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Submission of research report for graduation for the degree

MMed in Chemical Pathology

03/10/2023

TITLE:

THE STABILITY OF C-PEPTIDE AND INSULIN IN PLASMA AND SERUM SAMPLES UNDER DIFFERENT STORAGE CONDITIONS

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Degree: MMed in Chemical Pathology

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DECLARATION

I, Delhia Xikombiso Nkuna student nr 2302412, a candidate for the degree of MMed in Chemical Pathology, have submitted my research report entitled:

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

I declare that:

1. This research report was submitted with the acquiescence of the supervisors.

2. All of the work is my own, except as otherwise stated.

3. The substance (nor any part of it) has not been submitted in the past nor is being submitted for a degree in any other university

4. This research report has the approval of the Committee for Research on Human Subjects, and the Number of the Certificate of Approval is M1911105.

Signed 

Date 03/10/2023

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PUBLISHED ARTICLE

Delhia X. Nkuna, Siyabonga P. Khoza, Jaya A. George and Mpho R. Maphayi*

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

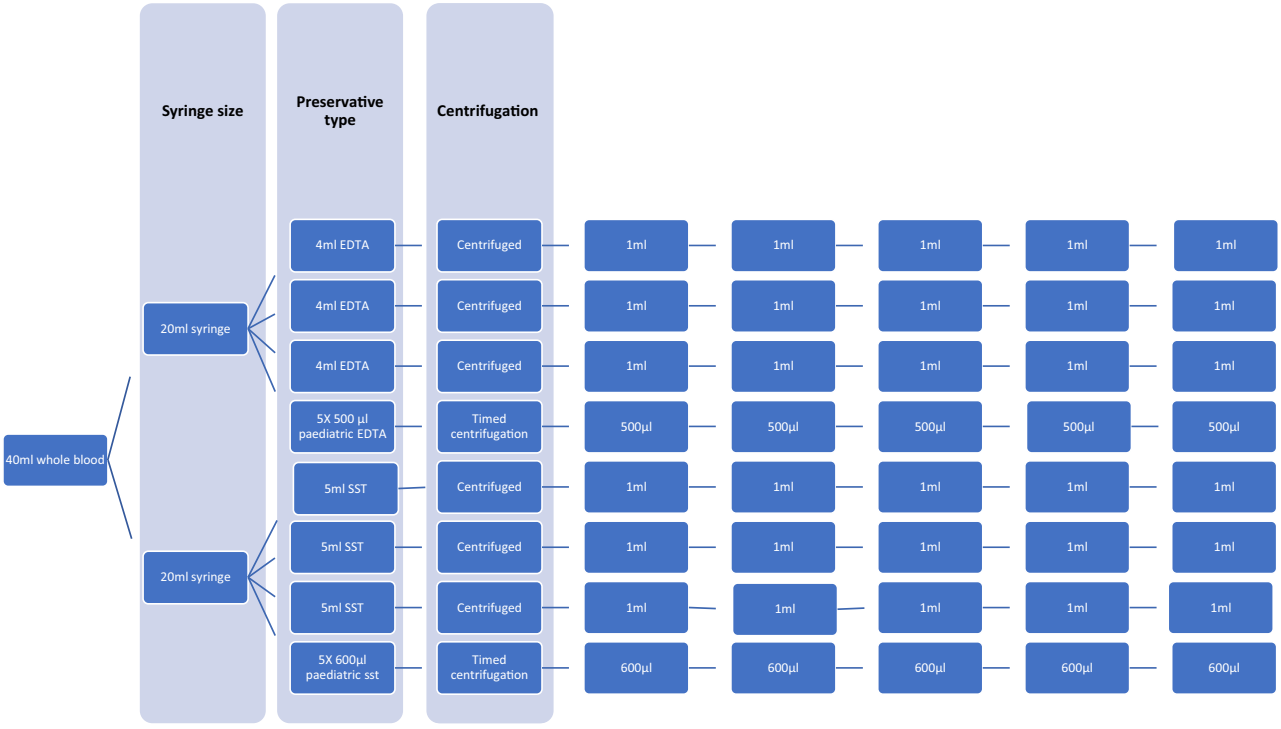

Figure 1: Sample collection procedure from each participant.

