The impact of five successive freezing-thawing cycles and storage at -20 °C for one, two, and three months on the stability of selected hormones

Mohanned Mohammed Bakir Al-Moosawi, Iman Midhat Abbas, Bushra Jaafar Abdulbaqi, Athraa Alauddin Abdullah

Abstract— Background: The storage conditions of serum specimens for a long time need to be established for the purpose of their use in reference interval studies, or epidemiologic studies. Experimental design frequently necessitates the use of frozen samples for retrospective studies. Blood specimens are frequently stored in central clinical laboratories for further analysis. In addition to that, sera may be used for more than a single study and undergo repeated freezing and thawing. Pre-analytical phase is the most critical part of the total analytical process which has significant effect on patient results in clinical chemistry testing. Most of the manufacturers of the diagnostic kits allow the usage of sera stored frozen and thawed only for once. Information on the stability of commonly used chemistry analytes in human sera in Iraq are less available. Stability studies of analytes after long-term storage compared with the fresh sample values, with estimation of recovery rates, are important to determine the effects of long-term storage. The determination of the significance of the change in the stability of an analyte after successive freezing – thawing cycles, and after different storage periods at -20 °C is important to interpret the results of our study.
Different methods can be used for this determination, but they are debatable.
Objectives: To determine the impact of five successive freezing-thawing cycles and storage at -20 °C for one, two, and three months on specimen stability of some hormones.
Methodology: The specimens collected from ten participants..Fasting venous blood was collected using gel & clot activator tubes. Sera of each subject were separated into 8 aliquots for each patient. Following baseline measurement (T_0), all were kept frozen until analysis for two experiments: Five samples of each subject were frozen at -20 °C and subjected to five successive freezing-thawing cycles. A group of sera were stored frozen at -20 °C for up to 1, 2 and 3 months respectively and then analyzed (F_1, F_2, F_3).
Assays were performed on the miniVIDAS analyze at the Central Public Health Laboratory in Baghdad, Iraq. FSH, LH, Prolactin, Testosterone, FT3, Ferritin, and TSH were measured.
The stability of the selected analytes was assessed by calculating the mean percentage deviation from the baseline measurement. The mean percentage deviation was reflected to the total allowable error obtained from Ricos et al. In addition, for each analyte; the Critical Change Value, and the Maximum Permissible Instability were calculated for each analyte to add additional resources for testing the stability of the selected analytes].
Results: All the analytes tested for stability did not exceed total allowable error except Testosterone which exceeded it the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months.
Testosterone exceeded the Critical Change Value after storage at -20 °C for 2 months, and the Maximum Permissible Instability at the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months. TSH and Ferritin exceeded the the Maximum Permissible Instability at the 4th and 5th freezing-thawing cycles.
Conclusion: There is an overall accepted stability for FSH, LH, Prolactin, FT3, Ferritin, and TSH when tested for the influence of freezing-thawing cycles and on storage at -20 °C for one, two, and three months. Testosterone showed instability after three freezing-thawing cycles and after storage at -20 °C for one, two, and three months.
Key words— Hormones, Stability, Freezing-thawing cycles, Storage time, Storage temperature, Total allowable error, Mean percentage deviation.

1 INTRODUCTION

THE storage conditions of serum specimens for a long time need to be established for the purpose of their use in reference interval studies, or epidemiologic studies [1].

The stability of blood samples during storage was defined as the capacity of sample material to retain the initial value of the quantity measured within specified limits and under specified conditions [4].

Experimental design frequently necessitates the use of frozen samples for retrospective studies. The

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planning for multiple experimental time points often results in samples that will be analyzed together at a later date and thus subjected to different periods of storage before analysis. Acquisition of samples after hours or on weekends often results in a few days of refrigeration before analysis. In addition, many investigators may not have access to –70 °C storage or may use frost-free –20 °C freezers without being aware that doing so may affect their experimental results. Studies of human blood samples, including stability analyses for refrigeration and the effects of freeze–thawing have resulted in guidelines for storage. [2], [3], [4].

Blood specimens are frequently stored in central clinical laboratories for further analysis. Parameters that may vary across collections include; i) power cut or voltage fluctuations of the freezers; and ii) using frost-free freezer that goes through numerous defrost cycles, resulting in the loss of stability of some analytes [1].

In addition to that, sera may be used for more than a single study and undergo repeated freezing and thawing [5].

Extra-analytical factors affecting the analysis process such as different transportation conditions, prolonged storage at high or low temperature, improper handling, are still sources of interest [6], [7]. Though it is highly recommended to study tests from freshly drawn serum or plasma, delay in the testing process or reuse of the samples for missing results that may cause false concentrations are sometimes inevitable [8], [9].

