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RESEARCH ARTICLE

Stability of Thyroid Function Test Analytes Whose Serum Levels Are Determined by Immunoassay

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ABSTRACT
We have performed stability studies on thyroid function tests, i.e., T4, T3, free T4, free T3 and thyroid stimulating hormone (TSH), over a five-day period on the sera of ten patients. We find that the levels for each analyte were stable; the mean coefficients of variation (CV) ranged from 3.2 % for TSH to 8.7% for T4. ANOVA statistical analysis of group means for each analyte over the five-day period indicated that there was no statistically significant difference in these means, confirming the reproducibility of values. On the other hand, the CVs are significantly larger than those found in our prior stability studies for other critical analytes such as electrolytes (sodium, potassium, chloride, calcium). In addition, significant variations in values over the five-day period occurred for T4 which showed the largest variations in values of the five analytes studied. In one patient, the values ranged from a low of <10 ug/dL to over 14 ug/dL over the five-day period. A two-tailed t test comparing the mean CV for TSH with the mean CVs of the other four analytes showed that the mean CV for TSH was statistically significantly lower than that for the other four analytes indicating that the assay for TSH yield the most precise results. These results parallel those obtained in our prior electrolyte study that showed very low CVs for sodium, potassium and chloride and a significantly higher CV for calcium. Since approximately half of total serum calcium is bound to albumin and since very high percentages of T4 and T3 (≥99%) are bound to thyroid binding globulin and albumin, protein binding of these analytes may introduce a possible source of assay imprecision.

Introduction

Loss of precision in the quantitative determination of analyte levels in body fluids, predominantly serum, can result from a number of different sources at the preanalytical, analytical and postanalytical levels. Most preanalytical causes are due to varying storage times of samples prior to quantitative analysis. Examples of this are glucose levels in sera that can decrease with time due to contact with red blood cells which metabolize this analyte [1-2]. Analytical causes of precision are often due to such factors as temperature fluctuations, carry over from prior samples, and small but important changes in dilution volumes used in indirect method determinations[1]. Postanalytical causes of loss of precision most frequently occur in stored samples maintained at refrigeration temperatures (approx 4°C). Values for analytes in stored samples that need to be re-analyzed may be affected by storage at low temperature and the re-heating process prior to analysis, possible protein denaturation during the storage period resulting in changes in ionic strength loss or gain of analyte binding to proteins and by other factors such as analyte metabolism. In addition, long-term storage of samples at freezing temperatures can result in protein denaturation and/or aggregation especially when a sample is successively frozen and thawed. This can affect values for T4, T3 and their free forms since T4 and T3 are almost completely bound to serum proteins, i.e., thyroid binding globulin (TBG), albumin and transthyretin.

While there has been much focus on the pre-analytical and analytical causes of loss of precision, less attention has been paid to post-analytical reproducibility. However, at least one percent of samples submitted to the clinical chemistry laboratory require re-analysis which is often initiated by the clinical staff for values that seem to be incompatible with current diagnoses[3]. Imprecision in repeat determinations of specific samples can result in confusion concerning diagnosis. This applies especially to analyte values that are "borderline," i.e., that lie close to the lower or upper limit values of the reference range[4]. Imprecision in these cases can result in false positive or false negative test results leading to an incorrect diagnosis. The same considerations apply to "add-on" testing to previously drawn stored samples[5].

It is therefore important to test reproducibility of analyte values for samples that have already been analyzed or on which "add-on" tests are requested but any of which may require reanalysis or first determinations during storage. We have undertaken on our clinical chemistry services at the University Hospital at SUNY Downstate Medical Center and our affiliate, Maimonides Medical Center, and our VA affiliate to test the precision of analyte stabilities over time for samples stored at low temperatures[3]. In our studies of electrolyte (i.e., sodium, potassium, chloride and calcium) stabilities over a nine-day period, we found the coefficients of variation (CV) for all of these analytes to be low[3]. The range of CV values for sodium, potassium and chloride was 0.32-1.2 percent. Interestingly, the range for calcium was significantly higher, i.e., 1.23-3.57 percent[3]. This difference could not be attributed to method variation. The first three analytes were assayed by a voltammetric method while calcium was assayed by a spectrophotometric assay. The latter method yields CV values on other analytes that are in the lower CV range suggesting that method differences were not responsible for the differences in CV values.

