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The stability of C-peptide and insulin in plasma and serum samples under different storage conditions

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Abstract

Objectives: C-peptide and insulin are peptide hormones and their stability is affected by a number of pre-analytical factors. The study aimed to investigate the impact of sample type, storage temperature and time delays before centrifugation and analysis on the stability of C-peptide and insulin.

Methods: Ten healthy non-diabetic adults in fasting and non-fasting state were enrolled. 40 mL of blood was collected from each participant into SST and dipotassium EDTA tubes. Samples were centrifuged immediately or at timed intervals (8, 12, 48 and 72 h). After baseline measurements on the Roche Cobas e602 analyzer using electrochemiluminescence immunoassays, aliquots were stored at room temperature (RT), 2–8 and −20 °C for 4 h to 30 days. The percentage deviation (PD) from baseline was calculated and a change greater than desirable biological variation total error was considered clinically significant.

Results: C-peptide was more stable in separated serum than plasma (PD of −5 vs. −13 %) samples stored at 2–8 °C for 7 days and was most unstable at RT when centrifugation was delayed (PD −46 % in plasma and −74 % in serum after 48 h). Insulin was more stable in plasma than in serum under the different storage conditions with a minimum PD of −1% when stored at −20 °C for 30 days. When samples were kept unspun at RT for 72 h, PD was −23 and −80 % in plasma and serum, respectively.

Conclusions: C-peptide was more stable in serum provided the sample was centrifuged immediately and stored in the fridge or freezer while insulin was found to be more stable in EDTA plasma.

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Keywords: C-peptide; insulin; sample stability

Introduction

Insulin and C-peptide are peptide hormones produced by the beta cells of the pancreatic islets. Insulin is involved in the regulation of blood glucose levels whereas the function of C-peptide is not well elucidated [1]. Accurate measurements of insulin and C-peptide are important because of their clinical use. These analytes are requested for assessment of insulin resistance, insulinoma and hypoglycaemia [1]. However, their stability is affected by a number of pre-analytical factors such as collection tube preservative type, storage temperature and time delay before analysis [2–4]. Stability is defined as the ability of a biochemical analyte of a sample or specimen material to retain its properties over time [5].

Studies looking at the stability of C-peptide and insulin in different sample types and storage conditions are limited. In addition, there have been contradictory results on stability of these analytes in tripotassium ethylenediaminetetraacetic acid (EDTA) plasma compared to in serum [2–4]. Oddoze and colleagues noted that when insulin was measured in serum, stability was prolonged up to 72 h when stored at 4 °C compared to at room temperature (RT) where it was only stable for 6 h. Stability of insulin in EDTA plasma at RT showed a slight improvement. The decrease in concentrations exceeded acceptable limits at 48 h in RT compared to 72 h at 4 °C. Furthermore, in this study, C-peptide was more stable at 4 °C up to 72 h in both EDTA plasma and serum separator tubes (SST) serum. The drawback to this study is lack of comparison of stability of C-peptide in SST and EDTA when centrifugation was delayed. Another study by McDonald et al. assessed the stability of insulin and C-peptide in both sample types (serum and plasma), as well as in uncentrifuged samples for 120 h and showed that C-peptide was more stable in EDTA (up to 36 h) than in SST when the samples were left unspun at room temperature [3]. However, the findings in Oddoze et al. study seem to suggest that the timing of centrifugation is pivotal in maintaining stability of C-peptide as they showed that C-peptide was more stable in centrifuged

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samples collected in SST compared to EDTA at room temperature [4].

Comparison of stability studies on insulin and C-peptide are made difficult by the different protocols and acceptable limits criteria used. In addition, stability in most studies was assessed over hours and for up to seven days [3, 4, 6]. Insulin and C-peptide were analyzed on different analyzers and at different sites, which introduces more variability unrelated to stability [3]. Only sample type (plasma and serum) and storage temperature (RT and 4 °C) were compared and delay in sample centrifugation was not assessed in some studies, which is often a challenge in our setting [4, 7, 8]. Furthermore, no clearly defined protocol was used for these studies [2–4]. Delay in centrifugation is often a challenge in our setting because we are a referral laboratory which receives uncentrifuged samples from other hospitals and clinics. The impact of these factors on stability of insulin and C-peptide is particularly relevant across the African continent and other low to middle income countries, where laboratory sample transportation to central laboratories may take up to several days and due to resource constraints samples may be batched prior to analysis [9].

Therefore, the aim of this study was to investigate the impact of sample type (plasma and serum), storage temperature and time delay before centrifugation and analysis on stability of insulin and C-peptide in human blood over a 30-day period following the recently published guidelines on stability studies.