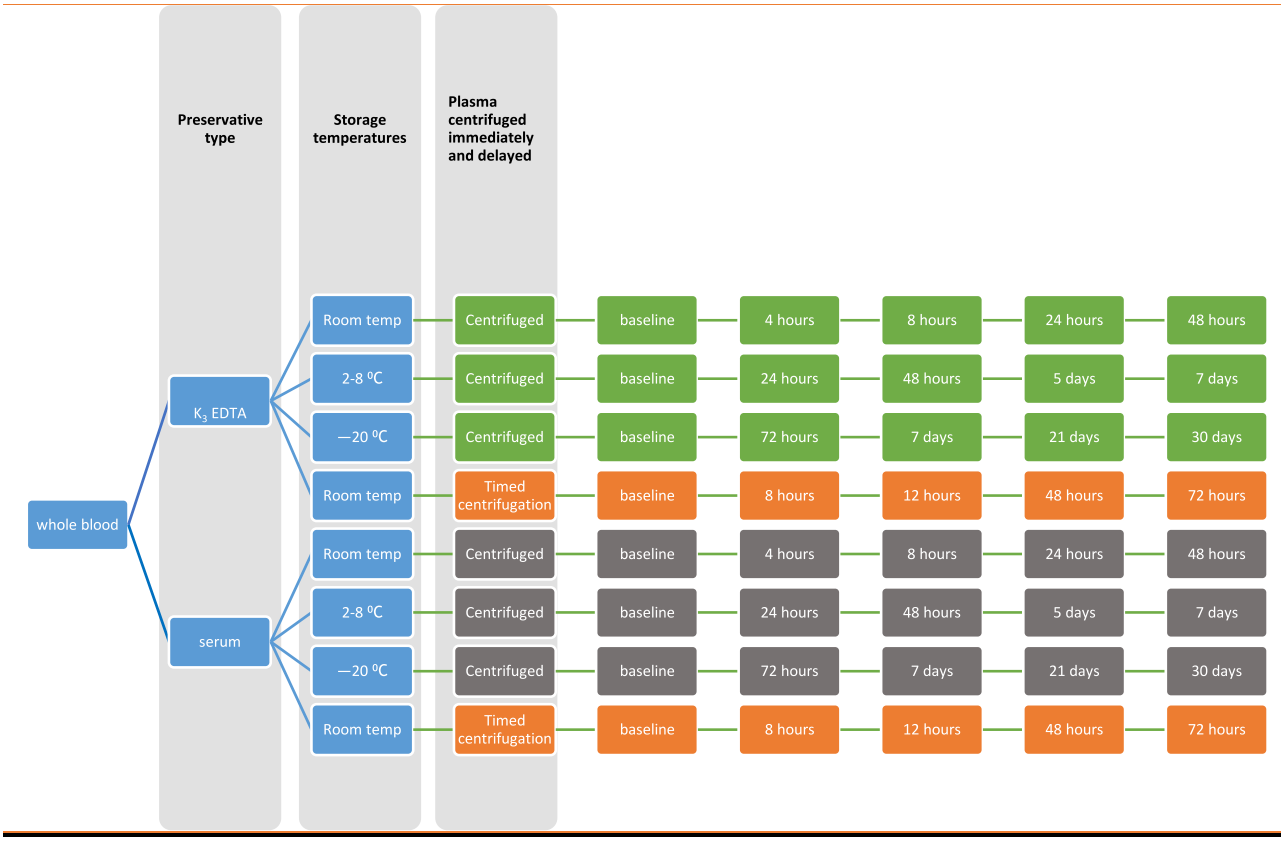

Figure 2: Flow diagram showing sample processing and analysis procedure over 30-day period. Stored as serum-grey, plasma-green and whole blood-orange.

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 S1, 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.

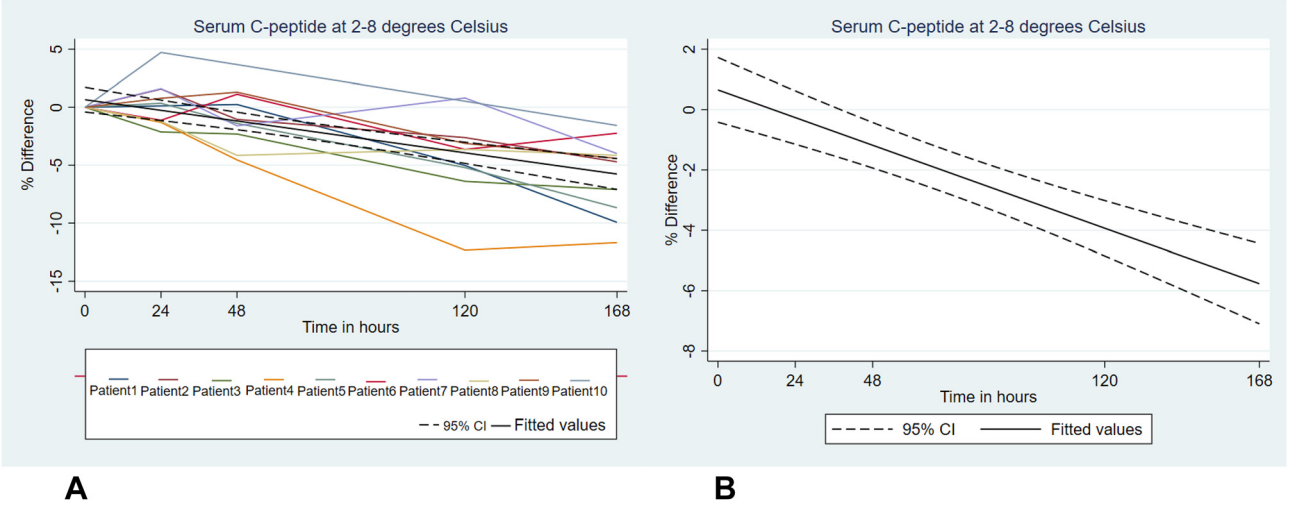

Figure 3: Serum C-peptide stored at 2–8 °C. Instability equation calculation using least square linear regression (bold line) with confidence intervals (dotted lines) for the mean of all ten patients results at each time point. (A) Shows all individual patients results while (B) shows cumulative patients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

Table 1: Stability of insulin in serum and plasma stored at room temperature, 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	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 %.

Stability of insulin (Table 1, 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 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

