

Stability of Stopped thyroid hormones in Enzyme Linked Immunosorbent Assay

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Abstract

Power outage is a common feature in the third world countries. Oftentimes after the preparation and completion/ and stopping of reaction in tube method of Immunometric assay there is power outage. One wonders what should be done with the set up. How long can the stopped reaction wait before reading and the result will be useful? This aspect is not included in the method sheet. To answer this question, the test is done as usual but the readings were taken at 0, 2hours, 1 day, 2 days, 3 days, 4 days and 5 days.

The results were compared. It was evident that when read within two hours, the result remain unchanged but after 24hours the slope is depressed and all the readings wane. Effort should be made to take the readings within 24hours for reliable result.

Keywords: Stability, thyroid hormones, hormonal assay.

Introduction

Time and again, in many laboratories in the third world countries, we are faced with power outages. When such outages occur after stopping the reaction and electricity is not returned until after some time, what should be done? Should the whole process be abandoned and restarted when electricity can be guaranteed or for how long can such set up be kept before reading so that the result obtained can be useful.

The importance of thyroid function test cannot be over emphasized (Paul 2000, Pearce 1985). The hypermetabolic state of normal pregnancy can mimic the features of thyroid disease and this need to be ascertained. In the work up for infertility, thyroid hormones come handy since hypothyroidism is associated with hyperprolactinemia (Marwaha 2008, Panear 2001, Parker 1985). Human chorionic gonadotrophin (hCG) can stimulate the thyroid gland during the first trimester because of its structural similarity to thyrotrophin (TSH). Increased elaboration of oestrogens reduces the clearance of thyroxin binding globulin, and this results in increased levels of thyroxin (T_4) and triiodotyronine (T_3). As pregnancy progresses, levels of albumin and free fatty acids do change and these affect the binding of T_4 and T_3 to carrier proteins, lowering the blood levels of free hormones (FT_4 and FT_3).

The relationship between the thyroid hormones and the binding proteins can be described by the law of mass action:

$$[FT_4] \times [\text{unbound TBG}] = k \times [T_4\text{- TBG}]$$

Where

FT_4 = the concentration of free T_4 in serum

[unbound TBG] = the concentration of unbound sites on TBG

k = reaction rate constant

$[T_4\text{- TBG}]$ = concentration of T_4 occupying TBG binding sites

An increase in the concentration of FT_4 or unbound TBG would drive the reaction to the right. Therefore, in situations where there is an excess of protein, more T_4 will be bound, and in situations where there is little protein available, less T_4 will be bound. Because free thyroid hormones are being routinely measured now, the low results in latter pregnancy may be termed and treated as ‘hypothyroidism’ by the unwary physician. Since there is an endemic deficiency of iodine with the attendant goiter in some communities like the thyroid belt of Nigeria, it becomes necessary to have a reference range locally as applicable elsewhere (Blonde 1984, Brent 1997, Kabyemala 1996). Thyroid work up is also needed when trying to establish the diagnosis of diabetes mellitus, malabsorption syndrome, Grave’s disease and a host of other diseases. This explains why the analytic method for thyroid hormones should be cheap, robust, simple and reproducible. When it comes to the third world, effort should be made to ensure sure tests can stand the test of time at all stages involved.

Methods

Analyses were done using the accompanying standards and control. Ten (10) samples were assayed for respective hormones and the optical densities were noted and corresponding concentrations read off the graph plotted with the standards. Readings were taken immediately after stopping the reaction, two hours thereafter, then daily for the next five days. Readings were taken and compared with the initial reading. The actual method followed in the immunometrics(2015) assay is as shown below for each hormone.

