Clinical Biochemistry 65 (2019) 38–44

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Impact of clinical sample handling and processing on ultra-low level measurements of plasma cytokines

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ABSTRACT

Objectives: In this study, we evaluated the impact of clinical sample handling and processing on IL-6, IL-10, IFNγ, and IL-2 measurements in plasma.
Design and methods: We collected whole blood samples and analyzed various pre-analytical parameters. We assessed the following: 1) cytokine stability in whole blood that was stored over a ten-hour period at room temperature and 4 °C; 2) cytokine stability in plasma over 6 h; 3) vigorous sample handling including repeated dropping and transport through a pneumatic transport system; and 4) freeze-thaw stability of cytokines in plasma. To ensure ability to measure IL-6, IL-10, IFNγ, and IL-2 levels in plasma, we used Simoa, an ultra-sensitive immunoassay platform.
Results: We show that whole blood storage at room temperature results in decreased cytokine levels and that whole blood storage at 4 °C results in greater cytokine stability. We also show that cytokines are stable when whole blood samples are subjected to vigorous sample handling. Lastly, we show that cytokines are stable in plasma over three freeze-thaw cycles.
Conclusions: Clinical sample handling and processing can affect measurements of IL-6, IL-10, IFNγ, and IL-2 in plasma. We believe this study will be a useful reference for future studies in which these cytokines are used as potential biomarkers.

1. Introduction

Cytokines are important immune signaling molecules that are associated with various diseases including cancer, neurological disorders, and autoimmune diseases [1]. Cytokines can exert their function at the local tissue level but can also be released and enter peripheral circulation. Changes in cytokine expression levels in blood have been shown to be biologically significant, making them attractive biomarker candidates for diagnosing disease, monitoring response to therapy, and monitoring disease progression. For example, cytokines can be used as part of a blood biomarker panel to assess rheumatoid arthritis disease activity [2]. Yet, despite the vast literature implicating cytokines with disease, the clinical utility of cytokines is still very limited.

Cytokine measurements in biological fluids are challenging for several reasons. First, cytokines vary in concentration between different individuals, sometimes by several orders of magnitude [3]. Second, blood cytokine levels do not necessarily correlate to local tissue expression levels due to the presence of soluble cytokine receptors, antibodies, and other factors that bind to cytokines and interfere with their detection. For example, IL-2 binding to its solubilized receptor, IL-2R, is known to interfere with immunoassays and may therefore lead to underestimated IL-2 levels [4]. Third, many cytokines are present at very low levels in the blood, and are difficult to measure using conventional techniques. Thus, due to limitations in analytical measurements of cytokines and difficulties in establishing cut-offs for healthy and disease states, the use of cytokines in disease diagnosis has been limited.

In addition to the challenges described above, variations in sample handling and processing in the clinical laboratory prior to analytical cytokine measurements can complicate accurate quantification [5]. Particularly, at ultra-low cytokine levels, small changes can have major consequences for reliable quantification. Downstream processing of whole blood can also affect cytokine measurements. For example, prior to plasma separation from whole blood, leukocytes can secrete cytokines in vitro and alter plasma levels [6]. Additionally, platelet activation can be accompanied by cytokine release, potentially resulting in apparently elevated cytokine levels in plasma that are not indicative of the underlying clinical condition [7]. Cytokine stability may also affect reliable quantification. Some cytokines have particularly short-half-lives, are temperature labile, and are subject to proteolytic degradation [8]. Plasma has been shown to have fewer matrix effects than serum when measuring a wide range of cytokines [9]. Additionally, heparin,

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https://doi.org/10.1016/j.clinbiochem.2019.01.001
Received 18 September 2018; Received in revised form 25 November 2018; Accepted 8 January 2019
Available online 08 January 2019