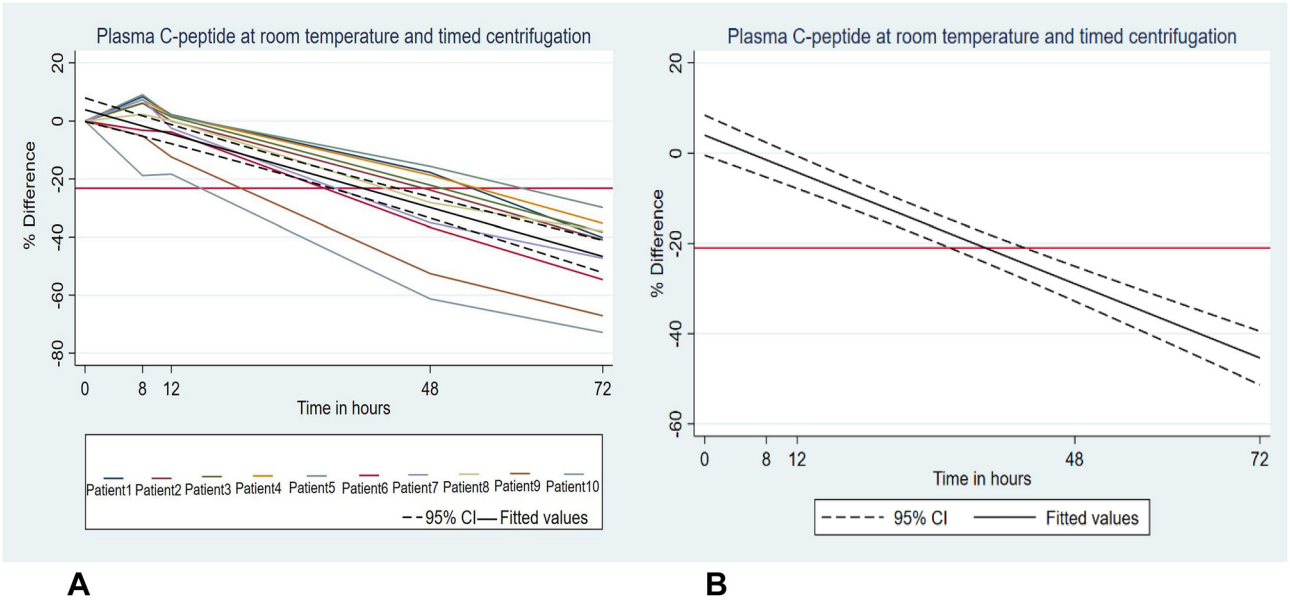

Figure 4: Plasma C-peptide stored at RT and centrifuged at the time of analysis. Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 21 % based on biological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

improves stability of C-peptide up to 36 h using a strict stability criterion of less than 10 % [3].

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.

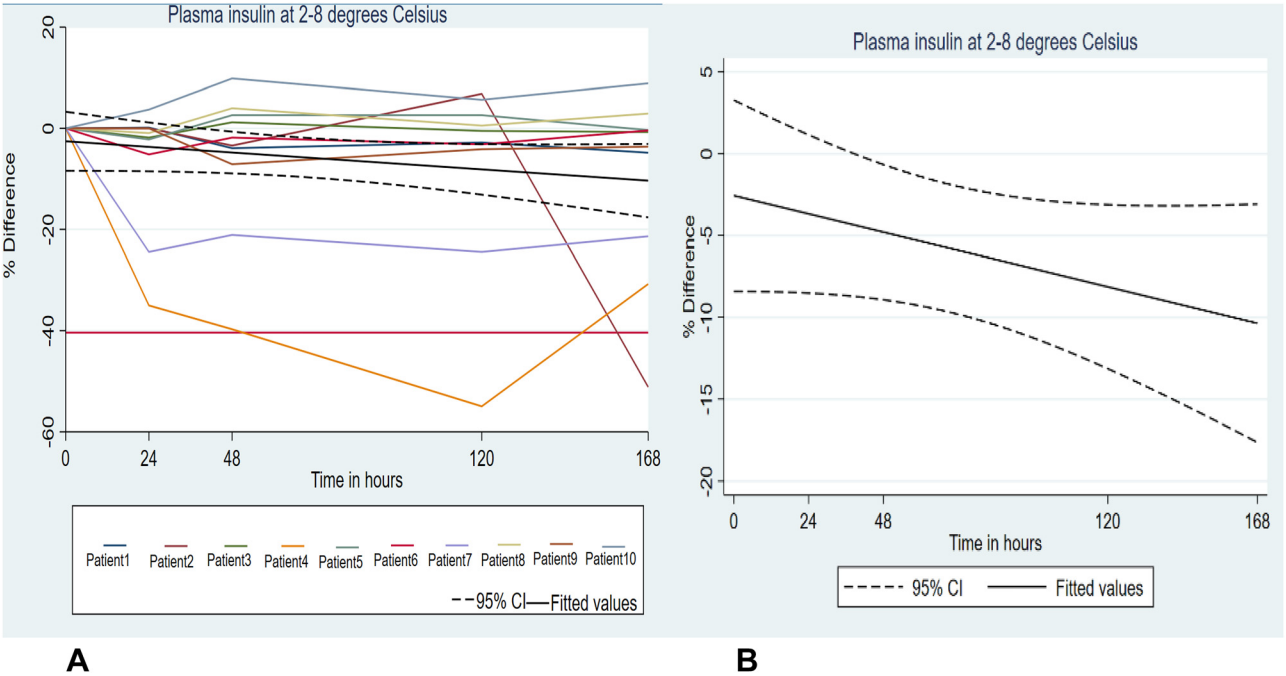

Figure 5: Plasma insulin when stored at 2–8 °C for 7 days (168 h). Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 36 % based on biological variation.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

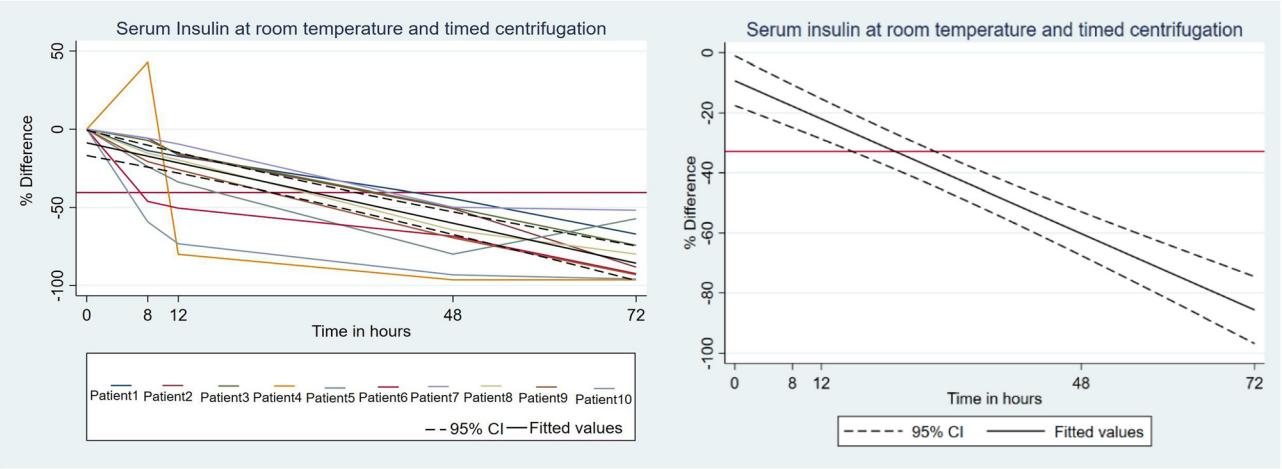

Figure 6: Serum insulin result stored at RT and centrifuged at the time of analysis. Instability equation calculation (bold line) using least square linear regression with confidence intervals (dotted line) for the slope. The horizontal red line represents the acceptability limit of 36 % based on biological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