Pre-analytical phase is the most critical part of the total analytical process which has significant effect on patient results in clinical chemistry testing [10]. In the real daily work; it is possible to reanalyze samples stored to confirm the previous results or to perform additional testing, however the stability of the analytes must be assured before giving results, or before establishing new investigations. More than that, analyzing unsuitable samples often causes more spending of funds and expanding the total testing process.

Most of the manufacturers of the diagnostic kits allow the usage of sera stored frozen and thawed only for once, and their advice is to divide sera into the single usage aliquots before freezing. However, the number and volume of the aliquots must be taken into account regarding the handling and freezer space costs while planning the studies.

There are various studies examined how storage conditions affected the stability of various serum components [5],[11], [12], [13]. However, information on the stability of commonly used chemistry analytes in human sera in Iraq are less available.

Biospecimens is the term used to describe materials taken from the human body, such as tissue, blood, plasma, and urine that can be used for cancer diagnosis and analysis. At the beginning of processing a biospecimen multiple aliquots should be created at the beginning of processing a biospecimen rather than delayed until the specific assay is conducted, as repeated freeze–thaw cycles may be detrimental in some cases [14].

The authors introduced the principle of single use only for samples, circumventing multiple freezing and thawing cycles [14].

Ideally, a "freeze–thaw stable" fluid biospecimen is not affected by thermal, mechanical, or chemical stress. Thus, the goal in storage of biospecimens is to minimize or halt these detrimental processes [15]. Storage encompasses both short and long-term storage of biospecimens, depending on their planned future use. Biospecimens contain degradative molecules (eg, proteases, lipases, nucleases) [16]. Long-term storage may result in aggregation, precipitation, or biochemical degradation of proteins (altering both structure and activity), ice damage, dehydration and increase in salt concentration resulting in osmotic damage, formation of water crystals, recrystallization after thawing, and toxicity from substances that are added to the biospecimens in the freezing state (cryoprotectants) or in the drying state (lyoprotectants) to protect the active ingredients [15]. These changes may cause the real biological variations to disappear. There is considerable variation among biomarkers in stability and recovery; therefore, different storage conditions may apply depending on the downstream analyses [14], [17]. Freeze–thaw cycles are a major concern, and may happen unintentionally during transport of frozen samples or freezer failure or intentionally because the biospecimens are thawed for analyses and then refrozen. It is advised to perform pilot studies and to carefully search the literature before measuring biomarkers on stored biospecimens. It is also important to have some biospecimens available only for quality control (QC) purposes, on which the same biomarkers are measured in fresh biospecimens repeatedly on a regular annual basis to monitor any critical change [14].

Stability studies of analytes after long-term storage compared with the fresh sample values, with estimation of recovery rates, are important to determine the effects of long-term storage. Recovery rates may increase or decrease after long-term storage, and thus, result in either increased or attenuated risk ratios, respectively, when assessing the associations of an analyte with a disease state. Hormone analytes are stable when serum samples are stored at −80°C up to 13 months [18], but various studies of longer term stability of hormone analytes have shown different stability patterns depending on the analyte, time, and temperature of storage [14], [19].

The determination of the significance of the change in the stability of an analyte after successive freezing – thawing cycles, and after different storage periods at -20 °C is important to interpret the results of our study.

Different methods can be used for this determination, but they are debatable.

One method is to use t-tests to determine main effect differences, but this statistical approach is too strict in clinical practice [20]. Many authors use stability criteria based on the coefficient of biological variation (CVb) and/or the

coefficient of analytical variation (CVa) in different formulas [20], [21], [22], [23]. Formulas like the critical change value (CCV), the maximum permissible instability (MPI) and the total allowable error (TEa) are examples.

The CCV is generally used to compare sequential results from one individual. The MPI was introduced by the German working group of extra-analytical quality assurance of the German United Society for Clinical Chemistry and Laboratory Medicine [25]. The permissible deviation should be smaller than half of the total error derived from the sum of biological and analytical variability in this method.

There is a wide agreement for using TEa for internal and external quality control procedures, but the theoretical basis is doubted by some, because bias and imprecision are combined into a single parameter adjustments [26], [27]. The six sigma concept for quality control proposes minimal, desirable and optimal specifications for the Tea [26]. In our study we used the widely used desirable concentration for all analytes.

2 Aim of the Study

To determine the impact of five successive freezing-thawing cycles and storage at -20 °C for one, two, and three months on specimen stability of some hormones

3 Materials and Methods

3.1 Subjects

This study included ten participants working at the Central Public Health Laboratory.

The specimens collected from each participant were for the laboratory testing included in our research study.

All the participants informed for the study and signed the informed consent. The procedures were in accordance with the guidelines of the scientific research committee at the Public Health Directorate / Ministry of Health / Baghdad / Iraq.