Materials and methods

The study was conducted at the National Health Laboratory Services (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). CMJAH is a tertiary hospital situated in the city of Johannesburg, South Africa. The CMJAH NHLS laboratory receives samples from CMJAH hospital and surrounding clinics and smaller hospitals.

Ethics approval

The study was approved by the University of Witwatersrand Human Research Ethics Committee (Clearance number M1911105) and followed the Declaration of Helsinki.

Study participants

Ten healthy non-diabetic adult staff volunteers (five males, five females), from the department of Chemical pathology were recruited for the study. To achieve a wide range of concentrations for C-peptide and insulin, five volunteers were requested to fast for eight to ten hours prior to blood collection, while five were post prandial (last meal ranging from 30 min to 2 h prior to blood collection). The volunteers completed questionnaires and were not on biotin supplements to avoid interference with insulin and C-peptide measurements. Written informed consent was obtained from all the participants prior to blood collection.

Sample collection

Approximately 40 mL of blood was collected from each participant in a single draw using syringe (Avacare Health). Aliquots were then transferred into eight Becton, Dickinson and Company (BD) SST (three five ml adult tubes, batch number 9127604 and five pediatric 600 µL tubes, batch number 9282035) and eight dipotassium EDTA (three four ml adult tubes, batch number 9092822 and five pediatric 500 µL tubes, batch number 9190053). The paediatric tubes were used for samples that were stored as whole blood to be centrifuged at different time points to avoid aliquoting whole blood. Adult tubes were used for samples that were centrifuged and stored as serum or plasma. In addition, using paediatric tubes minimized the amount of blood collected from each participant. All the adult tubes were centrifuged within 1 h of collection at 3,500 rpm for 10 min at RT. One of the pediatric tubes was centrifuged within 1 h and the remaining stored at RT and centrifuged at the time of analysis (Figure 1). The centrifuged samples were immediately aliquoted into Hitachi cups (1.5 mL) for baseline measurement. For the remaining centrifuged samples, 1 mL was aliquoted into Eppendorf tubes (1.8 mL) and Hitachi cups (1.5 mL) to avoid sample evaporation. Samples in Eppendorf tubes were stored in a freezer at –20 °C and one sample analyzed at different time points (72 h, day 7, day 21 and day 30) to avoid the effect of the freeze-thaw cycle. Similarly, samples in Hitachi cups were stored at room temperature and 2–8 °C and one sample analyzed at different time points (4 h, 8 h, 24 h and 48 h at RT and 24 h, 48 h, day 5 and day 7 at 2–8 °C) as shown on Figure 2.

Frozen samples were thawed once for 20 min to RT (maintained at 18–26 °C) before analysis. Electronic thermometers in the refrigerators were used to monitor the storage temperatures. The pediatric tubes that were not centrifuged within 1 h were stored at RT to centrifuge at the time of analysis at the 8 h, 12 h, 48 h and 72 h time intervals as shown on Figure 2.

Sample analysis

Samples were analyzed on the Roche Cobas e602 analyzer (Roche Diagnostics, 68298 Mannheim, Germany) using electrochemiluminescence immunoassay kits. Both insulin and C-peptide use sandwich immunoassays employing biotinylated monoclonal antibody and ruthenium labeled monoclonal antibody. The insulin assay is standardized to the 1st IRP WHO Reference Standard 66/304 from National Institute for Biological Standards and Control (NIBSC) while the C-peptide assay to the WHO international reference reagent for C-peptide of human insulin for immunoassay, IRR, code 84/510 from NIBSC. Analytical coefficient variation (CV) for insulin assay was 7 % at concentrations 22.7 and 75.6 µU/mL, and for C-peptide CV was 3.1 % at 2.03 ng/mL and 3.4 % at 10 ng/mL. The external quality controls for both analytes were within acceptable limits.

Statistical analysis

Results were captured on Microsoft Excel spreadsheet. Statistical analysis was done using Stata software version 16.64 bit (StataCorp LLC College Station, Texas, USA). Descriptive statistics were used to describe participants' characteristics. The D'Agositino-Pearson test