When receiving requests for performing repeat or "add-on" analyses on patient samples, we have noticed that a significant percentage of these requests concerned hormone assays especially involving thyroid function tests. As a result, we have extended our stability studies to include all thyroid function tests, viz, total T4, total T3, free T4, free T3 and TSH.

The literature on the stability of thyroid function analytes is sparse and somewhat contradictory. Recently published thyroid function test stability studies by Rush Medical Center (7/1/2021) state that T4, T3, and free T4 and free T3 can be stored at 4°C for up to six days while TSH can be stored for seven days without significant changes in the levels of these analytes (Rush Medical Laboratories Core Laboratory. Specimen Stability Chart. rush.edu. Published July 1, 2021. https://rml.rush.edu/Documents/Specimen%20Stability%20Chart%20(07.01.2021)%20FINAL.pdf).

However, other, earlier studies using DPC Immulite immunoassays on samples that were stored at refrigeration temperature concluded that serum samples tested for free T4 had unacceptable increases in changes of values by the fourth day of refrigerated storage, and, similarly, by the third storage day for free T3[6]. In addition, using a third generation TSH assay, it was found that serum values for this analyte remained stable only for three days[6] although in a prior study on serum TSH stability on samples stored at refrigeration temperatures, TSH was found to be stable for four days[7].

In contrast, a more recent study found that TSH assays performed on sera from fifteen patients that were immediately assayed, then assayed after standing at room temperature for 24h, and then assayed after storage at 4°C for one week, all gave values that were statistically insignificantly different[8]. An early comprehensive study of thyroid function test stabilities found significant differences in all thyroid function tests depending on ambient temperature[9]. On the other hand, an earlier radioimmunoassay for T4 on four samples from one hyperthyroid and three euthyroid patients indicated stable values for this analyte at room temperature for two weeks although use of two other methods based on use of TBG and resin showed significant increases in values over this period[10].

Further studies on frozen (-25°C) serum samples suggested that TSH, free T4, and free T3 can reliably be analyzed in samples stored for 23 years[11]. However, this conclusion was disputed by another study in which reanalysis of these hormones in 30 of the archived samples used in the prior study[12] showed significant changes in values after 8-11 years. Similar results were reported for TSH.

These prior studies present contradictory results that may be due to a variety of circumstances such as differences in analytical methods, differences in assay conditions such as ambient temperature, and different patient populations involved in the studies. There is therefore a clear need to determine systematically the stabilities of each thyroid function analyte in stored serum to define the time limits for reproducible results. We have therefore undertaken to perform stability studies for each of the five thyroid function analytes, T4, T3, free T4, free T3 and TSH from randomly selected serum samples stored at refrigeration temperatures over a five-day period.

Materials and Methods

Ten serum samples were randomly selected and de-identified as part of a quality improvement process in Clinical Chemistry at the Maimonides Medical Center. Each sample was assayed for T4, T3, free T4, free T3 and TSH using the Beckman Unicell DXI 800 analyzer. After the first set of assays was completed on a sample, this sample was stored at 4°C for 24h and was then re-assayed. This process was repeated over a five-day period. All samples were stored at 4°C when not assayed. All assays were performed at room temperature. Six of the samples were found to be quantity not sufficient (qns) at day 5, but eight samples were re-assayed for four successive days, and all samples were assayed for three successive days. ANOVA statistical analysis was performed on the group means and standard deviations for each of the five analytes over the five-day period to test for significant differences. Coefficients of variation were computed for each analyte for each sample; the means and standard deviations for the coefficients of variation were then computed; the values for the four thyroid hormone forms were compared with those for TSH using a two-tailed t test for statistical significance.