Fasting venous blood (totally 10.0 cc blood) was collected in the morning into a 10 mL gel & clot activator tubes (HEBEI XINLE SCI & TECH Vacutainer Systems, Xinle City, Hebei Province, China). The sample tubes were left in upright position for 30 min at room temperature for complete clot formation. All were then centrifuged at 1800 x g for 10 minutes (according to the instruction of the tube manufacturer). Serum samples were checked visually for hemolysis and lipemia for possible interferences [28], [29]. Sera of each subject were aliquoted into a 10-mL plain tube (AFCO - DISPO, Jordan), composing of 8 aliquots for each patient.

Following baseline measurement (T0), all were kept frozen until analysis for two experiments below: Five samples of each subject were frozen at -20 °C. After 24 hours, all frozen sera in the plain tubes of each patient were thawed at room temperature for approximately 1 h at room temperature until completely thawed, and then mixed properly with automatic pipettes before analysis (freeze-thaw 1). Samples were immediately re-frozen at -20 °C for the next study day. This cycle was repeated for five consecutive days (T1, T2, T3, T4, T5) to yield freeze- thaw processing.

A group of sera were stored frozen at -20 °C for up to 1, 2 and 3 months, and then analyzed for stability in singleton at three time intervals (F1, F2, F3).

3.2 Methods

Assays were performed on the miniVIDAS analyzer (bioMérieux S.A. 69280 Marcy l'Etoile / France) with bioMérieux reagents at the Hormones Unit / Clinical Chemistry Department of Central Public Health Laboratory in Baghdad, Iraq.

The following biochemical constituents were assayed: Follicle Stimulating Hormone (FSH), Lutenizing Hormone (LH), Prolactin, Testosterone, Free Triiodothyronine (FT3), Ferritin, and Thyroid Stimulating Hormone.

Quality control was performed each day before studying aliquots with control materials (bioMérieux S.A. 69280 Marcy l'Etoile / France).

3.3 Statistical analysis

The distribution of the variables was determined using Shapiro-Wilk normality test.

The stabilities of the analytes after freeze- thaw cycles and after freezing storage were assessed by calculating the mean percentage deviation (mean percentage deviation = [(Tx-T0)/T0)*100%] where

T0: the mean of samples of the ten participants (baseline measurement);

Tx: mean of samples of the ten participants tested after five successive freeze-thaw cycles (T1, T2, T3, T4, T5), and after one, two, three months freezing at-20 (F1, F2, F3) respectively.

The mean percentage deviations were given with a 95% confidence interval (CI) and were reflected to the total allowable error (TEa = 0.25 * (CVi2 + CVg2)0.5 +1.65 * 0.5 * CVi), obtained from Ricos et al [30]. CVi describes the coefficient of variation within a subject while CVg describes the coefficient of variation between subjects.

In addition, for each analyte the CCV was calculated using the analytical variation and the biological variation (CCV = 2.8 * √(CVa2 + CVb2) [31]. The analytical variation was derived from the manufacturer's product insert, and the biological variation was derived from Ricos et al [30]. Finally, the MPI was calculated (MPI = (0.5(CVa + CVi)) [25].

4 Results

A range of variables were investigated to measure the influence of five successive freezing-thawing cycles and freezing at -20 °C on the stability of the selected hormones.

All analytes were measured freshly after blood collection (basal measurement), after five successive freeze-thaw cycles (T1, T2, T3, T4, T5), and after one, two, three months freezing at -20 (F1, F2, F3).

The TEa, CCV, and MPI were presented in separated tables to make them more readable.

The results and variations for two experiments are presented in Table (1), (2), and (3), and in Figure (1).

Table 1, 2, and 3 show the mean percentage deviations for all the measured hormones.

In Table (1) the bold cells are used to represent the deviations that exceeded the TEa.

With the except of Testosterone, all the results of analytes that included in the experiment of the current study did not exceed the TEa at every freezing-thawing cycle and at storage at the three points of storage at -20 °C.

Testosterone exceeded the TEa at five points; the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months.