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

UNIVERSITY OF THE WITWATERSRAND

FACULTY OF HEALTH SCIENCES

SCHOOL OF PATHOLOGY

The stability of C-peptide and insulin in human whole blood, serum and plasma

Research Proposal

In partial fulfilment of the requirements for the degree Master of Medicine:

Chemical Pathology

Submitted by

Dr Dehlia Xikombiso Nkuna

Student number:

2302412

Supervisor:

Dr M Maphayi

Co-supervisor:

Dr S Dlamini

The Stability of C-peptide and Insulin in Human Whole Blood, Plasma and Serum

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Helen Joseph Hospital

Background

Insulin and C-peptide are peptide hormones produced in equal amounts by the beta cells of the pancreatic islets following sequential cleavage of pre-proinsulin and pro-insulin(1). However, they are found in blood circulation in different concentrations. Insulin has a shorter half-life of 3-5 minutes as it undergoes first metabolism in the liver where it is degraded. On the contrary, C- peptide has a longer half-life of 20-30 minutes as it does not undergo first pass metabolism by the liver(1). It is found in 5-10 times higher concentration than insulin. Furthermore, the excretion and degradation of C-peptide is by the kidney which is decreased in renal disease(1).

The function of insulin is to maintain glycaemic control by increasing cellular uptake of glucose by skeletal and adipose tissue where it is utilized(2) .C-peptide was thought to be biologically inactive but there is evidence to suggest that C-peptide has molecular and physiological effects. It has been shown to stimulate the sodium/potassium ATPase pump and increases intracellular calcium ions(3).

Both insulin and C-peptide measurements are frequently requested for a variety of clinical conditions and concentrations can be affected by preanalytical factors. One of these which is the type of sample collected which may affect the stability.

Measurement of C-peptide and insulin

Stability is defined as the ability of a sample material to maintain the initial concentration over period of time when sample is stored under defined conditions(4). There is no standardized approach to evaluate stability.

As peptide hormones, insulin and C-peptide stability is affected by a number of preanalytical factors such as preservative type and storage temperatures. Both serum and plasma are recommended sample types for analysis of these analytes(5-7). However, there is conflicting evidence on which sample type is better.

Some studies support the measurement of insulin and C-peptide in ethylenediaminetetraacetic acid (EDTA) plasma instead of serum(6, 8). In a study looking at the effect of storage and tube type on 81 analytes, insulin measured in serum was found to be stable for only 6 hours compared to plasma EDTA which was stable for 72 hours when stored at room temperature(6). The stability of C-peptide in serum was found to be lower at room temperature than at 4ºC, but when measured in plasma with EDTA preservative, it was found to be stable for 24hrs(6). Chelators such as EDTA are able to inhibit the activity of insulin degrading enzymes but not prevent the binding of the degrading enzyme to insulin itself (8). This favors the measurement of insulin on plasma with EDTA preservative as hemolysis induced insulin loss was prevented(8). Hemolysis affects insulin results in that insulin degrading enzymes that are found inside red blood cells can leak when a sample is haemolysed and therefore lead to falsely decreased insulin levels (8). The presence of an anticoagulant (EDTA /fluoride) in plasma has been shown to minimize haemolysis and prolong the stability of insulin for more than 5 days when stored at 4ºC as compared to serum (9).

In contrast, some studies have shown that insulin and C-peptide are stable in both plasma and serum(10, 11). Wu et al recommend that C-peptide and insulin be placed on ice directly after sampling and be centrifuged immediately when it reaches the laboratory (10).This is because both insulin and C-peptide concentrations rapidly decrease after sampling due to proteases present in the blood that breakdown insulin and C-peptide(10).

Wu et al found that C-peptide and insulin were stable when stored at 4ºC and -20ºC for five days in both separated serum and EDTA plasma, the same assay was used on all samples on 3 different instruments(10). When different methods in the measurement of C-peptide using serum and plasma samples were compared it was found that the mean values of the plasma and serum samples were comparable, however further studies were suggested to probe into this finding with larger sample size (11).

Findings of most studies agree that whole blood is not suitable for measurement of C-peptide and insulin due to the proteases released from the red blood cells(5, 7, 10, 11). It hs been shown that delayed separation of whole blood in an EDTA tube resulted in massive decreases in both insulin and C-peptide levels after 24hrs of analysis on 3 different platforms and early separation of whole blood resulted in better stability of C-peptide and insulin when compared to serum (5). These findings were confirmed by another study found that insulin decreased significantly when separation of cells from the plasma EDTA was delayed for up to 24hrs(7). EDTA plasma was still the preferred sample type provided that the sample was centrifuged immediately(5, 7).

There are no international recognized reference methods for C-peptide and insulin. However, Kohek et al showed that there was a difference in stability between serum and EDTA sample results, that were based on the different analytical methods. Available methods include immunoassays, high performance liquid chromatography and liquid chromatography mass spectrometry. However, most laboratories use different immunoassays, this is important as there will be lack of harmonization between laboratories(11).The most commonly used method is chemiluminescent immunoassay(12). High performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry(LC-MS) methods are fairly accurate but are time consuming and expensive and therefore reserved for research purposes (11).

Plasma or serum can be analysed with comparable results when using chemiluminescent enzyme immunoassay and time resolved flouroimmunoassay(11). EDTA plasma samples had falsely low results when using immunofluorometric assays when comparing to serum samples making serum samples more accurate(13).

Due to the lack of standardization reference intervals are also assay specific.

Research problem

Studies looking at the stability of C-peptide and insulin in different sample types and storage conditions are limited. For the most part, different studies agree that serum and plasma samples were more stable when stored at 4ºC, but no consensus could be reached as to whether plasma was a better sample type than serum in the measurement of C-peptide and Insulin levels.