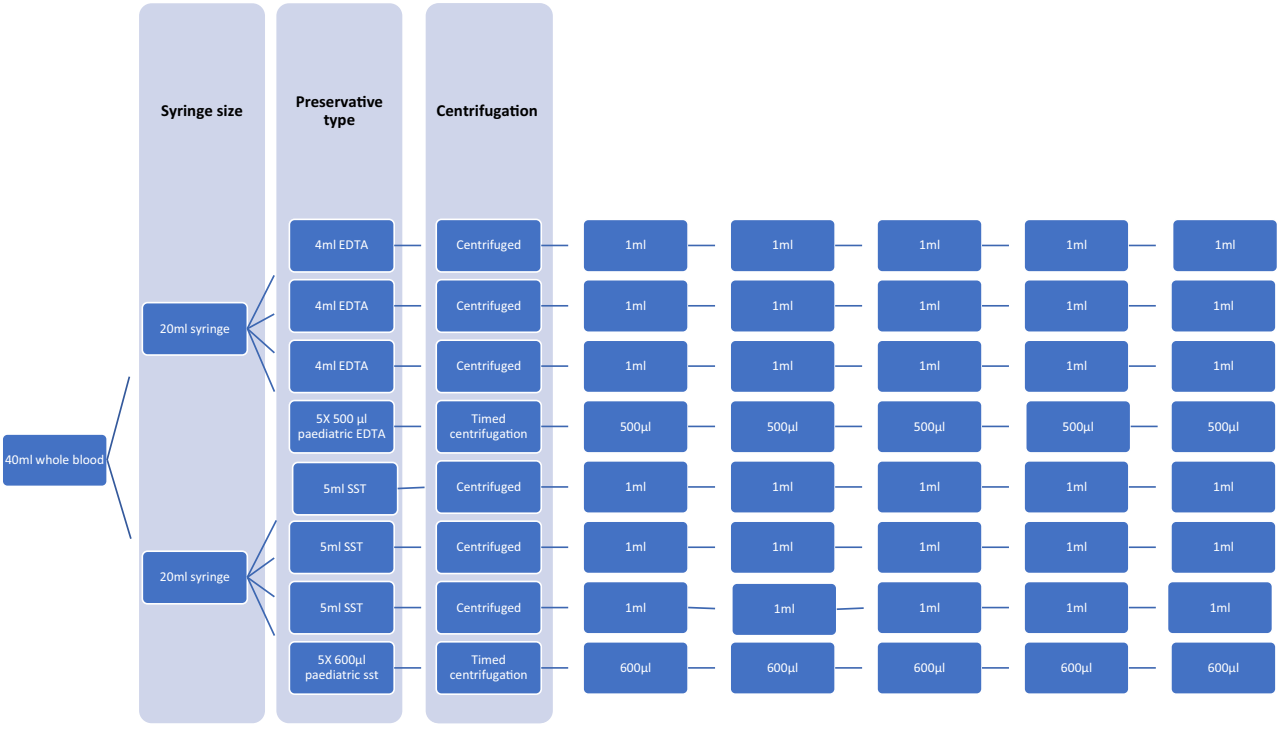


Figure 1: Sample collection procedure from each participant.