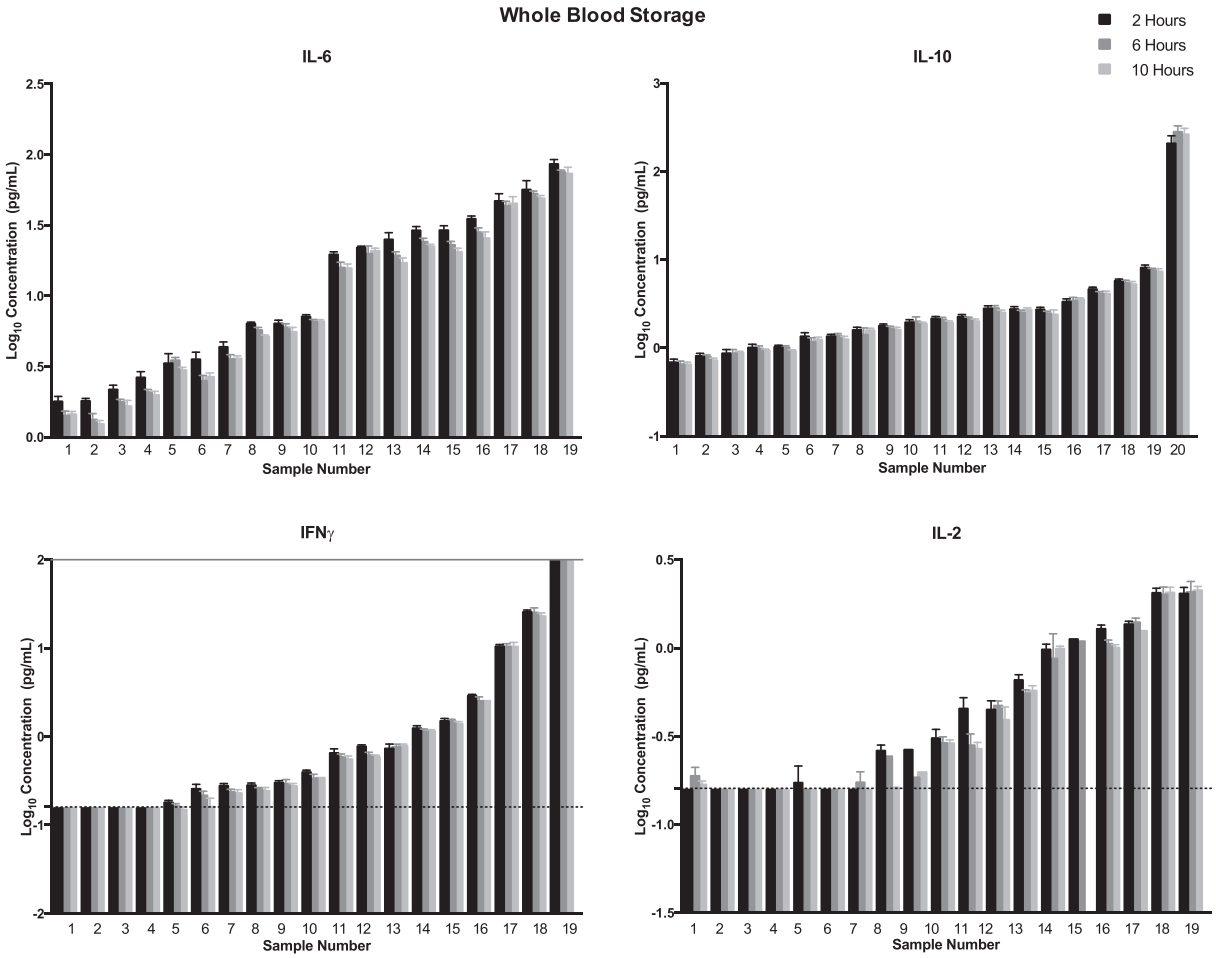

Fig. 1. Cytokine stability in whole blood. Whole blood was collected from different patients and stored at room temperature for 2, 6, or 10 h before being processed to plasma. Cytokines were then measured in plasma. Dotted line is the LLOQ and solid line is the ULOQ, indicated for markers where any sample concentration fell outside of either limit, with samples outside the measurable range set to the respective limit and excluded from statistical analysis. All samples were between LLOQ and ULOQ for IL-6 and IL-10.

Table 1
Degradation of cytokines when whole blood was stored at room temperature for 2, 6, or 10 h before being processed to plasma.