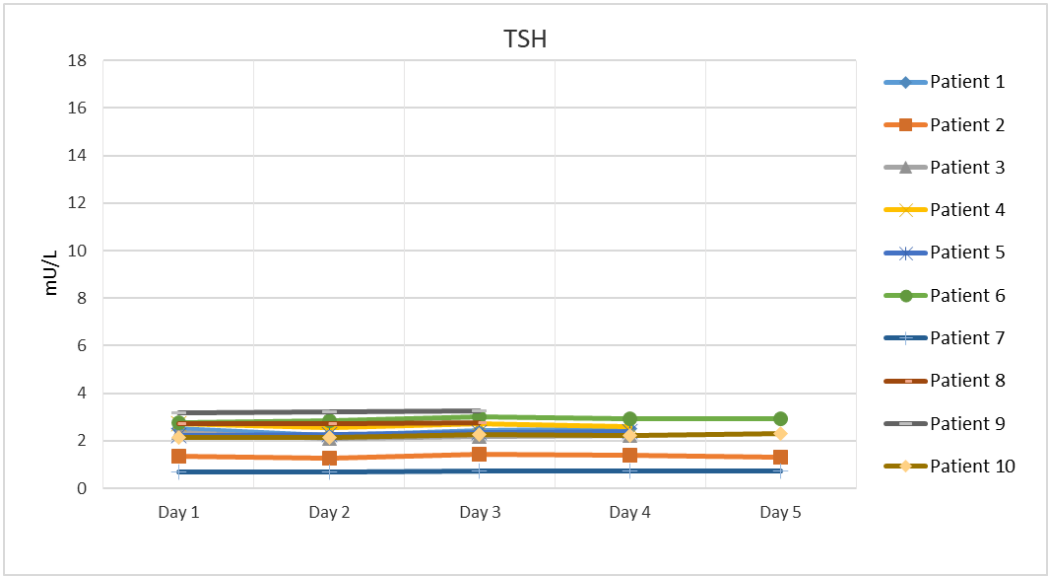
Results

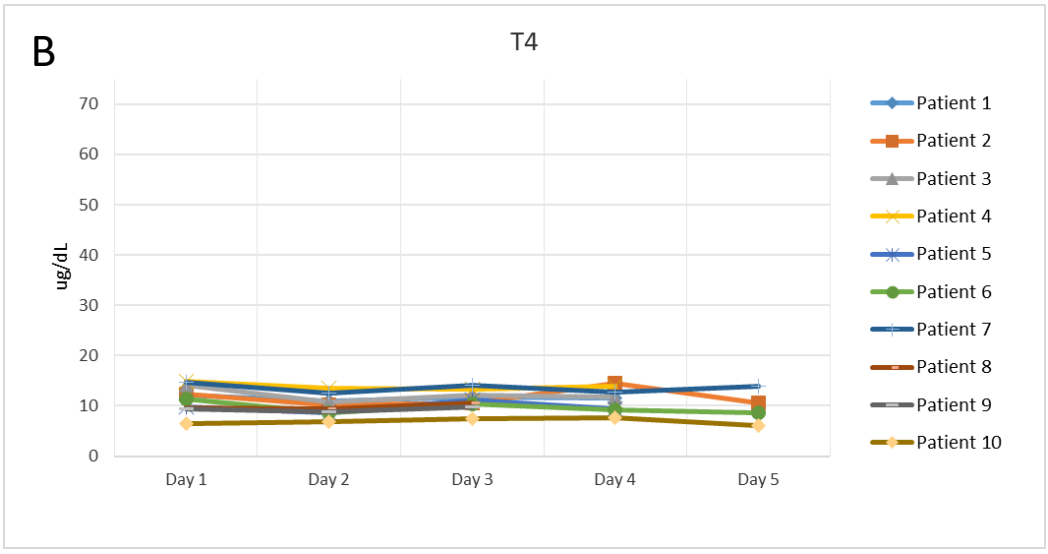
Plots of the values for each of the thyroid function analytes over the five-day time course are shown in Figures 1. Table 1 shows the mean CVs and the corresponding standard deviations for each analyte together with the ranges of the mean CVs. This table also shows the range of values obtained for each analyte together with the respective reference ranges. As can be seen in Table 1, T4 values were found to be either normal or high while T3 values occurred in low, normal and high ranges; free T4 and free T3 values were found to occur in the normal or high ranges. TSH values were found to occur exclusively in the reference range.

