

Z. Klin. Chem. Klin. Biochem.  
13. Jg. 1975, S. 571–574

## Radioimmunoassay of 3,3',5'-Triiodo-L-thyronine (Reverse T<sub>3</sub>) in Human Serum and its Application in Different Thyroid States

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(Eingegangen am 29. Juli/8. Oktober 1975)

**Summary:** A radioimmunoassay for the measurement of 3,3',5'-triiodo-L-thyronine (reverse T<sub>3</sub>, rT<sub>3</sub>) has been developed. The known limitations of this technique have been overcome by the use of the biologically relevant L-compound for the production of highly specific antisera and for preparing the standard curve. The high sensitivity of the assay (lower limit of detection 20 ng/l serum) was obtained by using <sup>125</sup>I-labelled rT<sub>3</sub> of maximum specific radioactivity. Mean serum rT<sub>3</sub> concentrations for various thyroid states were as follows: Normal subjects: 0.182 µg/l (0.280 nmol/l), hypothyroidism: 0.038 µg/l (0.058 nmol/l), hyperthyroidism: 0.522 µg/l (0.802 nmol/l), pregnant: 0.200 µg/l (0.307 nmol/l), newborn (cord serum): 2.11 µg/l (3.24 nmol/l). The method described should provide additional information with regard to the clarification of thyroxine metabolism.

*Radioimmunoassay für 3,3',5'-Triiod-L-thyronin (reverses T<sub>3</sub>) im menschlichen Serum und seine Anwendung bei verschiedenen Funktionszuständen der Schilddrüse*

**Zusammenfassung:** Es wird die Entwicklung eines radioimmunchemischen Verfahrens zur Bestimmung von 3,3',5'-Triiod-L-thyronin (reverses T<sub>3</sub>, rT<sub>3</sub>) im menschlichen Serum beschrieben. Durch die Verwendung der biologisch relevanten L-Verbindung zur Gewinnung hochspezifischer Antisera sowie zum Ansatz der Standardkurven wurden Nachteile der einzigen bisher beschriebenen Methode überwunden. Die hohe Empfindlichkeit der Methode (untere Nachweisgrenze 20 ng/l Serum) wurde durch Benutzung von <sup>125</sup>Jod-markiertem rT<sub>3</sub> mit maximaler spezifischer Radioaktivität erreicht. Für verschiedene Funktionszustände der Schilddrüse wurden die folgenden mittleren rT<sub>3</sub>-Serumkonzentrationen gemessen: Normalpersonen: 0.182 µg/l (0.280 nmol/l), Hypothyreose: 0,038 µg/l (0,058 nmol/l), Hyperthyreose: 0.522 µg/l (0,802 nmol/l), Schwangere: 0,200 µg/l (0,307 nmol/l), Neugeborene (Nabelschnurserum): 2,11 µg/l (3,24 nmol/l). Die beschriebene Methode dürfte es ermöglichen, weitere Informationen zur Klärung des Thyroxinmetabolismus zu erhalten.

In the recent years it has been clearly demonstrated that L-thyroxine (T<sub>4</sub>)<sup>1)</sup> is converted into 3,5,3'-triiodo-L-thyronine (T<sub>3</sub>) in the peripheral tissue of man (1, 2, 3) and it has been concluded that T<sub>3</sub> may be the biologically active hormone (3). The assumption that T<sub>4</sub> is a mere prohormone for T<sub>3</sub> derives from kinetic calculations which preclude that all available T<sub>4</sub> is converted by monodeiodination. This seems to be the case if a random deiodination to T<sub>3</sub> and to 3,3',5'-triiodo-L-thyronine (reverse T<sub>3</sub>, rT<sub>3</sub>) is assumed (3). rT<sub>3</sub> is currently regarded to be biologically inactive (4). However, since quantitative determinations of rT<sub>3</sub> were not possible, no data about the generation of rT<sub>3</sub> from T<sub>4</sub> are available at the present time.