	2 versus 6 h		2 versus 10 h		6 versus 10 h	
	P value	Average % degradation	P value	Average % degradation	P value	Average % degradation
IL-6	< 0.0001	15%	< 0.0001	18%	0.0258	4%
IL-10	0.0400	2%	0.0064	5%	0.0583	3%
IFNγ	0.0250	7%	0.0250	11%	0.211	5%
IL-2	0.0640	11%	0.0420	15%	0.638	4%

which is used to process whole blood to plasma, has been associated with endotoxin contamination, which can induce cytokine release from monocytes [10]. Therefore, whole blood processing and handling can affect cytokine measurements and obscure disease associations.

In this study, we evaluated the effects of clinical specimen handling and processing on measurements of IL-6, IL-10, IFNγ, and IL-2 in plasma. IL-6, IL-10, IFNγ, and IL-2 are often present at ultra-low levels in blood [11,12]. To ensure our ability to quantify low cytokine levels, we used an ultra-sensitive assay technology known as Single Molecule Arrays (Simoa) [13,14]. Simoa assays have been previously demonstrated for ultra-sensitive detection of proteins and nucleic acids [15–18] and their low limits of detection are accompanied by low CVs, which was important for this study. We used this method to study the stability of these cytokines under different clinical handling and processing conditions. We assessed various parameters including cytokine stability in whole blood that was stored over a ten-hour period at room temperature and 4 °C, cytokine stability in plasma over 6 h, vigorous sample handling including repeated dropping and transport through a pneumatic transport system, and freeze-thaw stability of cytokines in plasma. We show that in some cases, pre-analytical sample handling and processing can contribute to measurement variations. We conclude that it is important to consider pre-analytical parameters when measuring cytokines in blood for biomarker studies.

2. Materials and methods

2.1. Simoa assay description

Simoa assays are bead-based immunoassays with the major advance of signal detection by single molecule counting, which results in ultra-

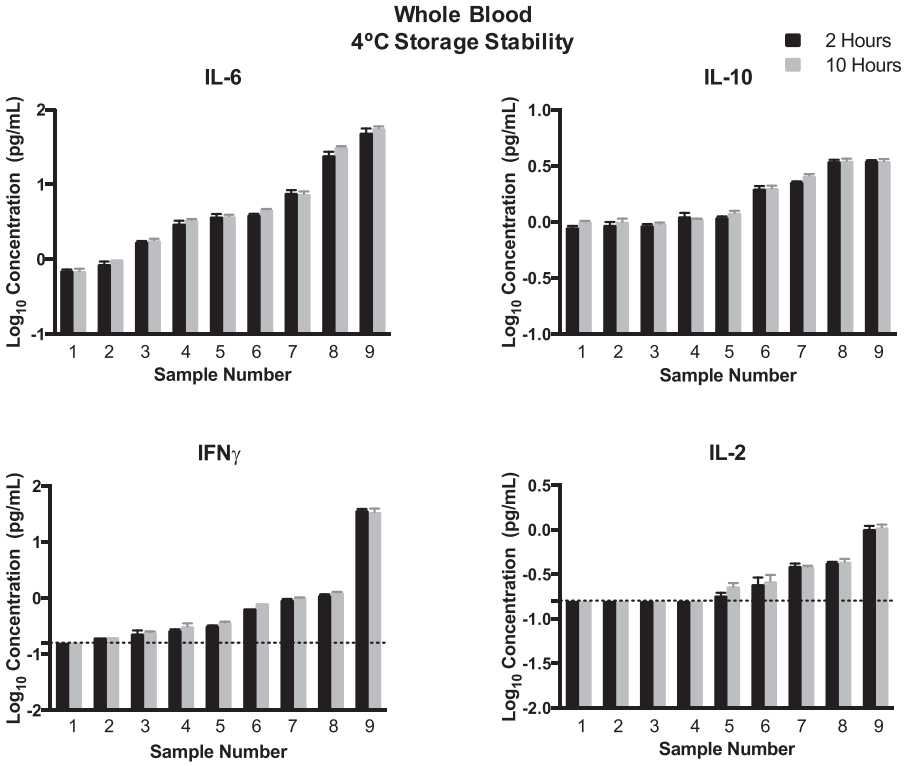

Fig. 2. Cytokine stability in whole blood when stored at 4 °C. Whole blood was collected and stored at 4 °C for 2 or 10 h before being processed to plasma. Cytokines were then measured in plasma. Dotted line is the lower limit of detection. *P* values are 0.0547, 0.0977, 0.195, 0.125 for IL-6, IL-10, IFNγ, and IL-2, respectively.