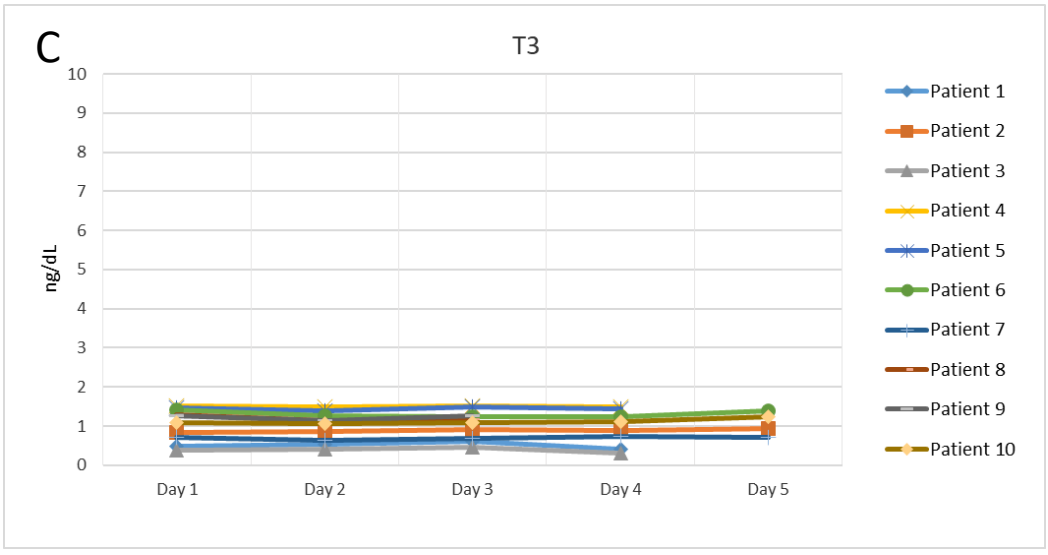
Table 1. Coefficients of Variation for Serum Assays forT4, T3, FT4, FT3 and TSH

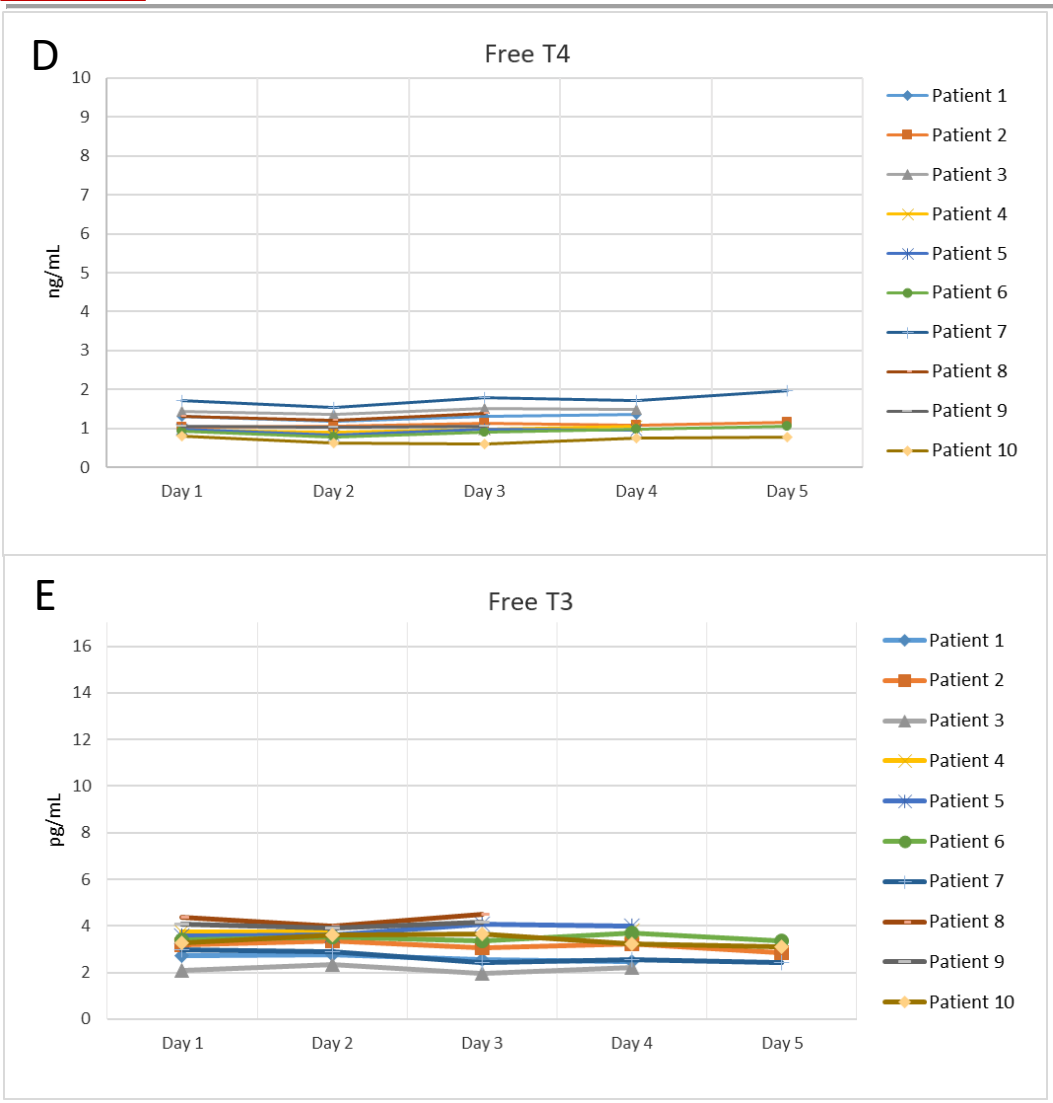
ANALYTE	MEAN CV	1 SD	CV RANGE	VALUE RANGE	REFERENCE RANGE
T4	0.087	0.04	0.039-0.156	6.9-14.9ug/dL	4.4-9.5 ug/dL
T3	0.073	0.055	0.01-0.167	0.31-1.52 ng/mL	0.72-1.35 ng/mL
FT4	0.071	0.034	0.015-0.131	0.70-3.26 ng/dL	0.58-1.67 ng/dL
FT3	0.058	0.025	0.008-0.098	1.97-4.52 pg/mL	2.53-3.87pg/mL
TSH	0.0327	0.014	0.011-0.056	0.7-3.26 mU/L	0.39-4.08 mU/L

