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Evaluation of the Manual Enzyme Immunoassay (EMIT) Procedure for Determination of Serum Thyroxine

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Summary: Our experience with the determination of thyroxine (T_4) in serum using a homogeneous enzyme immunoassay technique (EMIT, Syva Corp.) is reported. The intra-assay precision of the EMIT Manual Thyroxine Assay was investigated with 2 different calibrator concentrations and showed coefficients of variation varying from 5–2% for thyroxine concentrations of 40 and 120 μg/l thyroxine respectively. The inter-assay precision was investigated with different series of calibrators and serum specimens. Coefficients of variation for the calibrators varied from 35–5% in the range of 20–200 μg/l thyroxine and for the serum specimens in the range of 8–232 μg/l thyroxine from 50–4%. The recovery of various amounts of thyroxine added to thyroxine-free serum varied between 91–103%. The cross reactivity of structurally related compounds such as: monoiodothyronine, diiodothyronine, triiodothyronine, triiodothyroacetic acid and tetraiodothyroacetic acid was investigated.

Serum samples of 100 patients were analysed by EMIT and radioimmunoassay (T_4 RIA (PEG), Abbott Lab.). A good correlation was found between the EMIT and RIA assay (r = 0.96, slope = 0.96 and y-intercept = 3.37 μg/l).

Evaluation eines manuellen Enzymimmunassay (EMIT) für die Bestimmung von Thyroxin im Serum

Zusammenfassung: Wir berichten über unsere Erfahrungen mit einem homogenen Enzymimmunoassay (EMIT, Syva Corp.) zur Bestimmung von Thyroxin im Serum. Die Präzision in der Serie wurde für die manuelle EMIT-Methode mit zwei Kalibratorkonzentrationen untersucht und ergab Variationskoeffizienten von 5–2% für Thyroxinkonzentrationen von 40 und 120 μg/l. Die Präzision von Tag zu Tag wurde mit verschiedenen Serien von Kalibratoren und Serumproben untersucht; es ergaben sich Variationskoeffizienten von 35–5% im Bereich von 20–200 μg/l Kalibrierlösung und von 50–4% für Serumproben im Bereich von 8–232 μg/l Thyroxin. Die Wiederfindung verschiedener Mengen Thyroxin, die thyroxinfreiem Serum zugesetzt wurden, schwankte zwischen 91 und 103%. Die Kreuzreaktivität strukturell verwandter Verbindungen wie Mono-, Di- und Triiodthyronin sowie Tri- und Tetraiodthyroessigsäure wurde untersucht.

Serumproben von 100 Patienten wurden mit der EMIT-Methode und einem Radioimmunassay (T_4RIA (PEG), Abbott Lab.) untersucht. Es fand sich eine gute Korrelation zwischen Enzym- und Radioimmunassay (r = 0,96; Steigung = 0,96 und y-Intercept = 3,37 μg/l).

Introduction

The competitive protein binding assay described by *Murphy & Pattee* has provided the basis of many methods subsequently developed for the measurement of thyroxine (T_4) in serum (1). The principle of their procedure was based on the competition between radiolabeled T_4 and serum T_4 for binding sites on thyroxine binding globulin.

Their method allowed the direct quantitation of T_4 without interferences from organic or inorganic iodides. This test, however, requires an initial extraction of thyroxine from serum proteins. The variation in the recovery of thyroxine during this procedure greatly affects accuracy and precision of the results (2). The development of a radioimmunoassay (RIA) for T_4 has resulted in a strong increase in sensitivity and