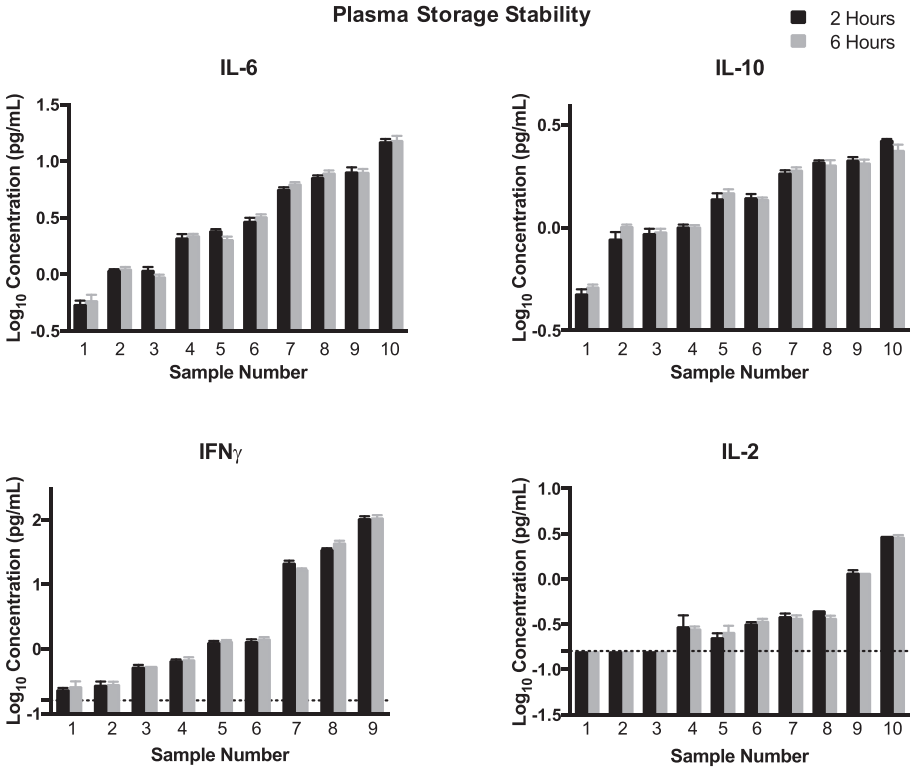

Fig. 3. Cytokine stability in plasma when stored at room temperature for 2 or 6 h. Cytokines were measured in plasma. Dotted line is the lower limit of quantification. *P* values are 0.232, > 0.999, 0.0977, and 0.469 for IL-6, IL-10, IFNγ, and IL-2, respectively.