Figures 1. Christian et al.









Figures 1. Serum sample values for each of the five thyroid function tests analytes by day during storage of the samples for five days at 4°C. Each sample has a unique color enabling its identification. A, TSH; B, T4; C, T3; D, Free T4; E, Free T3.

Figures 1 suggest overall that the values for each analyte are reproducible. However, Table 1 shows that there are some elevated CVs. Surprisingly, the highest CV was found for the most commonly assayed analyte, T4, whose mean CV was 8.7 percent with a wide standard deviation of almost half of this value. Inspection of the individual CV values for T4 revealed that sample 2 (orange plot in Figure 1B) was found to have a CV of 16 percent. This reflected a range of values from a low value of 10.12 on day 2 to a high value of 14.44ug/dL on day 4. All of the values for this sample were in the high range. However, the free T4 values for this sample were in the normal range (orange plot in Figure 1D). The TSH values for this sample were likewise in the normal range. Thus, the patient was euthyroid, and the consistently high, somewhat erratic, values for T4 were most likely caused by increased bound T4. Similarly, sample 6, whose CV was 13.1 percent, was found to have oscillating values for T4 (green plot in Figure 1B) which straddled the upper limit of the reference range, 9.5 ug/dL initially 11.44 (high) on day 1 which decreased to 8.54 (normal) on day 2, then to 10.38 (high) followed by normal values of 9.19 and 8.56 on days 4 and 5 respectively (green plot, Figure 1B). On the other hand, the free T4 was consistently in the normal range (green plot, Figure 1D), and the TSH was also in the normal range (green plot, Figure 1A) as described above. Thus, in this case, reliance on T4 values could confuse correct evaluation. The two reliable analytes are free T4 and TSH.

For sample 3, that had a CV of over 11 percent, the day 1 T4 value was 14.18 ug/dL, an elevated value, which then decreased on day 2 to 10.82 ug/dL also in the high range and then remained in this range though with higher values. Samples 1 and 3 were found to have high CVs for T3, but the range of values occurred in the normal range and represented relatively small changes in absolute value.

Because of the significant fluctuations of some of the values of some of the samples as discussed in the preceding paragraph and the high CVs associated with them, we tested the results to determine if the values for each sample over the five-day time period were statistically the same or different from one another. For this purpose, we used ANOVA statistics on the means and standard deviations for the results of each analyte for each day for all ten patients. The results are shown in Table 2. All p values for each analyte are seen to be significantly higher than the cutoff value of 0.05 indicating that the means for all patient values on each day were not statistically different from one another. Thus, despite fluctuations, the values for each analyte did not change in a statistically significant manner.