It was only recently that the natural existence of rT<sub>3</sub> in human serum could be established using a radioimmunological method (5). Some questions regarding the use of racemic D,L-rT<sub>3</sub> in this method led us to the development of a radioimmunoassay for the measurement of L-rT<sub>3</sub> in serum.

### Materials and Methods

#### Antisera

Highly specific antisera against L-rT<sub>3</sub> were raised in two rabbits by serial injections of L-rT<sub>3</sub> conjugates to bovine serum albumin, using the procedure described earlier for the production of antisera against T<sub>3</sub> and T<sub>4</sub> (6, 7). The antiserum selected for use in the assay of rT<sub>3</sub> was obtained 10 weeks after the initial injection. It was used in a final dilution of 1:18 000.

<sup>1)</sup> Abbreviations: rT<sub>3</sub> = 3,3',5'-triiodo-L-thyronine; D,L-rT<sub>3</sub> = 3,3',5'-triiodo-D,L-thyronine; T<sub>3</sub> = 3,5,3'-triiodo-L-thyronine; T<sub>4</sub> = L-thyroxine

The rT<sub>3</sub> conjugate to bovine serum albumin and all iodothyronines used in this study were provided by the courtesy of Dr. H. Steinmaus, Henning Berlin GmbH.

#### Preparation of <sup>125</sup>I-labelled rT<sub>3</sub>

A modification of the method of Weeke & Ørskov (8) for labelling thyroid hormones with chloramine T was used. All reagents were added in volumes of 20 µl each. 2 mCi <sup>125</sup>Iodine (Radiochemical Centre, Amersham), which was buffered with 20 µl 0.5 mol/l phosphate buffer pH 7.5, 2 µg (3.8 nmol) 3,3'-diiodo-L-thyronine and 100 µg (355 nmol) chloramine T (Merck, Darmstadt) were incubated for 30 seconds. Then 250 µg (1.32 µmol) sodium disulfite was added. The reaction mixture was transferred to a Sephadex G 25 (fine) column (12 × 1 cm) and eluted with 0.05 mol/l phosphate buffer pH 7.5. The radioiodinated rT<sub>3</sub> peak appeared after the peak of labelled 3,3'-diiodo-L-thyronine with yields between 59 to 70%. Inorganic <sup>125</sup>Iodine (4 to 5%) was observed in the first fractions of the eluate. The reaction products were identified by paper and thin layer chromatography using unlabelled iodothyronines as standards. The peak fraction of labelled rT<sub>3</sub> was rechromatographed on Sephadex G 25. The specific radioactivity of the purified product (approx. 3300 µCi/µg) approached the theoretical maximum value.

#### Extraction of sera

0.5 ml serum was mixed with 1 ml absolute ethanol. After centrifugation of the precipitated proteins, 300 µl aliquots of the supernatant which were equivalent to 100 µl of original serum, were evaporated to dryness and used for rT<sub>3</sub> assay. The extraction yield was estimated to be 80.2 ± 2.2% using labelled rT<sub>3</sub> as tracer. For recovery experiments, known amounts of unlabelled rT<sub>3</sub> were added to sera, which were then extracted in the routine way.

#### Assay procedure

All dilutions were made in 0.08 mol/l barbital buffer pH 8.6 containing 2 g/l bovine serum albumin and 200 mg/l sodium azide. Reagents were incubated as follows: Evaporated serum extract (representing 100 µl serum), or 100 µl standard solution (from 1.6 to 424 pg, i.e. 2.5 to 650 fmol rT<sub>3</sub>), 100 µl tracer solution (containing approx. 6 pg <sup>125</sup>I-rT<sub>3</sub> and 0.5 µl normal rabbit serum), 100 µl antiserum to rT<sub>3</sub> diluted 1:3000, and barbital buffer to give a final volume of 600 µl. After an incubation period of 2 hours at room temperature, 100 µl anti-rabbit gammaglobulin serum (donkey) was added and incubated at 4°C for 16 to 20 hours. After centrifugation, the precipitated radioactivity was measured. Corrections were made for non-specific binding which was usually between 2 and 3% of the total activity.