In our laboratory C-peptide and insulin levels are measured in serum collected in serum separator gel tubes as per manufacturers' recommendation. These analytes are said to be stable for 24 hours at 2-8º C (14). Our laboratory receives samples from across the country and samples may be collected in different tube types and may not be transported at 2-8 C.

Although the manufacturer also recommends lithium heparin and EDTA plasma as a suitable sample types for insulin and C-peptide, they do not provide information on the stability of the analytes. However, there is evidence to suggest that C-peptide may be more stable in EDTA plasma as compared to serum.

Therefore, the aim of this study is to investigate the impact of preservative type, storage conditions and duration on the stability of insulin and C-peptide.

Study objectives

1. To compare the results of insulin and C-peptide in serum, EDTA plasma and whole blood at different time intervals from baseline.
2. To compare the effect of different storage temperatures on insulin and C-peptide levels measured at different time intervals from baseline.

Methods

Study Participants

National Health Laboratory Services (NHLS) and University of the Witwatersrand Department of Chemical Pathology healthy adult staff members (>18 years) of both genders (5 males and 5 females) will be recruited into the study. They will be requested

to complete a questionnaire and samples will be collected after we obtain informed consent (see appendices 1 and 2). We will collect fasting samples from three participants and post-prandial samples from the other seven. This will give us a wide range of both insulin and C-peptide concentrations.

Inclusion criteria:

1. Participants more than 18 years' old
2. Consenting males and females

Exclusion criteria

1. Participants less than 18 years' old
2. Participants unable to sign consent form
3. Diabetic participants on insulin (insulin autoantibodies interferes with insulin measurement)
4. Participants taking biotin supplements (biotin interferers with insulin and C-peptide measurements)

Sample size

There are no defined guidelines on sample size for stability studies. Some studies looking at stability of insulin and C-peptide have used sample size ranging from 5 to 10(5,6). Based on this, we aim to recruit ten participants for this study.

Blood sample collection and processing

A single blood sample (40 ml) will be collected from each participant. Twenty ml into 4 EDTA tubes and 20ml into 4 serum separator tube (SST). Each tube is 5 ml. Samples from one EDTA and one SST tubes will be aliquoted as whole blood into 3 tubes each

and stored at room temperature to be centrifuged at time of analysis. The remaining serum and EDTA samples will be centrifuged immediately at 3000rpm for 10 min(5). These will be aliquoted and stored at room temperature, 2-8 and -20^0 Celsius. Three EDTA plasma will be aliquoted into 3 tubes each, making a total of 9 plasma aliquots per participant. Samples collected in the 3 SST (20ml of blood) will be aliquoted into 3 tubes each, making a total of 9 aliquots of serum per participant. The samples stored at room temperature will be analysed over 72 hrs. And those stored at 2-8 ºC will be analysed once a day for 7 days. Other samples will be stored at -20 ºC and analysed over 30 days as shown on Figure 1. We will measure baseline C-peptide and insulin concentrations in all samples.

Laboratory measurement

Samples will be analysed at Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service on the Roche analyser (Cobas e 602). The method of measurement for both insulin and C-peptide is electrochemiluminescence sandwich immunoassay. Biotinylated monoclonal insulin or C-peptide-specific antibody and a monoclonal insulin or C-peptide specific antibody labelled with ruthenium bind with human insulin or C-peptide to form a sandwich complex which is then coated with streptavidin microparticles. Application of voltage induces a chemiluminescent emission which is measured by a photomultiplier (15, 16).