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specificity for the thyroxine determinations in serum (3,4,5). By using antibodies specific to thyroxine, the T_4 radioimmunoassay method eliminated many of the interference problems associated with the competitive protein binding assay, thus improving the reliability of the test. Like competitive protein binding assay methods, however, the T_4 radioimmunoassay used radioactive components which have the disadvantages inherent to all radioisotopic methods when used in the routine clinical laboratory: special isotopic safety considerations, licensure requirements, decay of radiolabeled reagents, need for radiocounting equipment and in the method itself, the obligatory separation of antibody bound from unbound isotope. In 1975, *Ullman* et al. reported the development of an enzyme-multiplied immunoassay technique (EMIT)[1]) for thyroxine, which could simultaneously eliminate all the above outlined disadvantages without sacrifice of the speed, specificity and sensitivity previously characteristic only of the radioimmunological methods (6). The EMIT enzyme immunoassay for thyroxine used stable enzyme labels instead of radioisotopic labels. When the enzyme is activated, the chemical reaction that occurs can be monitored spectrophotometrically and since the enzymatic activity is inversely related to the total concentration of T_4 in the sample, quantitation of the total T_4 in serum can easily be performed. Furthermore, unlike radioimmunoassay procedures, the enzyme immunoassay for thyroxine is homogeneous and no separation step is required.

The procedure is straight forward and lends itself well to automation (6,7). Recently automated procedures for the determination of T_4 by EMIT on the AGA Autochemist (LKB, Bromma, Sweden) and the ABA-100 (Abbott, South Pasedena, California) have been reported (8,9,10). These automated EMIT thyroxine assays yielded results that agreed well with those obtained by a radioimmunoassay and are, according to the investigators, good alternatives to the commonly used RIA procedures. Until now, no study has been published on the evaluation of the EMIT Manual Thyroxine Assay. This EMIT thyroxine kit is particularly of interest for use in those clinical laboratories which have only a limited number of thyroxine determinations and/or do not have an expensive automatic analyzer. In this paper we present

(a) an evaluation of the EMIT Manual Thyroxine Assay with respect to precision, accuracy and specificity

(b) a comparison of results for patients' samples obtained with EMIT and T_4 RIA-PEG (Abbott Laboratories)[2]).

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[1]) EMIT^R, Syva Corp., Palo Alto, USA; Merckotest^R, from E. Merck, Darmstadt for Europe.

[2]) T_4 RIA (PEG); Abbott Laboratories, Diagnostic Division, North Chicago, USA.

Materials and Methods

Enzyme immunoassay (EMIT) of thyroxine

Reagents

EMIT Manual Thyroxine Assay Kit.
(Syva Corp., Palo Alto, USA; Merckotest, from E. Merck Darmstadt for Europe).

The reagents in the kit were accurately prepared and properly stored according to the manufacturer's instructions (11).

Instruments and settings

A Model 300 T spectrophotometer (Gilford, Oberlin, Ohio 44074), with digital readout was used to measure absorbances at 340 nm. The spectrophotometer was connected to a Model 2400 Timer-Printer (Syva, Palo Alto, CA) for printing the absorbance reading at a precise time (9 seconds) after activation by the flow-cell sampling control.

The temperature of the flow cell was set at 25.0 °C. The sample volume was adjusted to 0.70 ml. Diluter dispensers from Syva were used for the addition of the various reagents.

A Model TX_9 thermostatic waterbath (Tamson, Zoetermeer, The Netherlands) was used for incubation of the samples at 37.00 ± 0.05 °C. Eppendorf micropipettes of 50 µl with disposable tips were used to deliver serum samples, thyroxine serum calibrators and the serum pretreatment solution. The assay was carried out in glass test tubes of 12 × 75 mm. A Genie Vortex mixer (Scientific Industries, Mineola, N.Y.) was used for mixing the content of each tube after the successive additions of the reagents. A timing device (Tamson, Zoetermeer, The Netherlands) was employed for the timing of the 15 second interval and the 15 minutes of incubation time.

Procedure

The determination of thyroxine with the EMIT Manual Thyroxine Assay Kit was performed according to the manufacturer's directions (11). Contrary to these directions, however, the contents of each tube were each time mixed separately after the successive additions of the various reagents, instead of vortexing a whole rack at once as recommended in the directions. This modification was used to prevent inadequate mixing of the components.