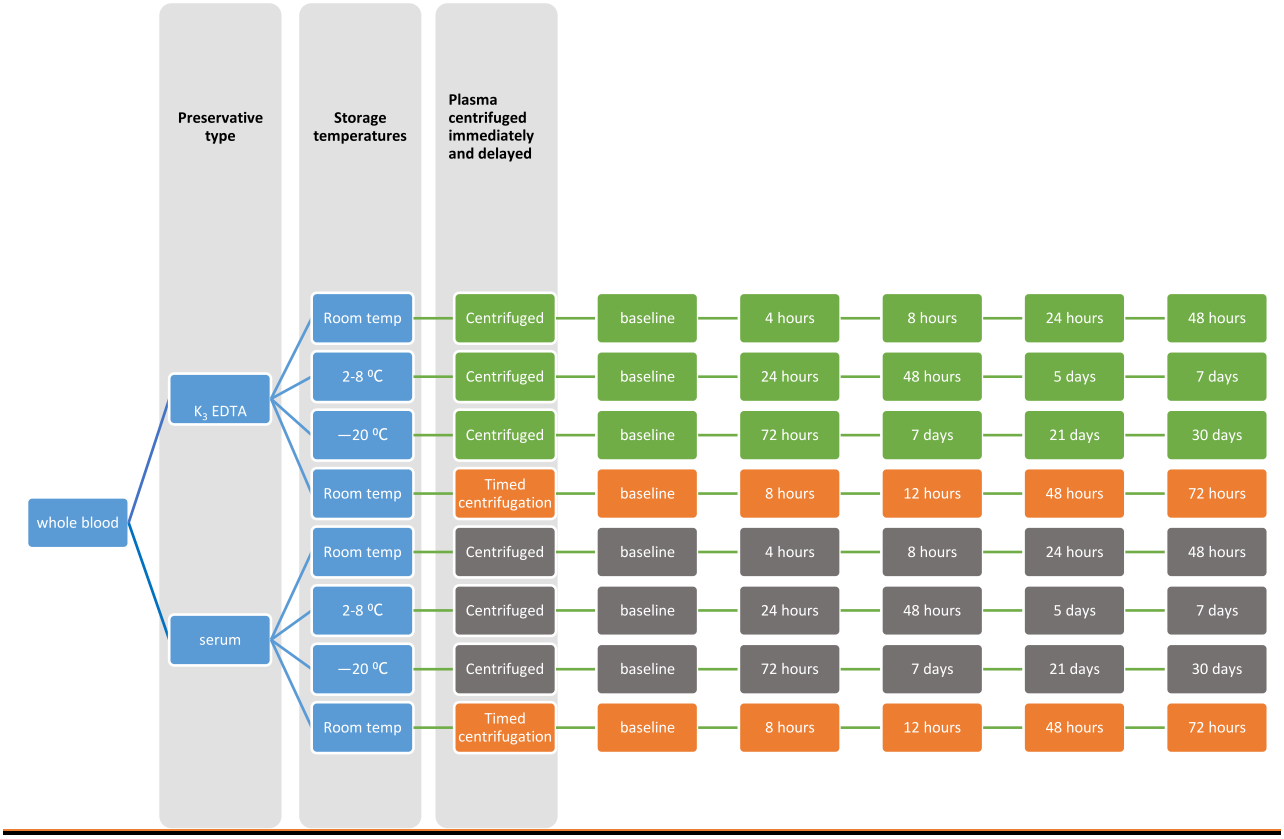


Figure 2: Flow diagram showing sample processing and analysis procedure over 30-day period. Stored as serum-grey, plasma-green and whole blood-orange.

was used to assess normal distribution and Tukey test for outlier detection. Parametric data was reported as mean and standard deviation (SD) and nonparametric data as median and interquartile range (IQR). Categorical data was reported as absolute value and percentages.

The percentage deviation (PD) at different time intervals from baseline was calculated as [5, 10]:

$$\text{PD} = \frac{\text{Result at time x} - \text{Baseline result}}{\text{Baseline result}} \times 100$$

A change from baseline greater than desirable biological variation total error was considered clinically significant.

The insulin biological variation values were derived from the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) biological variation database with the total error being ±36 %. The C-peptide biological variation values were derived from Westgard biological variation database (2014 update) with the total error being ±21 % as it was not available on EFLM biological variation database [11, 12].

Linear regression analysis was performed to determine the instability equation:

$$\text{PD} = a \times \text{time}$$

with a being the slope of the equation. The regression analysis was a forced through zero equation as recommended [10]. A p-value less than 0.05 was considered statistically significant.

Results

Participants

The participants' characteristics are summarized on Table S1 (see Supplementary Material). A total of ten participants were enrolled in the study. The majority of participants were Black Africans (70 %) and the mean age was 39 years (SD ± 10.2).

Stability of C-peptide (Table 1, Figures 3 and 4)

Stored at RT and delayed centrifugation

When there was a delay in centrifugation in samples stored at room temperature, C-peptide deteriorated faster in both sample types although in EDTA it was within acceptable limits at 12 h. At 72 h, the PD from the baseline was −74 and −46 % in serum and plasma respectively, which was unacceptable.

Stored at RT

C-peptide concentrations decreased significantly in both sample types over 48 h (p=0.001). The PD from baseline in serum was −29 % and in plasma −31 %.

Stored at 2–8 °C

C-peptide concentrations decreased less in serum than in plasma. The PD in serum was −0.87 % and in plasma −3.70 %. At day seven, PD was −6 % in serum and −13 % in plasma.

Stored at −20 °C

The PD change from the baseline was within acceptable limits throughout this period with PD from the baseline not exceeding 7 % for both sample types on day 30.

Stability of insulin (Table 2, Figures 5 and 6)

Stored at RT and delayed centrifugation

When sample centrifugation was delayed, insulin showed better stability in plasma than in serum over the 72-h period. The PD from the baseline measurement was more than three times in serum (−80 %) than in plasma (−23 %).

Stored at RT

There was a significant decrease in insulin concentration in serum at 48 h (p=0.001) with PD from baseline in serum of −67 vs. −21 % in plasma.

Stored at 2–8 °C

Even at fridge temperatures, the decrease in insulin concentration in serum samples was more than in plasma with PD from the baseline of −19 % in serum and −10 % in plasma at day seven of storage. Of note is that plasma results of participants (patients) two and four at time point 168 h (day seven) might have skewed the data.

Stored at −20 °C

Insulin concentration dropped by 21 % over a period of 30 days in serum. PD in plasma was lower with an increase seen between day 21 and 30 from −11 to +1 %.

Discussion

In this study, we investigated the effect of sample type, storage temperature, delayed centrifugation and analysis on stability of insulin and C-peptide. To our knowledge, this is the first study conducted following approved published guidelines on

Table 1: Stability of C-peptide in serum and plasma stored at RT, 2–8 °C and −20 °C and measured at different time intervals.