The relative cross-reactivity of L-T<sub>4</sub> with the antiserum was routinely determined in each assay by setting up a dose-response curve of T<sub>4</sub> with concentrations ranging from 2.4 to 30 ng/tube (3.1 to 39 pmol/tube). Serum rT<sub>3</sub> values were corrected for the cross-reactivity effects of the T<sub>4</sub> in the individual serum. T<sub>3</sub> and T<sub>4</sub> concentrations of test sera were measured by radioimmunoassay as previously described (6, 7).

## Results

The assay specificity was measured by studying the ability of various iodothyronines to compete with <sup>125</sup>I-rT<sub>3</sub> for the binding to the antiserum. The highest relative cross-reactivity was found for 3,3'-diiodo-L-thyronine (1.0%). L-T<sub>3</sub> and D-T<sub>3</sub> showed values of 0.020 and 0.032%, respectively. While the cross-reactivity usually is assessed by determining the amount of each compound required to cause a 50% displacement of <sup>125</sup>I-rT<sub>3</sub> originally bound, cross-reactivity data of L-T<sub>4</sub> were estimated for the whole range of the rT<sub>3</sub> standard

curve. Because of the non-parallelism of rT<sub>3</sub> and T<sub>4</sub> inhibition curves (Fig. 1) relative cross-reactivity values of T<sub>4</sub> ranged from 0.1 to 0.025%. In Figure 1 a regular standard curve of rT<sub>3</sub> has been compared with a dose-response curve of T<sub>4</sub>, a serum dilution curve of hyperthyroid serum and results of a typical recovery experiment obtained by the addition of known amounts of unlabelled rT<sub>3</sub> to normal serum. Under the conditions pertinent in the assay system the lower limit of detection was 1.6 pg/tube rT<sub>3</sub> (2.5 fmol/tube) which is equivalent to approx. 20 ng/l serum (31 pmol/l). The dose-response curve of extracts of a hyperthyroid serum, which was diluted stepwise with saline before extraction, is not exactly parallel to the rT<sub>3</sub> standard curve. The slightly flatter slope of this curve could be due to the increasing relative cross-reactivity of T<sub>4</sub> with decreasing T<sub>4</sub> concentrations. The non-parallelism could also be caused by increasing extraction yields in diluted sera during ethanol extraction. The mean recovery of unlabelled rT<sub>3</sub> added to three normal sera in amounts ranging from 0.060 to 0.800 µg/l serum (0.092 to 1.23 nmol/l) was 100.5 ± 2.4% (range 94.6 to 104.6%).

Scatchard plot analyses (Fig. 1) on the chosen antiserum yielded an effective equilibrium constant (9) of 0.98 × 10<sup>10</sup> l/mol. The binding site concentration (expressed in terms of rT<sub>3</sub> binding capacity) in the undiluted antiserum was calculated to be 1.15 µmol/l (0.75 mg/l). Intraassay reproducibility, assessed by measuring 32 different samples in triplicate in the same assay was 5.2 ± 1.1% (mean ± SD). Interaassay reproducibility of 6 samples in three successive assays was 10.7 ± 1.5%.

Table 1 presents results of serum rT<sub>3</sub>, T<sub>4</sub> and T<sub>3</sub> levels for various thyroid states. The mean rT<sub>3</sub> concentration in 45 normal subjects was 0.182 µg/l (0.280 nmol/l).