TABLE 1 STABILIY OF ANALYTES WHEN ASSESSED AGAINST DESIRABLE TOTAL ALLOWABLE ERROR

Analyte	Desirable TEa	Mean T_0	Parameter	T_1	T_2	T_3	T_4	T_5	F_1	F_2	F_3
FSH mIU/mL	21.19	4.647	Mean	4.702	4.709	4.741	4.820	4.919	4.473	4.965	4.505
			CI	(+3.0,+6.3)	(+3.0,+6.3)	(+3.1,+6.3)	(+3.1,+6.4)	(+3.2,+6.5)	(+2.6,+6.0)	(+3.1,+6.7)	(+2.9,+6.1)
			Mean % Deviation	1.18	1.33	2.02	2.02	5.85	-3.74	6.84	-3.05
LH mIU/mL	27.92	3.355	Mean	3.357	3.479	3.567	3.659	3.733	3.625	3.676	3.444
			CI	(+2.1,+ 4.5)	(+2.2,+ 4.7)	(+2.2,+ 4.8)	(+2.3,+ 4.9)	(+2.4,+ 5.0)	(+2.3,+ 4.9)	(+2.3,+ 4.9)	(+2.2,+ 4.6)
			Mean % Deviation	0.05	3.69	6.31	9.06	11.26	8.04	9.56	2.65
Prolactin ng/mL	29.4	14.68	Mean	15.075	15.592	15.856	15.873	16.092	16.072	16.247	15.040
			CI	(+11.8,+18.4	(+11.9,+19.3)	(+12.4,+19.3	(+12.4,+19.3	(+12.4,+19.6	(+12.6,+19.6	(+12.8,+19.7)	11.6,18.2
			Mean % Deviation	2.69	6.21	8.01	8.12	9.61	9.48	10.67	2.45
Testosterone ng/mL	13.61	0.246	Mean	0.254	0.255	0.267	0.314	0.331	0.305	0.355	0.304
			CI	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.2,+ 0.4)	(+0.1,+ 0.4)	(+0.1,+ 0.4)	(+0.2,+ 0.7)	(+0.1,+ 0.4)
			Mean % Deviation	3.15	3.60	8.55	**27.47**	**34.23**	**23.87**	**44.14**	**23.42**
Free T3 pmol/L	11.3	3.864	Mean	3.808	3.862	3.876	3.878	3.904			
			CI	(+3.40,+4.21	(+3.32,+4.40)	(+3.20,+4.54	(+3.34,+4.41	(+3.41,+4.39	NA	NA	NA
			Mean % Deviation	-1.44	-0.05	0.31	0.36	1.03			
TSH µIU/mL	23.7	3.752	Mean	3.877	3.895	3.895	4.445	4.470			4.747
			CI	(-2.8,+10.5)	(-3.0,+10.8)	(-3.0,+10.8)	(-3.2,+12.1)	(-3.2,+12.1)	NA	NA	(-1.7,+11.2)
			Mean % Deviation	3.33	3.79	3.79	18.45	19.12			10.79
Ferritin ng/mL	16.9	13.18	Mean	13.79	13.84	14.05	14.71	14.89	13.54		
			CI	(-18.8,+46.5)	(-19.0,+46.7)	(-17.8,+45.9)	(-18.2,+47.6)	(-18.7,+48.7)	(-18.7,+45.8)	NA	NA
			Mean % Deviation	4.62	4.95	6.54	11.57	12.96	2.70		

Note: Mean % deviations in relation to T0; Bold cells exceed total allowable error

T1 – T5 Freezing-thawing cycles; F1 – F3 Storage at -20 °C for 1, 2, and 3 months; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; TSH: Thyroid Stimulating Hormone; CI: 95% Confidence Interval.

The results and variations for two experiments when using CCV as the decision limit are presented in table (2).

Bold cells are used to represent the deviations that exceeded the CCV. Again we found that Testosterone exceeded the CCV but at one point; after storage at -20 °C for 2 months.

TABLE 2 STABILITY OF ANALYTES WHEN ASSESSED AGAINST CRITICAL CHANGE VALUE

Analyte	CCV	Mean T_0	Unit	T_1	T_2	T_3	T_4	T_5	F_1	F_2	F_3
FSH mIU/mL	32.3	4.647	Mean	4.702	4.709	4.741	4.820	4.919	4.473	4.965	4.505
			CI	(+3.0,+6.3)	(+3.0,+6.3)	(+3.1,+6.3)	(+3.1,+6.4)	(+3.2,+6.5)	(+2.6,+6.0)	(+3.1,+6.7)	(+2.9,+6.1)
			Mean % Deviation	1.18	1.33	2.02	2.02	5.85	-3.74	6.84	-3.05
LH mIU/mL	65.2	3.355	Mean	3.357	3.479	3.567	3.659	3.733	3.625	3.676	3.444
			CI	(+2.1,+ 4.5)	(+2.2,+ 4.7)	(+2.2,+ 4.8)	(+2.3,+ 4.9)	(+2.4,+ 5.0)	(+2.3,+ 4.9)	(+2.3,+ 4.9)	(+2.2,+ 4.6)
			Mean % Deviation	0.05	3.69	6.31	9.06	11.26	8.04	9.56	2.65
Prolactin ng/mL	64.8	14.68	Mean	15.075	15.592	15.856	15.873	16.092	16.072	16.247	15.040
			CI	(+11.8,+18.4	(+11.9,+19.3)	(+12.4,+19.3	(+12.4,+19.3	(+12.4,+19.6	(+12.6,+19.6	(+12.8,+19.7)	11.6,18.2
			Mean % Deviation	2.69	6.21	8.01	8.12	9.61	9.48	10.67	2.45
Testosterone ng/mL	41.8	0.246	Mean	0.254	0.255	0.267	0.314	0.331	0.305	0.355	0.304
			CI	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.2,+ 0.4)	(+0.1,+ 0.4)	(+0.1,+ 0.4)	(+0.2,+ 0.7)	(+0.1,+ 0.4)
			Mean % Deviation	3.15	3.60	8.55	27.47	34.23	23.87	**44.14**	23.42
Free T3 pmol/L	28.6	3.864	Mean	3.808	3.862	3.876	3.878	3.904			
			CI	(+3.40,+4.21	(+3.32,+4.40)	(+3.20,+4.54	(+3.34,+4.41	(+3.41,+4.39	NA	NA	NA
			Mean % Deviation	-1.44	-0.05	0.31	0.36	1.03			
TSH µIU/mL	55.1	3.752	Mean	3.877	3.895	3.895	4.445	4.470			4.747
			CI	(-2.8,+10.5)	(-3.0,+10.8)	(-3.0,+10.8)	(-3.2,+12.1)	(-3.2,+12.1)	NA	NA	(-1.7,+11.2)
			Mean % Deviation	3.33	3.79	3.79	18.45	19.12			10.79
Ferritin ng/mL	41.6	13.18	Mean	13.79	13.84	14.05	14.71	14.89	13.54		
			CI	(-18.8,+46.5)	(-19.0,+46.7)	(-17.8,+45.9)	(-18.2,+47.6)	(-18.7,+48.7)	(-18.7,+45.8)	NA	NA
			Mean % Deviation	4.62	4.95	6.54	11.57	12.96	2.70		