Sample type	No. of participants	Basal mean (range), mIU/L	4 h	8 h	24 h	48 h	Instability equation, R	p-Value
Stored at RT								
Serum	10	3.70 (1.25–8.55)	−0.61 %	−1.33 %	−5.44 %	−29.16 %[a]	%PD=−0.6 time, r=−0.76	0.001
Plasma	10	3.64 (1.27–8.42)	−13.24 %	−5.77 %	−21.53 %[a]	−30.67 %[a]	%PD=−0.6 time, r=−0.58	<0.001
Stored at 2–8 °C								
			1 day	**2 days**	**5 days**	**7 days**		
Serum	10	3.67 (1.25–8.35)	+0.34 %	−0.87 %	−4.70 %	−5.85 %	%PD=−0.03 time, r=−0.68	<0.001
Plasma	10	3.66 (1.26–8.3)	−4.02 %	−3.70 %	−12.11 %	−13.39 %	%PD=−0.08 time, r=−0.38	0.022
Stored at −20 °C								
			3 days	**7 days**	**21 days**	**30 days**		
Serum	09	3.47 (1.27–8.41)	−2.37 %	−1.34 %	−2.42 %	−6.53 %	%PD=−0.15 time, r=−0.52	<0.001
Plasma	10	3.62 (1.21–8.26)	−1.47 %	−0.54 %	−3.31 %	−6.25 %	%PD=−0.19 time, r=−0.39	0.001
Stored RT and delayed centrifugation								
			8 h	**12 h**	**48 h**	**72 h**		
Serum	10	3.71 (1.25–8.5)	−5.78 %	−15.34 %	−58.19 %[a]	−74.41 %[a]	%PD=−1.10 time, r=−0.95	<0.001
Plasma	10	3.64 (1.21–8.23)	+1.98 %	−2.94 %	−31.16 %[a]	−46.44 %[a]	%PD=−0.70 time, r=−0.88	<0.001

[a]PD exceeded acceptability limit of 21 %.

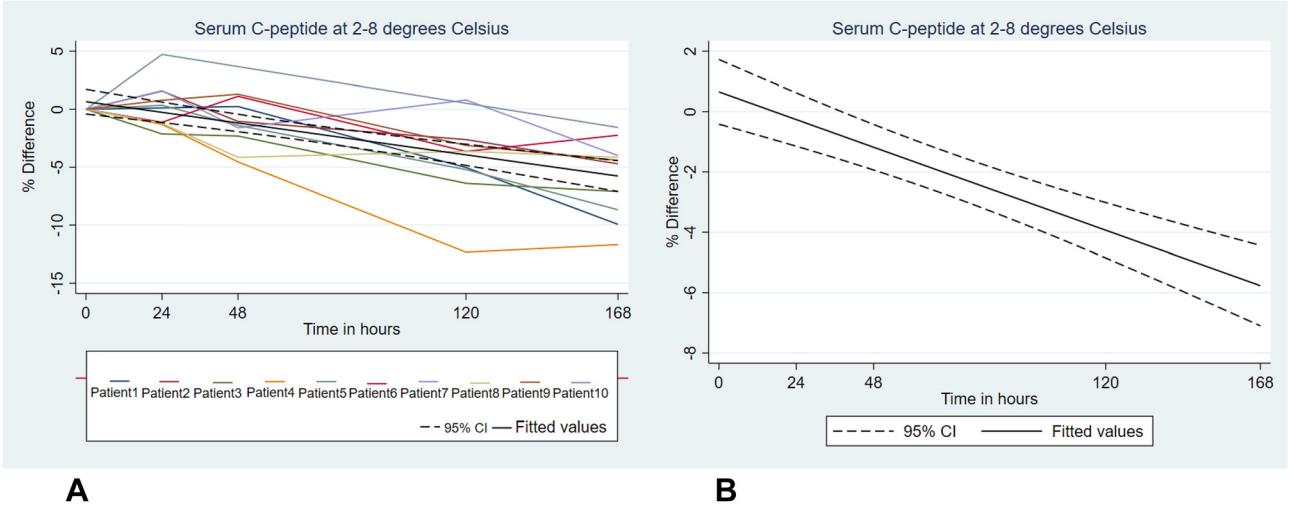


Figure 3: Serum C-peptide stored at 2–8 °C. Instability equation calculation using least square linear regression (bold line) with confidence intervals (dotted lines) for the mean of all ten patients results at each time point. (A) Shows all individual patients results while (B) shows cumulative patients results. Acceptability limit of 21 % not presented on the Figure as it was not exceeded.

sample stability for insulin and C-peptide [10]. Our results showed that serum provided a better stability for C-peptide in centrifuged samples stored at RT, fridge temperature (2–8 °C) and at −20 °C for 24 h, 7 days and 30 days, respectively. The finding that serum stored in fridge temperature is preferred for C-peptide is in keeping with other studies and manufacturers' recommendations [7, 13, 14]. However, when centrifugation and separation is delayed, we found that EDTA improved the stability of C-peptide compared to SST for a short period even when samples are stored at RT. In this study stability of C-peptide was acceptable up to 12 h, beyond 48 h it was unacceptable. McDonald et al. showed that EDTA improves stability of C-peptide up to 36 h using a strict stability criterion of less than 10 % [3].