T3 EIA kit(per manufacturer)

▶ high performance

- detection limit 0.23 nmol/L (0.15 ng/mL)
- within-assay CV less than 15% between 0.2 - 15 nmol/L (0.13 - 10 ng/mL)

▶ low cost

- all reagents are provided - only test tubes and water are required

- uses inexpensive, robust equipment
- each kit is sufficient for 100 assay tubes

▶ **convenient**

- direct serum assay
- magnetic separation
- shelf life of 9 months at 4-8 °C

Principle

The assay is a direct competitive EIA for the quantitative measurement of Total Triiodothyronine (Total T3) in human serum or plasma. A specific agent displaces T3 from binding proteins, making it available for antibody binding. T3 then equilibrates with fluorescein labelled T3 (T3 Derivative) in binding to a limited amount of alkaline phosphatase labelled anti-T3 antibody. An anti-fluorescein antibody bound to magnetic particles then separates the T3/fluorescein T3-antibody complex from unbound components. A further incubation with substrate produces colour in inverse proportion to the amount of T3 present. The assay has four main stages:

Displacing agent, alkaline phosphatase labelled anti-T3 antibody, and fluorescein-T3 derivative are incubated with sample (100 µL) for 15 minutes at 37 °C.

Anti-fluorescein antibody coupled to magnetic particles is added and incubated for 10 minutes at 37 °C. Anti-T3 antibody is then isolated by means of a magnetic wash step.

A coloured enzyme substrate is incubated with the particles for 15 minutes at 37 °C. The presence of alkaline phosphatase causes a colour change from yellow to pink. The reaction is terminated by addition of Stop Solution.

The tubes are then placed in the spectrophotometer or colorimeter. The optical density (at 550nm or 492nm) of each tube can be measured and the results calculated using a data processing program.

T4 EIA kit(per manufacturer)

high performance

- detection limit 6.5 nmol/L (5 ng/mL)
- within-assay CV less than 15% between 10-380 nmol/L (8 - 300 ng/mL)

low cost

- all reagents are provided - only test tubes and water are required
- uses inexpensive, robust equipment
- each kit is sufficient for 100 assay tubes

convenient

- direct serum assay
- magnetic separation
- shelf life of 9 months at 4-8 °C

Principle

The assay is a direct competitive EIA for the quantitative measurement of Total Thyroxine (Total T4) in human serum or plasma. A specific agent displaces T4 from binding proteins, making it available for antibody binding. T4 then equilibrates with fluorescein labelled T4 (T4 Derivative) in binding to a limited amount of alkaline phosphatase labelled anti-T4 antibody. An anti-fluorescein antibody bound to magnetic particles then separates the T4/fluorescein T4-antibody complex from unbound components. A further incubation with substrate produces colour in inverse proportion to the amount of T4 present. The assay has four main stages:

Displacing agent, alkaline phosphatase labelled anti-T4 antibody, and fluorescein-T4 derivative are incubated with sample (50 µL) for 15 minutes at 37 °C.

Anti-fluorescein antibody coupled to magnetic particles is added and incubated for 10 minutes at 37 °C. Anti-T4 antibody is then isolated by means of a magnetic wash step.

A coloured enzyme substrate is incubated with the particles for 15 minutes at 37 °C. The presence of alkaline phosphatase causes a colour change from yellow to pink. The reaction is terminated by addition of Stop Solution.

The tubes are then placed in the spectrophotometer or colorimeter. The optical density (at 550nm) of each tube can be measured and the results calculated using a data processing program.

TSH EIA kit(per manufacturer)

high quality

- developed for the WHO Collaborating Centre in London
- performs well in national EQA Scheme

high performance

- detection limit 0.1 mIU/L WHO IRP 80/558
- between-assay CV better than 10% in range 1 - 50 mIU/L

low cost

- all reagents are provided - only test tubes and water are required
- uses inexpensive, robust equipment
- each kit is sufficient for 100 assay tubes

convenient

- magnetic separation
- shelf life of 1 year at 4-8 °C

Principle

The assay is an immunometric ('sandwich') EIA for the quantitative measurement of TSH in human serum or plasma. TSH in the sample is bound by 2 monoclonal anti-TSH antibodies directed at different epitopes. One antibody is attached to magnetic particles and the other is labelled with alkaline phosphatase. The assay has four main stages:

Magnetic anti-TSH is incubated with sample (100 µL) for 15 minutes at 37 °C followed by a magnetic wash step.