Note: Mean % deviations in relation to T0; Bold cells exceed Critical Change Value

Table (3) illustrates the results and variations for two experiments when using MPI as the decision limit.

Bold cells are used to represent the deviations that exceeded the MPI. Again Testosterone exceeded the MPI at five points; the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months. TSH and Ferritin exceeded the MPI at the 4th and 5th freezing-thawing cycles.

TABLE 3 STABILITY OF ANALYTES WHEN ASSESSED AGAINST MAXIMUM PERMISSIBLE INSTABILITY

Analyte	MPI	Mean T_0	Unit	T_1	T_2	T_3	T_4	T_5	F_1	F_2	F_3
FSH mIU/mL	7.25	4.647	Mean	4.702	4.709	4.741	4.820	4.919	4.473	4.965	4.505
			CI	(+3.0,+6.3)	(+3.0,+6.3)	(+3.1,+6.3)	(+3.1,+6.4)	(+3.2,+6.5)	(+2.6,+6.0)	(+3.1,+6.7)	(+2.9,+6.1)
			Mean % Deviation	1.18	1.33	2.02	2.02	5.85	-3.74	6.84	-3.05
LH mIU/mL	13.4	3.355	Mean	3.357	3.479	3.567	3.659	3.733	3.625	3.676	3.444
			CI	(+2.1,+ 4.5)	(+2.2,+ 4.7)	(+2.2,+ 4.8)	(+2.3,+ 4.9)	(+2.4,+ 5.0)	(+2.3,+ 4.9)	(+2.3,+ 4.9)	(+2.2,+ 4.6)
			Mean % Deviation	0.05	3.69	6.31	9.06	11.26	8.04	9.56	2.65
Prolactin ng/mL	12.85	14.68	Mean	15.075	15.592	15.856	15.873	16.092	16.072	16.247	15.040
			CI	(+11.8,+18.4)	(+11.9,+19.3)	(+12.4,+19.3)	(+12.4,+19.3)	(+12.4,+19.6)	(+12.6,+19.6	(+12.8,+19.7)	11.6,18.2
			Mean % Deviation	2.69	6.21	8.01	8.12	9.61	9.48	10.67	2.45
Testosterone ng/mL	10.38	0.246	Mean	0.254	0.255	0.267	0.314	0.331	0.305	0.355	0.304
			CI	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.1,+ 0.3)	(+0.2,+ 0.4)	(+0.1,+ 0.4)	(+0.1,+ 0.4)	(+0.2,+ 0.7)	(+0.1,+ 0.4)
			Mean % Deviation	3.15	3.60	8.55	**27.47**	**34.23**	**23.87**	**44.14**	**23.42**
Free T3 pmol/L	7.2	3.864	Mean	3.808	3.862	3.876	3.878	3.904	NA	NA	NA
			CI	(+3.40,+4.21	(+3.32,+4.40)	(+3.20,+4.54	(+3.34,+4.41	(+3.41,+4.39)			
			Mean % Deviation	-1.44	-0.05	0.31	0.36	1.03			
TSH µIU/mL	11.6	3.752	Mean	3.877	3.895	3.895	4.445	4.470	NA	NA	4.747
			CI	(-2.8,+10.5)	(-3.0,+10.8)	(-3.0,+10.8)	(-3.2,+12.1)	(-3.2,+12.1)			(-1.7,+11.2)
			Mean % Deviation	3.33	3.79	3.79	**18.45**	**19.12**			10.79
Ferritin ng/mL	9.3	13.18	Mean	13.79	13.84	14.05	14.71	14.89	13.54	NA	NA
			CI	(-18.8,+46.5)	(-19.0,+46.7)	(-17.8,+45.9)	(-18.2,+47.6)	(-18.7,+48.7)	(-18.7,+45.8)		
			Mean % Deviation	4.62	4.95	6.54	**11.57**	**12.96**	2.70		