Radioimmunoassay (RIA) of thyroxine with use of polyethylene glycol (PEG) precipitation

Reagents

Test kit with ^{125}I thyroxine.
(T_4 RIA (PEG) Diagnostic Kit (Abbott Laboratories, Diagnostics Division, North Chicago, Il 60064, USA).

Instrument and setting

The radioactivity of the ^{125}I thyroxine bound to the antiserum and from the ^{125}I thyroxine solution in the total count tubes was counted in an automatic gamma counting system (Baird Atomic TRI-Gamma, The Hague, The Netherlands).

Time setting of the instrument was 1.20 minutes in which about 30,000 counts were obtained from the total count test tubes.

Procedure

The determination of thyroxine with the T_4 RIA (PEG) kit was performed according to the manufacturer's instructions (12). In our procedure serum samples and standard were incubated with the antiserum for 2 h at room temperature (22 to 25 °C).

Sample preparation

Human sera were stored at −20 °C until used. Thyroxine-free serum was prepared by adding 15 g of Dowex 50 W or Amberlite IRA 400 cation exchanger to 40 ml of serum. After mixing, the mixture was stored overnight at 4 °C. This treatment was repeated once. The removal of thyroxine (more than 99% by this procedure) was checked by addition of a trace amount of ^{125}I-T_4 to the serum before the procedure was started. Using this procedure no changes in the total protein content of the

serum occurred; no differences were found in the electrophoretic pattern of the serum proteins and in the TBG content. A number of spiked serum samples with thyroxine concentrations of 40, 80, 120 and 200 μg/l respectively were prepared by diluting an aqueous stock solution of 2000 μg/l *L*-thyroxine with thyroxine-free serum.

Results

Standard curves

In figure 1 a mean standard curve for the EMIT Manual Thyroxine method is presented. This curve is the average of 11 standard curves, which were run on several days during a 2 month time period. The calibrators used for the construction of these curves were from 6 kits with the same lot number. Figure 2 illustrates the mean standard curve (n=5) for the T_4 RIA (PEG) method, which was obtained during the 2 months evaluation period for the EMIT kit. The mean fractions of $^{125}I-T_4$ bound to the antiserum for the 5 calibrators were plotted on the Y-axis versus the calibrator concentrations on the X-axis. The calibrators used for the construction of the curve were from 4 kits with the same lot number.

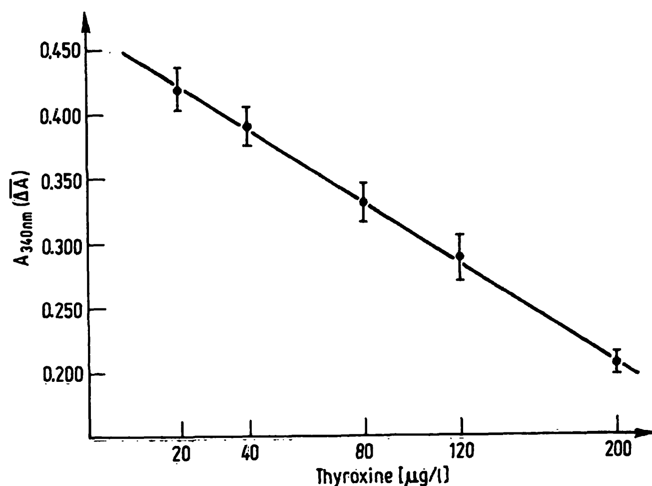

Fig. 1. Mean EMIT standard curve for thyroxine. Each closed circle is the average of 11 determinations.

Precision

Intra-Assay Precision

The intra-assay variation of the EMIT Manual T_4 Assay was determined by 10 replicate analyses of calibrators containing 40 and 120 μg/l of thyroxine. The coefficient of variation (CV) was 4.8% at a T_4 level of 39.6 ± 1.9 μg/l (mean ± SD) and 2.4% at 120.4 ± 2.9 μg/l.

