k$ of pairs for which the value of [*Ag] can be calculated is then given by

$$k = n(n - 1)/2 \qquad (9)$$

and Eq. (*8*) can be rewritten in the form

$$[^*\mathrm{Ag}]_i = \frac{[\mathrm{Ag}]_x B_x - [\mathrm{Ag}]_{x-1} \cdot B_{x-1}}{B_{x-1} - B_x} , \qquad (10)$$

where $x = 2, \ldots, n$ and $i = 1, \ldots, k$.

For the average value of the radioindicator concentration calculated from the experimental data corresponding to $n$ points complying with the condition (*7*) we obtain

$$\frac{\sum_{i=1}^{k} [^*\mathrm{Ag}]_i}{k} = \overline{[^*\mathrm{Ag}]} , \qquad (11)$$

where $\overline{[^*\mathrm{Ag}]}$ is the required value of the average concentration of antigen added to the reaction mixture in the form of the radioindicator.

## RESULTS AND DISCUSSION

Using Eq. (*11*) the radioindicator concentration can be calculated and the specific activity $a_s$ of the radioindicator is then given by Eq. (*5*) using the usual experimental data obtained by the radioimmunoanalysis. Eq. (*7*) means that in the reactions of the $x$-th and $(x - 1)$-th standard the identical amount of thyroxine is bound into

the complex with the antibody in the reaction mixture containing the standard solution, radioindicator, and antiserum. This case can be detected by a characteristic dependence of $[B]_x$ *vs* $[Ag]_x$ using the transformation introduced in[17]. If the condition given by Eq. (7) is fulfilled in more points of the calibration graph this transformation gives curves of the type *B* in Fig. 1. This curve was obtained in the radioimmunoanalysis with the antiserum diluted in the ratio 1 : 800; in the case of a lower or higher dilution of the antiserum the curves *1* and *3*, respectively, were obtained (Fig. 1). Radioimmunoanalytical data, required for the subsequent calculations, that were measured for these three experiments, are listed in Table II. Eq. (8) was applied to these results even though the theoretical justification for the use of this

TABLE II

Radioimmunoanalytical data ($B_x$ in MBq/l) required for the computation of the concentration of added radioindicator

| Standard | *r* | | |
|---|---|---|---|
| | 400 | 800 | 1400 |
| 1 | 1·332 | 0·825 | 0·480 |
| 2 | 1·242 | 0·537 | 0·274 |
| 3 | 1·129 | 0·442 | 0·193 |
| 4 | 0·922 | 0·354 | 0·174 |
| 5 | 0·731 | 0·286 | 0·073 |
| 6 | 0·362 | 0·134 | 0·030 |

TABLE III

Comparison of the radioimmunoanalytically determined specific activity ($a_s$ in TBq/g) with the values determined by the reference methods (values of $T_r$ is given in MBq/l, [*Ag] and SD in nmol. $.l^{-1}$ and CV in %)

| Method | $T_r$ | [*Ag] | SD | CV | *n* | $a_s$ |
|---|---|---|---|---|---|---|
| Calculated | – | – | – | – | – | 6·17 |
| $^{131}$I (RIA) | – | – | – | – | – | 6·23 |
| *r* = 400 | 6·436 | 4·435 | 3·987 | 89·7 | 6 | 1·86 |
| *r* = 800 | 6·178 | 1·18 | 0·112 | 9·49 | 5 | 6·74 |
| *r* = 1 400 | 6·641 | – | – | – | – | – |

relation is fulfilled only for the majority of radioimmunoanalytical points for the antibody dilution $r = 800$. Table III presents the results of this treatment of the radioimmunoanalytical data. The average concentration of the radioindicator and the SD of this value were calculated using Eq. (*8*). In the case of the antiserum diluted in the ratio 1 : 400 the concentration value was determined with a high value of SD, the variation coefficient reaches the value of 89·7%. Similarly, this method yields an exceptionally low value of the specific activity if compared with the two reference methods. On the other hand, in the case of the antiserum dilution in the ratio 1 : 800, where the use of this requation is theoretically justified, we obtained a much lower average value of the radioindicator concentration and, namely, with a much lower SD (CV = 9·49%). A good agreement between the determined specific

TABLE IV

Radioimmunoanalytical data ($B_x$ in MBq/l) required for the calculation of the concentration of added radioindicator

| Standard | *r* | | |
|---|---|---|---|
| | 333 | 500 | 333 |
| 1 | 1·408 | 1·910 | 3·580 |
| 2 | 1·316 | 1·695 | 3·038 |
| 3 | 0·974 | 1·153 | 2·390 |
| 4 | 0·881 | 0·963 | 2·054 |
| 5 | 0·623 | 0·710 | 1·643 |
| 6 | 0·496 | 0·542 | 1·284 |

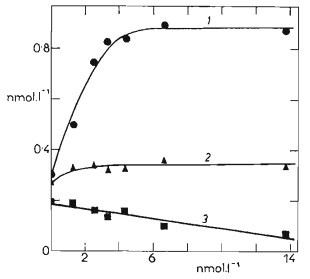


FIG. 1

Dependence of the values of $[B]_x$ on the concentration of antigen added in the form of standard solutions. Antiserum dilution: 1 1 : 400; 2 1 : 800; 3 1 : 1 400

activity and the values of reference methods has been obtained. In the case of 1 : 1 400 dilution Eq. (*8*) cannot altogether be used because it yields negative values of concentrations. This is the consequence of the fact that Eq. (*7*) is not valid and, moreover, the antigen concentration bound into the complex with the antibody decreases with the increasing concentration of the standard (Fig. 1, curve 3).