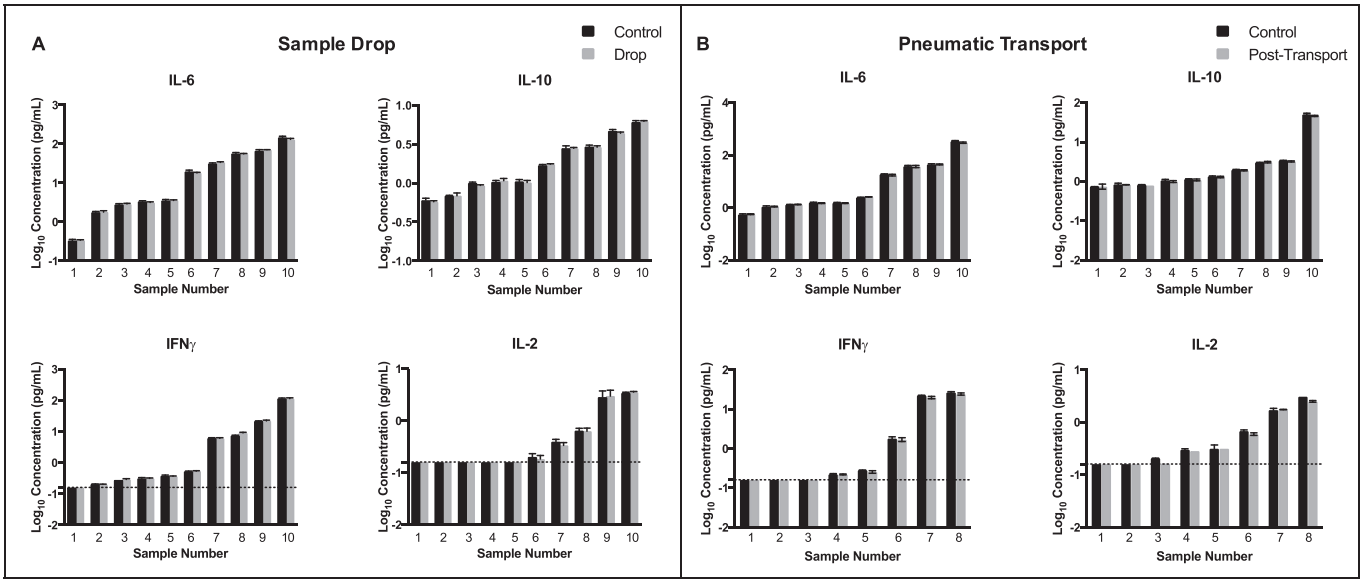

Fig. 4. Cytokine stability in whole blood when subjected to vigorous sample handling. A. Samples were dropped five times from a height of five feet. The control samples were not dropped. *P* values are 0.846, 0.695, > 0.999, and 0.813 for IL-6, IL-10, IFNγ, and IL-2, respectively. B. Samples were transported through a pneumatic transport system five times. Control samples were not transported through the pneumatic transport system. *P* values are 0.432, 0.0645, 0.0625, and 0.156 for IL-6, IL-10, IFNγ, and IL-2, respectively. Cytokines were measured in plasma. Dotted line is the lower limit of quantification.

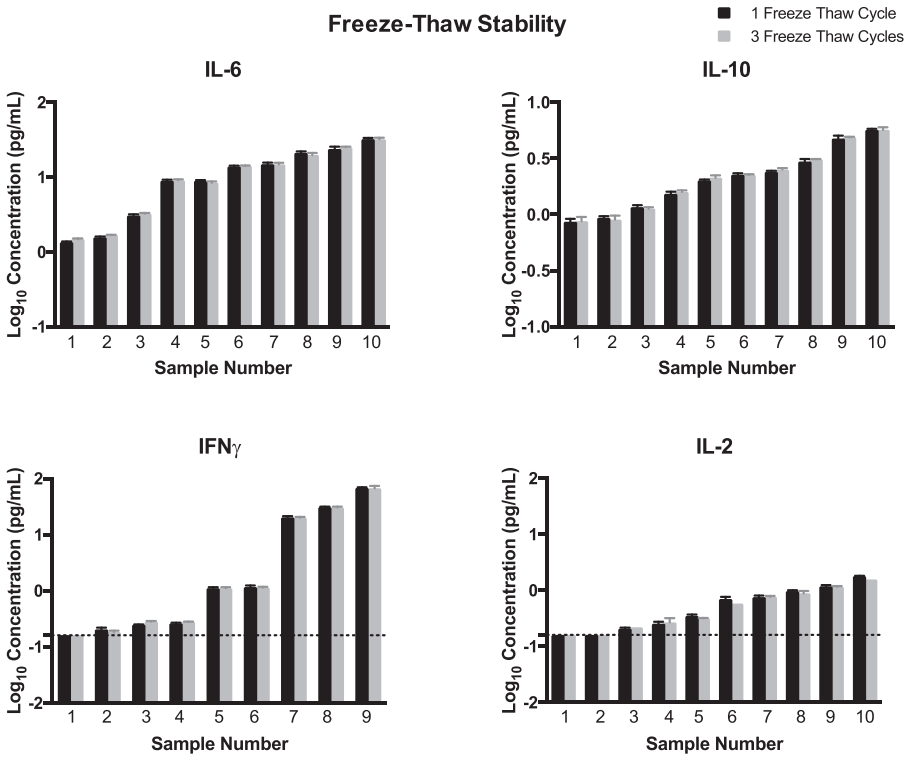

Fig. 5. Freeze-thaw stability of cytokines in plasma. Samples were subjected to either one or three freeze-thaw cycles. Cytokines were measured in plasma. Dotted line is the lower limit of quantification. P values are 0.695, 0.131, 0.195, and 0.313 for IL-6, IL-10, IFNγ, and IL-2, respectively.