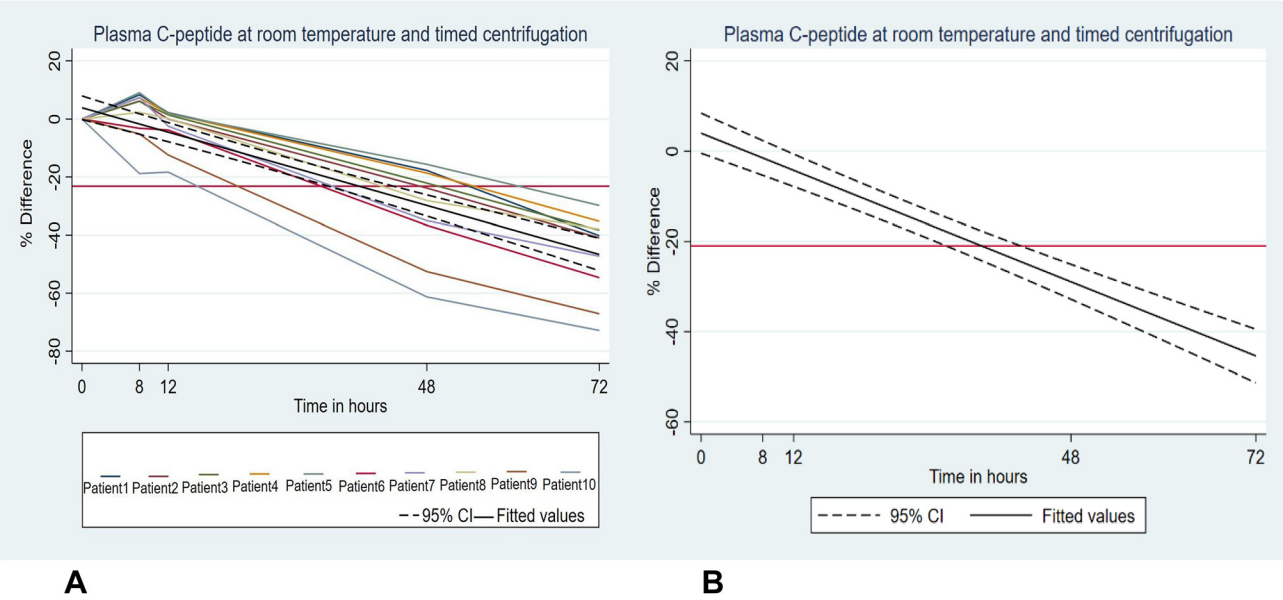


Figure 4: Plasma C-peptide stored at RT and centrifuged at the time of analysis. Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 21 % based on biological variation. (A) Shows all individual patients results while (B) shows cumulative patients results.

Table 2: Stability of insulin in serum and plasma stored at room temperature, 2–8 °C and −20 °C and measured at different time intervals.

Stored at RT								
Sample type	**No. of participants**	**Basal mean (range), mIU/L**	**4 h**	**8 h**	**24 h**	**48 h**	**Instability equation, R**	**p-Value**
Serum	10	24.26 (4.57–92.8)	−17.68 %	−38.40 %[a]	−43.80 %[a]	−66.58 %[a]	%PD=−1.2 time, r=−0.65	0.001
Plasma	10	25.38 (4.71–91.4)	−11.81 %	−7.81 %	−18.79 %	−20.53 %	%PD=−0.35 time, r=−0.36	0.001
Stored at 2–8 °C								
			1 day	**2 days**	**5 days**	**7 days**		
Serum	10	24.61 (4.50–92.82)	−14.05 %	−14.20 %	−18.64 %	−18.85 %	%PD=−0.08 time, r=−0.17	0.154
Plasma	10	25.73 (4.69–93.76)	−6.57 %	−5.95 %	−7.44 %	−10.14 %	%PD=−0.04 time, r=−0.20	0.134
Stored at −20 °C								
			3 days	**7 days**	**21 days**	**30 days**		
Serum	09	21.41 (4.33–94.36)	−33.75 %	−25.69 %	−28.16 %	−21.38 %	%PD=−0.25 time, r=−0.121	0.029
Plasma	10	25.47 (5.17–92.97)	−4.96 %	−1.33 %	−10.80 %	+0.72 %	%PD=−0.04 time, r=−0.05	0.608
Stored at RT and delayed centrifugation								
			8 h	**12 h**	**48 h**	**72 h**		
Serum	10	24.66 (4.52–95.37)	−15.49 %	−34.18 %	−66.67 %[a]	−79.61 %[a]	%PD=−1.05 time, r=−0.81	<0.001
Plasma	10	25.19 (4.82–92.28)	−11.90 %	−7.57 %	−18.89 %	−23.37 %	%PD=−0.28 time, r=−0.45	<0.001

[a]PD exceeded acceptability limit of 36 %.