Inter-Assay Precision

The interassay (day-to-day) variation for both the EMIT and RIA T_4 method was determined by calculating the coefficient of variation for each calibrator concentration from the mean standard curve of each method (figure 1 and 2). The mean value ± SD of the absorbance (EMIT) or percentage bound radioactivity (RIA) of each calibrator was translated into a thyroxine concentration and resulting coefficients of variation (CV%) were plotted versus the thyroxine calibrator concentrations in figure 3. Furthermore, the inter-assay variation was assessed by the replicate analysis of 5 patient samples of various thyroxine concentrations on 10 days during a 1 month period. The coefficient of variation was 50.0% at a T_4 level of 8 ± 4 μg/l (mean ± SD), 6.7% at 69 ± 5, 6.3% at 85 ± 5, 5.2% at 168 ± 9 and 4.1% at 232 ± 10 μg/l.

Sensitivity

The EMIT Manual Thyroxine Assay has been designed for optimum sensitivity in the assay range 20 to 200 μg/l. Outside this range amounts of about 10 μg/l can be determined with a coefficient of variation of ± 50% and of 240 μg/l with a coefficient of variation of 5%.

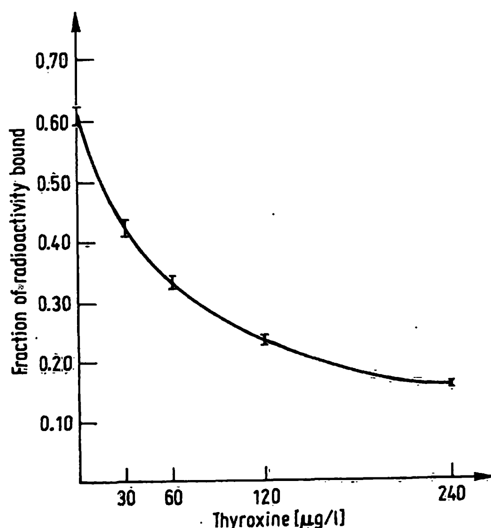

Fig. 2. Mean standard curve for thyroxine obtained with the T_4-RIA (PEG) method.
Each closed circle is the average of 10 determinations.

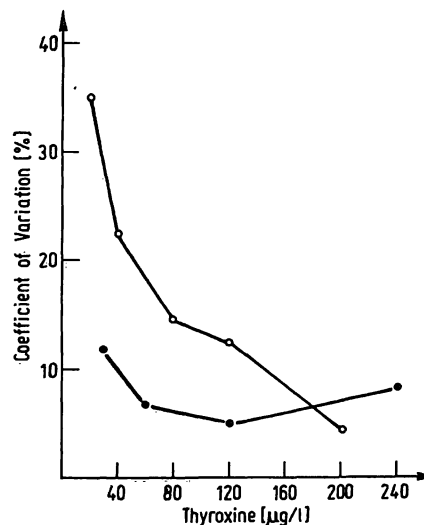

Fig. 3. Precision for the EMIT and T_4 RIA (PEG) method calculated from the mean standard curves.
o——o, EMIT method; •——•, T_4 RIA (PEG) method.

Accuracy

The results for the recovery of thyroxine by the EMIT and RIA method are shown in table 1. Thyroxine was added to thyroxine-free sera to give concentrations of 40, 80, 120 and 200 μg/l. The recovery at the various concentrations was adequate for both methods: 97–107% with RIA and 91–103% with EMIT.

Tab. 1. Recoveries of thyroxine added to thyroxine-free serum.

Thyroxine concentration (μg/l)	RIA Method Found[a] (μg/l)	RIA Method Recovery (%)	EMIT Method Found[a] (μg/l)	EMIT Method Recovery (%)
0	16[b]	–	–	–
40	59	107	56	100
80	99	104	93	96
120	138	102	139	103
200	209	97	198	91
Mean recovery		102		98

a) Each value is the mean of 7 analyses.
b) T_4 concentration of thyroxine-free serum determined by RIA.