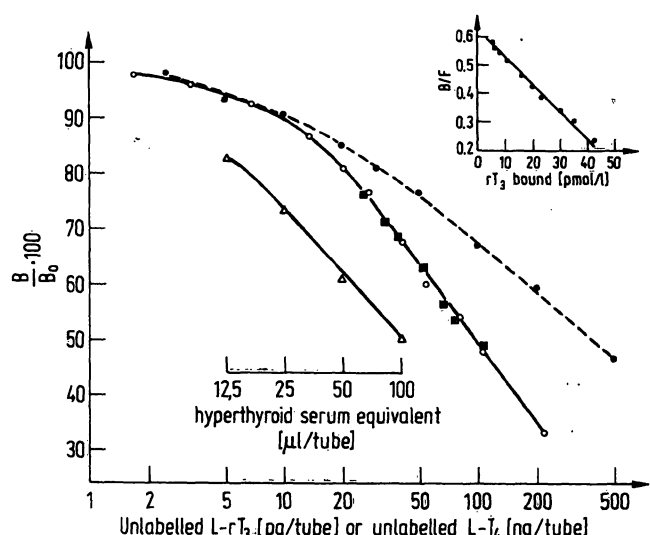


Fig. 1. Dose-response curves of L-rT<sub>3</sub> (—○—), L-T<sub>4</sub> (---●---) and of serial dilutions of hyperthyroid serum (—△—). Results of a typical recovery experiment by adding known amounts of unlabelled L-rT<sub>3</sub> to normal serum (■). The inset shows a Scatchard plot of L-rT<sub>3</sub> binding to the antiserum used.

Tab. 1. Serum rT<sub>3</sub>, T<sub>4</sub> and T<sub>3</sub> concentrations in various thyroid states

Thyroid Status		Serum Concentrations (μg/l)	
		Mean ± SD	Range
Normal subjects n = 45	rT <sub>3</sub>	0.182 ± 0.118	0.021 – 0.463
	T <sub>4</sub>	96.7 ± 17.0	62 – 137
	T <sub>3</sub>	1.33 ± 0.26	0.73 – 1.89
Hypothyroidism n = 15	rT <sub>3</sub>	0.038	*) <0.020 – 0.122
	T <sub>4</sub>	24.6 ± 16.8	6.7 – 45.3
	T <sub>3</sub>	0.38	<0.12 – 0.78
Hyperthyroidism with elevated T <sub>4</sub> n = 28	rT <sub>3</sub>	0.522 ± 0.348	0.155 – 1.22
	T <sub>4</sub>	219 ± 42	154 – 340
	T <sub>3</sub>	4.02 ± 1.71	1.88 – 7.60
Pregnants n = 12	rT <sub>3</sub>	0.200 ± 0.118	0.041 – 0.458
	T <sub>4</sub>	144 ± 17	115 – 170
	T <sub>3</sub>	1.91 ± 0.36	1.24 – 2.47
Newborn (cord serum) n = 22	rT <sub>3</sub>	2.11 ± 0.42	1.55 – 3.19
	T <sub>4</sub>	145 ± 26	91.3 – 205
	T <sub>3</sub>	0.34 ± 0.18	<0.12 – 0.63

\*) Samples containing undetectable quantities of rT<sub>3</sub> were assigned a value of zero for calculating group means.

Pregnants have slightly increased rT<sub>3</sub> concentrations were significantly lower than in normal subjects. In 6 (40%) out of 15 hypothyroid patients, rT<sub>3</sub> values were below the detection limit of 0.020 μg/l. Hyperthyroid patients with increased T<sub>4</sub> showed clearly elevated rT<sub>3</sub> concentrations. An extremely high mean rT<sub>3</sub> value of 2.11 μg/l (3.24 nmol/l) has been measured in newborn cord sera. While in hypothyroidism and hyperthyroidism rT<sub>3</sub> values have shown some overlap with the normal range, the lowest value in 22 cord sera (1.55 μg/l) was distinctly higher than the upper limit (1.22 μg/l) of the hyperthyroid range.