In addition, we showed that EDTA provided a better stability for insulin in centrifuged samples stored at RT, fridge temperature (2–8 °C) and at −20 °C for 48 h, 7 days, and 30 days, respectively. Even when there was a delay in centrifugation and separation, the EDTA samples were still suitable for insulin analysis up to 48 h at RT.

Previous studies demonstrated that insulin is stable in EDTA at room temperature, at fridge temperature, and 4 °C

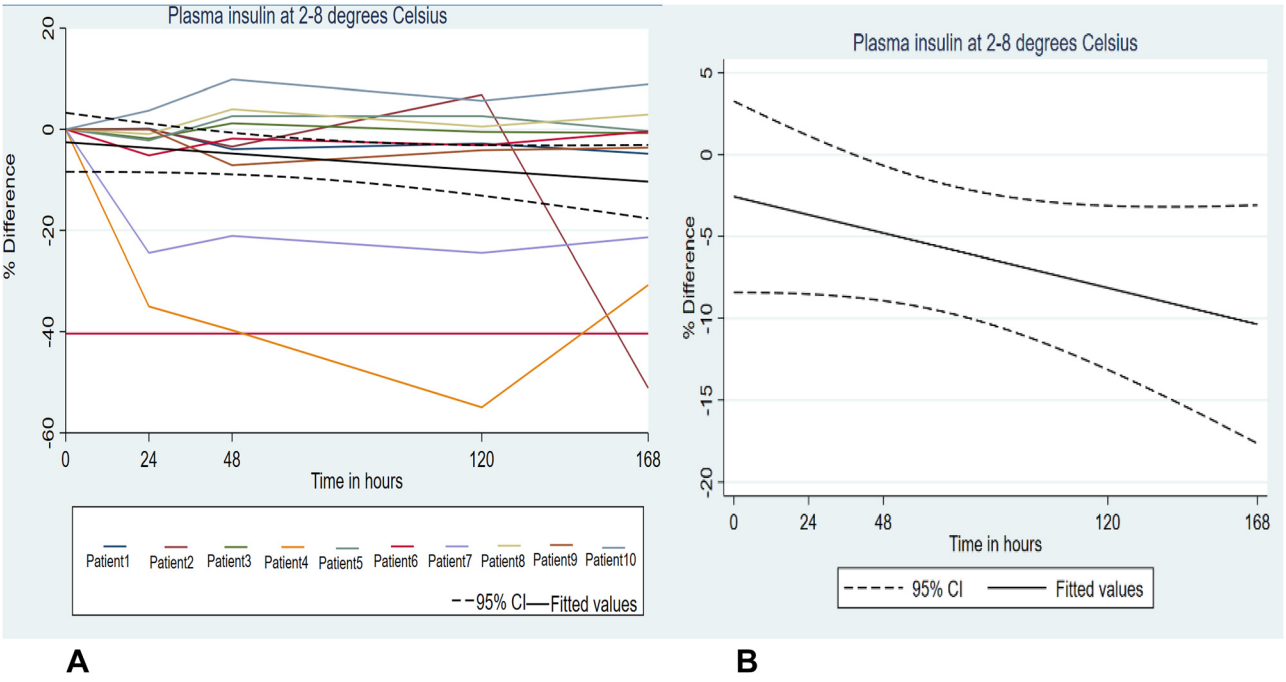


Figure 5: Plasma insulin when stored at 2–8 °C for 7 days (168 h). Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 36 % based on biological variation. (A) Shows all individual patients results while (B) shows cumulative patients results.