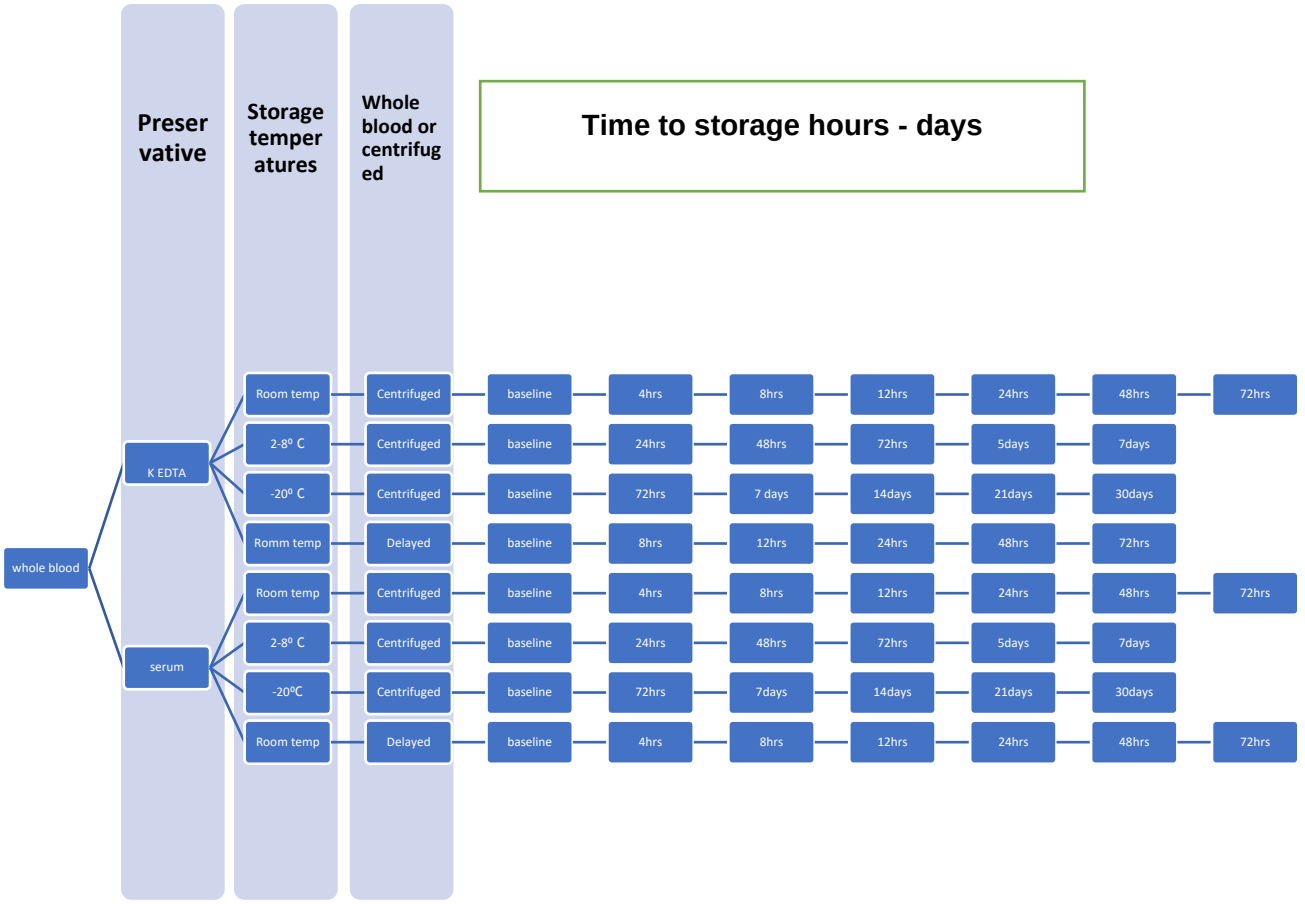


Figure 1. Diagram explaining samples collection and processing procedure over a 30-day period

Statistical analysis

Results will be captured on Microsoft Excel spreadsheet. Statistical analysis will be done using Statistica 2019 version software.

The percentage change at different time intervals from baseline will be calculated (Baseline result – Result at time/Baseline result x100). A change from baseline greater than biological variation total error and reference change value will be considered clinically significant.

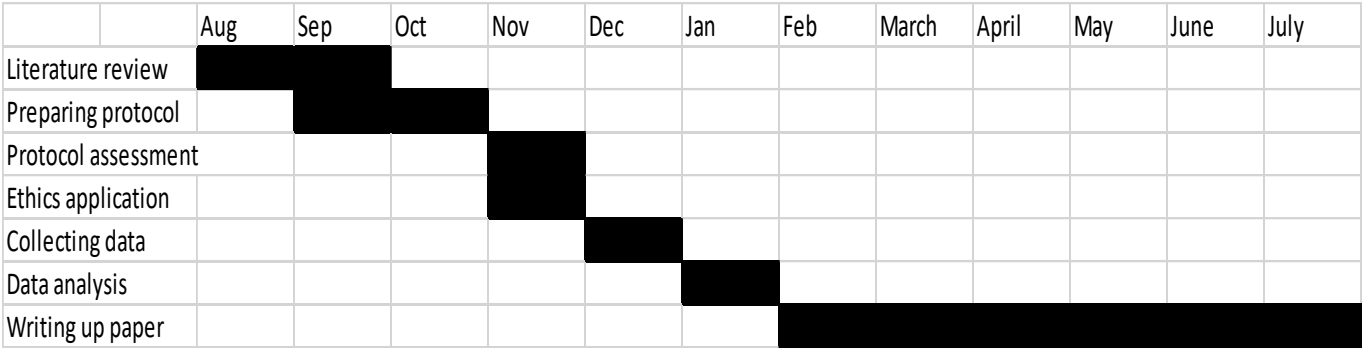
The differences between baseline and results at 72 hours will be compared using paired T test. Should the data not be evenly distributed then the non-parametric equivalent will be applied which is the Wilcoxon signed rank test.

ANOVA will be used to compare the mean between the 3 sample types i.e. EDTA whole blood, EDTA plasma and serum. Should the data not be evenly distributed then the non-parametric equivalent will be applied which is Kruskall –Wallis.

Ethics Approval

An application will be submitted to the University of Witwatersrand Human Research Ethics Committee.

Timeline



Budget

Proposed budget

Test	Unit Cost	Number	Total Cost
Insulin	R116.61	510	R59 471.10
C-peptide	R116.61	510	R59 471.10
Cryovials		500	R2500.00

EDTA tubes	R1.00	100	R100.00
Serum separator tubes	R1.00	100	R100.00
Publication			R40000
			R161 642.20

Funding

We will be applying to the NHLS Research Trust for R90 000 and Roche diagnostics will supply the reagents kits.

Potential challenges

- Storage space for study samples will be a challenge. To overcome this, prior arrangement with the chemistry laboratory manager and supervisors for allocation of storage space will be made.
- Losing or misplacing samples during the study period, this can be reduced by correct labelling and storage of each tube

Outcomes

The following outcomes are expected from this research:

1. Training in research

2. MMed degree

3. Publication

4. Possible translation in to recommendation regarding optimal pre-analytical sample handling procedures for the tests (insulin and C-peptide)

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ETHICS CLEARANCE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Delhia Xikombiso Nkuna

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1911105

NAME: Dr Delhia Xikombiso Nkuna
(Principal Investigator)
DEPARTMENT: Chemical Pathology
National Health Laboratory Service
Charlotte Maxeke Johannesburg Academic Hospital

PROJECT TITLE: The stability of C-peptide and insulin in human whole blood, plasma and serum

DATE CONSIDERED: 29/11/2019

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr Mpho Maphayi and Dr Sipho Dlamini

APPROVED BY: CB Penny
Dr CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL: 03/02/2020

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized
to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **November** and will therefore be due in the month of **November** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 03/02/2020

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

TURN-IT IN REPORT

RE: TURN-IT IN REPORT

Please take note that the article was already published online by the time the turn-it in report was generated.

This resulted in an increase of the similarity index on the turn-it in report, most notably source 1 and 2 that is referencing my own article.

Subtracting these from the total similarity score reveals only 4% similarity.