Specificity

The specificity of the EMIT and RIA method was tested by the determination of the cross-reactivity of compounds structurally related to thyroxine. Thyroxine-free sera containing 100 μg/l of each compound were prepared and subsequently measured by both methods. The apparent thyroxine content of each sample is summarized in table 2 together with the serum concentration range of each compound normally found in human serum.

Comparison

Calibrators

Five sets of EMIT thyroxine calibrators with the same lot number were analyzed with the RIA procedure and 5 sets of RIA calibrators were assayed with the EMIT method. The results of the analyses with both methods are presented in table 3 and 4. The RIA analysis of the EMIT calibrators, as well as the EMIT analysis of the RIA calibrators, showed good agreement between the nominal stated and found concentrations. Deviations from the labeled values ranged from 0.1 to 5.1% for the EMIT calibrators and from 0.3 to 10.9% for the RIA calibrators. The difference between the nominal stated and observed concentration for the RIA calibrator of 240 μg/l (table 4) is probably due to a lower sensitivity of the EMIT method in the determination of concentrations higher than 200 μg/l. The difference in matrix of the RIA and EMIT calibrators (equine and human serum) showed no marked influence on the EMIT and RIA assayed values (table 3 and 4).

Tab. 3. RIA analysis of EMIT Thyroxine calibrators.

EMIT thyroxine calibrator concentration (μg/l)	RIA assayed values[a] (μg/l)	Deviation[b] (%)
20	20.2 ± 2.4	+ 1.0
40	41.4 ± 3.3	+ 3.5
80	80.1 ± 2.9	+ 0.1
120	125.8 ± 8.0	+ 4.8
200	210.1 ± 19.8	+ 5.1

a) Mean ± SD of 12 determinations
b) Deviation from stated value in %

Tab. 4. EMIT assay of RIA thyroxine calibrators.

RIA thyroxine calibrator concentration (μg/l)	EMIT assayed value[a] (μg/l)	Deviation[b] (%)
0	3.0 ± 3.3	
30	31.0 ± 4.7	+ 3.3
60	61.6 ± 5.2	+ 2.7
120	119.6 ± 9.2	– 0.3
240	214.0 ± 9.2	–10.9

a) Mean ± SD of 9 determinations
b) Deviation from stated values in %

Patient samples

The results of a comparative evaluation of 100 patient's sera obtained with the EMIT and the RIA method are shown in figure 4. For the comparative study serum samples were selected with thyroxine concentrations ranging from 0–200 μg/l. The analyses of specimens by

Tab. 2. Cross-reactivity of compounds related to thyroxine in the EMIT and RIA thyroxine assay.

Compound	Concentration (μg/l)	Apparent thyroxine content in (μg/l)[a] EMIT	Apparent thyroxine content in (μg/l)[a] RIA	Normal range of serum concentration (μg/l)
Thyroxine-free serum	–	–	14	–
3-monoiodothyronine	100	12	15	?
3,5-diiodothyronine	100	15	11	0.05
3,3',5-triiodothyronine	100	140	61	0.98–2.0
3,3',5-triiodothyroacetic acid	100	17	15	0.1
3,3',5,5'-tetraiodothyroacetic acid	100	170	82	0.75–2.9

a) Each value is the mean of 4 determinations.
?) no reliable data available in the literature.

