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Radioimmunoassay of 3,3',5'-Triiodo-L-thyronine (Reverse T_3) in Human Serum and its Application in Different Thyroid States

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Summary: A radioimmunoassay for the measurement of 3,3',5'-triiodo-L-thyronine (reverse T_3 , rT_3) 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 ^{125}I -labelled rT_3 of maximum specific radioactivity. Mean serum rT_3 concentrations for various thyroid states were as follows: Normal subjects: 0.182 $\mu\text{g/l}$ (0.280 nmol/l), hypothyroidism: 0.038 $\mu\text{g/l}$ (0.058 nmol/l), hyperthyroidism: 0.522 $\mu\text{g/l}$ (0.802 nmol/l), pregnant: 0.200 $\mu\text{g/l}$ (0.307 nmol/l), newborn (cord serum): 2.11 $\mu\text{g/l}$ (3.24 nmol/l). The method described should provide additional information with regard to the clarification of thyroxine metabolism.

Radioimmunoassay für 3,3',5'-Trijod-L-thyronin (reverses T_3) 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'-Trijod-L-thyronin (reverses T_3 , rT_3) 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 ^{125}Jod -markiertem rT_3 mit maximaler spezifischer Radioaktivität erreicht. Für verschiedene Funktionszustände der Schilddrüse wurden die folgenden mittleren rT_3 -Serumkonzentrationen gemessen: Normalpersonen: 0.182 $\mu\text{g/l}$ (0.280 nmol/l), Hypothyreose: 0.038 $\mu\text{g/l}$ (0.058 nmol/l), Hyperthyreose: 0.522 $\mu\text{g/l}$ (0.802 nmol/l), Schwangere: 0.200 $\mu\text{g/l}$ (0.307 nmol/l), Neugeborene (Nabelschnurserum): 2.11 $\mu\text{g/l}$ (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_4)¹⁾ is converted into 3,5,3'-triiodo-L-thyronine (T_3) in the peripheral tissue of man (1, 2, 3) and it has been concluded that T_3 may be the biologically active hormone (3). The assumption that T_4 is a mere prohormone for T_3 derives from kinetic calculations which preclude that all available T_4 is converted by monodeiodination. This seems to be the case if a random deiodination to T_3 and to 3,3',5'-triiodo-L-thyronine (reverse T_3 , rT_3) is assumed (3). rT_3 is currently regarded to be biologically inactive (4). However, since quantitative determinations of rT_3 were not possible, no data about the generation of rT_3 from T_4 are available at the present time.

It was only recently that the natural existence of rT_3 in human serum could be established using a radioimmunological method (5). Some questions regarding the use of racemic *D,L*- rT_3 in this method led us to the development of a radioimmunoassay for the measurement of *L*- rT_3 in serum.

Materials and Methods

Antisera

Highly specific antisera against *L*- rT_3 were raised in two rabbits by serial injections of *L*- rT_3 conjugates to bovine serum albumin, using the procedure described earlier for the production of antisera against T_3 and T_4 (6, 7). The antiserum selected for use in the assay of rT_3 was obtained 10 weeks after the initial injection. It was used in a final dilution of 1:18 000.

¹⁾ Abbreviations: rT_3 = 3,3',5'-triiodo-L-thyronine; *D,L*- rT_3 = 3,3',5'-triiodo-*D,L*-thyronine; T_3 = 3,5,3'-triiodo-L-thyronine; T_4 = *L*-thyroxine

The rT_3 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 ^{125}I -labelled rT_3

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 μ l each. 2 mCi ^{125}I iodine (Radiochemical Centre, Amersham), which was buffered with 20 μ l 0.5 mol/l phosphate buffer pH 7.5, 2 μ g (3.8 nmol) 3,3'-diiodo-L-thyronine and 100 μ g (355 nmol) chloramine T (Merck, Darmstadt) were incubated for 30 seconds. Then 250 μ g (1.32 μ mol) sodium disulfite was added. The reaction mixture was transferred to a Sephadex G 25 (fine) column (12 $\times$ 1 cm) and eluted with 0.05 mol/l phosphate buffer pH 7.5. The radioiodinated rT_3 peak appeared after the peak of labelled 3,3'-diiodo-L-thyronine with yields between 59 to 70%. Inorganic ^{125}I 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_3 was rechromatographed on Sephadex G 25. The specific radioactivity of the purified product (approx. 3300 μ Ci/ μ g) 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 μ l aliquots of the supernatant which were equivalent to 100 μ l of original serum, were evaporated to dryness and used for rT_3 assay. The extraction yield was estimated to be $80.2 \pm 2.2\%$ using labelled rT_3 as tracer. For recovery experiments, known amounts of unlabelled rT_3 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 μ l serum), or 100 μ l standard solution (from 1.6 to 424 pg, i.e. 2.5 to 650 fmol rT_3), 100 μ l tracer solution (containing approx. 6 pg ^{125}I - rT_3 and 0.5 μ l normal rabbit serum), 100 μ l antiserum to rT_3 diluted 1:3000, and barbital buffer to give a final volume of 600 μ l. After an incubation period of 2 hours at room temperature, 100 μ l 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_4 with the antiserum was routinely determined in each assay by setting up a dose-response curve of T_4 with concentrations ranging from 2.4 to 30 ng/tube (3.1 to 39 pmol/tube). Serum rT_3 values were corrected for the cross-reactivity effects of the T_4 in the individual serum. T_3 and T_4 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 ^{125}I - rT_3 for the binding to the antiserum. The highest relative cross-reactivity was found for 3,3'-diiodo-L-thyronine (1.0%). L - T_3 and D - T_3 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 ^{125}I - rT_3 originally bound, cross-reactivity data of L - T_4 were estimated for the whole range of the rT_3 standard