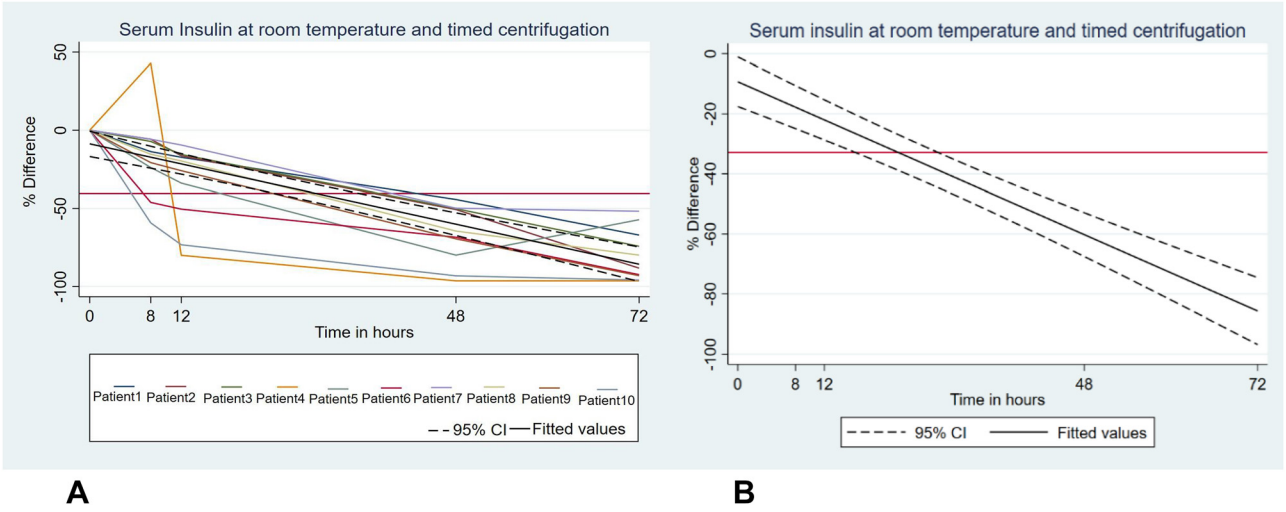


Figure 6: Serum insulin result stored at RT and centrifuged at the time of analysis. Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 36 % based on biological variation. (A) Shows all individual patients results while (B) shows cumulative patients results.

for 5 days [3, 8]. Yet, these studies did not evaluate the stability of insulin at –20 °C. A study by Chevenne et al. showed that the use of ion chelators like EDTA did not have any effect on reducing insulin degradation, while storing the samples at 4 °C may reduce activity of the insulin degrading enzyme [6].

In contrast, our results showed that insulin is more stable in EDTA samples stored as whole blood even at RT which suggests that EDTA itself has a stabilizing effect on insulin. Although EDTA is a better preservative for insulin, storage at cold temperatures improves the stability of insulin and this is thought to be due to the reduced activity of insulin degrading enzymes at fridge temperatures [6]. Our results showed that serum samples were not suitable for insulin analysis even when centrifugation was performed immediately despite

manufacturer recommending serum samples [14]. Using serum samples that were centrifuged immediately and stored at 4 °C, Atanasovski et al. found that insulin was most unstable in the first 24 h. This finding was attributed to the cross-reactivity of the Roche insulin assays with pro-insulin which is unstable in serum [7]. Instabilities of insulin observed in serum samples is attributed to the intracellular proteases released in serum that may cause insulin degradation [3]. When delays in shipping the samples to the central laboratory is anticipated, EDTA is recommended.

We also looked at the stability of insulin and C-peptide at −20 °C in serum and EDTA plasma. As an academic center, we receive samples that have been stored at freezer temperatures of −20 °C for routine analysis or research purposes. We found that C-peptide was stable in both EDTA plasma and serum for the entire study period of 30 days while insulin was only stable in EDTA plasma. This suggests that in addition to separation from cellular components, storage temperature has a significant impact on stability of C-peptide.

Nevertheless, comparison of stability studies conducted prior to the release of published guidelines is difficult due to variability in how these studies were conducted and non-standardized acceptance criteria for allowable difference used. The introduction of the set guidelines is likely to improve comparability among stability studies [10].

This study has several strengths. Firstly, it was conducted following the recently published guidelines on sample stability studies and both fasting and post prandial samples were used to obtain a wide range of concentrations. Secondly, stability was assessed over a longer period (30 days) at various storage temperatures, including −20 °C which may have implications when storing samples for research purposes. Lastly, the impact of delayed centrifugation was also assessed, which is most relevant for referral samples in our setting.

The limitations of this study: sample analysis was performed only on the Roche platform, and findings may not apply to other platforms. Due to limited sample volume and cost, sample analysis was not performed from primary tubes and aliquots made were not run in replicates as recommended by the CRESS report [10]. Furthermore, for freezer temperature, primary tubes could not be used to avoid hemolysis during the thawing process.

Conclusions

C-peptide was more stable in serum samples provided the sample was centrifuged immediately, the best storage conditions for C-peptide was fridge storage and −20 °C. Insulin was more stable in plasma at different storage conditions than in serum. The best storage conditions for insulin is fridge storage and −20 °C. We recommend that C-peptide should be analyzed in serum and insulin in plasma. The samples should be centrifuged immediately on arrival to the laboratory, if there's a delay in analysis samples should be stored at cold temperatures to ensure adequate stability.

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Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The study was approved by the University of Witwatersrand Human Research Ethics Committee (Clearance number M1911105) and followed the Declaration of Helsinki.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/cclm-2023-0339).