high sensitivity. Antibody-coated capture beads are added in large excess to a sample containing low concentrations of target analyte molecules. Poisson statistics dictate that either one or zero target protein molecules will bind to each bead. The beads are then incubated with a biotinylated detection antibody and streptavidin-β-galactosidase, forming an enzyme-labeled immunocomplex. Then the beads are loaded onto an array of 50 fL sized wells in which each well can hold only one bead. A fluorogenic substrate is added and the wells are sealed with oil, producing a locally high concentration of fluorescent product, thus enabling single molecule quantitation by counting active wells. At high target molecule concentrations, fluorescence intensity of the array is used to determine target concentration, thereby extending the dynamic range of the assay. The signal output is measured on the Simoa instrument using the standard unit of average enzymes per bead (AEB) [19,20]. All Simoa consumables and reagents were purchased from Quanterix Corp.

2.2. Preparation of reagents and Simoa assay setup

Antibody-coated capture beads were diluted in Sample/Detector Diluent (SDD) to a concentration of 20,000 beads/μL. Biotinylated detection antibodies were diluted in SDD to the desired concentration. Detection antibody concentrations are provided in Table S2. Streptavidin-β-galactosidase (SβG) Concentrate was diluted to 150 pM in SβG Diluent. Recombinant protein standards (R&D Systems, Table S1) were serially diluted to desired concentrations in SDD. Human specimens were diluted in SDD. The reagents including beads, detection antibodies, and SβG were placed in plastic bottles. The samples, including calibrators and samples, were loaded onto a 96-well plate. All reagents (capture beads, detection antibodies, SβG, enzyme substrate Resorufin β-D-galactopyranoside (RGP), Wash Buffer 1, Wash Buffer 2, and Simoa Sealing Oil) were loaded onto the Simoa HD-1 Analyzer (Quanterix) based on the manufacturer's instructions. Either three or two step assay configurations were chosen. Additional information on assay configuration and incubation times for each assay is provided in Table S2. In a three-step assay configuration, 25 μL of bead solution and a chosen volume of sample (see Table S2) was pipetted into a reaction cuvette and allowed to incubate. The beads were then pelleted with a magnet and the supernatant was removed. Following several washes, 100 μL of detection antibody was added and incubated. The beads were then pelleted with a magnet and the supernatant was removed. Following a series of washes, 100 μL of SβG was added and incubated. The beads were washed, re-suspended in RGP solution, and loaded onto the array. In a two-step assay configuration, 25 μL of bead solution, a chosen volume of sample (See Table S2), and 20 μL of detection antibody were pipetted into a reaction cuvette and allowed to incubate. The beads were then pelleted with a magnet, and the supernatant was removed. Following several washes, 100 μL of SβG was added and incubated. The beads were washed, re-suspended in RGP solution, and loaded onto the array. The array was then sealed with oil and imaged. Images of the arrays were analyzed and AEB (average enzymes per bead) values were calculated by HD-1 Analyzer software. Detailed information on the Simoa HD-1 Analyzer has been previously reported [20].

2.3. Clinical sample processing

Plasma samples used in this study were obtained from excess whole blood remaining after complete blood counts (CBCs) in the clinical hematology laboratory or from healthy volunteers at Brigham and Women's Hospital. Excess patient samples were chosen at random based on the availability of sufficient excess material after CBC. Peripheral blood was drawn in 2.0 mL lavender EDTA plasma whole blood collection tubes (BD Vacutainer). Whole blood was centrifuged at 2000*g* for 15 min at room temperature and plasma was then immediately aliquoted and frozen at −80 °C such that the time from blood collection to freeze did not exceed 4 h (unless otherwise specified).