Note: Mean % deviation in relation to T0; Bold cells exceed Maximum Permissible Instability

The mean percentage change in concentrations during freezing-thawing cycles and during storage at -20 °C for three months period are illustrated in Figure (1) and (2) respectively.

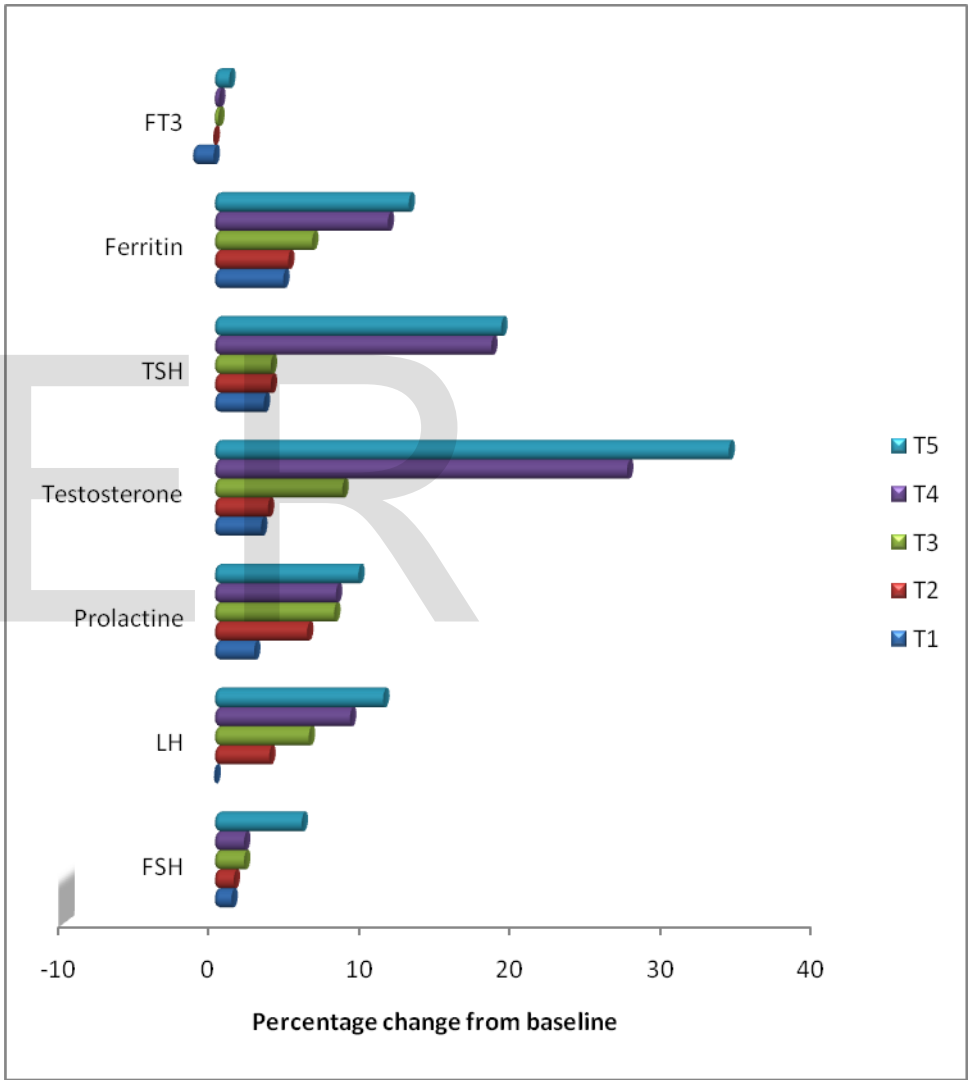

Fig 1 Mean percentage change in concentrations during five successive freezing-thawing cycles