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Xikombiso Nkuna

Stability of C-peptide and insulin manuscript - final edited (1).docx

by Delhia Nkuna

Submission date: 09-Jul-2023 05:59PM (UTC+0200)
Submission ID: 2128485516
File name: Stability_of_C-peptide_and_insulin_manuscript_-_final_edited_281_29.docx (680.55K)
Word count: 4126
Character count: 22105

The Stability of C-peptide and Insulin in Plasma and Serum Samples Under Different Storage Conditions

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 to ensure wide range of analytes concentrations 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~~hr~~,12~~hr~~, 48~~hr~~ and 72 hours~~hr~~). After baseline measurements on the Roche Cobas e602 analyzer using electrochemiluminescence immunoassays, aliquots were stored at room temperature (RT) , 2 – 8 °C and — 20 °C for 4 hours 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% versus -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 48hrs). 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 hours, PD was - 23% and -80-% in plasma and serum, respectively.

Conclusion:

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.

Keywords: insulin, C-peptide, sample stability

NOMENCLATURE

SST – Serum separator tube

EDTA - Ethylenediaminetetraacetic acid

RT – Room temperature

PD – Percentage deviation

NHLS - National Health Laboratory Services

CMJAH - Charlotte Maxeke Johannesburg Academic Hospital

BD - Becton, Dickinson and Company

NIBSC - National Institute for Biological Standards and Control

WHO – World Health Organisation

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Table 1. Baseline characteristics of participants

Table 2. Stability of C-peptide in serum and plasma stored at room temperature, 2-8°C and -20°C and measured at different time intervals

Table 3. Stability of insulin in serum and plasma stored at room temperature, 2-8°C

and -20°C and measured at different time intervals

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Figure 1. Sample collection procedure from each participant

Figure 2. Flow diagram showing sample processing and analysis procedure over 30-day period.

Figure 3A and 3B: Serum C-peptide stored at 2 – 8 °C

Figure 4A and 4B: Plasma C-peptide stored at RT and centrifuged at the time of analysis

Figures 5A and 5BA. Plasma insulin when stored at 2 – 8 °C for 7 days (168 hours)

Figures 6A and B: Serum insulin result stored at RT and centrifuged at the time of analysis

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 72hrs when stored at 4°C compared to at room temperature (RT) where it was only stable for six hours. Stability of insulin in EDTA plasma at RT showed a slight improvement. The decrease in concentrations exceeded acceptable limits at 48hrs in RT compared to 72hrs at 4°C. Furthermore, in this study, C-peptide was more stable at 4°C up to 72hrs 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 120hrs and showed that C-peptide was more stable in EDTA (up to 36hrs) 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 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]. Delay in centrifugation is often a challenge in our setting because we are a referral lab which receives sample from other hospitals and clinics as whole blood. Furthermore, no clearly defined protocol was used for these studies [2-4]. 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 minutes to two hours 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. Aliquots were then transferred into eight Becton, Dickinson and Company (BD) SST (three five ml adult tubes and five pediatric 600 μl tubes) and eight dipotassium EDTA (three four ml adult tubes and five pediatric 500 μl tubes). Paediatric tubes were used to minimize amount of blood collected from participants and sample volume requirements for the tests. All the adult tubes were centrifuged within one hour of collection at 3500 rpm for 10 min at RT. One of the pediatric tubes was centrifuged within one hour 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, 1mls were

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 hours, 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 hours, 8 hours,24 hours and 48 hours at RT and 24 hours, 48 hours, day 5 and day 7 at 2 – 8 °C) as shown on Figure 2.

Frozen samples were thawed once at 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 1hr were stored at RT to centrifuge at the time of analysis at the 8hr ,12hr, 48hr and 72hr time intervals as shown on Figure 2.

Commented [MM1]: Thawed for how long? Ask Xiko.

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 μU/ml and 75,6 μU/ml respectively, 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 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]:

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

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:

PD = *a* x 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 1 (see Supplementary). 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 2, 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 12hrs. At 72hrs, 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 48hrs (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.

~~**Stored at RT and delayed centrifugation**~~

Stability of insulin (Table 3, 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 72hr 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 48hrs (p=0.001) with PD from baseline in serum of -67% versus -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.

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%.

~~**Stored at RT and delayed centrifugation**~~

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

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 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 hours, 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 12hrs, beyond 48hrs it was unacceptable. McDonald et al showed that EDTA improves stability of C-peptide up to 36hrs using a strict stability criterion of less than 10% [3].

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 hours, 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 48hrs at RT.

However, our results showed that insulin is more stable in EDTA plasma even at RT

than in serum which suggest that EDTA 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 24hrs. 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 central laboratory is anticipated, EDTA is recommended~~preferred~~.

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 , limitations analysis was not done from primary tubes and aliquots made were not run in replicates as recommended by the CRESS report. [10]. Furthermore, for freezer temperature using primary tube may have caused hemolysis during the thawing processing.

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Conclusion

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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Conflicting interests

None

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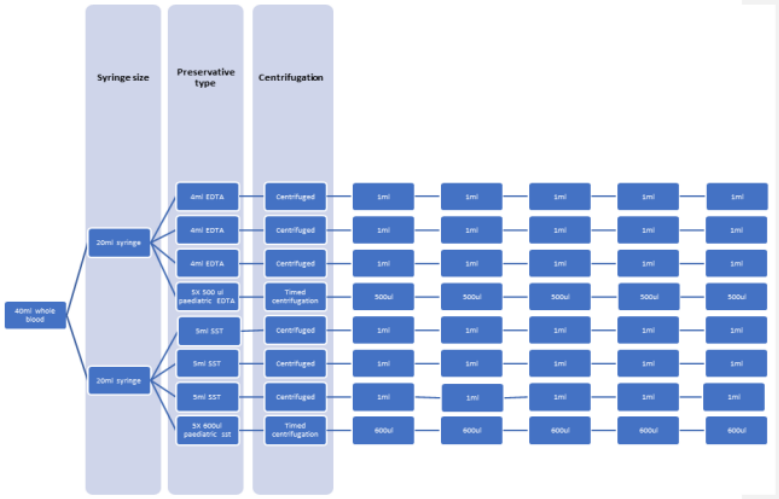

Figure 1: Sample collection procedure from each participant



Figure 2: Flow diagram showing sample processing and analysis procedure over 30-day period.