To test the stability of cytokines in whole blood, whole blood from lavender top specimens was obtained 2 h after venipuncture, aliquoted into three tubes, and either processed immediately (for a total of 2 h from blood draw) or left at room temperature for an additional 4 or 8 h (for a total of 6 or 10 h after blood draw, respectively), and then processed to plasma as described above, and frozen at −80 °C. To test the stability of cytokines in plasma, whole blood from lavender top specimens was obtained 2 h after venipuncture, immediately processed to plasma, and either frozen (for a total of 2 h from blood draw) or left out at room temperature for an additional 4 h (for a total of 6 h from blood draw) then frozen at −80 °C. To further test cytokine stability in whole blood, four samples of whole blood were processed to plasma as described above either immediately or 4 h following blood draw.

To test effects of vigorous specimen handling, whole blood from lavender top tubes was split and half the sample was subjected to five consecutive drops from five feet or five transports in the hospital's pneumatic tube system. For multiple freeze/thaw cycles, plasma was thawed for 1 h at room temperature and re-frozen and the cycle was repeated each day on two consecutive days. This study was approved by the Partners Human Research Committee.

2.4. Data analysis

Human samples along with calibration curves were measured using the Simoa HD-1 Analyzer. The calibration curves were fit using a 4PL fit, as shown below, with a $1/y^2$ weighting factor.

$$Y(x) = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B}$$

The calibration curves were used to determine concentrations of the unknown samples. This analysis was done automatically using the software provided by Quanterix with the Simoa HD-1 Analyzer. The limit of detection (LOD) was calculated as the mean of the background plus three times the standard deviation. The LOD was calculated from eight experiments on different days. Calibrator concentrations were adjusted to optimize quantitation of cytokines in human plasma specimens. The lower and upper limits of quantitation (LLOQ, ULOQ) were defined as the lowest and highest calibrators, respectively, measured in triplicate with coefficients of variation (CVs) < 20% across six experiments on different days. The LLOQ also met the criterion of having signal at least 10 standard deviations above the zero calibrator (background). The assay characteristics are shown in Table S3. Calibration curves are shown in supplementary Fig. 1. All specimens were diluted four-fold in buffer and assayed in duplicate or triplicate. Error bars represent standard deviation of triplicate measurements. We tested for differences in categorical variables by a Wilcoxon test using GraphPad Prism (San Diego, CA). Samples outside the reportable range (LLOQ and ULOQ) are set to the respective limit in the figures and excluded from the statistical analysis.

3. Results and discussion

3.1. Cytokine stability in whole blood and plasma

We first assessed the variability in plasma cytokine measurements when whole blood was stored over a period of 2, 6, or 10 h. Whole blood contains white blood cells that can secrete cytokines, which may result in increased cytokine levels. On the other hand, lymphocyte-mediated internalization or binding to lymphocyte-derived soluble receptors may result in decreased cytokine levels [21]. Cytokine degradation, unfolding, or instability in whole blood may also result in decreased cytokine levels. In the clinical laboratory, whole blood is collected and stored at either room temperature or 4 °C before being further processed to plasma or serum. To assess cytokine stability in whole blood, we collected whole blood, stored it at room temperature, and then processed to plasma after 2, 6, or 10 h. We then measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 1). We observe significant decreases in all four cytokine levels over the ten-hour time-period ($p < .05$) (Table 1). Decreases in levels were larger for all cytokines when whole blood samples were stored for 10 h compared to when they were stored for 6 h prior to processing to plasma. Therefore, we conclude that storage of whole blood at room temperature can substantially affect cytokine measurements.

We then assessed the variability in cytokine measurements when whole blood was stored at 4 °C. We collected blood, stored it at 4 °C, and then processed to plasma after 2 or 10 h. We then measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 2). We observe that IL-6, IL-10, IFNγ, and IL-2 are substantially more stable when whole blood samples are stored at 4 °C compared to when they are stored at room temperature, even after 10 h of storage.

Lastly, we assessed the stability of cytokines in plasma. We collected

whole blood and processed to plasma within 2 h. We either immediately froze or stored the plasma samples at room temperature for an additional 4 (for a total 6 h after blood draw). We then measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 3). We observe that overall, cytokines are stable over 6 h in plasma; however, we observed substantial decreases in IL-6 levels in two samples.