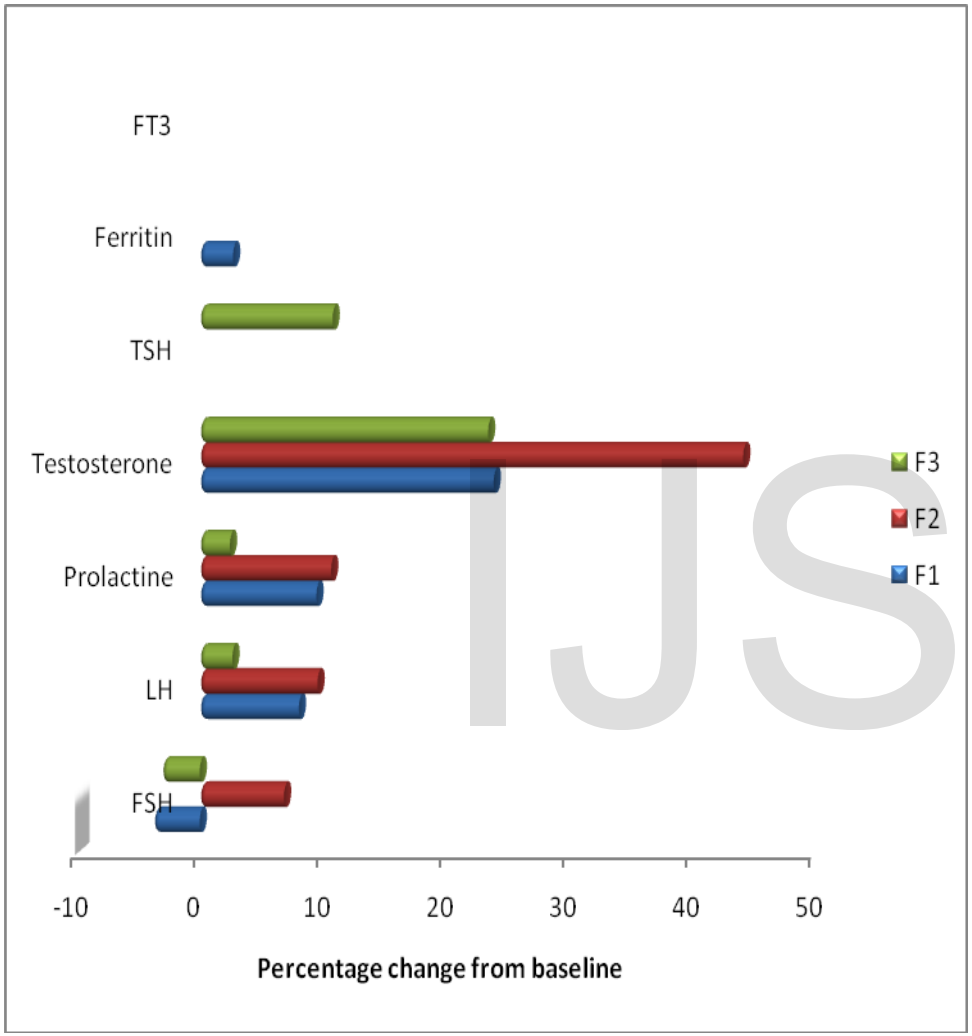

Figure 2 Mean percentage change during storage at -20 °C for 1, 2, and 3 months

5 Discussion

A high quality of laboratory analysis is crucial to diagnose and monitor patients, and it is one of the technical requirements of ISO15189.

It is important to deal carefully while managing the pre analytical phase of some analytes as they show greater variation than the desirable acceptable error.

In this study, we investigated the influence of freezing-thawing cycles and storage at -20 °C for three months period on the stability of some hormones. The stability of the selected analytes was tested against the TEa, CCD, and MPI.

The manufacturer of the diagnostic kits used in this study recommends the avoidance of successive freezing and thawing.

For FSH, FT3, LH, Ferritin, and TSH the manufacturer recommends the storage of the samples in (-25 ±6 °C) for long storage duration without defining specific duration [44], [45], [48], [49].

The manufacturer recommends the storage of samples for Prolactin and Testosterone at (-25 ±6 °C) for two months with a note for Testosterone samples to have slight variances with no clinical consequence [46], [47].

Our study showed that all the selected analytes were stable when reflected against TEa with the except of Testosterone, all the results of analytes that included in the experiment of the current study did not exceed the TEa at every freezing-thawing cycle and at storage at the three points of storage at -20 °C. Testosterone exceeded the TEa at five points; the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months.

The results and variations for two experiments when using CCV as the decision limit showed that Testosterone exceeded the CCV but at one point; after storage at -20 °C for 2 months. We must keep in mind that CCV method deals with within-subject variation and not with between subject variations [25], therefore it was considered less suitable to use in our study.

When using MPI as the decision limit Testosterone exceeded the acceptable limit at five points; the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months. TSH and Ferritin exceeded the MPI at the 4th and 5th freezing-thawing cycles.

When using MPI the decision limits become narrow. More than that, the working group warns that the stability of constituents in the sample can be considerably reduced under pathological conditions. These small ranges are unworkable in daily practice of most clinical laboratories [25].

Previous results showed controversial results although the studied hormones showed an overall acceptable stability.