The magnetic particles are incubated with alkaline phosphatase labelled anti-TSH for 1 hour at 37 °C followed by 2 magnetic wash steps.

A coloured enzyme substrate is incubated with the particles 1 hour at 37 °C. The presence of alkaline phosphatase causes a colour change from yellow to pink. The reaction is terminated by addition of Stop Buffer.

The tubes are then placed in the spectrophotometer or colorimeter. The optical density (at 550nm or 492nm) of each tube can be measured and the results calculated using a data processing program.

Sample collection and assay

Ten (10) ml of blood was collected from the antecubital veins of subjects between 7:00 and 8:00 am on the days of collection. The samples were centrifuged at 3000rpm for 5minutes and the plasma frozen at -20°C until analysis.

The assays were done using the enzyme immunoassay (EIA) system employing the kits manufactured by Immunometrics (UK) Ltd. The inter-assay coefficients of variation for all hormones were less than 9%.The reference ranges for non-pregnant women as given by the manufacturer are as follows: TSH (0.48-3.78MIU/L), FT₄ (10.23-19.10pmol/L) and FT₃ (3.53-6.54pmol/L).

Statistics

Data analysis was by the EXCEL software package and the one way ANOVA package.

Result

The result is as plotted in the accompanying table and graphs.

	Fresh	2Hrs	Day1	Day2	Day3	Day4	Day5
1	0.030	0.031	0.035	0.047	0.037	0.047	0.032
2	0.049	0.050	0.050	0.044	0.034	0.034	0.030
3	0.110	0.110	0.090	0.088	0.081	0.072	0.069
4	0.133	0.132	0.130	0.122	0.100	0.101	0.100
5	0.321	0.321	0.310	0.274	0.237	0.229	0.209
6	1.325	1.324	1.311	1.285	1.195	1.105	1.090
QC	0.134	0.134	0.131	0.142	0.101	0.109	0.117
T1	0.092	0.092	0.090	0.098	0.081	0.083	0.073
T2	1.480	1.480	1.410	1.610	1.560	1.520	1.520
T3	0.162	0.162	0.159	0.144	0.135	0.117	0.111
T4	0.082	0.081	0.080	0.084	0.069	0.077	0.074
T5	0.038	0.038	0.035	0.043	0.035	0.042	0.043
T6	0.125	0.125	0.124	0.125	0.123	0.122	0.120
T7	0.375	0.376	0.370	0.374	0.370	0.373	0.370
T8	0.060	0.060	0.060	0.058	0.056	0.058	0.050
T9	1.000	1.000	1.000	0.980	1.005	0.970	0.960
T10	0.187	0.187	0.189	0.185	0.180	0.180	0.179