In the studies described above, we processed whole blood to plasma 2 h or more after blood draw from excess clinical material. This time frame is compatible with standard procedures in the clinical laboratory, where processing whole blood to plasma immediately after blood draw is often not possible. Nevertheless, to explore cytokine stability immediately after blood draw we processed four whole blood samples to plasma either immediately after blood draw or after storage for 4 h at room temperature (Supplementary Fig. 2). We observe decreases in the first 4 h after blood draw in levels of IL-6 (an average 25% decrease) and IL-10 (an average 8% decrease). Thus, to prevent variations in cytokine measurements due to sample processing and handling, whole blood should be refrigerated and then processed to plasma and frozen with minimal delay. To ensure that variation arising from sample handling and processing does not exceed 25%, samples should be processed less than 6 h from blood draw if stored at room temperature or less than 10 h from blood draw if stored at 4 °C.

3.2. Cytokine stability in whole blood following vigorous handling

In the clinical laboratory, whole blood samples are often handled vigorously, and therefore, we assessed the impact of vigorous sample handling on cytokine measurements. For example, blood samples are commonly delivered to the clinical laboratory via a pneumatic transport system. During this process, blood samples are subjected to differences in air pressure, accelerations and decelerations, shaking, and vibrations, which may affect cytokine levels in vitro. We collected whole blood and aliquoted it into two tubes. The first tube was the control while the second tube was subjected to repeated dropping from a height of five feet, leading to mild hemolysis, in which the plasma samples are visibly hemolyzed but still translucent. We then processed the samples to plasma and measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 4A). We observe that there are no differences in cytokine levels between the control and the dropped samples. Hemolysis may affect immunoassay performance via release of cellular components that may interfere with measurements [22]. For example, others have shown that hemolysis can affect plasma measurements of troponin [23]. However, we show that mild hemolysis has no effects on IL-6, IL-10, IFNγ, and IL-2 levels.

We then assessed the effects of pneumatic transport on cytokine measurements. Similar to the previous experiment, we collected whole blood and partitioned it into two samples. The first sample was the control while the second sample was subjected to five rounds of pneumatic transport. We then processed the samples to plasma and measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 4B). Similar to the drop tests, we observe that there are no differences in cytokine measurements between the control samples and those subjected to pneumatic transport. Thus, vigorous sample handling does not affect IL-6, IL-10, IFNγ, and IL-2 measurements.

3.3. Freeze-thaw stability

Plasma samples often must undergo several freeze-thaw cycles prior to analysis. The process of freeze-thawing plasma may affect the stability of cytokines. Therefore, we assessed the effects of sample freeze-thaw on cytokine measurements. Plasma was thawed for 1 h at room temperature and frozen. This process was repeated twice for two consecutive days, resulting in a total of three freeze-thaw cycles. The control samples were subjected to only one freeze-thaw cycle. We then measured IL-6, IL-10, IFNγ, and IL-2 levels in plasma using the Simoa assays (Fig. 5). We observe no substantial differences between the control and case samples and therefore conclude that IL-6, IL-10, IFNγ, and IL-2 are stable when plasma samples undergo three freeze-thaw cycles.

4. Conclusions

In this study, we assessed various clinical sample handling and processing parameters on IL-6, IL-10, IFNγ, and IL-2 measurements in plasma. We show that storage of whole blood at room temperature over a period of hours can result in decreased cytokine levels. We also show that whole blood storage at 4 °C results in greater cytokine stability. Physical processes, such as dropping and pneumatic transport, as well as freeze-thawing do not seem to affect cytokine levels. While the measurements performed here were done with only four cytokines, it is likely that similar results would be obtained with other cytokines and proteins. Some large biomarker studies have failed previously due to unforeseen pre-analytical variations that should have been characterized prior to searching for biomarker-disease associations [24]. Therefore, when measuring biomarkers for clinical applications, in addition to assessing the robustness of the analytical method, it is important to ensure that minimal measurement variations arise from sample handling and processing.

Conflict of interest

The authors declare the following competing financial interest: David R. Walt is the scientific founder and a board member of Quanterix Corporation. All other authors declare no competing financial interest.

Acknowledgements

LC and DRW were funded by DARPA (HR0011-12-2-0001; Pass-through-entity: Univ. of North Carolina Chapel-Hill, subaward 5055065). AK and SEFM were supported by the BWH Department of Pathology. We thank the volunteers who donated their blood for stability studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.01.001.

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