The finding of Reyna R and her colleagues [34] agrees with our findings. They found that there is no consistent or predictable alteration in the results of FSH, LH, and prolactin when evaluated as a function of repeated freeze/thaw or the storage temperature of human serum.

Mannisto and his colleagues concluded that TSH, and FT3 can reliably be analyzed in samples stored for 23 years at -25 °C [11]. Our findings showed that TSH was reasonably stable when stored at -20 °C for three months.

Livesey JH and colleagues found that LH, FSH, TSH, and prolactin hormones were stable during five freeze-thaw cycles [39]. A finding agrees with our study.

Kathy J. Helzlsouer and her colleagues found that no effects on serum FSH, and LH hormone levels after repeated freeze-thaw cycles, up to three cycles were observed [42]. Again these findings agree with our study.

Ann W. Hsing and her colleagues found that the effect of freezing and thawing on LH, FSH, Testosterone, and Prolactin hormones are not likely to be catastrophic [5]. The findings of Testosterone in our study differ from Hsing et.al findings and this could be explained by the fact of difference in the measurement method which results in different performance specifications and to the difference in statistical methods used.

Multiple freeze–thaw cycles did not affect TSH in study done by Jacquelien J Hillebrand and her colleagues [32]. Although that the freezing point in this study was similar to that in our study but we do not have enough information regarding the measurement method and statistical methods used to study the stability.

Holl K and her colleagues showed that long-term storage (up to 22 years) did not materially affect the testosterone level in women in the median age of their first pregnancy with serum samples drawn during the first trimester [19]. This study differs from our study in the studied participants and the measurement procedure which may result in different performance characteristics.

In the study of Randi E. Gislefoss and colleagues; testosterone and thyroid stimulating hormone (TSH) showed overall statistically significant changes in serum levels when robust to 10 repeated thaws compared to baseline level [33]. Although that this finding disagree with our study but we must keep in mind the difference in the number freezing thawing cycles and the different statistical method was used to investigate the stability of the analytes.

Wickings and Nieschlag assayed four aliquots of plasma after 33 freeze-thaw cycles. They concluded that repeated freezing and thawing of plasma samples did not affect the plasma concentrations of testosterone [37]. A different measurement procedure was used in this study which means different performance characteristics especially that the results were compared against the intra-assay coefficient of variation.

George W. Comstock and his colleagues believed that repeated freezing to -70 °C and thawing has no meaningful effects on the plasma and serum concentrations of a FSH, LH, and Testosterone [38]. This was a different model as we studied the analytes at a freezing point of -20 °C, and freezing the samples at -70 provides more stability to hormones [41].

Nirmal and Lydia detected significantly lower TSH and increased FT4 and FT3 concentrations in the stored samples after 8-11 yr at -80 C when tested against the methodological performances in EQA [40]. A different storage time and temperature as well as different way in investigating the stability were used in this study.

Testosterone is subjected to many pre-analytical factors. Intensive physical activity (within 12 hours before blood sampling) may affect homeostasis for Testosterone. Hemolysis has been shown to cause negative interference with concentration of Testosterone. Some blood collection tubes with clot activators based on silica particles affect the measurement of Testosterone. Testosterone exhibits rhythmic variation in its circulating concentration [43]. All these factors may contribute to the variability of results among the research studies.

The small participant's group size is a limitation of the current study. Secondly our measurements were done with the defined autoimmune analyzer using its original reagents. Therefore, these results might not be reproducible with other testing systems.

It is important to notice that many authors concluded that storage at -20 °C may not be acceptable for sex hormones. However, long-term storage at -70 °C seems to be acceptable for most sex hormones [41].

As we used TEa as the main factor to study the stability of the analytes in the current study; some authors showed that TEa is overestimated by Westgard [35]. Oosterhuis said that the maximum allowable imprecision and bias have been derived separately from data on biological variation for different purposes, and the combination of these maximum values into a single expression has no theoretical basis and leads to gross overestimation of TEa [36].

So it is important to keep in mind the fact of different statistical methods and different models used to study the stability of the analytes in the previous researches.

6 Conclusion

In this study, we investigated the influence of freezing-thawing cycles and storage at -20 °C for three months period on the stability of seven analytes. The stability of the selected analytes was tested against the Total Allowable Error (TEa), Critical Change Value (CCD), and Maximum Permissible Instability (MPI). There is an overall accepted stability of the studied analytes when tested for the influence of freezing-thawing cycles and on storage at -20 °C for one, two, and three months.

The exception was with Testosterone which exceeded the TEa and MPI at five points; the 4th and 5th freezing-thawing cycles, and after storage at -20 °C for 1, 2, and 3 months, and exceeded the CCV after storage at -20 °C for 2 months.
TSH and Ferritin exceeded the decision limit at the 4th and 5th freezing-thawing cycles when reflected to MPI.

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