These results were verified also for another radioindicator and for a new set of standard thyroxine solutions (Table I, set 2). The respective radioimmunoanalytical data are given in Table IV and the corresponding results of the application of Eqs (*8*) and (*5*) are summarized in Table V. Antiserum dilutions $r = 333$, $r = 500$, and again $r = 333$ were used but in the last case the volume activity of the same radioindicator was much higher than in the first case of the same dilution. The results obtained

TABLE V

Comparison of the radioimmunoanalytically determined specific activity ($a_s$ in TBq/g) with the values determined by the reference methods (the value of $T_r$ is given in MBq/l, [*Ag] and SD in nmol/l, and CV in %)

| Method | $P_r$ | [*Ag] | SD | CV | $n$ | $a_s$ |
|---|---|---|---|---|---|---|
| Calculated | — | — | — | — | — | 7·68 |
| $^{131}$I (RIA) | — | — | — | — | — | 7·02 |
| $r = 333$ | 6·26 | 4·049 | 1·77 | 43·71 | 6 | 1·99 |
| $r = 500$ | 10·51 | 1·95 | 0·059 | 3·07 | 5 | 6·937 |
| $r = 333$ | 19·16 | 3·78 | 0·424 | 11·42 | 5 | 6·649 |

TABLE VI

Determination of the specific activity ($a_s$ in MBq/l) by several independent radioimmunoanalyses (the value of $T_r$ is given in MBq/l, [*Ag] and SD in nmol/l, and CV in %) at $r = 800$

| No | $P_r$ | [*Ag] | SD | CV | $n$ | $a_s$ |
|---|---|---|---|---|---|---|
| 1 | 8·139 | 1·654 | 0·343 | 20·7 | 5 | 6·33 |
| 2 | 5·978 | 1·272 | 0·201 | 15·8 | 6 | 6·05 |
| 3 | 6·202 | 1·337 | 0·087 | 6·51 | 6 | 5·97 |
| 4 | 6·837 | 1·3708 | 0·212 | 15·46 | 5 | 6·42 |
| 5 | 6·555 | 1·3245 | 0·34 | 25·67 | 5 | 6·37 |
| 6 | 6·839 | 1·469 | 0·157 | 10·68 | 5 | 5·99 |
| 7 | 6·12 | 1·2872 | 0·283 | 21·98 | 6 | 6·12 |

in the first two cases are analogous to the preceding experiments – in the case of low dilution a high average concentration of the radioindicator with high CV and a disagreement between the calculated specific activity and the values determined by the reference methods have been obtained. On the other hand, in the case of $r = 500$ a good agreement has been observed. Increasing the volume activity of the radioindicator introduced into the reaction mixture in the third case we have tried to achieve a similar state as in the case of $r = 500$, *i.e.*, to achieve the approximately constant value of $[B_x]$ *vs* $[Ag]_x$. We have succeeded only partially (Fig. 2) as proved also by the value of the variation coefficient and by the slightly lower value of the calculated specific activity in comparison with the values determined by the reference methods.

Finally, we have made several independent radioimmunoanalyses with the antiserum dilution of $r = 800$ and with small variation of the volume activities of the radioindicator. Standards of the set No 1 (Table I) were used. The experimental results are given in Table VI. The calculated average specific activity from these experiments is equal to 6·178 TBq $g^{-1}$ (with the variation coefficient 3·07%) and is in excellent agreement with the values obtained by the reference methods (Table III).

It can be concluded that the worked-out method is – due to its simplicity and sufficient precision – suitable for the determination of the specific activity of radioindicators used in the radioimmunoanalysis of univalent (from the point of view of immunochemistry) haptenes as, *e.g.*, thyroxine. It is much simpler than the radioimmunoanalytical method using the different energies of gamma radiation of $^{131}I$ and $^{125}I$ and it is more precise than the computational method (from the distribution of the radiochemical forms of $^{125}I$ in the mixture after labelling) which cannot include into the computation the corrections for the loss of the initial amount of the

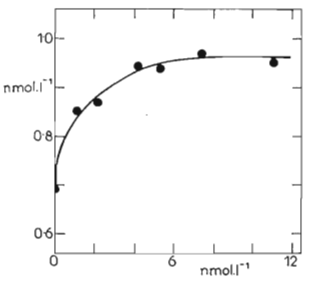


FIG. 2

Dependence of the values of $[B]_x$ on the concentration of antigen added in the form of standard solutions (antiserum dilution 1 : 333, increased volume activity of the radioindicator)

antigen used for labelling in course of the operations and, moreover, it gives substantially incorrect results if diiodo derivatives are present[6]. However, the assumption of the heterogeneity index[19] of the used antiserum close to unity is very important.

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