Table 1: Absorbance of Standards and Tests for TSH in an Assay

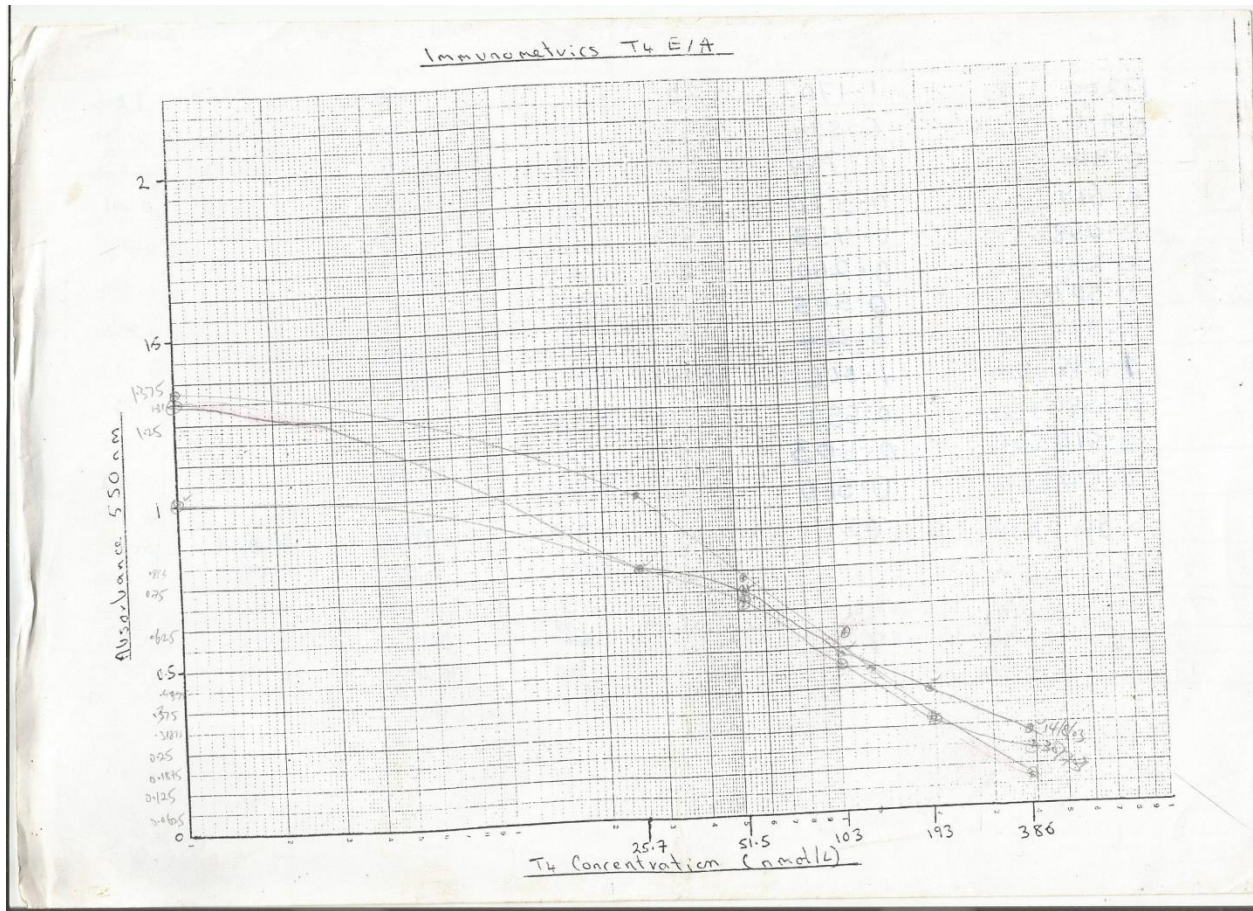


Fig1: T₄ plots over 5days

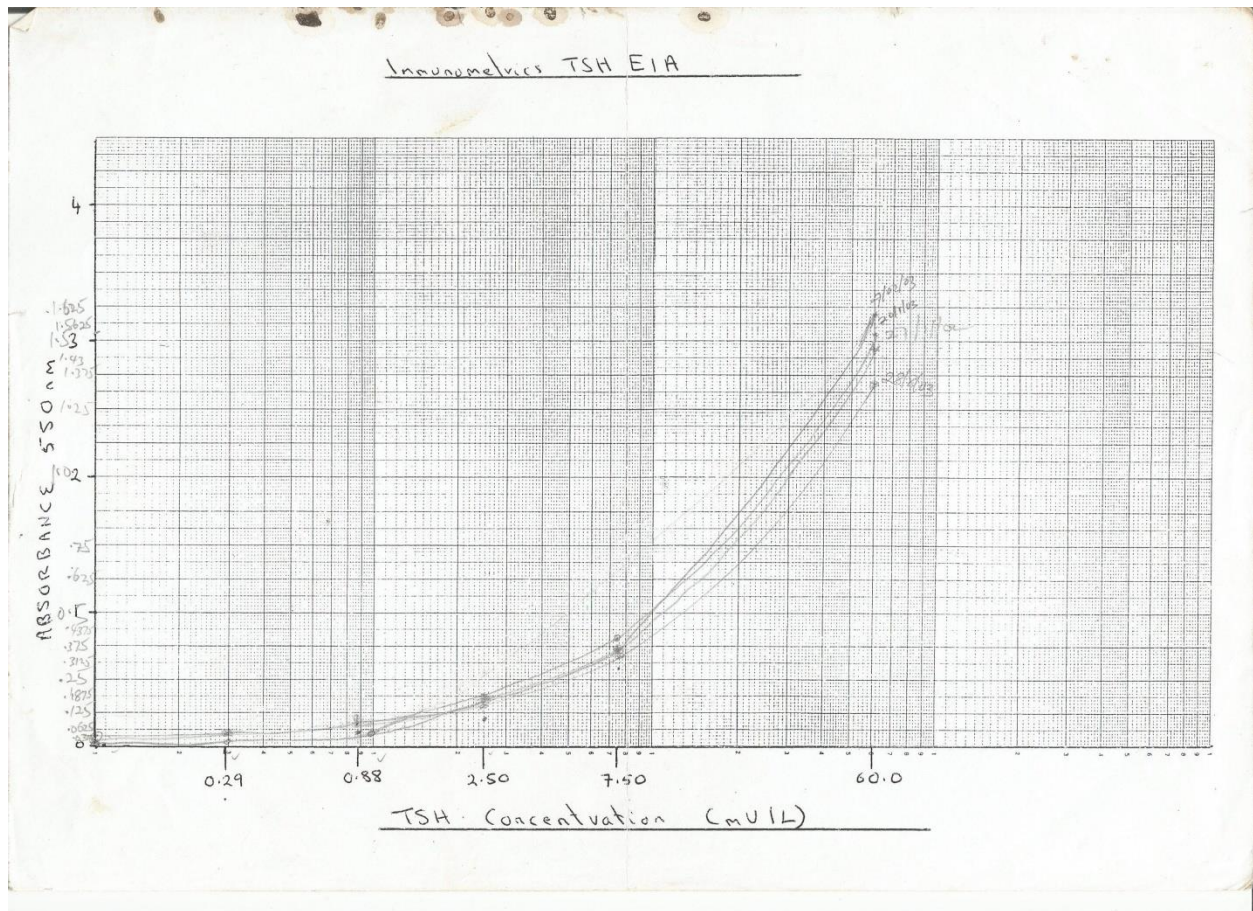


Fig2: TSH Plots over 5days

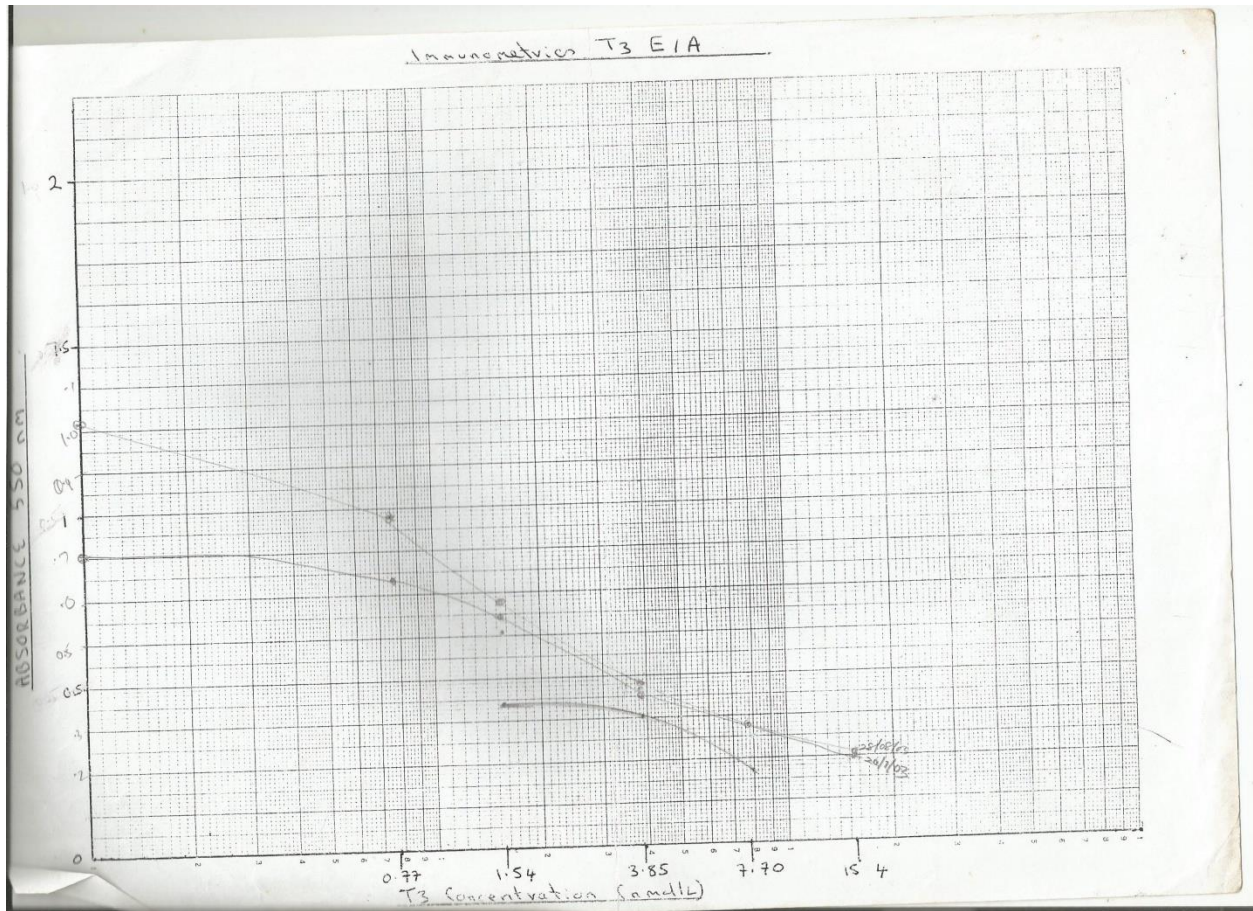


Fig3: T₃ Plots over 5days.

Discussion and Conclusion:

From the graphs, it can be seen that after stopping the reaction, absorbances and values of these hormones remain unchanged over two hours. Soon thereafter, for TSH and T₄, there is a depression of the slope. The good thing about this is that the values of the tests as well as that of the standard tend to remain the same. This happens in a much predictable manner. In case of T₃ however, the changes after two hours is an outright reduction despite maintaining the slope.

This whole exercise might make no sense to those in the developed countries and other places where there is a stand-by generator dedicated to the laboratory. In the laboratory in Nigeria where this study was carried out, there was no backup power supply and power supply from the main grid was epileptic. Some equipment were damaged either by power surge or power outage.

The stability study done by the manufacturers is that of how long the reagent could be used after opening the bottles/vials. We need them to look into this area of stability after stopping the reaction.

From the findings in this study, after stopping the reaction with stop solution (NaOH), when read within two hours, no depreciation in the test result (Table 1). However, the usefulness of the result decreases each day that they are left to stand.

A study is already under way to study the stability of some hormones after stopping the reaction using the automate ELISA method. This is important since it is becoming more popular and easily done in many parts of Nigeria.

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