## Discussion

Chopra (5) recently reported a radioimmunological method which for the first time allowed measurements of rT<sub>3</sub> in human serum. In that method, conjugates of racemic D,L-rT<sub>3</sub> to human serum albumin were used for antibody production and D,L-rT<sub>3</sub> was used in the standards. Therefore, this author stated that the true L-rT<sub>3</sub> concentrations in human serum might be somewhat different than recorded. The aim of the present studies was to obtain a radioimmunoassay of high specificity and sensitivity for the measurement of low quantities of L-rT<sub>3</sub> in human serum. The antisera to L-rT<sub>3</sub> produced in two rabbits using bovine serum albumin conjugates showed high titres, which allowed final dilutions of 1:15000 and 1:18000 in the assay, respectively. The most critical factor regarding specificity is the cross-reacting effect of T<sub>4</sub> which is present in normal serum in concentrations more than 200 times higher than rT<sub>3</sub>. The cross-reactivity of T<sub>4</sub> corresponding to serum concentrations up to 250 μg/l was between

0.10 and 0.07%. This is comparable to the value of 0.06% observed by Chopra (5). At T<sub>4</sub> concentrations of 450 ng/tube which displaced 50% of the labelled rT<sub>3</sub> originally bound, a cross-reactivity value of 0.025% was measured in our system. The cross-reactivity of 3,3'-diiodo-L-thyronine (1.0%) was tenfold lower than the value found by Chopra, probably due to the higher purity of our compound. The high sensitivity of the present method (lower limit of detection 1.6 pg/tube) was mainly due to the maximum specific radioactivity of the <sup>125</sup>I-rT<sub>3</sub> tracer – 3300 μCi/μg as compared with 500 μCi/μg (5).

In order to avoid interferences of serum proteins, especially of thyroxine binding globulin in the assay of rT<sub>3</sub>, serum samples were extracted with ethanol and the evaporated extracts were incubated. Contrary to Chopra's results, we found a depression of binding values and a flatter slope of the standard curve by using non-evaporated ethanol extracts for incubation. This effect was mainly observed with low rT<sub>3</sub> concentrations, thus decreasing the sensitivity of the assay in the hypothyroid and normal range of serum rT<sub>3</sub> levels. The technical inconvenience of serum extraction might be overcome by employing unextracted serum in the assay. Preliminary experiments using 8-anilino-1-naphthalene sulfonic acid as an inhibitor of thyroxine binding globulin offers the possibility of estimating rT<sub>3</sub> directly in unextracted serum samples.

Our studies confirm the results reported previously (5) that serum rT<sub>3</sub> concentrations in man are very low. However, serum rT<sub>3</sub> levels in hypothyroidism, normals, hyperthyroidism and pregnant measured in our system are clearly lower than those found by Chopra (5), whereas in cord serum markedly higher rT<sub>3</sub> values were observed. Therefore, it seems unlikely that with our technique generally lower values are obtained. Chopra (5) implied that the results furnished by his method „should be interpreted to reflect relative changes in various situations rather as absolute (true) serum rT<sub>3</sub> values” mainly because only D,L-rT<sub>3</sub> was available and because the cross-reaction of 3,3'-diiodo-thyronine was 10% in his system. Since the above obstacles could be virtually eliminated in our assay system it may be speculated that these rT<sub>3</sub> values approach the “true” rT<sub>3</sub> concentrations in serum. The data on serum rT<sub>3</sub> levels in the newborn indicate that degradation of T<sub>4</sub> in peripheral tissue may be different in the newborn from that in the adult. This might imply that at least in the fetus, rT<sub>3</sub> is the major product of T<sub>4</sub> deiodination (10) in contrast to the assumed random deiodination of T<sub>4</sub> in the adult (3).

## Acknowledgement

We are indebted to Dr. S. Zabransky, Pediatric Clinic, Free University Berlin, for kindly providing the cord sera. This work was supported by the Deutsche Forschungsgemeinschaft (Schl. 95/3).

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