curve. Because of the non-parallelism of rT_3 and T_4 inhibition curves (Fig. 1) relative cross-reactivity values of T_4 ranged from 0.1 to 0.025%. In Figure 1 a regular standard curve of rT_3 has been compared with a dose-response curve of T_4 , a serum dilution curve of hyperthyroid serum and results of a typical recovery experiment obtained by the addition of known amounts of unlabelled rT_3 to normal serum. Under the conditions pertinent in the assay system the lower limit of detection was 1.6 pg/tube rT_3 (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_3 standard curve. The slightly flatter slope of this curve could be due to the increasing relative cross-reactivity of T_4 with decreasing T_4 concentrations. The non-parallelism could also be caused by increasing extraction yields in diluted sera during ethanol extraction. The mean recovery of unlabelled rT_3 added to three normal sera in amounts ranging from 0.060 to 0.800 μ g/l serum (0.092 to 1.23 nmol/l) was $100.5 \pm 2.4\%$ (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^{10} l/mol. The binding site concentration (expressed in terms of rT_3 binding capacity) in the undiluted antiserum was calculated to be 1.15 μ mol/l (0.75 mg/l). Intraassay reproducibility, assessed by measuring 32 different samples in triplicate in the same assay was $5.2 \pm 1.1\%$ (mean $\pm$ SD). Interassay reproducibility of 6 samples in three successive assays was $10.7 \pm 1.5\%$.

Table 1 presents results of serum rT_3 , T_4 and T_3 levels for various thyroid states. The mean rT_3 concentration in 45 normal subjects was 0.182 μ g/l (0.280 nmol/l).

Fig. 1. Dose-response curves of L - rT_3 (—○—), L - T_4 (---○---) and of serial dilutions of hyperthyroid serum (—△—). Results of a typical recovery experiment by adding known amounts of unlabelled L - rT_3 to normal serum (■). The inset shows a Scatchard plot of L - rT_3 binding to the antiserum used.

Tab. 1. Serum rT₃, T₄ and T₃ concentrations in various thyroid states

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

*) Samples containing undetectable quantities of rT₃ were assigned a value of zero for calculating group means.

Pregnants have slightly increased rT₃ concentrations were significantly lower than in normal subjects. In 6 (40%) out of 15 hypothyroid patients, rT₃ values were below the detection limit of 0.020 μg/l. Hyperthyroid patients with increased T₄ showed clearly elevated rT₃ concentrations. An extremely high mean rT₃ value of 2.11 μg/l (3.24 nmol/l) has been measured in newborn cord sera. While in hypothyroidism and hyperthyroidism rT₃ 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 rT₃ in human serum. In that method, conjugates of racemic D,L-rT₃ to human serum albumin were used for antibody production and D,L-rT₃ was used in the standards. Therefore, this author stated that the true L-rT₃ 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-rT₃ in human serum. The antisera to L-rT₃ 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 T₄ which is present in normal serum in concentrations more than 200 times higher than rT₃. The cross-reactivity of T₄ 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 T₄ concentrations of 450 ng/tube which displaced 50% of the labelled rT₃ 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 ¹²⁵I-rT₃ 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 rT₃, 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 rT₃ concentrations, thus decreasing the sensitivity of the assay in the hypothyroid and normal range of serum rT₃ 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 rT₃ directly in unextracted serum samples.

Our studies confirm the results reported previously (5) that serum rT₃ concentrations in man are very low. However, serum rT₃ 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 rT₃ 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 rT₃ values” mainly because only D,L-rT₃ 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 rT₃ values approach the “true” rT₃ concentrations in serum. The data on serum rT₃ levels in the newborn indicate that degradation of T₄ in peripheral tissue may be different in the newborn from that in the adult. This might imply that at least in the fetus, rT₃ is the major product of T₄ deiodination (10) in contrast to the assumed random deiodination of T₄ in the adult (3).

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References

1. Braverman, L. E., Ingbar, S. H. & Sterling, K. (1970), *J. Clin. Invest.* **49**, 855–864.
2. Braverman, L. E., Vagenakis, A., Downs, P., Foster, A. E., Sterling, K. & Ingbar, S. H. (1973), *J. Clin. Endocrinol. Metab.* **52**, 1010–1017.
3. Surks, M. I., Schadow, A. R., Stock, J. M. & Oppenheimer, J. H. (1973), *J. Clin. Invest.* **52**, 805–811.
4. Pittman, J. A., Brown, R. W. & Register, H. B. (1962), *Endocrinology* **70**, 79–83.
5. Chopra, I. J. (1974), *J. Clin. Invest.* **54**, 583–592.
6. Meinhold, H. & Wenzel, K. W. (1974), *this J.* **12**, 477–486.
7. Meinhold, H. & Wenzel, K. W. (1974), *Horm. Metab. Res.* **6**, 169–170.
8. Weeke, J. & Ørskov, H. (1973), *Scand. J. Clin. Lab. Invest.* **32**, 357–360.
9. Ekins, R. P., Newman, G. B., Piyasena, R., Banks, P. & Slater, J. D. H. (1972), *J. Steroid Biochem.* **3**, 289–304.
10. Chopra, I. J. (1975), 7th International Thyroid Conference, Boston, Abstract 87.

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