Table 2. Group means, Standard Deviations and ANOVA p values[1]

ANALYTE	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	P VALUE[1]
T4 (ug/dL)	11.482±2.705	10.035±1.989	11.096±1.845	11.350±2.415	9.749±3.20	0.525
T3 (ng/mL)	1.046±0.414	0.993±0.367	1.041±0.361	0.951±0.448	1.073±0.306	0.978
FT4 (ng/mL)	1.152±0.284	1.053±0.282	1.164±0.345	1.174±0.320	1.245±0.513	0.868
FT3 (pg/mL)	3.336±0.656	3.372±0.533	3.31±0.874	3.154±0.662	2.928±0.401	0.798
TSH (mU/L)	2.267±0.734	2.204±0.739	2.313±0.747	2.126±0.709	1.811±0.982	0.837

[1]p values ≥ 0.05 denote no statistical difference between the means of the groups.

Table 1 also shows that the lowest CV with the lowest standard deviation obtained for all five analytes was for TSH (CV= 3.27%, SD=0.014). This result is shown in Figure 2 where the CVs and standard deviations are plotted on the Y-axis for each of the five analytes represented on the X-axis. These results suggest that the CV for TSH is statistically significantly lower than the CVs for the other four analytes. To determine if this was the case, we compared the CV means and standard deviations of the other four analytes with those for TSH using the two-tailed t-test. The results of this comparison are shown in Table 2. This table shows that all p values are less than 0.05 indicating that the low CV for TSH is statistically significantly lower than for the other four thyroid analytes.

Figure 2. Christian et al.

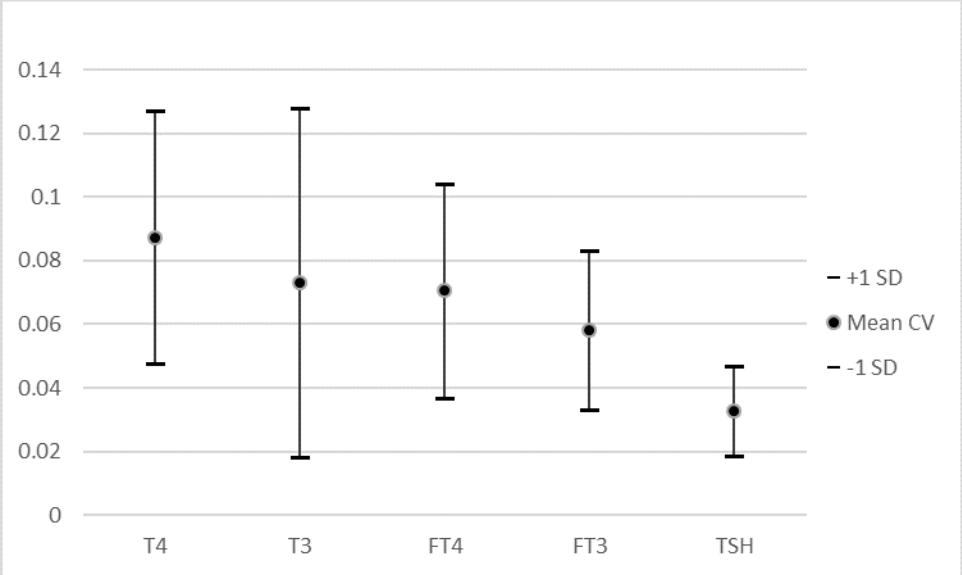


Figure 2. Plots of the means and standard deviations of correlation coefficient values for each of the five thyroid function analytes. The means with their standard deviations are plotted on the Y axis for each analyte identified on the X-axis.

Table 3. Statistical Significance of Mean CV Values for TSH vs the Other Thyroid Function Tests.

Comparison of Mean CV of TSH With Mean CV of:	P Value[1]
T4	0.0007
T3	0.0375
FT4	0.0049
FT3	0.0120

[1]Using two-tailed T-test. P values < 0.05 are considered to show statistically significant differences between two means given their respective standard deviations.