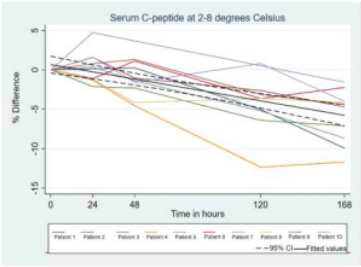

Figure 3A

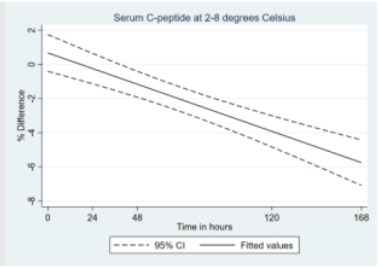

Figure 3B

Figure 3A and 3B: 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.

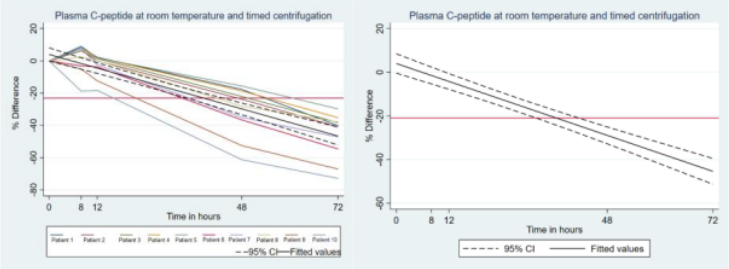

Figure 4A Figure 4B

Figure 4A and 4B: 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.

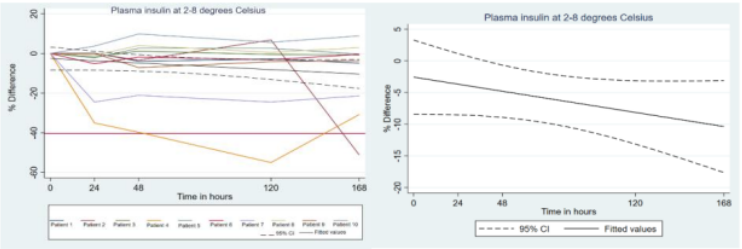

Figure 5A **Figure 5B**

Figures 5A and 5BA. Plasma insulin when stored at 2 – 8°C for 7 days (168 hours). 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.

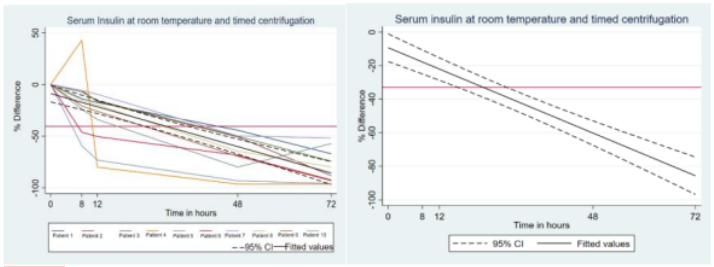

Figure 6A **Figure 6B**

Figures 6A and B: 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.

Table 2. Stability of C-peptide in serum and plasma stored at RTroom ~~temperature~~, 2-8°C and -20 °C and measured at different time intervals

Sample type	No. of participants	Basal mean (mIU/L) Range	4 hours	8 hours	24 hours	48 hours	Instability equation, R	P-value
Stored RT								
Serum	10	3.70 (1.25-8.55)	-0.61%	-1.33%	-5.44%	-29.16%*	%PD = -0.6time, r = -0.76	0.001
Plasma	10	3.64 (1.27-8.42)	-13.24%	-5.77%	-21.53%*	-30.67%*	%PD = -0.6time, 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 = -0.03time, r = -0.68	<0.001
Plasma	10	3.66 (1.26-8.3)	-4.02%	-3.70%	-12.11%	-13.39%	%PD= -0.08time, 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 = -0.15time, r=-0.52	<0.001
Plasma	10	3.62 (1.21-8.26)	-1.47%	-0.54%	-3.31%	- 6.25%	%PD = -0.19time, r= -0.39	0.001
Stored RT and delayed centrifugation								
			8 hours	12 hours	48 hours	72 hours		
Serum	10	3.71 (1.25-8.5)	-5.78%	-15.34%	-58.19%*	-74.41%*	%PD = -1.10time, r=-0.95	<0.001
Plasma	10	3.64 (1.21-8.23)	+1.98%	-2.94%	-31.16%*	-46.44%*	%PD = -0.70time, r=-0.88	<0.001

*PD exceeded acceptability limit of 21%

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Table 3. Stability of insulin in serum and plasma stored at room temperature, 2-8°C and -20°C and measured at different time intervals

Sample type	No. of participants	Basal mean, Range (mIU/L)	4 hours	8 hours	24 hours	48 hours	Instability equation, R	P-value
			Stored at RTroom temperature					
Serum	10	24.26 (4.57-92.8)	-17.68%	-38.40%*	-43.80%*	-66.58%*	%PD = -1.2time, r=-0.65	0.001
Plasma	10	25.38 (4.71-91.4)	-11.81%	-7.81%	18.79%	-20.53%	%PD = -0.35time, 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 = -0.08time,r=-0.17	0.154
Plasma	10	25.73 (4.69-93.76)	-6.57%	-5.95%	-7.44%	-10.14%	%PD = -0.04time, 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= -0.25time, r=-0.121	0.029
Plasma	10	25.47 (5.17-92.97)	-4.96%	-1.33%	-10.80%	+0.72%	%PD=-0.04time, r=-0.05	0.608
			Stored at RT and delayed centrifugation					
			8 hours	**12 hours**	**48 hours**	**72 hours**		
Serum	10	24.66 (4.52-95.37)	-15.49%	-34.18%	-66.67%*	-79.61%*	%PD = -1.05time , r=-0.81	<0.001
Plasma	10	25.19 (4.82-92.28)	-11.90%	-7.57%	-18.89%	-23.37%	%PD = -0.28time ,r=-0.45	<0.001

*PD exceeded acceptability limit of 36%

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