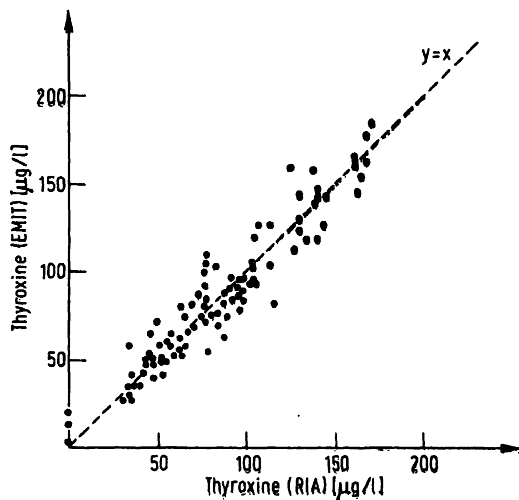

Fig. 4. Correlation between T_4 values as measured by the T_4 RIA method and by the EMIT method.

$y = 0.96\,x + 3.37$
$r = 0.955$
$n = 100$

EMIT and RIA were performed in duplicate and the results were averaged. As none of these methods could be indicated as more accurate, we calculated a regression line from an orthogonal instead of a linear regression analysis. This regression analysis of the paired data from 100 sera gave a correlation coefficient of 0.96, a slope of 0.96, and a y-intercept of 3.37 µg/l. The mean value for thyroxine in the patient's sera was 88.1 µg/l for the EMIT method and 88.5 µg/l for the RIA method.

Diagnosis of thyroid status

The comparisons between the EMIT method and RIA method in the diagnostic classification of 100 patients, based on the normal ranges of 45–120 µg/l for EMIT (11) and 50–130 µg/l for the RIA assay (12), showed a diagnostic agreement in 89 out of the 100 patients. In addition, the difference of diagnosis (hypothyreotic, by RIA and euthyreotic by EMIT) of 5 patients was only caused by the variation of the low borderline values (45–50 µg/l) of the normal ranges of both methods.

Discussion

Our data in this study indicate that the EMIT Manual Thyroxine Assay, when used exactly according to the manufacturer's instructions, gives results that agree with those obtained by the T_4 RIA (PEG) method.

Over a 2 month time period the variation in the mean standard curves, shown in figure 1 and 2, for both the EMIT and the RIA method was acceptable.

However, by plotting these standard deviations as coefficients of variation in the concentration versus the calibrator concentrations (fig. 3), it can be seen that especially the use of a mean EMIT standard curve for the calculation of the concentration of the samples is not acceptable. Therefore using the EMIT method for the thyroxine determination, it is essential to run a calibration curve each day of analysis. The intra-assay precision of the EMIT Method is satisfactory with a coefficient of variation ranging from 2.4–4.8%. Except for the specimen with a T_4 level of 8 µg/l, which had a coefficient of variation of 50%, the interassay variations in values obtained by EMIT for the same specimens were quite acceptable (coefficient of variation 4.1–6.7%) during the 4 weeks in which this variable was measured. The analytical recovery for both methods was good and varied for EMIT from 91–103% and for the RIA method from 97–107%. Cross reactions for triiodothyronine and tetraiodothyroacetic acid were observed for the EMIT as well as the RIA assay. However from the levels normally found for the 2 compounds in human serum it can be concluded that they will have no clinically significant influence on the serum thyroxine concentration measured with both methods. From the good correlation (r = 0.955) between serum thyroxine concentrations obtained by the EMIT and RIA method it can be concluded that these assay techniques are equivalent. In addition, there was satisfactory agreement between the two methods in the diagnosis of the thyroid status. Thus, the EMIT Manual Thyroxine Assay is a good alternative for RIA methods and is especially suitable in hospital laboratories which may not have facilities for radioimmunoassay. Furthermore, compared to the T_4 RIA (PEG) procedure, the EMIT Manual Thyroxine Assay has the advantage that the technique is simpler and faster and can be adapted more easily to automation. The total time of analysis of 40 unknown specimens in duplicate together with 5 calibrators in duplicate took about 4 h in the EMIT procedure and about 6 h in the RIA assay. For the reasons described in the introductory paragraphs and in the present study, we recommend the EMIT assay for analysis of serum thyroxine in the range of 20–200 µg/l.

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