Discussion

Thyroid dysfunction is one of the most common endocrine disorders seen in clinical practice and represents approximately 30–40% of the patients seen in an endocrine clinic[13]. In addition, thyroid dysfunction can affect metabolic, cardiovascular, and neurologic function, and recent studies have reported subclinical thyroid anomalies to approach over 40%[14]. Furthermore, thyroid study "add-ons" represent a substantial component of the clinical chemistry and endocrinology testing in both inpatient (3 percent) and outpatient (8 percent) facilities[5]. Thus, a robust understanding of thyroid stability studies over time is critical in appropriately interpreting thyroid tests for patient diagnosis and management.

The mean CVs for all of the five thyroid function test analytes suggest that the values for these analytes are reproducible. This conclusion is reinforced by the finding that the group means for the values of each analyte remained statistically constant over the five-day period. A surprising finding was the relatively large changes in T4 values in some of the samples which could result in incorrect evaluation if a repeat determination of a sample found to have a high T4 is subsequently found to have a normal value that might also apply to an "add-on" test result. This phenomenon was not observed for free T4 or for TSH suggesting that these latter analytes are more reliable for patient evaluation than T4. It is also of interest that the analyte whose mean CV value was statistically the lowest of the mean CV values for the five analytes was that for TSH.

This latter finding raises the question as to possible reasons why TSH values are the most stable over the five-day observation period. This result is reminiscent of our previous finding on electrolyte stabilities[3] that sodium, potassium and chloride values had low CVs that were significantly lower than the CV for calcium. The difference between these electrolytes is that approximately 50 percent of serum calcium is bound to albumin while the other ions are not strongly bound to protein. All thyroid hormones are tightly bound to three different proteins, i.e., albumin, thyroid binding globulin (TBG) and transthyretin.

In a prior study of serum levels of thyroid function analytes in patients treated for hypothyroidism[15], many of the patients were found to be euthyroid (normal free T4 and TSH) but were clinically found to be hypothyroid. Analysis of thyroid function analytes by liquid chromatography-mass spectroscopy-mass spectroscopy (LC-MS-MS) revealed that the concentration of free T4 and total T4 were lower than that determined by immunoassay. The study found that free T4 levels were strongly influenced by levels of binding proteins such as TBG that caused false elevations[15].

In studies of thyroid function analyte stabilities in samples stored at low temperatures (in the current study, 4°C), the samples must be allowed to equilibrate to room temperature prior to analysis. Changes in temperature can result in significant changes in free T4 and/orT4 concentrations[16]. Studies on the dependence of the dissociation constant for the binding of T4 to TBG reveal that there is at least a threefold increase in this constant when the temperature increases from 4°C to 25°C[16]. Much of this temperature sensitivity of this constant to temperature is due to well-known structural features of TBG in which movement of a reactive loop out of a beta-sheet called the A sheet at low temperatures results in exposure of a T4 binding site. At higher temperatures re-entry of the loop blocks this site resulting in a decreased affinity of the protein for T4[16-18].

Thus, if the assay is performed at an ambient temperature that is higher than 25°C, such as at 30°C, there could be a significant increase in free T4. The reverse would hold at temperatures lower than 25°C. We are currently further investigating the possible role of protein binding on the reproducibility of assays for these analytes.

Conclusion

Serum levels, as determined by immunoassay, for the five thyroid function analytes, i.e., T4,T3, free T4, free T3 and TSH are stable over at least a five day period. The coefficients of variation (CVs) for the first four analytes are significantly higher than that for TSH.

A possible source of this increase in values may be due to the extensive binding of T4 and T3 to serum proteins so that their determinations may be influenced by changes in their binding affinity constants with such conditions as temperature. Thus, care should be taken when performing repeat or "add-on" analyses on serum samples to ensure that samples reach assay temperatures prior to analysis.

References

1. Lifshitz M. *Henry's Clinical Diagnosis and Management by Laboratory Methods*. 24th ed. (Mcpherson RA, Pincus MR, eds.). Elsevier; 2022:22-34.

2. Seydafkan S, Minkowitz J, Li G, et al. Short Communication: Stability of Glucose Levels in Serum and Plasma. Ann Clin Lab Sci. 2021;51(4):580-583.

3. Garcia R, Li G, Wang Z, Cabanero M, Pincus MR. Stability of electrolyte determinations on the Siemens Advia 1800 analyzer. J Clin Lab Anal. 2012;26(5):372-375. doi:10.1002/jcla.21533

4. Levitan D, Harper AE, Sun Y, et al. Do Serum Creatinine Levels Show Clinically Significant Fluctuations on Serial Determinations on the Siemens Advia 1800 Analyzer?. J Clin Lab Anal. 2017;31(1):e22012. doi:10.1002/jcla.22012

5. Nelson LS, Davis SR, Humble RM, Kulhavy J, Aman DR, Krasowski MD. Impact of add-on laboratory testing at an academic medical center: a five year retrospective study. BMC Clin Pathol. 2015;15:11. Published 2015 Jun 7. doi:10.1186/s12907-015-0011-7

6. Hajjiri F, Azmi D, Ibrahim Subhi Q, Hamid. Effect of Storage Temperature on the Stability of Free T4, Free T3 and Third Generation TSH Results by DPC Immulite 2000. Journal of the Bahrain Medical Society. 2003;15(4):204-207.

7. Koliakos G, Gaitatzi M, Grammaticos P. Stability of serum TSH concentration after non refrigerated storage. Minerva Endocrinol. 1999;24(3-4):113-115.

8. Basanta G, Gupta SP, Gautam S, Ashwini KN, Lal Das BK, et al. (2017) Effect of Delay Separation and Short Term Storage of Serum on Thyroid Stimulating Hormone (TSH). Open J Thyroid Res 1(1): 007-009. DOI: 10.17352/ojtr.000003

9. Oddie TH, Klein AH, Foley TP, Fisher DA. Variation in values for iodothyronine hormones, thyrotropin, and thyroxine-binding globulin in normal umbilical-cord serum with season and duration of storage. Clinical Chemistry. 1979;25(7):1251-1253. doi:10.1093/clinchem/25.7.1251

10. Nye L, Yeo TH, Chan V, Goldie D, Landon J. Stability of thyroxine and triiodothyronine in biological fluids. J Clin Pathol. 1975;28(11):915-919. doi:10.1136/jcp.28.11.915

11. Mannisto T, Surcel HM ., Bloigu A, et al. The Effect of Freezing, Thawing, and Short- and Long-Term Storage on Serum Thyrotropin, Thyroid Hormones, and Thyroid Autoantibodies: Implications for Analyzing Samples Stored in Serum Banks. Clinical Chemistry. 2007;53(11):1986-1987. doi:10.1373/clinchem.2007.091371

12. Panesar NS, Lit LC. Stability of serum thyroid hormones following 8-11 years of cold storage. Clin Chem Lab Med. 2010;48(3):409-412. doi:10.1515/CCLM.2010.067.

13. Diab N, Daya NR, Juraschek SP, et al. Prevalence and Risk Factors of Thyroid Dysfunction in Older Adults in the Community. Scientific Reports. 2019;9(1). doi:10.1038/s41598-019-49540-z.

14. Rashad NM, Samir GM. Prevalence, risks, and comorbidity of thyroid dysfunction: a cross-sectional epidemiological study. Egypt J Intern Med. 2019;31:635-641. doi:https://doi.org/10.4103/ejim.ejim_22_19

15. Welsh KJ, Soldin SJ. DIAGNOSIS OF ENDOCRINE DISEASE: How reliable are free thyroid and total T3 hormone assays?. Eur J Endocrinol. 2016;175(6):R255-R263. doi:10.1530/EJE-16-0193.

16. Qi X, Chan WL, Read RJ, Zhou A, Carrell RW. Temperature-responsive release of thyroxine and its environmental adaptation in Australians. Proc Biol Sci. 2014;281(1779):20132747. Published 2014 Jan 29. doi:10.1098/rspb.2013.2747

17. Zhou A, Wei Z, Read RJ, Carrell RW. Structural mechanism for the carriage and release of thyroxine in the blood. Proc. Natl Acad. Sci. USA. 2006;103 (13): 321–326. (doi:10.1073/pnas.0604080103)

18. Qi X et al. Allosteric modulation of hormone release from thyroxine and corticosteroid-binding globulins. J. Biol. Chem. 2011;286:16 163–173. (doi:10.1074/jbc.M110.171082)